Application of *Hibiscus Sabdariffa* Extract to Manage Obesity

A Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Manisha Singh

B.Tech. (Food Technology), National College of Food Science and Technology, Tribhuvan University, Nepal

M. Sc. (Food Engineering and Bioprocess Technology), Asian Institute of Technology, Thailand

School of Science

College of Science, Technology, Engineering and Maths

RMIT University

December 2022

Declaration

I certify that except where due acknowledgement has been made, this research is that of the author alone; the content of this research submission is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

In addition, I certify that this submission contains no material previously submitted for award of any qualification at any other university or institution, unless approved for a joint-award with another institution, and acknowledge that no part of this work will, in the future, be used in a submission in my name, for any other qualification in any university or other tertiary institution without the prior approval of the University, and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of any published works contained within this thesis resides with the copyright holder(s) of those works.

I give permission for the digital version of my research submission to be made available on the web, via the University's digital research repository, unless permission has been granted by the University to restrict access for a period of time.

Manisha Singh

09 December 2022

Copyright

Manisha Singh

2022

Copyright Notices

Notice 1

Notice 2

Acknowledgement

I wish to thank my Senior Supervisor Prof. Benu Adhikari for his patience, motivation, and continuous support during my candidature. His guidance helped me in planning and conducting research, drafting publications, and compiling thesis. I also wish to thank my Associate supervisors Dr. Thilini Thrimawithana and Assoc. Prof. Ravi Shukla for their timely and valuable advice and invaluable guidance. My sincere gratitude also goes to Prof. Charles Stephen Brennan for the guidance he offered during my publication. The completion of this study could not have been possible without their continuous support, encouragement, and belief in me.

I wish to extend my sincere gratitude to RMIT University for providing me scholarship and fee waiver which enabled me to carry out this research. I am also grateful to Lee Etherington, Managing Director of Wild Hibiscus Flower Company, NSW, Australia for donating unlimited roselle calyces for this study.

I thankfully acknowledge the support of technical staff at RMIT University including Mina Dokouhaki, Sanaz Salehi, Lillian Chuang, Yan Chen, Spiros Tsaroumis, Sophie Loone, Adam Vogrin, Zoon Chan, Nadia Zakhartchouk, Stephen Grist, Chaitali Dekiwadia and Sanje Mahasivam during the entire candidature.

A special thanks goes to my friends and colleagues from RMIT University- Pranita Mhaske, Lizebona August, Dao Nguyen, Diah Ikasari, Bao Loc Pham, Kingshuk Dhali, Mehran Ghasemlou, Zijia Zhang, Ziyu Wang, Yanyi Huang, Billy Lo, Gbemisola Fadimu, Nabeela Dullu, Chintha Lankatillake, Nee Edirisinghe, Kanjana Singh and Sudip Dhakal for their moral support and helpful advice.

Finally, I am extremely thankful to my family- my parents (Neelam Maharjan and Sanu Bhai Maharjan) and my sister (Rojeesha Singh) for their unconditional love, support, and their unwavering faith in me. I owe everything to them.

Table of contents

List of abbreviations

4-MUO	4-Methylumbelliferyl oleate
ACC	Acetyl coenzyme A carboxylase
Acetyl-CoA	Acetyl-coenzyme A
AMPK	Adenosine monophosphate -activated protein kinase;
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCAAs	Branched-chain amino acids
BCS	Bovine calf serum
C/EBP α	CCAAT/enhancer binding protein-α
CNS	Central nervous system
COMT	Catechol- O -methyltransferase
CPT-1	Carnitine palmitoyl transferase 1
DAPI	4,6-diamidino-2-phenylindole, dilactate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNSA	3,5-dinitro salicylic acid
DPPH	2, 2-Diphenyl-1-picrylhydrazyl free radical
DXM	Dexamethasone
EE	Ethanol extract
EGCG	Epigallocatechin-3-gallate
FA	Fatty acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter
hADSCs	Human adipose-derived stem cells
HCA	Hydroxycitric acid
HPLC-UV	High performance liquid chromatography with ultraviolet detector

IBMX	3-isobutyl-1-methylxanthine
IR	Insulin receptor kinase
K-HCA	Potassium hydroxycitrate
LDL	Low Density Lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
MCH	Melanin-concentration hormone
ME	Methanol extract
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
PBS	Phosphate-buffered saline
PGC-1-α	Proliferator-activated-receptor- gamma-coactivator1-α
PPAR-α	Peroxisome proliferator-activated receptor-α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
SCFAs	Short chain fatty acids
SIRT 1	Sirtuin 1
SNS	Sympathetic nervous system
SREBP-1c	Sterol-regulatory-element- binding-protein-1c
TCA	Tricarboxylic acid cycle
TFC	Total flavonoid content
TG	Triglycerides
TPC	Total phenolic content
TPTZ	2,4,6-Tris(2-pyridyl)- s -triazine
UCP1	Uncoupling protein1
UPLC-DAD	Ultra-Performance Liquid Chromatography with diode array detector
WAT	White adipose tissue
WE	Water extract
ρ-NPG	4-nitrophenyl β-D-glucopyranoside

List of figures

Page

List of tables

Page

List of Explanatory notes

The following notes briefly delineate the points that were taken into consideration during the writing of this thesis.

i. Attempts have been made to use British spellings in the text except in the published journal articles where the journal guidelines have been followed.

ii. Symbols or abbreviations, used in place of a lengthy name or expression, have been defined or explained in appropriate places as far as practicable.

iii. Wherever possible, SI units have generally been used in expressing results throughout this thesis.

iv. APA referencing format has been followed in this Thesis except in the published articles where journal guidelines have been followed.

Summary

Global rise in obesity has necessitated continued effort to develop suitable therapies for its effective management. Although a number of synthetic medications with anti-obesity properties are commercially available, they are associated with wide range of risks and side effects. Many plant-derived compounds with potential preventive and therapeutic effects against obesity are identified with negligible side effects. Phenolic compounds from plant sources have shown to possess promising anti-obesity effects assessed through cell line, animal, and clinical trials. In addition, hydroxycitric acid, commonly extracted from *Garcinia cambogia* is marketed as weight managing supplement. Considering the presence of high concentration of phenolic compounds and hydroxycitric acid, extract of *Hibiscus sabdariffa* can be considered as suitable material for quantifying anti-obesity effects. Therefore, this PhD study aimed to optimize the extraction of phenolic compounds and hydroxycitric acid from *H. sabdariffa* followed by the determination of their anti-obesity potential individually and in combination. For this purpose, *in vitro* studies i.e., inhibition of digestive enzymes and cell line studies on inhibition of adipogenesis were undertaken.

The first experimental chapter focused on the extraction of bioactive compounds (phenolic compounds and hydroxycitric acid) and determining their physicochemical characteristics. The type and concentration of solvents and extraction temperature varied to maximise the yield and antioxidant potency of phenolic compounds. Spectrophotometric and chromatographic methods were used for determining phenolic and flavonoid contents, and antioxidant activity. Low temperature (23°C) and organic solvents (methanol and ethanol) were found to be suitable medium for extracting phenolic and flavonoid compounds with higher antioxidant activity. The antioxidant activity of these phenolic compounds was found to be stable for 5 weeks at -30^{o}C. Hydroxycitric acid was extracted in the form of potassium salt to

overcome its instability. The purity of extracted potassium hydroxycitrate was 180.14 μg/mg of dry extract.

The second experimental chapter focused on determining the inhibition of digestive enzymes by the phenolic compounds and potassium hydroxycitrate individually and in combination. For this, three enzymes associated with obesity (α-amylase, α-glucosidase and pancreatic lipase) were selected and the inhibition potential of the extract was determined through *in vitro* assays. All phenolic extracts extracted using aqueous and organic (methanol, ethanol, and ethyl acetate) solvents showed different degree of inhibition against these enzymes. The ethyl acetate, methanolic and ethanolic extracts showed higher inhibition efficacy (IC_{50}) for α-amylase (3.69 mg/mL), α-glucosidase (1.59 mg/mL) and pancreatic lipase (1.88 mg/mL) respectively as compared to aqueous extract (5.74, 3.65 and 4.25 mg/mL) which could be attributed to the higher concentration of phenolic compounds in the organic extracts. These phenolic compounds had binding affinity with these enzymes at their active sites which reduced their activity. Potassium hydroxycitrate did not show inhibition against these enzymes when used alone or in combination with phenolic compounds.

The third experimental chapter assessed the inhibition of adipogenesis/adipocyte differentiation by extracted phenolic compounds and potassium hydroxycitrate in human adipose-derived stem cells (hADSCs). Adipogenesis was chemically induced in hADSCs for 21 days and the cells were treated with different concentration of phenolic extracts and potassium hydroxycitrate during first three days of differentiation. The lipid accumulation after completion of adipogenesis was quantified. Phenolic extracts showed significant reduction (45-95%) in the lipid accumulation in these cells at the concentration of 1 mg/mL, however, potassium hydroxycitrate had no inhibiting effect on adipogenesis in hADSCs. The adipogenic inhibition by phenolic compounds was reflected on the down regulation of gene expression of two key adipogenic markers [peroxisome proliferator-activated receptor- γ (PPAR-γ) and aP2].

The incorporation or presence of hydroxycitrate in phenolic extract did not show additional inhibition of adipogenesis in hADSCs.

The fourth experimental chapter focussed on developing suitable encapsulating shell material for these phenolic compound-rich extracts of *H. sabdariffa* in order to extend their shelf life and also to make them available in easy-to-handle bead form. Encapsulation of phenolic extract was achieved through ionic gelation using alginate-chitosan hybrid matrix. The concentration of alginate, chitosan and extract was optimized to obtain the highest encapsulation efficiency. The maximum encapsulation efficiency (91.9%) of phenolic compounds was achieved with 3% alginate, 0.2% chitosan and 1% extract concentrations. The capsule beads had a smooth surface with no cracks and pores on the surface. The alginate-chitosan matrix created through ionic gelation preserved antioxidant activity of phenolic compounds (81%) as compared to the non-encapsulated extracts (71%). This matrix also contributed to the sustained release of phenolic compounds by delivering more than 50% of the compounds to the simulated intestinal condition.

These findings from this research provide scientific basis for application of phenolic compound rich extracts of *H. sabdariffa* as natural therapeutic agent to manage obesity.

CHAPTER 1

Introduction

1. Introduction

1.1 Background

Obesity is a global public health problem which is continuously increasing globally due to urbanization, sedentary lifestyle, and higher consumption of energy dense foods. About 38% of world's adults population was overweight or obese in 2016 (World Health Organization, 2021). And it is predicted that overweight or obese adult population will reach to 51% by 2035 (World Obesity Federation, 2023). Reduced calorie intake in combination with increased physical activity can reduce the risk of obesity; however, it is not an effective approach for all individuals (Tiwari & Balasundaram, 2022). Genetic factors are also accounted for over 40% of heritable obesity (Flores-Dorantes et al., 2020). In addition to lifestyle changes, excess body weight can be managed with pharmacological agents such as orlistat (Xenical, Alli), phentermine-topiramate (Qsymia), naltrexone-bupropion (Contrave), liraglutide (Saxenda), semaglutide (Wegovy), and setmelanotide (IMCIVREE) (Feingold, 2020). However, the use of these medications is limited due to their unacceptable side effects. In the past, some of the approved drugs have been discontinued even after regulatory approval because of their acute side effect (Müller et al., 2022). Therefore, exploration of natural, safer at the same time effective anti-obesity ingredient is vital to manage obesity.

Natural bioactive compounds are becoming increasingly popular in pharmaceutical and food industries due to their health promoting characteristics (Srivastava et al., 2021). Plant-based bioactive compounds have been traditionally used by humans as therapeutic ingredients. Currently, about 25% of the pharmaceutical ingredients and their derivatives are derived from plants (Patra et al., 2018). Plant based bioactive compounds are metabolic products isolated from various plant components (e.g., roots, fruits, leaves, barks). These compounds improve physiological functions or reduce the risk of chronic diseases in humans (Loi et al., 2020). Natural bioactive compounds can be categorized into alkaloids, capsaicinoids, carotenoids,

glucosinolates, phytosterols, polyphenols and phenolic compounds, polysaccharides, saponins, terpenoids, triterpenes and tocopherols (Pai et al., 2022). These compounds are known to possess various biological effects such as antioxidant activity, receptor modulation, regulation of enzyme and gene expression (Santos et al., 2019). These biological effects along with their chemical diversity and low toxicity make them suitable therapeutic candidates (Patra et al., 2018).

Among these plant-based bioactive compounds, polyphenols and phenolic compounds have emerged as one of the promising therapeutic groups due to their abundance in plants and multiple physiological effects. Polyphenols and phenolic compounds are naturally occurring secondary metabolites present in plants and provide protection against pathogenic aggression and ultraviolet radiation. Several epidemiological research have revealed that plant polyphenols can impart anti-obesity, anticancer, antidiabetic, antioxidant, cardioprotective and neuroprotective effects (Mutha et al., 2021; Yan et al., 2021).

Hibiscus sabdariffa (family Malvaceae) is a well-known flowering plant which is also known as rosella or roselle. This shrub is widely cultivated all over the world for its traditional uses in food, cosmetic, and medicinal applications. It produces brilliant red calyces that allows them to be used as a red colouring agent in beverages, jams, and jellies. The characteristic red colour of these calyces is contributed by the anthocyanins. The calyces are uniquely acidic in taste because of organic acids such as citric acid, hibiscus acid, hydroxycitric acid, malic acid, and tartaric acids (Izquierdo-Vega et al., 2020). Roselle calyces are a good source of bioactive phytochemicals such as phenolic compounds, organic acids, alkaloids, saponins and glycosides (Janson et al., 2021). The most dominant phenolic compounds in roselle include caffeic acid, catechin, chlorogenic acid, cinnamic acid, gallic acid, hydroxy benzoic acid, quercetin, rutin and anthocyanins (Banwo et al., 2022). Cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside are the two major anthocyanins present in calyces of *H. sabdariffa* (Grajeda-

Iglesias et al., 2016; Morales-Luna et al., 2019). Scientific literature has acknowledged the therapeutic properties of *H. sabdariffa* are imparted by these phytochemicals and organic acids.

Several *in vitro* and *in vivo* studies support the fact that *H. sabdariffa* has anti-obesity potential (Chang et al., 2014; Janson et al., 2021; Omar et al., 2018; Prasomthong et al., 2022). *H. sabdariffa* is proposed to impart anti-obesity function through muti-targeted mechanisms (Fig 1.1) such as appetite regulation, promoting energy expenditure, modulation of lipid metabolism, enzyme inhibition, modulation of gut microbiome and inhibition of adipogenesis/adipocyte differentiation (Herranz-López et al., 2017; Ojulari et al., 2019; Serna et al., 2022; Silva et al., 2022). Kao et al. (2016) have linked phenolic compounds in *H. sabdariffa* extract to inhibition of lipogenesis and adipogenesis in hamsters fed with a high-fat diet. Similarly, Chang et al. (2014) attributed polyphenols in *H. sabdariffa* extract for the reduced body weight and fat accumulation in humans. Delphinidin-3-sambubioside, one of the major anthocyanins present in *H. sabdariffa,* was also found to regulate the lipid metabolism resulting into reduced body weight gain, visceral and abdominal fat and decreased hepatic lipid deposits in high fat diet fed Sprague-Dawley rats (Long et al., 2021). Morales-Luna et al. (2019) reported that organic acids of *H. sabdariffa* (hydroxycitric acid, hibiscus acid and dimethyl hibiscus acid) are responsible for anti-obesity effects in wister male rats rather than anthocyanins.

Hydroxycitric acid (HCA) is an organic acid structurally identical to citric acid found in *Hibiscus sabdariffa* but richly present in *Garcinia cambogia.* It is unstable in its free acid form hence extracted and stabilized as a calcium or potassium salt or as a lactone (Márquez et al., 2012). HCA is one of the supplements marketed for antiobesity therapeutics for weight management (Mena-Garcia et al., 2022). The mechanisms through which HCA exert antiobesity effects are suggested to be appetite suppression, reduction of de novo lipogenesis, acceleration of fatty acid oxidation and downregulation of obesity related gene expression

(Chuah et al., 2013; Tomar et al., 2019). Other studies have also validated the potential of HCA from *Garcinia cambogia* in inhibiting ATP citrate lyase and fatty acid synthase mRNA expression levels contributing to accelerated fatty acid oxidation (Han et al., 2016; Li et al., 2017). However, some of these studies presented a conflicting and weak result on the weight loss effect of HCA (Mena-Garcia et al., 2022). Nevertheless, the studies on extraction of hydroxycitric acid from *H. sabdariffa* and their anti-obesity effects are limited.

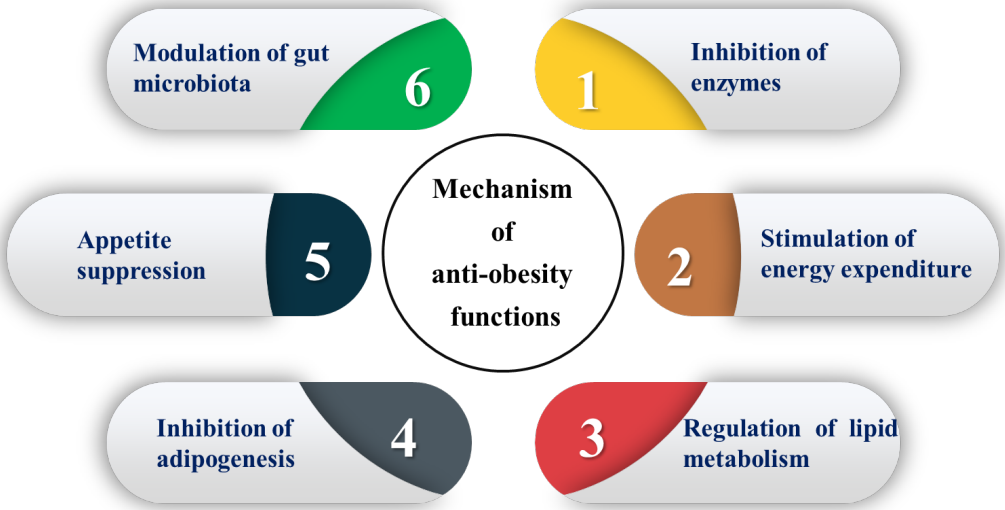


Figure 1.1 Schematic representation of muti-target anti-obesity mechanisms through which *H. sabdariffa* extracts are likely to act

Phenolic compounds and polyphenols are unstable compounds and are susceptible to temperature, light and pH induced degradation (Sun et al., 2017). In addition, phenolic compounds can undergo degradation under highly acidic condition prevailing in gastrointestinal tract (Qin et al., 2022). This necessitates the development of a robust technology which can protect their properties from the harsh environments encountered during processing, storage, and consumption. Encapsulation in a polymeric matrix has been proposed as a suitable option for preservation of functional properties of many bioactive compounds (Bourbon et al., 2016). There are several methods of encapsulation such as coacervation, emulsions, extrusion, fluidized bed coating, ionic gelation and liposomes that can be used for

phenolic compounds (Mehta et al., 2022). Amongst these, ionic gelation, which can be carried out at ambient environment, can be the most appropriate one considering the heat sensitive nature of the polyphenols and phenolic compounds. It is a simple technique involving the formation of complexes through interaction of charged biopolymer and an oppositely charged solution (Naranjo-Duran et al., 2021). Alginate and chitosan have been largely exploited as a promising material for encapsulation because they are readily available and also are food grade (Szczęsna et al., 2021).

The potential of *H. sabdariffa* extracts to be used as an anti-obesity ingredient prompted the need for determining efficacy of anti-obesity function of phenolic compounds and hydroxycitric acid extracted from it. A greater understanding of the mechanism of action of these compounds in the management of obesity will broaden their application in both food and pharmaceutical industries. It is possible that phenolic compounds and hydroxycitric acid may work in tandem to deliver synergistic, additive effects or only one of them can be dominant the other exerting no effect. These aspects are also explored in this thesis which will provide the basis for developing more effective formulation to manage obesity.

1.2 Research questions

In the above context, this thesis addresses the following research questions.

1. What are the optimum process parameters for extraction of phenolic compounds from the calyces of *H. sabdariffa* and what is their storage stability?
2. Can hydroxycitric acid be extracted from *H. sabdariffa* and what is the purity of the extracted hydroxycitric acid?
3. What is the inhibitory potency of phenolic compounds and hydroxycitric acid on obesity related enzymes (α-amylase, α-glucosidase, and lipase) and can this potency improve when they are used in combination?

4. Do phenolic compounds and hydroxycitric acid inhibit the adipocyte differentiation and can this be improved when they are used in combination?
5. Does encapsulation protect the antioxidant activity of phenolic compounds and improve the release behaviour in simulated human digestive system?

1.3 Research aims

This research aimed to advance the science to enable the application of both phenolic compounds and hydroxycitric acid extracted from *H. sabdariffa*, for the management of obesity. It proceeds by hypothesising that their combination may bring additional effectiveness, or one component can be dominant over other, and no additive effect may be realised. The specific objectives of the thesis were as follows.:

1. Extract and characterise phenolic compounds and potassium hydroxycitrate from calyces of *H. sabdariffa* and study the storage stability of phenolic compounds
2. Determine the extent and nature of inhibition of α-amylase, α-glucosidase and pancreatic lipase by phenolic compounds and potassium hydroxycitrate, individually and in combination
3. Investigate the anti-obesity potential of phenolic compounds and potassium hydroxycitrate by quantifying inhibition of adipocyte differentiation in human adipose-derived stem cells, individually and in combination
4. Develop a suitable polymeric shell matrix to encapsulate phenolic compounds to improve their stability and release behaviour

1.4 Expected outcomes

This study proceeded by expecting to quantify the anti-obesity effects of phenolic compounds and potassium hydroxycitrate from *H. sabdariffa*. It also expected to validate the mechanism

of action of these compounds (in extract) for the treatment of obesity through chemical and *in vitro* studies. This study also expected to reveal whether the combination of phenolic compounds and potassium hydroxycitrate would bring additional effectiveness on anti-obesity potential. If the anti-obesity properties of *H. sabdariffa* extract was found to be effective, then it would provide scientific foundation for application of the extract and its components (phenolic compounds and hydroxycitric acid) as therapeutic agent to manage obesity. Therefore, the finding of this study would be particularly significant in managing obesity which is one of the most pervasive health problems now.

1.5 Outline of the thesis

This thesis is assembled into seven chapters, a brief outline of each is presented below.

Chapter 1 presents a brief background and sheds light on the existing knowledge relevant to this research. It provides an overview on the global status of obesity and the potential of natural bioactive compounds including phenolic compounds and hydroxycitric acid on managing this issue. This chapter also presents the research questions, research aims and expected outcomes of this research.

Chapter 2 documents a comprehensive literature review on areas related to this research project. It reviews the different classes of polyphenols and their possible mechanisms of action to exert anti-obesity function. The findings on antiobesity effects of natural ingredients assessed through *in vitro, in vivo,* and clinical trials and reported in the literature are summarised. The challenges associated with the application of natural ingredients including polyphenols is also reviewed. The content of this paper is published in Future Foods (Singh et al., 2020).

Chapter 3 documents outcome of studies on the extraction of phenolic compounds and potassium hydroxycitrate from *H. sabdariffa* and characterization of the extracts through

chemical and chromatographic methods. The extraction parameters for phenolic compounds were optimized and storage stability of these extracts is assessed. The content of this chapter is published in Future Foods (Singh et al., 2021).

Chapter 4 documents the outcome of investigation on the enzyme inhibition potential of phenolic compounds and potassium hydroxycitrate against three main digestive enzymes linked to obesity (α-amylase, α-glucosidase, and pancreatic lipase). The mechanism of inhibition by these compounds is established by the molecular docking approach. The formation of various bonding (particularly hydrogen bond) between compounds and the specific site of enzymes is established. The content of this chapter is published in Food Bioscience (Singh et al., 2022).

Chapter 5 documents the results of investigation on the effect of phenolic compounds and potassium hydroxycitrate on the adipogenesis in human adipose-derived stem cells. The impact on lipid accumulation and regulation of adipogenic marker genes (PPAR-γ and aP2) during adipogenesis are also examined. The content of this chapter is published in International Journal of Food Science and Technology (Singh et al., 2023).

Chapter 6 provides the details of studies on the encapsulation of phenolic compounds of *H. sabdariffa* in alginate-chitosan matrix. The ionic gelation process involved is optimised. The encapsulation efficiency and physicochemical properties of the resulting microcapsules are evaluated. The *in vitro* release study of phenolic compounds in simulated gastric fluid and simulated intestinal fluid is determined and discussed. The antioxidant activity of phenolic compounds released in the gastro-intestinal fluids is also determined and explained.

Chapter 7 presents the discussion on the main findings or gist of all experimental chapters (Chapters 3-6) in an integral manner. It highlights the contributions made by this study to the existing body of knowledge in relevant field. It also provides recommendations for future works.

References

Banwo, K., Sanni, A., Sarkar, D., Ale, O., & Shetty, K. (2022). Phenolics-Linked Antioxidant and Anti-hyperglycemic Properties of Edible Roselle (Hibiscus sabdariffa Linn.) Calyces Targeting Type 2 Diabetes Nutraceutical Benefits in vitro. Frontiers in Sustainable Food Systems, 6. doi:10.3389/fsufs.2022.660831

Bourbon, A. I., Cerqueira, M. A., & Vicente, A. A. (2016). Encapsulation and controlled release of bioactive compounds in lactoferrin-glycomacropeptide nanohydrogels: Curcumin and caffeine as model compounds. Journal of Food Engineering, 180, 110-119. doi:https://doi.org/10.1016/j.jfoodeng.2016.02.016

Chang, H. C., Peng, C. H., Yeh, D. M., Kao, E. S., & Wang, C. J. (2014). Hibiscus sabdariffa extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. Food Funct, 5(4), 734-739. doi:10.1039/c3fo60495k

Chuah, L. O., Ho, W. Y., Beh, B. K., & Yeap, S. K. (2013). Updates on Antiobesity Effect of Garcinia Origin (-)-HCA. Evidence-Based Complementary and Alternative Medicine, 2013, 751658. doi:10.1155/2013/751658

Feingold, K. R. (2020). Obesity and Dyslipidemia. In A. B. Feingold KR, Boyce A, et al. (Ed.), Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.

Flores-Dorantes, M. T., Díaz-López, Y. E., & Gutiérrez-Aguilar, R. (2020). Environment and Gene Association With Obesity and Their Impact on Neurodegenerative and Neurodevelopmental Diseases. Frontiers in Neuroscience, 14. doi:10.3389/fnins.2020.00863

Grajeda-Iglesias, C., Figueroa-Espinoza, M. C., Barouh, N., Baréa, B., Fernandes, A., de Freitas, V., & Salas, E. (2016). Isolation and Characterization of Anthocyanins from Hibiscus sabdariffa Flowers. Journal of Natural Products, 79(7), 1709-1718. doi:10.1021/acs.jnatprod.5b00958

Han, J., Li, L., Wang, D., & Ma, H. (2016). (−)-Hydroxycitric acid reduced fat deposition via regulating lipid metabolism-related gene expression in broiler chickens. Lipids in Health and Disease, 15(1), 37. doi:10.1186/s12944-016-0208-5

Herranz-López, M., Olivares-Vicente, M., Encinar, J. A., Barrajón-Catalán, E., Segura-Carretero, A., Joven, J., & Micol, V. (2017). Multi-Targeted Molecular Effects of Hibiscus sabdariffa Polyphenols: An Opportunity for a Global Approach to Obesity. Nutrients, 9(8), 907. doi:10.3390/nu9080907

Izquierdo-Vega, J. A., Arteaga-Badillo, D. A., Sanchez-Gutierrez, M., Morales-Gonzalez, J. A., Vargas-Mendoza, N., Gomez-Aldapa, C. A., . . . Madrigal-Santillan, E. (2020). Organic acids from roselle (Hibiscus sabdariffa L.)-A brief review of its pharmacological effects. Biomedicines, 8(5), 100. doi:10.3390/biomedicines8050100

Janson, B., Prasomthong, J., Malakul, W., Boonsong, T., & Tunsophon, S. (2021). Hibiscus sabdariffa L. calyx extract prevents the adipogenesis of 3T3-L1 adipocytes, and obesity-related insulin resistance in high-fat diet-induced obese rats. Biomedicine & Pharmacotherapy, 138, 111438. doi:https://doi.org/10.1016/j.biopha.2021.111438

Kao, E.-S., Yang, M.-Y., Hung, C.-H., Huang, C.-N., & Wang, C.-J. (2016). Polyphenolic extract from Hibiscus sabdariffa reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. Food & Function, 7(1), 171-182. doi:10.1039/C5FO00714C

Li, L., Peng, M., Ge, C., Yu, L., & Ma, H. (2017). (-)-Hydroxycitric Acid Reduced Lipid Droplets Accumulation Via Decreasing Acetyl-Coa Supply and Accelerating Energy Metabolism in Cultured Primary Chicken Hepatocytes. Cellular Physiology and Biochemistry, 43(2), 812-831. doi:10.1159/000481564

Loi, M., Paciolla, C., Logrieco, A. F., & Mulè, G. (2020). Plant Bioactive Compounds in Pre- and Postharvest Management for Aflatoxins Reduction. Frontiers in Microbiology, 11. doi:10.3389/fmicb.2020.00243

Long, Q., Chen, H., Yang, W., Yang, L., & Zhang, L. (2021). Delphinidin-3-sambubioside from Hibiscus sabdariffa. L attenuates hyperlipidemia in high fat diet-induced obese rats and oleic acid-induced steatosis in HepG2 cells. Bioengineered, 12(1), 3837-3849. doi:10.1080/21655979.2021.1950259

Márquez, F., Babio, N., Bulló, M., & Salas-Salvadó, J. (2012). Evaluation of the Safety and Efficacy of Hydroxycitric Acid or Garcinia cambogia Extracts in Humans. Critical Reviews in Food Science and Nutrition, 52(7), 585-594. doi:10.1080/10408398.2010.500551

Mehta, N., Kumar, P., Verma, A. K., Umaraw, P., Kumar, Y., Malav, O. P., . . . Lorenzo, J. M. (2022). Microencapsulation as a Noble Technique for the Application of Bioactive Compounds in the Food Industry: A Comprehensive Review. Applied Sciences, 12(3). doi:10.3390/app12031424

Mena-Garcia, A., Bellaizac-Riascos, A. J., Rada-Mendoza, M., Chito-Trujillo, D. M., Ruiz-Matute, A. I., & Sanz, M. L. (2022). Quality Evaluation of Dietary Supplements for Weight Loss Based on Garcinia cambogia. Nutrients, 14(15). doi:10.3390/nu14153077

Morales-Luna, E., Pérez-Ramírez, I. F., Salgado, L. M., Castaño-Tostado, E., Gómez-Aldapa, C. A., & Reynoso-Camacho, R. (2019). The main beneficial effect of roselle (Hibiscus sabdariffa) on obesity is not only related to its anthocyanin content. Journal of the Science of Food and Agriculture, 99(2), 596-605. doi:https://doi.org/10.1002/jsfa.9220

Müller, T. D., Blüher, M., Tschöp, M. H., & DiMarchi, R. D. (2022). Anti-obesity drug discovery: advances and challenges. Nature Reviews Drug Discovery, 21(3), 201-223. doi:10.1038/s41573-021-00337-8

Mutha, R. E., Tatiya, A. U., & Surana, S. J. (2021). Flavonoids as natural phenolic compounds and their role in therapeutics: an overview. Future Journal of Pharmaceutical Sciences, 7(1), 25. doi:10.1186/s43094-020-00161-8

Naranjo-Duran, A. M., Quintero-Quiroz, J., Rojas-Camargo, J., & Ciro-Gomez, G. L. (2021). Modified-release of encapsulated bioactive compounds from annatto seeds produced by optimized ionic gelation techniques. Sci Rep, 11(1), 1317. doi:10.1038/s41598-020-80119-1

Ojulari, O. V., Lee, S. G., & Nam, J. O. (2019). Beneficial Effects of Natural Bioactive Compounds from Hibiscus sabdariffa L. on Obesity. Molecules, 24(1). doi:10.3390/molecules24010210

Omar, M. H., Shamsahal, N., Muhammad, H., Wan Ahmad, W. A. N., & Wasiman, M. I. (2018). Anti-obesity and haematological effects of Malaysia Hibiscus sabdariffa L. aqueous extract on obese Sprague Dawley rats. Functional Foods in Health and Disease, 8(6). doi:10.31989/ffhd.v8i6.407

Pai, S., Hebbar, A., & Selvaraj, S. (2022). A critical look at challenges and future scopes of bioactive compounds and their incorporations in the food, energy, and pharmaceutical sector. Environmental Science and Pollution Research, 29(24), 35518-35541. doi:10.1007/s11356-022-19423-4

Patra, J. K., Das, G., Fraceto, L. F., Campos, E. V. R., Rodriguez-Torres, M. d. P., Acosta-Torres, L. S., . . . Shin, H.-S. (2018). Nano based drug delivery systems: recent developments and future prospects. Journal of Nanobiotechnology, 16(1), 71. doi:10.1186/s12951-018-0392-8

Prasomthong, J., Limpeanchob, N., Daodee, S., Chonpathompikunlert, P., & Tunsophon, S. (2022). Hibiscus sabdariffa extract improves hepatic steatosis, partially through IRS-

1/Akt and Nrf2 signaling pathways in rats fed a high fat diet. Scientific Reports, 12(1), 7022. doi:10.1038/s41598-022-11027-9

Qin, W., Ketnawa, S., & Ogawa, Y. (2022). Effect of digestive enzymes and pH on variation of bioavailability of green tea during simulated in vitro gastrointestinal digestion. Food Science and Human Wellness, 11(3), 669-675. doi:https://doi.org/10.1016/j.fshw.2021.12.024

Santos, D. I., Saraiva, J. M. A., Vicente, A. A., & Moldão-Martins, M. (2019). 2 - Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In F. J. Barba, J. M. A. Saraiva, G. Cravotto, & J. M. Lorenzo (Eds.), Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds (pp. 23-54): Woodhead Publishing.

Serna, A., Marhuenda, J., Arcusa, R., Pérez-Piñero, S., Sánchez-Macarro, M., García-Muñoz, A. M., . . . López-Román, F. J. (2022). Effectiveness of a polyphenolic extract (Lippia citriodora and Hibiscus sabdariffa) on appetite regulation in overweight and obese grade I population: an 8-week randomized, double-blind, cross-over, placebo-controlled trial. European Journal of Nutrition, 61(2), 825-841. doi:10.1007/s00394-021-02678-x

Silva, M., Cueva, C., Alba, C., Rodriguez, J. M., de Pascual-Teresa, S., Jones, J., . . . Bartolomé, B. (2022). Gut microbiome-modulating properties of a polyphenol-enriched dietary supplement comprised of hibiscus and lemon verbena extracts. Monitoring of phenolic metabolites. Journal of Functional Foods, 91, 105016. doi:https://doi.org/10.1016/j.jff.2022.105016

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. Future Foods, 1-2, 100002. doi:https://doi.org/10.1016/j.fufo.2020.100002

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from Hibiscus sabdariffa. Future Foods, 4, 100087. doi:https://doi.org/10.1016/j.fufo.2021.100087

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2022). Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of Hibiscus sabdariffa. Food Bioscience, 50, 101992. doi:https://doi.org/10.1016/j.fbio.2022.101992

Singh, M., Thrimawithana, T., Shukla, R., Brennan, C. S., & Adhikari, B. (2023). Impact of phenolic extracts and potassium hydroxycitrate of Hibiscus sabdariffa on adipogenesis: a cellular study. International Journal of Food Science & Technology, 58(3), 1204-1218. doi:https://doi.org/10.1111/ijfs.16269

Srivastava, N., Singh, A., Kumari, P., Nishad, J. H., Gautam, V. S., Yadav, M., . . . Kharwar, R. N. (2021). Chapter 21 - Advances in extraction technologies: isolation and purification of bioactive compounds from biological materials. In R. p. Sinha & D.-P. Häder (Eds.), Natural Bioactive Compounds (pp. 409-433): Academic Press.

Sun, H.-N., Mu, T.-H., & Xi, L.-S. (2017). Effect of pH, heat, and light treatments on the antioxidant activity of sweet potato leaf polyphenols. International Journal of Food Properties, 20(2), 318-332. doi:10.1080/10942912.2016.1160410

Szczęsna, W., Tsirigotis-Maniecka, M., Szyk-Warszyńska, L., Balicki, S., Warszyński, P., & Wilk, K. A. (2021). Insight into multilayered alginate/chitosan microparticles for oral administration of large cranberry fruit extract. European Polymer Journal, 160, 110776. doi:https://doi.org/10.1016/j.eurpolymj.2021.110776

Tiwari, A., & Balasundaram, P. (2022). Public health considerations regarding obesity. In StatPearls [Internet]: StatPearls Publishing.

Tomar, M., Rao, R. P., Dorairaj, P., Koshta, A., Suresh, S., Rafiq, M., . . . Venkatesh, K. V. (2019). A clinical and computational study on anti-obesity effects of hydroxycitric acid. RSC Adv, 9(32), 18578-18588. doi:10.1039/c9ra01345h

World Health Organization. (2021). Obesity and overweight. Retrieved from https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight

World Obesity Federation. (2023). World Obesity Atlas 2023. Retrieved from https://data.worldobesity.org/publications/?cat=19

Yan, B., Chen, Z. S., Hu, Y., & Yong, Q. (2021). Insight in the Recent Application of Polyphenols From Biomass. Frontiers in Bioengineering and Biotechnology, 9. doi:10.3389/fbioe.2021.753898

CHAPTER 2

Managing obesity through natural polyphenols: A review

Future Foods 1–2 (2020) 100002

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo

Managing obesity through natural polyphenols: A review

Manisha Singh [a], Thilini Thrimawithana [b], Ravi Shukla [a,c], Benu Adhikari [a,*]

[a] School of Science, RMIT University, Melbourne, VIC 3083, Australia
[b] Discipline of Pharmacy, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC 3083, Australia
[c] NanoBiotechnology Research Laboratory (NBRL), School of Science, RMIT University, Melbourne, VIC 3001, Australia

ARTICLE INFO

Keywords:
Obesity, Fat metabolism
Natural polyphenols
Mechanism of action
Polyphenol-gut microbe interaction

ABSTRACT

Obesity is a pervasive global health problem needing urgent research and regulatory attention. Although synthetic anti-obesity drugs are available, they come with considerable risk of adverse effects and variable outcomes. Thus, there is a growing trend globally in using natural compounds for management of obesity. There is a considerable body of knowledge, supported by rigorous experimental data, that natural polyphenols can be effective and safer alternative for managing obesity through food and food fortification. This review documents the status of research on anti-obesity mechanisms and critically evaluates the effectiveness of natural polyphenolic compounds that are currently used for management of obesity. It summarizes different mechanisms of anti-obesity action associated with the enzymes, energy expenditure, appetite suppression, adipocyte differentiation, lipid metabolism and gut microbiota by various natural polyphenols. It also critically evaluates the *in vitro*, animal and clinical experimental data that support the anti-obesity potential of natural polyphenols. Most of the published data indicate that natural phenolic compounds can be effectively utilized as food or fortified foods, to manage obesity. However, there is still a need for systematic and targeted clinical studies before these natural compounds can be used as the mainstream therapy for managing obesity.

1. Introduction

In recent years, the increase in incidence of overweight and obesity among all age groups has become a serious public health problem. In 2016, over 1.9 billion adults worldwide were categorized as overweight and more than 34% of this population were obese (World Health Organization, 2020). Obesity is considered as a multifactorial complex disease caused due to behavioural, genetic and environmental factors (Rodriguez-Perez et al., 2017). It is caused by an increased calorie consumption and/or reduced calorie expenditure due to sedentary lifestyles or genetic susceptibility (Romo Vaquero et al., 2012).

Obesity can be controlled by the consumption of low calorie diet and increased level of physical exercise; however, these approaches do not provide rapid results therefore many people choose medications for effective and rapid results (Urbatzka et al., 2018). Currently, pharmacotherapeutic options available for the management of obesity are orlistat (Xenical™), lorcaserin (Belviq®), phentermine/topiramate (Qsymia™), naltrexone/bupropion (Contrave™) and liraglutide (Saxenda® and Victoza®). These medicines are approved by US Food and Drug Administration (FDA) and Korean Ministry of Food and Drug Safety (Kim, 2019; Srivastava & Apovian, 2018). European Medicines Agency (EMA) and Therapeutic Goods Administration have only approved orlistat, naltrexone/bupropion and liraglutide (Haslam, 2016). Orlistat acts as a lipase inhibitor whereas remaining four have anorexigenic effect (Jackson et al., 2015). However, few cases of kidney and liver failures have been reported with the use of orlistat for weight reduction (Filippatos et al., 2008). Similarly, use of phentermine can result in high blood pressure, high pulse rate and palpitation whereas topiramate can cause memory impairment and paresthesia (Kang & Park, 2012). In addition, these medications are not suitable for all ages and people with certain co-morbidities.

Bariatric surgery is another approach for treating severe obesity through surgery of gastrointestinal tract (Smith et al., 2011). However, the cost of surgery and after-surgery care is high (Shah et al., 2016). Moreover, it is also linked with risk of acute kidney injury (Thakar et al., 2007), increased incidence of marginal ulcers, bowel obstructions, gall stones and nutritional deficiencies (Ma and Madura, 2015). Hence, there are continuous efforts being made to discover and evaluate natural compounds with minimal side effects for weight management.

A wide range of natural dietary phytochemicals including polyphenols, terpenoids, organosulfurs and phytosterols have shown potential as anti-obesity agents (Gonzalez-Castejon and Rodriguez-Casado, 2011). These phytochemicals can modulate inflammatory, oxidative, and cell proliferative processes that are responsible for initiation of several metabolic disorders including obesity (Kim et al., 2013). Recent *in vitro* and *in vivo* studies have reported that several bioactive compounds,

* Corresponding author.
E-mail address: benu.adhikari@rmit.edu.au (B. Adhikari).

https://doi.org/10.1016/j.fufo.2020.100002
Received 6 June 2020; Received in revised form 22 August 2020; Accepted 15 September 2020

polyphenols in particular, extracted from plants are effective in preventing diet-induced obesity (Yun, 2010). More than 8000 polyphenolic compounds with different structure and function are identified in plant kingdom (Dai and Mumper, 2010). Of these phenolic compounds catechin, chlorogenic acid, coumaric acid, gallic acid, quercetin, resveratrol, rutin etc. have shown to possess anti-obesity effects through *in vitro* and *in vivo* studies (Hsu and Yen, 2008). Dietary polyphenols have been linked with suppression of food intake, reduced lipogenesis, elevated lipolysis, prevented fatty acid oxidation and inhibited adipogenesis and apoptosis. These pharmacological effects, specifically, lipid metabolism and adipogenesis are the most important contributors in the management of obesity (Wang et al., 2014). It is believed that polyphenols possess subtle effects through multiple mechanisms and biochemical targets collectively leading to substantial health benefits (Li et al., 2018). This indicates that polyphenols are potential compounds for treating obesity and they can be fortified into suitable food products for potential anti-obesity effects.

There is an increasing research focus in finding polyphenolic compounds with high efficacy and low side-effects for the management of obesity. In this context, this review documents and critically evaluates the anti-obesity potential of naturally occurring polyphenolic compounds with a special focus on their mechanisms of action in regulating obesity. This paper initially presents distribution of polyphenolic compounds with proven anti-obesity effects followed by description and critical evaluation of possible mechanisms with which they impart anti-obesity functions. The evidence of anti-obesity functions of polyphenols is drawn from published studies that have used both *in vitro* and *in vivo* experimental methods.

2. Bio-distribution of natural polyphenols in food

Polyphenols, also known as phenolic compounds, comprise of one or more aromatic rings bearing at least a hydroxyl substituents and may possess other functional groups like esters, glycosides and methyl ethers (Bhuyan and Basu, 2017). Depending on quantity of aromatic rings and structural moiety attached to them, polyphenols can be divided into different categories (Pandey and Rizvi, 2009). The primary phenolic compounds from plants include phenolic acids, flavonoids, tannins, stillbenes, coumarins, lignans and lignins (Naczk and Shahidi, 2006). Table 1 summarizes the major categories, subcategories, and structures of polyphenolic compounds along with their three main sources. Most of them are synthesized through shikimate pathway as secondary metabolites in plants and are part of plants' defence mechanism (Tsao, 2010). In plants, polyphenols contribute to colour, flavour, oxidative stability, antioxidative properties and those linked with cell wall provide mechanical strength to it (Naczk and Shahidi, 2006).

Even though the lack or inadequate intake of polyphenol by human is not linked with disorders or diseases; however, there are sufficient evidences that adequate consumption of polyphenols brings about many health benefits (Fraga et al., 2019). Consumption of polyphenols provide important physiological benefits such as antibacterial, anti-proliferative, antioxidant, antiviral etc. and beneficial effects against cardiovascular disease, diabetes mellitus, neurodegenerative disease, osteoporosis, and obesity (Baboota et al., 2013). Most of these beneficial properties of polyphenols are believed to be contributed by their free radical scavenging ability forming stable complexes and blocking further chemical reactions (Sroka and Cisowski, 2003). Likewise, they can also prevent the production of hydrogen peroxide or scavenge them and provide protection against oxidative stress that can enhance the immune responses.

3. Mechanism of anti-obesity action of polyphenols

Several *in vitro*, animal and human trials have been performed to evaluate the anti-obesity potential of dietary polyphenols. The outcomes of these studies have indicated that they impart anti-obesity function through various mechanisms (Cory et al., 2018). The mechanisms through which polyphenols impart anti-obesity functions, either individually or in certain combination, include inhibition of enzymes, stimulation of energy expenditure, appetite suppression, inhibition of adipocyte differentiation, regulation of lipid metabolism and modulation of gut microbiota (Liu et al., 2020; Rodriguez-Perez et al., 2017). Fig. 1 collectively presents the main mechanisms through which natural polyphenols exert anti-obesity functions. These mechanisms are explained in the ensuring sections.

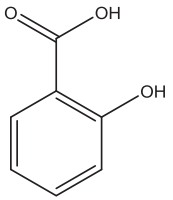
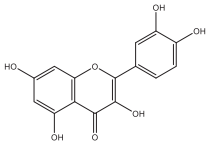
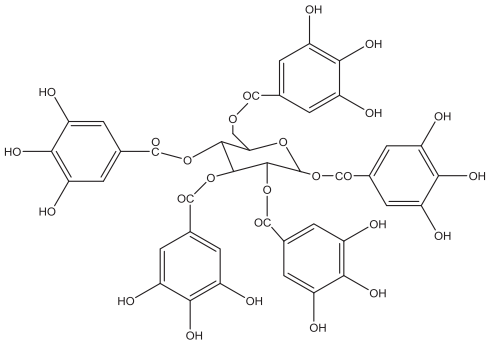
3.1. Inhibition of digestive enzymes

Inhibition of enzymes is considered a key mechanism by which these compounds exert their effects. Polyphenols are known to hinder the activity of key digestive enzymes (amylase, glucosidase and lipase) that in-turn reduce digestion of carbohydrates and fats lowering the energy intake. Amylase and glucosidase are the key enzymes for hydrolysis of starch (carbohydrates). Amylase, secreted by pancreas and salivary glands, acts on α-1, 4 glycosidic bonds present in starch producing maltose and oligosaccharides. Oligosaccharides are then hydrolysed by glucosidase to produce glucose in the small intestine for the transportation into the blood through glucose transporter (Nyambe-Silavwe et al., 2015). Excess glucose leads to higher blood glucose concentration that stimulates pancreas to secret insulin in order to utilize the glucose from blood. At this stage, three different glucose utilizing pathways are initiated: glycolysis (breakdown of glucose), glycogenesis in the liver and muscles (conversion of glucose to glycogen), and de novo lipogenesis in liver and adipose tissue (Wong et al., 2016). De novo lipogenesis is termed for synthesis of fatty acids from carbohydrates which are then esterified into triglyceride for storage in adipose tissue. Most of the triglycerides synthesized in liver are transported to adipose tissue as very low density lipoprotein which is then resynthesized as triglycerides in adipose tissue (Duwaerts and Maher, 2019).

Lipase are digestive enzymes of fats, triglycerides and phospholipids. In human, there are two types of lipases; the pre-duodenal which includes lingual and gastric; and the extra-duodenal comprising of pancreatic, hepatic, lipoprotein and endothelial lipases (Tucci et al., 2010). Pancreatic lipase is considered as the principal lipolytic enzyme hydrolysing 50–70% of total dietary fats. Lingual lipase is responsible for digesting only a small portion of ingested fat. Gastric lipase, which is produced in response to food intake, mechanical or sympathetic stimulation, hydrolyses 10–30% of total dietary fat. These lipases hydrolyse dietary fats into monoglycerides and fatty acids that form mixed micellar structures with cholesterol, bile salts and lysophosphatidic acid which then pass into the enterocytes. Subsequently, triglycerides are resynthesized within enterocyte and stored in adipose tissues as the source of energy. Therefore, surplus carbohydrate and fats, unused by the energy metabolism of the body is converted to triglycerides and fatty acids and are deposited in adipose tissue leading to obesity as demonstrated in Fig. 2 (Birari & Bhutani, 2007). Therefore, inhibition of these digestive enzymes stands out as one of the effective mechanism for the treatment of obesity (Fig. 2).

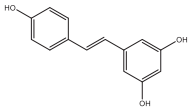
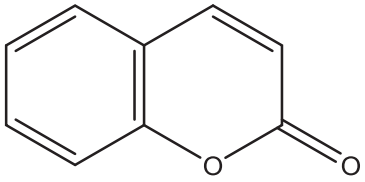
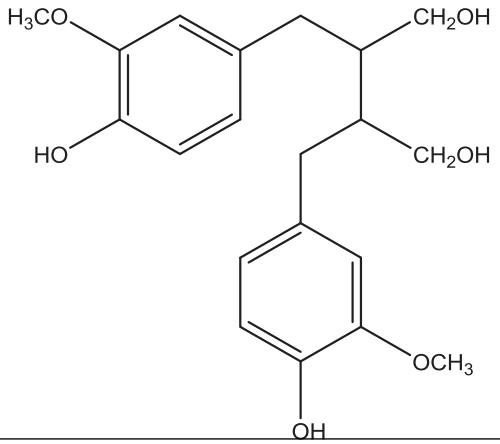
The digestion of fat by lipase is greatly affected by the surface characteristics of lipid micelles. Most lipase inhibitors preferentially adsorb onto (or penetrate) the lipid surface and make the surface unavailable to the lipase (Bitou et al., 1999). Lipase inhibition can also be due to the change in physicochemical properties of oil-water interface causing desorption of lipase from the triglyceride substrate (Gargouri et al., 1984). Sometimes lipases can be activated or denatured by adsorption (or penetration) on to the lipid micellar surface (Bitou et al., 1999). The inhibition of gastrointestinal lipase by natural polyphenolic compounds can be reversible or irreversible. Irreversible inhibition occurs when inhibitors interact with lipase to form stable intermediate and inactivates it (Yun, 2010). In the case of reversible inhibition, inhibitors weakly bind with the lipase and they may also interact with the lipid substrate (Tsujita et al., 2007). For instance, flavonoids such as isoginkgetin, bilobetin and ginkgetin from *Ginkgo biloba* can easily dock into the catalytic

Table 1
Main categories, structure and sources of natural phenolic compounds

Major categories	Structure	Subcategories	Compounds	Top three sources (mg/ 100 g)	References
Phenolic acid		Hydroxy-benzoic acids	Ellagic acid, Gallic acid, p-hydroxybenzoic acid, Vanillic acid, Protocatechuic acids, Syringic acid	Black berry (8-27), Raspberry (6-10) and Black currant (4-13)	Luna-Guevara et al. (2019); Manach et al. (2004)
		Hydroxycinnamic acid	Chlorogenic acid. Ferulic acid	Blueberry (200-220), Kiwi (60-100) and Cherry (18-115)	
Flavonoids		Anthocyanidins	Delphinidin, Pelargonidinm Cyanidin, Malvidin	Aubergine (750), Black currant (130-400) and Blackberry (100-400)	Li et al. (2018); Manach et al. (2004)
		Flavanols	Catechin, Epicatechin. Epigallocatechin, Epigallocatechin gallate, Procyanidins	Chocolate (46-61), Beans (35-55) and Apricot (10-25)	
		Flavanones	Hesperidin, Naringenin	Orange juice (22-69), Grape fruit juice (10-65) and Lemon juice (5-30)	
		Flavones	Apigenin, Chrysin, Luteolin	Parsley (24-185), Celery (2-14) and Capsicum pepper (0.5-1)	
		Flavonols	Quercetin, Kaempferol, Myricetin, Isorhamnetin, Galangin	Yellow onions (35-120), Curly kale (30-60) and Leek (3-23)	
		Isoflavonoids	Genistein, Daidzein	Soy flour (80-180), Miso (25-90) and Tofu (8-70)	
Tannins		Condensed tannins	Proanthocyanidins	Cacao beans (6100-8100), Grape seeds(2180-6050), Sorghum (413-5333)	Luna-Guevara et al. (2019); Smeriglio et al. (2017)
		Hydrolyzable tannins	Gallotannins, Ellagitannins	Raspberries (160-326), Black berries (150-270), Pomegranate (58-177)	

(*continued on next page*)

Table 1 (*continued*)

Major categories	Structure	Subcategories	Compounds	Top three sources (mg/ 100 g)	References
Stillbenes			Resveratrol, Pterostillbene, Piceatannol	Southeast China grape (*Vitis chunganensis)* (113), Cowberry (3) and Cranberry (1.9)	Błaszczyk et al. (2018); Li et al. (2018)
Coumarins		Simple coumarins Furocoumarins Pyracoumarins	Hydroxycoumarin, Esculetin, Esculin, Scopoletin, Scopolin, Umbelliferone Bergamottin, Psoralen, Xanthotoxin, Bergapten, Isopimpinellin, Marmesin, Scopoletin, Deltoin Decursinol, Khellactone, Praeruptorin B, Acetylkhellactone, Praeruptorin F, Anomalin	Jujube (*Zizyphus jujube*) (300), Corn (200), Lavender (150)	Luna-Guevara et al. (2019); Matos et al. (2015)
Lignans		Furofuran Furan Dibenzylbutane, Dibenzylbutyrolactol Dibenzylbutyrolactones Aryltetralin, Arylnaphthalene Dibenzocyclooctadiene		Flaxseed (301), Sesame seed (39.3) and Curly kale (2.3)	Luna-Guevara et al. (2019); Milder et al. (2005)

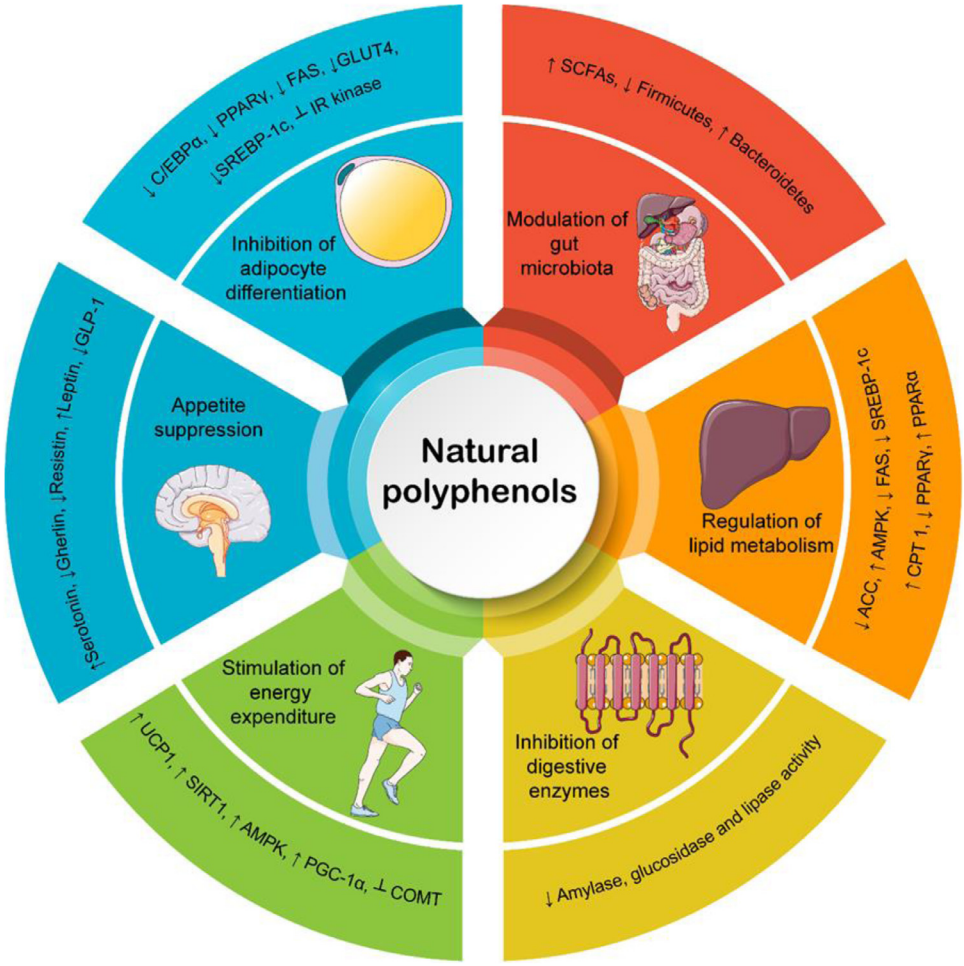


Fig. 1. Different mechanisms and their associated changes in anti-obesity function of natural polyphenols. ACC, acetyl coenzyme A carboxylase; AMPK, adenosine monophosphate -activated protein kinase; COMT, catechol-*O*-methyltransferase (COMT) Carnitine palmitoyl transferase 1 (CPT-1); C/EBPα, CCAAT/enhancer binding protein; FA, fatty acid; FAS, fatty acid synthase; GLP-1, glucagon-like peptide 1; GLUT4, glucose transporter; IR kinase, insulin receptor kinase; PGC-1α; proliferator-activated-receptor-gamma-coactivator1-α; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; SCFAs, short chain fatty acids; SIRT 1, sirtuin 1; SREBP-1c, sterol-regulatory-element-binding-protein-1c; TG, Triglycerides; UCP1, uncoupling protein 1. Note: ↑ increase, ↓ decrease, ⊥ inhibit.

cavity of pancreatic lipase leading to the formation hydrogen bonds with amino acids in the catalytic triad of pancreatic lipase (Liu et al., 2018). They exhibited the inhibition activity of pancreatic lipase with the IC_{50} value ranging from 1.64 to 3.81 μg/ mL. The formation of hydrogen bonds by the O-atom at C-3 site and the phenolic group at C-5 site of polyphenols plays important role in inhibiting lipase activity (Liu et al., 2018).

You et al. (2011) extracted polyphenols from the fruit and skin of whole muscadine grape (*Vitis rotundifloia*) and revealed pancreatic lipase inhibition of methanolic extract with IC_{50} values of 16.90 and 11.15 mg/mL, respectively. Cyanidin and cyanidin-3, 5-diglucoside are the two major lipase inhibitors in grape fruit extract (You et al., 2011). Polyphenolic extracts rich in ellagitannins and proanthocyanidin from a variety of berries including cloudberry, raspberry and strawberry were also found to alter the *in vitro* pancreatic lipase activity quite effectively. Polyphenols from cloudberry presented inhibitory activity against pancreatic lipase at an EC_{50} concentration of 5 μg/ mL (McDougall et al., 2009). The porcine pancreatic lipase was inhibited by walnut polyphenolic extract with IC_{50} value of 163 μg/ mL and it also significantly reduced the body weight gain by 13.52% after 8 weeks of treatment in obese mice (Shi et al., 2014). Similarly, a flavonol glycoside extracted from a wild *Alpinia galangal* called galangin exhibited pancreatic lipase inhibition with IC_{50} value of 48.20 mg/ mL. Ingestion of galangin at the dosage of 50 mg/ kg orally for 6 weeks resulted in approximately 40% reduction in body weight in female rats when compared to the control (Kumar & Alagawadi, 2013). Rahim et al. (2015) reported that gallic acid, epigallocatechin and epigallocatechin gallate possess high pancreatic lipase inhibitory effect with IC_{50} value of 387.2, 237.3 and 391.2 μM, respectively.

Polyphenol rich extract from *Terminalia paniculata* comprising of gallic acid, ellagic acid and quercetin showed significant inhibition of lipase and α-amylase (78% and 81%, respectively) at the concentration of 250 μg/ ml (Ganjayi et al., 2017). Liu et al. (2013) also found that flavonoids from *Nelumbo nucifera* leaves were capable of inhibiting α-amylase, α-glucosidase and porcine pancreatic lipase at the IC_{50} concentration of 0.38, 2.20 and 1.86 mg/ mL, respectively which is comparable with that of acarbose and orlistat, which are commercial enzyme inhibitors. A novel flavanone extracted from YingDe black tea was shown to inhibit α-glucosidase at IC_{50} value of 10.2 μM, almost similar to that of acarbose (11.8 μM) (Zhou et al., 2017). Since, polyphenols from various natural sources have shown inhibiting activity against amylase, glucosidase and lipase with comparatively lower IC_{50} values, they can be considered as a potential alternative of synthetic drugs for treating obesity. However, most of the studies were performed with porcine pancreatic lipase and the result may not replicate with human pancreatic lipase. Therefore, systematic research using human enzymes are expected in the future.

3.2. Appetite suppression

Regulation of obesity by controlling appetite involves neurological and hormonal processes. Hormones such as dopamine, serotonin and histamine are linked with the regulation of appetite (Chantre & Lairon, 2002). Hypothalamus plays a central role in regulating appetite by communicating with brainstem and other brain rewarding system. It senses the peripheral metabolic signals and controls the food intake by regulating the feeding behaviour (Roh et al., 2016). Similarly, adipose tissue also produces bioactive adipokines like as

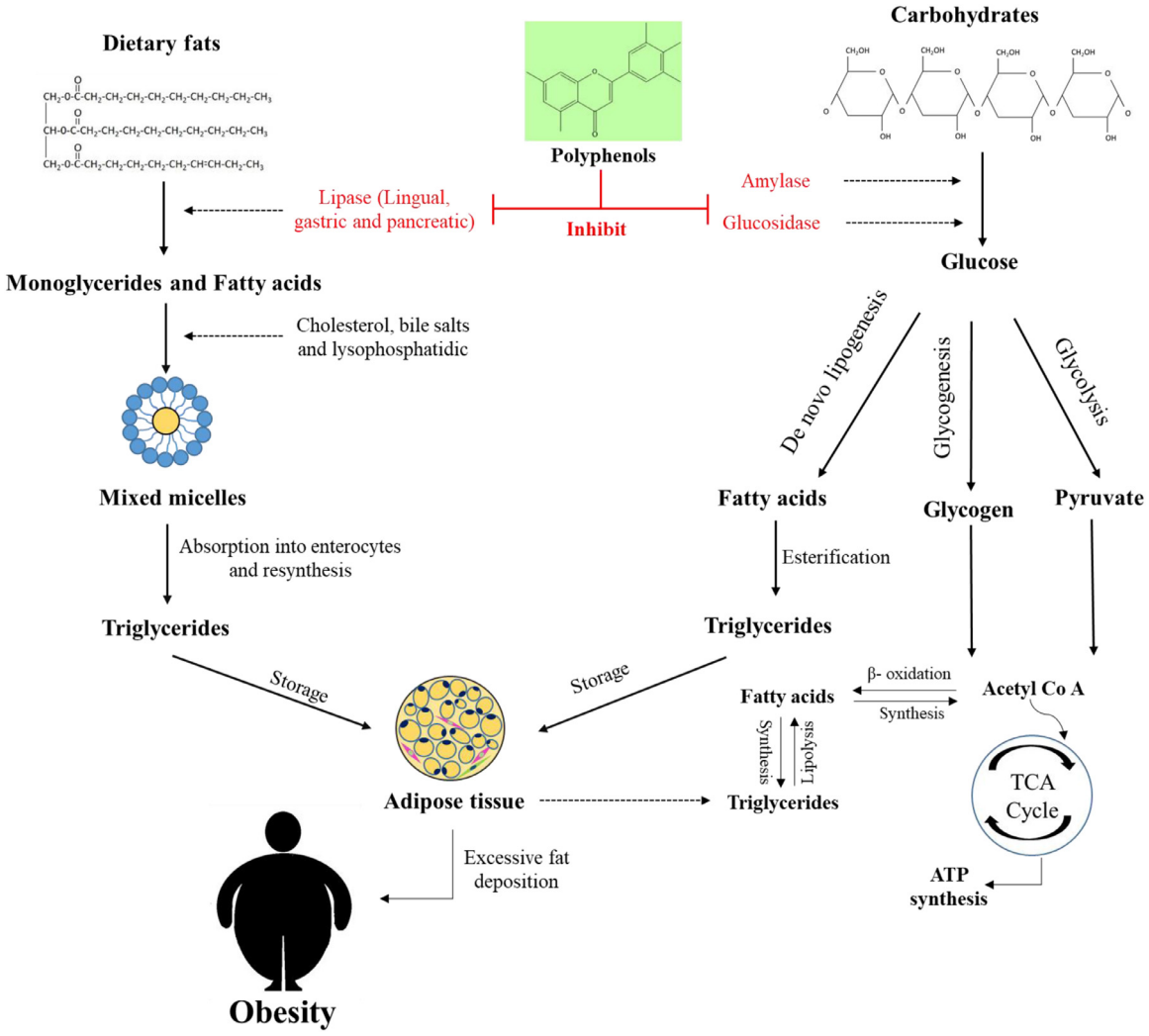


Fig. 2. Metabolism of carbohydrates and fats in human body and the role of digestive enzymes. ATP, adenosine triphosphate; TCA, tricarboxylic acid cycle.

adiponectin, leptin and apelin which regulate energy balance and glucose homeostasis (Kalupahana et al., 2012). Leptin is a cytokine-like hormone that is secreted by white adipose tissue depending on the mass of body fat (Friedman and Halaas, 1998). It regulates "brain gut axis" by activating the central nervous system (CNS) receptors in hypothalamus. Activation of these receptors suppress intake of food and stimulates energy expenditure pathways (Singla et al., 2010). Insulin, a pancreatic secretion, also provides signal to the CNS to regulate energy homeostasis and induce long-term inhibitory effects on food intake. Both the insulin and leptin signals communicate with each other to suppress the energy intake. In addition, numerous neuropeptides and neurotransmitters in the hypothalamus help to regulate homeostatic control of energy balance through energy intake and utilizing pathways (Ashrafi, 2007). Adiponectin is an adipokines that improves the hepatic insulin activities, increases glucose uptake in the liver and skeletal muscles, reduces flow of fatty acids into the liver and enhances fatty acid oxidation (Singla et al., 2010). Moreover, stimulation of adiponectin also enhances the activities of adenosine monophosphate -activated protein kinase (AMPK) that boosts fatty acid oxidation by inhibiting the activity of acetyl coenzyme A carboxylase (ACC) and dropping the malonyl-CoA content (Yamauchi et al., 2002).

Various gut hormones such as cholecystokinin, glucagon-like peptide 1 (GLP-1), oxyntomodulin, pancreatic polypeptide and peptide tyrosine-tyrosine are responsible for transferring the satiety signals to the brain. The gut hormone ghrelin is a physiological hunger hormone which effectively transmits hunger signals and stimulates the food intake (Yu and Kim, 2012). An appetite suppressing compound must be able to act on the hunger control centres of the brain and provide the feeling of fullness, ultimately reducing the intake of food. The alternative mechanism of appetite suppression can be melanin-concentration hormone (MCH) receptor antagonism (Bays, 2004). MCH is a endogenous appetite stimulating hormone and a reduction in MCH or its receptors is linked with decrease in body weigh in mice (Yu and Kim, 2012).

Plant-based phenolic compounds suppress appetite either by slowing down secretion of appetite-stimulating hormones, modulating MCH receptors or by inactivation of appetite sensors (Geoffroy et al., 2011). Extracts rich in flavonoids from various plants such as tea plant (*Camellia sinensis*) (Moon et al., 2007), bitter orange (*Citrus aurantium*) (Klontz et al., 2006) and Indian cactus (*Caralluma fimbriata*) (Kuriyan et al., 2007) are shown to have appetite-suppressing characteristics. The extract of Bushman's hat (*Hoodia gordonii)* increased the adenosine triphosphate (ATP) content in hypothalamic neurons in rat that may trigger the appetite response and positively aid in regulation of food intake (MacLean & Luo, 2004).

Supplementation of an isocaloric diet containing polyphenol rich extract from *Lippia citriodora* and *Hibiscus sabdariffa* to obese individuals significantly reduced body weight up to 3.48 kg as compared to placebo group and reduced the hunger and appetite after two months of intervention (Boix-Castejon et al., 2018). The authors attributed this positive effect to reduction of resistin circulation which is linked with insulin re-

sistance, normalization of leptin expression and regulation of ghrelin and GLP-1 levels. These findings provide strong evidence that polyphenols can regulate food intake by modulating adipohormones, expression of gut peptide along with the modulation of AMPK in different tissues (Boix-Castejon et al., 2018). Chen et al. (2016) have reported the appetite suppressing effect of epigallocatechin-3-gallate (EGCG)-rich extract from green tea. The appetite suppression was achieved through the inhibition of ghrelin secretion and increasing the adiponectin levels. Intake of green tea extract (856.8 mg EGCG per day) for 12 weeks resulted in significant decrease in body weight, waist circumference, total cholesterol and low-density lipoprotein levels in overweight and obese women. Phenolic compound-rich aqueous extract of medicinal plant *Carum carvi L.* significantly reduced the appetite levels and an 8.7% decline in carbohydrate intake in obese women over 90 days of intervention as compared to the placebo group. In addition, it also significantly decreased the anthropometric indices (waist circumference, waist to hip ratio, mid-upper arm circumference and thigh circumference) in the experimental group (Kazemipoor et al., 2016). Anthocyanins from *Glycine max* (L.) Merr. seed coat significantly reduced body weight gain by 15.16% and food intake by 19.10% in Male Sprague–Dawley rats when administered through intra-gastric route for 40 days. The key mechanism for reduction of food intake was highlighted as through the regulation of neuropeptide Y and γ–amino butyric acid receptor in hypothalamus (Badshah et al., 2013).

The appetite suppressing effect of polyphenol rich extracts in human has been supported by clinical trials as mentioned above. Therefore, these natural polyphenols can be explored as effective anti-obesity ingredients for the management of obesity.

3.3. Stimulation of energy expenditure

Basal metabolic rate, thermic effect of food and physical activity thermogenesis are the three categories that contribute to total daily energy expenditure (Balaji et al., 2016). Thermogenesis provides heat energy to maintain body temperature. White adipose tissue (WAT) and brown adipose tissue (BAT) are the two main adipose tissues in mammals. BAT, a unique mammalian fat tissue, plays a vital role in regulating adiposity by releasing excess energy through non-shivering thermogenesis. Thermogenesis in BAT is regulated by uncoupling protein 1 (UCP1) which reduces proton gradient and uncouples oxidation from ATP synthesis (Cannon & Nedergaard, 2004). BAT is activated by sympathetic pathways through β3-adrenoreceptors in response to diet and other factors (Wood Dos Santos et al., 2018). UCP3 which is homologous to UCP1 also facilitates thermogenesis through the regulation of leptin, thyroid hormones and β3-adrenergic agonists and potentially exerts the anti-obesity action (Gong et al., 1997). Therefore, regulation of energy expenditure through the activation of BAT by modulation of UCP is physiologically sound strategy for the control of obesity (Lowell and Spiegelman, 2000).

Polyphenolic compounds can act as effective thermogenic regulators and enhancers of energy expenditure. Phenolic compounds such as curcumin, quercetin, resveratrol, isoflavones, gallic acid and flavonol stimulate thermogenesis by interfering with AMPK, sirtuin 1 (SIRT1), proliferator-activated-receptor-gamma-coactivator1-α (PGC-1α), catechol-*O*-methyltransferase (COMT) and sympathetic nervous system (SNS) which play important role in transcriptional regulation and physiology of adipose tissue (Mele et al., 2017). The (-) –epigallocatechin-3-gallate (EGCG), a major bioactive polyphenol from green tea, has shown to enhance the thermogenesis and energy expenditure (Zhou et al., 2018). The supplementation of 1% EGCG in high-fat diet based on lard in mice for 4 weeks increased the expression of genes UCP1 and PGC-1α in BAT and resulted into increased thermogenesis (Zhou et al., 2018). The polyphenolic extract of mango (gallotannin derivatives) suppressed the adipogenesis and increased thermogenesis in 3T3-L1 adipocytes as evidenced from the increased expression of thermogenic markers (UCP1, SIRT1 and AMPK) (Fang et al., 2018). It is shown that resveratrol significantly slowed down the gain in body mass in non-human primate (grey mouse lemur) by increasing satiety (reducing energy intake by 13%) and increasing energy expenditure (increase of resting metabolic rate by 29%) (Dal-Pan et al., 2010). *In vivo* studies in rhesus monkey on supplementation of resveratrol with diet rich in fat and sugar further corroborated that resveratrol can reduce the size of adipocytes and enhance the expression of SIRT1 in visceral white adipose tissues (Jimenez-Gomez et al., 2013). Cyanidin and malvidin from fermented black carrot extract caused rise in energy expenditure in ovariectomized female rats when fed with high fat diet containing 2% fermented black carrot for 12 weeks (Park et al., 2015).

3.4. Inhibition of adipocyte differentiation

Adipocytes, which are main cellular constituents of adipose tissue, play a vital role in energy balance and lipid homeostasis. While excess energy derived from food is stored as droplets of triglycerides in WAT, the BAT utilizes stored energy as required by thermogenesis (Tong and Hotamisligil, 2001). Adipocytes are derived from mesenchymal precursor cells through the process of transformation of undifferentiated mesenchymal cell into preadipocyte and then to mature adipocytes. Excessive adipogenesis and adipocyte differentiation causes accumulation of large number of adipocytes leading to obesity (Ali et al., 2013). There are several stages in adipocyte differentiation and any of these stages can be successfully interfered by number of transcriptional factors to manage obesity (Lefterova and Lazar, 2009). Some of the important transcription factors involved in adipocyte differentiation are members of proliferator-activated receptor (PPAR), CCAAT/enhancer binding protein (C/EBP) and sterol regulatory element binding proteins (SREBP) (Brey et al., 2009). Adipose tissue derived fatty acid synthase (FAS) can play a significant role in adipocyte differentiation, therefore inhibition of FAS can suppress the adipogenesis and ultimately lead to reduction in adipose tissue (Schmid et al., 2005).

Curcumin, resveratrol, epigallocatechin-3-gallate and genistein are able to interfere or inhibit one or more stages of adipogenesis (Mohamed et al., 2014). These phytochemicals can alter the life cycle of adipocytes through the suppression of preadipocyte proliferation and mitogenesis, inhibition of adipocyte differentiation and adipogenesis and induction of mature adipocyte apoptosis. These physiological mechanisms can be tailored to develop promising approaches to manage and control obesity (Wang et al., 2014). It is shown that hydroxylated poly-methoxyflavones from citrus peels interfered with the adipocyte-specific transcriptional regulators and ultimately inhibited the adipocyte differentiation in 3T3-L1 adipocytes (Lai et al., 2013). *In vivo* study in male F344 rats indicated that quercetin was able to inhibit the adipogenesis by supressing the transcription of adipogenic markers in muscle progenitor cells (Funakoshi et al., 2018).

Polyphenolic extract from cocoa showed high potential in inhibiting adipogenesis in 3T3 L1 pre-adipocytes and was able to significantly reduce weight gain in obese mice fed with a high fat diet (Min et al., 2013). Polyphenols from cocoa underwent preferential binding with insulin receptor (IR) kinase and inhibited its activity. They also suppressed isobutylmethylxanthine, dexamethasone and insulin (MDI) induced phosphorylation that initiate pre-adipocyte proliferation (Min et al., 2013). It is reported that aqueous *Hibiscus sabdariffa* extracts are able to inhibit adipocyte differentiation by interfering with adipogenic transcriptional factors in 3T3-L1 preadipocytes stimulated with MDI hormonal mixture (Kim et al., 2007).

Phenolic compounds from black chokeberry extract suppressed the adipocyte differentiation by hindering major adipocyte transcription markers including peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein (C/EBPα) and sterol-regulatory-element-binding-protein-1 (SREBP1c) in 3T3-L1 preadipocytes (Kim et al., 2018). Carvacrol, a phenolic monoterpenoid, impaired adipogenic differentiation in 3T3-L1 and Whartons jelly derived mesenchymal stem cells. This inhibition was achieved through

the reduction of autophagy and expression of carbohydrate response element binding protein associated with regulation of adipose differentiation and lipogenesis (Spalletta et al., 2018). Piceatannol, a naturally occurring stilbene from wine was found to suppress adipocyte differentiation in human mesenchymal stem cells by reducing the expression of major adipogenic markers [PPARγ, fatty acid synthase (FAS) and glucose transporter type 4] (Carpene et al., 2018). Lee et al. (2016) studied on the effect of black soybean testa extract which is rich in anthocyanin (12.58 mg/ g) on the overweight or obese individuals for 8 weeks. They administered 2.5 g extract per day through two capsules each before three daily meals and discovered significant decrease in waist circumference, hip circumference, triglycerols and LDL cholesterol (Lee et al., 2016). They proposed that these changes can be through the modulation of adipogenesis and lipolysis. As many polyphenols have shown strong ability in inhibiting adipocyte differentiation, which is an important mechanism for managing the obesity, they can be used as natural anti-obesity agents. However, most of the studies have been conducted in 3T3-L1 cells and only few are reported on human cells. These studies also applied polyphenols directly to the cell cultures which can be completely different when compared to human physiology. Therefore, the results from these cell cultures should be correlated by conducting targeted human trials.

3.5. Regulation of lipid metabolism

Lipid metabolism is a multi-stage process of synthesis, storage and degradation of fatty acids, triglycerides and cholesterol where various enzymes and hormones are involved. It can be classified into cholesterol metabolism and metabolism of fatty acids and triglycerides. Cholesterol in human is either derived from diet or synthesized in the liver. The level of cholesterol in human body is regulated by physiological synthesis, transportation, absorption and excretion (Wang et al., 2018b). Acetyl-coenzyme A (Acetyl-CoA) is the precursor of cholesterol and its synthesis is regulated by SREBP1a, SREBP2, 3-hydroxy-3-methylglutaryl coenzyme A reductase and Low Density Lipoprotein (LDL) receptor (Karagianni & Talianidis, 2015). Activation of SREBP-1c is triggered by stress in endoplasmic reticulum that enhances the transcription of lipogenic enzyme genes mainly FAS and stearoyl-CoA desaturase (Salvado et al., 2015). AMPK is the principal regulator of lipid synthesis pathways and is capable of reducing the synthesis of fatty acid by interfering with SREBP-1c and fatty acid synthase. Activation of AMPK stimulates the oxidation of fatty acid in the liver and inhibits the synthesis of cholesterol (Srivastava et al., 2012). Similarly, carnitine palmitoyl transferase 1A (CPT1A) can also accelerate the oxidation of fatty acids and reduce the levels of hepatic triglyceride. PPARα can regulate the fatty acid oxidation and cholesterol breakdown in lipid metabolism (Takei et al., 2017). Therefore, modulation of these factors can affect lipid metabolism and impart desirable effects in preventing obesity (Fig 3).

Phenolic compounds can activate AMPK and PPARα that can lead to inhibition of acetyl-CoA carboxylase (ACC) and fatty acid synthase. These activities collectively lead to reduction in fatty acid synthesis and increase in fatty acid oxidation, thereby decreasing lipid accumulation and oxidative stress (Fig 3). Polyphenols from *Hibiscus sabdariffa* exhibited these anti –obesity properties by improving lipid metabolism (Herranz-Lopez et al., 2017). In a clinical trial, dietary supplement that combined polyphenol rich extract from *Hibiscus sabdariffa* and *Lippia citriodora* was supplied to obese and overweight subjects (Herranz-Lopez et al., 2019). It was shown that this extract significantly reduced the body weight, abdominal circumference and body fat. Further, the extract also significantly decreased the concentration of total cholesterol and low-density lipoprotein in both over weight and obese subjects. The supplementation of dietary polyphenols from blueberry in high fat diet significantly dropped the expression of FAS, and SREBP-1 and enhanced that of CPT 1 and PPARα in liver of mice as compared with only high fat diet fed mice (Jiao et al., 2018). Along with blueberry polyphenol, quercetin, epigallocatechin-3-gallate, resveratrol and curcumin can activate the AMPK by phosphorylation (Jiao et al., 2018). Similarly, feeding of green tea polyphenols to high fat diet fed mice resulted in significant reduction of cholesterol, triglyceride, low density lipoprotein and insulin levels when compared to those fed with fat diet alone (Wang et al., 2018a). Peng et al. (2020) also reported that the polyphenols from *Solanum nigrum* significantly decreased the body weight and body fat in high fat diet supplemented mice in 10 weeks treatment. They found that these polyphenols regulated the lipid metabolism by triggering the lipolysis through activation of PPARα and CPT-1 and suppressing the FAS to block lipogenesis. Exactly same mechanism through the activation of AMPK phosphorylation was proposed by Park et al. (2015) in ovariectomized female rats. They revealed that fermented black carrot extract rich in cyanidin and malvidin were able to significantly reduce the body weight gain by 32.83% and LDL cholesterol by 29.58% in ovariectomized female rats as compared to control (Park et al., 2015).

Istek and Gurbuz (2017) stated that blueberry anthocyanins were able to reduce the body weight by 11.34% when overweight or obese individuals were fed with 50 g of blueberries as a substitute of equal amount of carbohydrate after 1st six weeks of treatment per day for 12 week. In addition, significant reduction in body fat by 20.89%, total cholesterol by 14.75% and LDL cholesterol (18.3%) were also reported from the baseline. Administration of Melinjo (*Gnetum gnemon L.*) seed extract (rich in resveratrol) every morning for 14 days to healthy young male caused improvement in total adiponectin level, high molecular weight adiponectin and ratio of high molecular weight adiponectin to total adiponectin. All of these values were found to be dependent on the concentration of melinjo seed extract as their level was higher at the administration of 300 mg/ day as compared to 150 mg/ day (Oniki et al., 2020). They proposed that besides having insulin sensitizing effect, adiponectin can also suppress lipogenesis and increase β-oxidation which supports the anti-obesity effect. Jamar et al. (2020) supplemented 5 g of anthocyanin rich-freeze dried pulp powder of *Euterpe edulis* Mart. everyday to obese individuals with body mass index between 30 to 39.9 kg/ m^2. After 6 weeks of treatment, they observed significant reduction in body fat and improvement in HDL cholesterol and serum adiponectin.

3.6. Modulation of gut microbiota

Gut microbiota is an entire ecosystem of microorganisms residing in the gastrointestinal tract. Bacteroidetes and Firmicutes occupy 90% of the gut microbiota while the rest is occupied by actinobacteria, proteobacteria and verrucomicrobia (Kumar Singh et al., 2019). Diet plays a major role in the diversity of bacterial population in the gut. The growth and proliferation of firmicutes is favoured by high carbohydrate and fat rich diets whereas low fat diets increase bacteroidetes (Zhang and Yang, 2016). High fat diets and obesity is linked with a weak gut microbial diversity (Liu et al., 2020). Gut microbiota can reduce obesity through energy generation from prebiotics, reduction in fatty acid oxidation, lower production of bile acids, satiety effect and enhanced lipogenesis (Dahiya et al., 2017). Furthermore, modulation of gut microbiota by high fat diet causes reduction in short-chain fatty acids (SCFAs) and increase the production of lipopolysaccharide (LPS) and branched-chain amino acids (BCAAs). All of these facilitate the storage of fat which leads to obesity (Liu et al., 2020). Supplementation of polyphenols may positively interfere with the microbial diversity in gut and produce metabolites that help to control obesity.

Zhao et al. (2017) added quercetin and resveratrol in high-fat-diet fed to rats and discovered that these polyphenols modulated the composition of gut microbiota by decreasing the ratio of firmicutes-to-bacteroidetes. The authors also observed a significant decrease in body weight gain, weight of visceral adipose tissue and dimension of adipocyte after 10-weeks of treatment. An *in vivo* study was performed by Cheng et al. (2017) to observe the effect of (−)-epigallocatechin 3-O-(3-O-methyl) gallate from oolong tea on intestinal microbiota of high

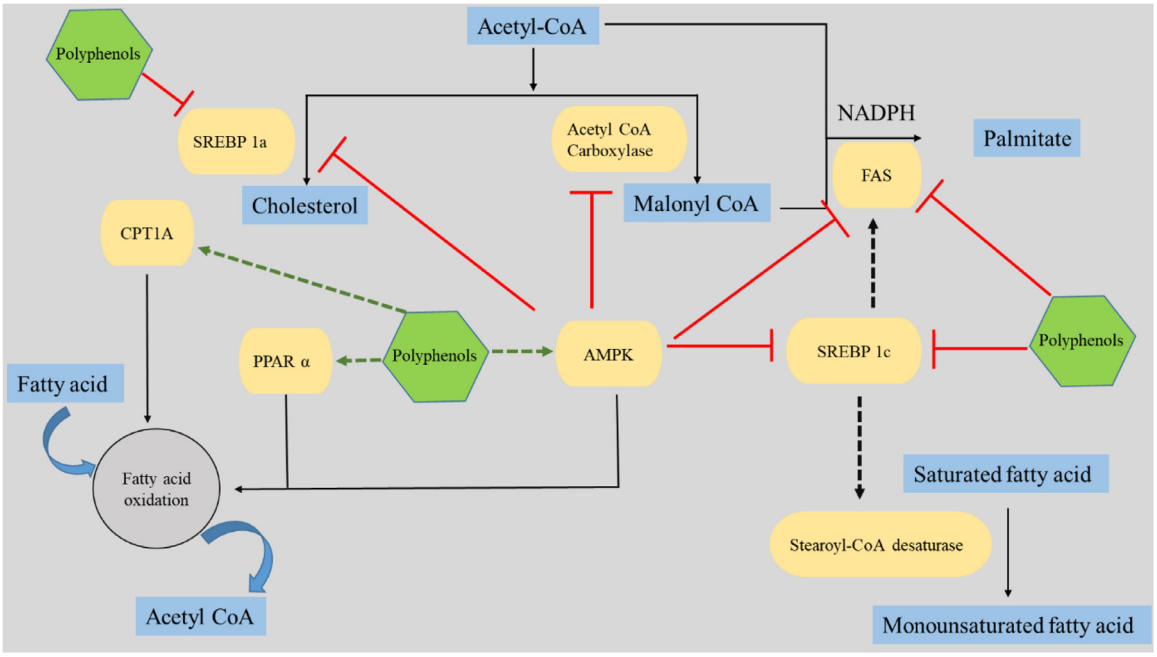


Fig. 3. Effect of natural polyphenols on the lipid metabolism. ACC, acetyl coenzyme A carboxylase; AMPK, adenosine monophosphate -activated protein kinase; Carnitine palmitoyl transferase 1 (CPT-1); FAS, fatty acid synthase; PPARα, peroxisome proliferator-activated receptor α; SREBP-1c, sterol-regulatory-element-binding-protein-1c. Note: ⇢ activates, ⊣ inhibit

fat diet fed mice. They observed significant reduction in firmicutes and increase in bacteroidetes resulting to a lower firmicutes/ bacteroidetes ratio when supplemented with (−)-epigallocatechin 3-O-(3-O-methyl) gallate as compared to only high fat diet in a 8-week trial. Moreover, there was also an increase of short chain fatty acid producing bacteria. Similarly, polyphenolic extract from blueberry also altered the gut microbiota diversity and significantly slowed down the gain of body weight in mice when the extract was supplemented in their high fat diet compared to the high fat diet alone (Jiao et al., 2018). The above mentioned studies propose that polyphenolic compounds from different plant sources are capable of modulating the gut microbiota and help control or manage the obesity.

4. Critical analysis of *in vitro, in vivo* and clinical trials of natural polyphenolic compounds used for treating obesity

The anti-obesity properties and potential of polyphenolic compounds are currently being extensively studied. The polyphenols and polyphenolic extracts from many natural sources discussed in previous section that have been tested *in vitro, in vivo* and clinically trailed to determine their anti-obesity effect and mode of action either individually or in combination were summarized in Tables 2, 3 and 4. These researches have also adequately considered required treatment dose and time to achieve desired anti-obesity effects.

The chemical structure of polyphenols, their interaction with other compounds and the dose used for treatment or intervention play vital role in their bioavailability and bioactivity (D'Archivio et al., 2007). The daily intake of polyphenols depends upon the type and composition of diet and it greatly varies among individuals. Plant foods contain higher concentration of polyphenols and the people consuming more plant based foods have higher intakes of polyphenols as compared to those who consume more of animal based (Burkholder–Cooley et al., 2016). It is reported that the average polyphenol consumption in European countries; France, Spain, Italy, Brazil and Finland is between 283–1100 mg per day whereas in Japan it is nearly 1500 mg per day (Chiva–Blanch and Badimon, 2017). Dietary polyphenol intake by elderly Japanese was reportedly 1492 mg/ day of which 79.1% came from drinking green tea and coffee (Taguchi et al., 2015). It is reported that in 2015, the incidence of obesity among adults was fairly low in Japan (3.7%) as compared to European countries (9.8–24.8%) including France, Spain, Italy, Brazil and Finland (OECD, 2017). The lower rate of obesity in Japanese population as compared to European people may be due to the higher intake of polyphenols in their diet. Flavonoids and phenolic acids are the two highly ingested classes of polyphenols and contributed to 62% and 35.5%, respectively in the total polyphenol intake of institutionalised elderly population in Spain (Gonzalez et al., 2014). Another research conducted in Polish Arm also presented that flavonoids and phenolic acids were the two major polyphenol categories which contributed to almost 97% in the daily polyphenol intake of Polish Arm (Grosso et al., 2014).

Ingestion of 10-100 mg of individual phenolic compound either alone or in foods can lead to concentration up to 1 μM of that compound, mostly as metabolites, in the human plasma (Scalbert & Williamson, 2000). For example, intake of 68 mg of quercetin equivalents from onion resulted in 0.74 μM of quercetin in plasma of which only negligible amount (less than 1.4 %) was excreted in urine (Hollman et al., 1997). Similarly, when 82 mg of epigallocatechin from green tea infusion was ingested, the plasma epigallocatechin reached to 0.67 μM of which 3.6 % was excreted from urine (Lee et al., 1995). Moreover, 19 mg of genistein from soy milk resulted in 0.74 μM of genistein in plasma with 19.8% in urine (Xu et al., 1994). It is reported that about one fourth of ingested polyphenols may not be absorbed by body and excreted in urine (Scalbert & Williamson, 2000).

The maximum concentration of polyphenol used in the *in vitro* and clinical trials as mentioned in Table 3 and 4 is 900 mg per day in humans and 200 mg/ kg body weight/ day in mice which is within the average daily intake of these polyphenols. Several *in vitro* and *in vivo* experimental trials have confirmed the significant anti-obesity effect at these concentrations as presented in Table 3 and 4. Therefore, these polyphenolic extracts derived from plants can have great potential for treating

Table 2
In vitro experiments on polyphenolic compounds depicting anti-obesity function with applicable mechanism of action

Plant source	Active component	Dose and treatment time	Anti-obesity effect	Mechanism of action	Reference
Cloudberry	Polyphenolic extract	0.5 and 1 µg/ µL extract and incubation for 2 h	Porcine pancreatic lipase inhibition with EC_{50} concentration of 5 µg phenols/ mL	Inhibition of pancreatic lipase	McDougall et al. (2009)
Skin and fruit of *Vitis rotundifloia*	Anthocyanin	100 µL of different extract and incubation for 30 min	Highest porcine pancreatic lipase inhibition by methanolic extract of fruit and skin at IC_{50} of 16.90 and 11.15 mg/mL, respectively	Inhibition of pancreatic lipase	You et al. (2011)
Alipinia galangal Willd	Galangin	100, 250, 500, 750 and 1000 mg/ mL of galangin and incubation for 30 min	Galagin presented IC_{50} value of 48.20 mg/ mL for pancreatic lipase inhibition	Inhibition of pancreatic lipase	Kumar and Alagawadi (2013)
-	Gallic acid (GA), Epigallocatechin (EGC), Epigallocatechin-3 gallate (EGCG)	10 µl of each component at different concentration and incubation for 30 min	GA, EGC, and EGCG showed significant porcine pancreatic lipase inhibition activity with IC_{50} values of 387.2, 237.3, and 391.2 μM respectively	Inhibition of pancreatic lipase	Rahim et al. (2015)
Ginko biloba	Isoginkgetin, bilobetin, ginkgetin	1, 10, 100 µM of each component and incubation for 40 min	Porcine pancreatic lipase inhibition with the IC_{50} values ranging from 2.90 μM to12.78 μM	Inhibition of pancreatic lipase	Liu et al. (2018)
Red rice bran	Aqueous and ethanolic extract	25 µl of 0, 12.5, 25, 37.5, 50 and 62.5 mg/mL extracts for 30 min	Lipase inhibition was higher in ethanolic extract (76.36%) than in aqueous extract.	Inhibition of pancreatic lipase	You et al. (2018)
Nelumbo nucifera	Flavonoids	0.1 g/ ml extract for 5 min, 20 min and 20 min for lipase, α-glucosidase and α-amylase activity	Inhibition of porcine pancreatic lipase, α-glucosidase and α-amylase with IC_{50} values of 0.38, 2.20 and 1.86 mg/ ml respectively	Inhibition of pancreatic lipase, α-glucosidase and α-amylase	Liu et al. (2013)
YingDe black tea	Flavanones	6 serial concentrations for 15 min	Inhibition of α-glucosidase with IC_{50} value of 10.2 μM	Inhibition of α-glucosidase	Zhou et al. (2017)
Citrus peel	Hydroxylated polymethoxyflavones (HPMFs)	0.25% and 1% HPMEs orally for 8 days in 3T3-L1 cells	Inhibition of lipid accumulation in adipocytes by 51–55%.	Suppression of adipogenesis by interfering with the adipocyte-specific transcriptional regulators and multiple signalling pathways	Lai et al. (2013)
Cocoa	Polyphenol extract	100 and 200 µg/mL in 3T3-L1 cells	i. Reduction in lipid accumulation by 30% and 72% in treatment with 100 and 200 µg/mL respectively ii. Significant decline in the expression levels of PPAR-γ, C/EBPa, aP2 and fatty acid synthase	i. Inhibition of adipogenesis by altering expressions of adipogenesis mediated proteins and/ or genes ii. Inhibiting the activity of insulin receptor kinase	Min et al. (2013)
-	Piceatannol	50 µM for 7 days in Human adipose mesenchymal stem cells and 3T3-L1 cells	Inhibition of 26-80% lipid storage in human mesenchymal stem cells and decrease in lipid content by 65% in 3T3-L1 adipocytes	Suppression of adipocyte differentiation by down regulation of expression of major adipogenic markers (PPARγ, FAS and glucose transporter GLUT4)	Carpene et al. (2018)

(*continued on next page*)

Table 2 (*continued*)

Plant source	Active component	Dose and treatment time	Anti-obesity effect	Mechanism of action	Reference
Mangifera indica L.	Pyrogallol (PG) Mango polyphenols (MG)	2.5, 5, 10, 20 mg/ L for 6 days in 3T3-L1 cells	i. Lipid accumulation in adipogenesis was reduced by 38.5% and 24.8% by 10 mg/L of MG and 20 mg/L of PG respectively ii. Up-regulation of gene and protein expressions of UCP1, Sirt1, LKB1 and AMPK	Suppression of adipogenesis and increase in thermogenesis	Fang et al. (2018)
-	Carvacrol	10 to 50 µM for 7 and 17 days in 3T3-L1 cells and Whartons jelly's derived mesenchymal stem cells	Reduction of adipogenic differentiation by 40% and 30% in 3T3-L1 and Whartons jelly's derived mesenchymal stem cells, respectively	Inhibition of adipocyte differentiation through the reduction of autophagy and expression of carbohydrate response element binding protein	Lai et al. (2013); Spalletta et al. (2018)

Table 3
I*n vivo* experiments on polyphenolic compounds depicting anti-obesity function with applicable mechanism of action

Plant source	Active component	Study model	Dose and treatment time	Anti-obesity effect	Mechanism of action	Reference
Alipinia galangal Willd	Galangin	Female albino Wistar rats	50 mg/ kg/ day orally for 6 weeks	Resulted in 40.02% reduction in body weight as compared to control rats	Inhibition of pancreatic lipase	Kumar and Alagawadi (2013)
Walnut	Polyphneolic extract	High-Fat Diet-Induced Obese Mice	200 µg/ g body weight orally for 8 weeks	Significantly decreased the body weight gain by 13.52%	Inhibition of pancreatic lipase	Shi et al. (2014)
-	Resveratrol	Male grey mouse lemurs	200 mg/kg/day orally for 4 weeks	Decrease in body mass gain through induced resting metabolic rate by 29% and reduced energy intake by 13%	Activation of energy expenditure and reduction in food intake	Dal-Pan et al. (2010)
Fermented black carrot	Cyanidin and Malvidin	Ovariectomized female Sprague–Dawley rats (n = 40)	High fat diet containing 2% black carrot fermented with *Aspergillus oryzae* for 12 weeks	Significant reduction in body weight gain by 32.83%, triglyceride by 24.4% and LDL by 29.58% as compared to control.	Stimulation of energy expenditure and modulation of lipid metabolism	Park et al. (2015)
Green tea	EGCG	Male C57BL/6J mice	1 % of dietary supplementation orally for 4 weeks	i. Significant decrease in body weight gain, blood glucose and triglyceride levels ii. Reduced lipid accumulation in adipose tissues	Stimulation of energy expenditure through enhanced thermogenesis	Zhou et al. (2018)
Cocoa	Polyphenol extract	Male C57BL/6N mice	40 and 200 mg/ kg body weight orally for 5 weeks	Decrease in body weight gain	Inhibition of adipogenesis	Min et al. (2013); Zhou et al. (2018)
-	Quercetin	*In vitro* muscle satellite cells from male F344 rats	0, 5, 10 and 30 µM for 6 days	i. Decrease in level of triglycerides ii. mRNA levels of PPAR-γ, FABP4, Pax7 and MyoD was reduced.	Inhibition of adipogenesis by supressing the transcription of adipogenic markers in muscle progenitor cells	Funakoshi et al. (2018)

(*continued on next page*)

Table 3 (*continued*)

Plant source	Active component	Study model	Dose and treatment time	Anti-obesity effect	Mechanism of action	Reference
Blueberry	Purified polyphenol extract	Male C57BL/6J mice	200 mg/ kg body weight/ day for 12 weeks orally with high fat diet	i. Inhibited body weight gain and reduced triglycerides, LDL and leptin ii. Significant reduction in expression of PPARγ, FAS, and SREBP-1 and rise in expression of CPT 1 and PPARα expression levels	Regulation of lipid metabolism and gut microbiota	Jiao et al. (2018)
Green tea	Polyphenol extract	C57BL/6J mice	0.05, 0.2 and 0.8% (w/w) for 8 weeks orally as a solution in water	Reduction in total cholesterol, triglyceride, low density lipoprotein, glucose (GLU) and insulin (INS) levels	Regulation of lipid metabolism	Wang et al. (2018a)
Solanum nigrum	Polyphenol extract rich in quercetin, catechin, rutin, epicatechin, epigallocatechin gallate and gallic acid	Male C57BL/6 mice	0.5, 1 and 2% supplemented orally with high fat diet for 10 weeks	Reduction in body weight and body fat	Regulation of lipid metabolism by triggering the lipolysis through activation of PPARα and CPT-1 and suppressing the FAS to block lipogenesis.	Peng et al. (2020)
Glycine max (L.) Merr. seed coat	Anthocyanins	Male Sprague–Dawley rats (n = 18)	6 mg/kg and 24 mg/kg through intra-gastric administration for 40 days	Significant decrease in body weight gain by 15.76% and daily food intake by 19.10% as compared to control	Reduction in food intake through regulation of neuropeptide Y and γ–amino butyric acid receptor in hypothalamus	Badshah et al. (2013)
-	Resveratrol (R) and quercetin (Q)	Male Wistar rats (n=32)	15 mg of R and 30 mg orally per kg body weight per day for 10 weeks	Significant decrease in body weight gain, visceral adipose tissue weight and adipocyte sizes	Modulation of gut microbiota	Zhao et al. (2017)

obesity without the fear of adverse effects as is the case in synthetic anti-obesity drugs. However, further *in vivo* studies and clinical trials involving multiple research organisations are required before they can be used as medication or supplements for weight management.

5. Challenges associated with the use of polyphenols to manage obesity

5.1. Effect of processing on polyphenols

Food materials contain many polyphenols at varying concentrations and they undergo significant changes during processing (Manach et al., 2004). Polyphenols are highly sensitive to both physical stressors (temperature and light) and chemical reactions (oxygen, pH and enzyme). Anthocyanins are unstable at alkaline environment (pH > 7.0) and undergo degradation depending on their substituent groups. The exposure of anthocyanins to high temperature during food processing has shown to degrade them into phenolic acids and phloroglucinaldehyde (Ifie & Marshall, 2018). In contrast, hydrothermal processing (e.g., extrusion cooking) was shown to release the intact phenolic acids present in cereal grains by rupturing the cell wall (Zielinski, Kozlowska, & Lewczuk, 2001). A significant increase in both free and ester-bound phenolic acids for ferulic acid, ρ-coumaric acid and vanillin was observed when the cereal grains were subjected to high temperature during extrusion, except for caffeic and sinapic acids.

Different thermal processing methods are expected to have different impact on the phenolic compounds. Thermal treatment of potatoes and fruit juice-soy milk beverages caused reduction in their chlorogenic acid content. The oven baking caused higher degradation of chlorogenic acid than microwave heating in potatoes (Dao & Friedman, 1992; Morales-de la Pena et al., 2011). Boiling caused the highest degree of degradation of quercetin present in onions and tomatoes followed by microwave heating and then frying (Pandey & Rizvi, 2009). Conversely, carotenoids in carrots, broccoli and zucchini were more sensitive to frying than boiling (Miglio et al., 2008). Peeling, dehulling and grinding may contribute to the loss of polyphenols to some extent. The clarification and stabilization of juice can remove the flavonoids. It is shown that the maceration process can increase the polyphenol content in juice by increasing their diffusion to the juice from the fruit solid (Manach et al., 2004). These observations indicate that the polyphenol content and its bioavailability in processed foods are greatly affected by the nature and intensity of the processing methods used. In order to retain highest possible content of polyphenols, novel processing methods such as high hydrostatic pressure, irradiation, ohmic heating, pulsed electric field and ultrasound have are considered as an alternative to the conventional thermal and mechanical processing methods (Khan et al., 2018).

5.2. Biotransformation of polyphenols in gut and their bioavailability

Polyphenols have poor bioavailability in human body compared to other macro- and micro-nutrients (Appeldoorn et al., 2009). Polyphe-

Table 4
Clinical trials on polyphenolic compounds depicting anti-obesity function with applicable mechanism of action

Plant source	Active component	Study model	Dose and treatment time	Anti-obesity effect	Mechanism of action	Reference
Hibiscus sabdariffa (HS) and *Lippia citriodora* (LC)	Polyphenolic extract with 3.5% anthocyanins and 15% verbascoside (% dry weight, w/w)	Obese and overweight individuals (n = 47)	500 mg of LC (35%)–HS (65%) orally per day for 60 days	i. Significant reduction in anthropometric measurements and body weight ii. Decrease in hunger and appetite	Suppression of appetite through regulation of resistin, leptin, ghrelin and glucagon-like peptide-1 levels	Boix-Castejon et al. (2018)
Green tea	EGCG	Obese and overweight individuals (n = 77)	856.8 mg per day orally for 12 weeks	Significant decrease in body weight by 1.43%, total cholesterol level by 7.49% and low-density lipoprotein cholesterol by 10.10%	Appetite suppression through the regulation of ghrelin secretion and adiponectin levels	Chen et al. (2016)
Red orange, grapefruit, sweet orange and guarana	Extract containing at least 90% of total polyphenols expressed as catechin	Overweight individuals (n = 95)	Two capsules of 450 mg dry extract packaged in red gelatine capsules per day for 12 week	Significant reduction in body weight (3.28%) and abdominal body fat (9.73%) as compared to placebo	-	Dallas et al. (2014)
Black soybean testa	Extract containing 12.58 mg of anthocyanins/ g of extract	Overweight/ obese individuals (n=63)	2.5 g extract per day through two capsules each orally before three daily meals for 8 weeks	Significant decline in waist circumference, hip circumference, triglyceride and LDL cholesterol	Modulation of adipogenesis and lipolysis	Lee et al. (2016)
Hibiscus sabdariffa (HS) and *Lippia citriodora* (LC)	Polyphenolic extract with 10% anthocyanins and 25% verbascoside (% dry weight, w/w)	Obese and overweight individuals (n = 46)	500 mg of LC (65%)–HS (35%) orally per day for 8 weeks	Reduction in body weight by 5.4% abdominal circumference by 7.97% and percentage of body fat by 1.33%	Regulation of lipid metabolism through activation of AMPK	Herranz-Lopez et al. (2019)
Blueberry	Total anthocyanins 12.83 mg/L	Overweight/ obese adult individuals (n=54)	50 g carbohydrate substituted with equal amount of blueberries after 1st six weeks of treatment per day for 12 weeks	Reduction in body weight by 11.34%, significant reduction in body fat (20.89%), total cholesterol (14.75%) and LDL cholesterol (18.3%) from baseline	Regulation of lipid metabolism	Istek and Gurbuz (2017)
Gnetum gnemon L. seed	Extract containing minimum 20% resveratrol derivatives	Healthy young male (n= 42)	150 and 300 mg per day orally for 14 days	Increase in the adiponectin level	Regulation of lipid metabolism	Oniki et al. (2020)
Euterpe edulis Mart.	Pulp rich in anthocyanins	Obese individuals (n = 35)	5 g of pulp powder per day orally for 6 weeks	Significant reduction of body fat by and significant increase in HDL cholesterol and adiponectin	Alterations in lipid metabolism	Jamar et al. (2020)

nols are mostly present as esters, glycosides or polymers in food which makes their absorption and bioavailability quite low (Ifie & Marshall, 2018). Only simple polyphenols such as caffeic acid, gallic acid and quercetin, accounting 5 to 10% of total ingestion, are generally absorbed in small intestine. These simpler polyphenols diffuse through enterocyte and reach to hepatocytes where they undergo biotransformation into various metabolites which are then distributed to the target organs. A substantial percentage (>90 %) of complex polyphenols then reaches to colon and interacts with gut microflora. The gut microflora can transform the complex polyphenols into a number of intermediate compounds and smaller phenolic acids and increase their bioactive potential. These small compounds are absorbed from the colon and get transported to liver through portal vein where they are biotransformed into glucuronides, sulfates and methyl metabolites. They are ultimately distributed to various organs (Correa et al., 2019; Edwards et al., 2017). For example, it was reported that all of the ingested caffeic acid and one third of ingested chlorogenic acid was found to absorb through the small intestine (Olthof et al., 2001). When ellagitannins are consumed and reach the intestine, they undergo hydrolysis and produce ellagic acid. Ellagic acid is metabolized by gut microflora to urolithins with varying number of hydroxyl groups (Cardona et al., 2013). Vitaglione et al. (2007) showed that microflora in human colon can produce bioactive metabolites including protocatechuic acid from anthocyanins; which possess antioxidative, anti-inflammatory, anticar-

cinogenic and neuroprotective effects in humans (Vitaglione et al., 2007).

Similarly, polyphenols and their metabolites can exert prebiotic and antipathogenic functions and positively influence the diversity of microflora population in gut and offer beneficial effect to the host. The polyphenolic compounds from tea are shown to suppress the growth of pathogenic microorganisms such as *Escherichia Coli, Listeria monocytogenes, Helicobacter pylori, Pseudomonas aeruginosa, Salmonella typhimurium* and *Staphylococcus aureus* (Duda–Chodak et al., 2015). Intake of red wine polyphenols and anthocyanins has shown to enhance the growth of beneficial *Bifidobacterium* and *Enterococcus* species (Duda–Chodak et al., 2015). Therefore, the interaction between the polyphenol and the gut microflora can improve the bioavailability of the former by transforming them into bioactive metabolites. On the other hand, polyphenols can also help the growth of beneficial bacterial population in the gut. Hence, there is a two-way interactive relationship between polyphenols and gut microorganisms that leads to desirable health benefits (Fraga et al., 2019).

The biotransformation of these polyphenols by indigenous enzymes in the gut can have both positive or negative affect on their bioavailability (Laparra and Sanz, 2010). The bioavailability of polyphenols is also greatly influenced by the composition of food, the interaction of polyphenols with other components, gut and intestinal metabolism and physiological condition of host (D'Archivio et al., 2010). To derive desired bioactive function from polyphenols, they should be consumed in sufficient dose, digested, absorbed and finally transported to the targeted tissues or organs (Dominguez–Avila et al., 2017).

Emerging processing technologies such as encapsulation (Fang and Bhandari, 2010) and synergistic interactions can be employed to enhance the bioavailability of polyphenolic compounds (Scheepens et al., 2010). The combination of two or more phenolic compounds may impart additive or synergistic effect on health-promoting properties and ultimately enhance the effectiveness (Brglez Mojzer et al., 2016). Sharma et al. (2018) found that the combination of *Garcinia cambogia* and pear pomace extracts exhibited additive to moderately synergistic effect on inhibition of adipocyte differentiation and accumulation of lipid in 3T3-L1 cells.

5.3. Toxicity concerns of polyphenols

Generally, the polyphenolic compounds derived from food are considered to be safe because they are part of human diet. A number of human trials involved with the consumption of polyphenols at different concentration for different length of time showed that they are safe to consume. It was reported that some polyphenols produced mild side effects such as headache, gastrointestinal problems, dizziness and rashes (Renaud and Martinoli, 2019). A study involving ingestion of curcumin up to 4 g per day by Alzheimer's patients for 48 weeks reported that 13% of the study population in curcumin group withdrew due to gastrointestinal side effects such as black stool and diarrhoea (Ringman et al., 2012). Polyphenols may exhibit harmful or toxic effect at higher concentrations, to certain vulnerable populations and in certain disease conditions (Lambert et al., 2007). Although there are proven beneficial effects of polyphenols, they can also impart antinutritive or prooxidative effects at high doses (Luna-Guevara et al., 2019). There are reports showing that there is a some degree of negative effect of polyphenols on deoxyribonucleic Acid (DNA) (Azqueta and Collins, 2016), hepatotoxicity (Bonkovsky, 2006), nephrotoxicity (Rasheed et al., 2017), carcinogenicity/ genotoxicity or thyroid toxicity (Mennen et al., 2005) associated with the higher doses/concentration of certain polyphenols. Murakami (2014) reported that higher dose of green tea polyphenols in diet ranging from 0.5 to 1% caused adverse health effects in mice as compared to low and medium doses. High doses of green tea polyphenols induced carcinogenesis, intestinal inflammation, nephrotoxicity and dysfunction of kidney and liver in mice (Murakami, 2014). Shoji et al. (2004) performed toxicological study of apple polyphenol extract and concluded that the polyphenolic extract at 2000 mg/kg did not present any significant toxicity effects in rats.

Therefore, a suitable dose level should be formulated for each polyphenol to derive desired health benefits and avoid unintended effects (Luna–Guevara et al., 2019).

6. Incorporation of polyphenols in foods

There is a growing trend of people getting more inclined towards functional foods rather than synthetic medications. Therefore, bioactive polyphenols with anti-obesity potential may be incorporated into food to treat and prevent the obesity. However, low water solubility and poor stability against heat, oxygen and light restricts the incorporation of these polyphenols into commercial products. The efficacy of these products for the desired therapeutic target greatly depends upon the stability, solubility, release kinetics and bioavailability and the efficacy can reduce substantially during food processing, handling and storage. Encapsulation of these polyphenols can be one of the promising approaches to enhance the stability, preserve the bioactivity and for desired targeted release. Several technologies of encapsulation including coacervation, co-crystallization, spray drying, freeze drying, emulsification, ionic gelation. have been described (Parisi et al., 2014). A new concept termed "nanofood" which involves the use of nanotechnologies for formulating innovative and functional foods is getting more popularity that enhances the stability and bioavailability of encapsulated bioactive compound (Dima et al., 2020). The nanoparticles of green tea catechins encapsulated in zein presented significant improvement in gastrointestinal stability (*in vitro)* and better cell permeability properties for potential sustained release (Bhushani et al., 2017). Similarly, Lopez de Dicastillo et al. (2019) encapsulated açaí fruit extract in zein to enhance thermal stability and bioaccesibility. They reported a reduction in the degradation of phenolics through encapsulation from 10% to 5% after sterilization at 121 ˚C and from 50% to 20% after baking at 180 ˚C. Moreover, the bioaccesibility study showed higher concentration of polyphenols in encapsulated fruit extract after simulated *in vitro* digestion than with the non-encapsulated ones.

Kiss et al. (2019) developed a bread fortified with three dried leafy vegetable powders rich in polyphenols (*Amaranthus viridis, Solanum macrocarpon*, and *Telfairia occidentalis*) and claimed to have enhanced the polyphenol content and antioxidant property in the fortified product than in the control. Moumita et al. (2018) formulated a curd using green tea infused in soy-fortified bovine milk which can be useful for patients with hypercholesterolemia. Mayneris-Perxachs et al. (2019) researched on the influence of supplementation of hesperidin and naringenin in biscuit on metabolic syndrome of rats fed with obesogenic cafeteria diet. They observed significant reduction in body weight, body fat, total cholesterol, LDL- cholesterol as well as oxidative stress and concluded it to be a promising functional food product for the improvement of metabolic syndrome.

The bioactive extract rich in polyphenols from wasted red pepper was encapsulated in whey protein isolate and sunflower oil and fortified into yoghurt at the concentration of 10% (Seregelj et al., 2019). During the storage period of 21 days, the polyphenol retention was higher (123.73%) in yoghurt fortified with encapsulated bioactive extract along with the superior sensory attributes as compared to the control yoghurt (115.48%) fortified with non-encapsulated extract. The rise in polyphenols during storage may be attributed by the degradation of conjugated polyphenols and protein-polyphenol aggregates (Seregelj et al., 2019). This study evidenced the advantage of encapsulation for retaining polyphenols during storage and handling. Hence, encapsulation techniques may be employed to improve the stability and bioavailability of polyphenols so that polyphenols can be integrated into the food systems effectively for the desired therapeutic purposes. Moreover, selection of suitable encapsulating material that compliments with the anti-obesity potential of bioactive compounds may be a challenging task and requires an in-depth studies.

7. Concluding remarks

There is an increasing awareness on the health benefits of naturally derived plant polyphenols. Researchers and manufactures are investing time and resources to discover and characterise natural phenolic compounds to prevent or manage obesity which is a pervasive health problem. Large and insightful body of knowledge on polyphenols is available in the literature covering the physicochemical properties and mechanism of natural polyphenols that underpins the prevention, treatment and management of obesity. This review summarizes types, physicochemical characteristics, mechanism of action and efficacy of natural polyphenols from diverse sources as anti-obesity compounds. This review also highlights the advances made in science that underpin the formulation of polyphenols as natural anti-obesity agents including their bioavailability, dose-effect relationship and avoiding side effects. It is expected that more rigorous studies, including human trials, will continue in the future and will provide evidence-based and more effective use of natural phenolic compounds either as functional foods or supplements to prevent and manage obesity in humans.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The first author acknowledges the scholarship support from RMIT University. This work did not receive any funding from public, commercial, or not-for-profit sectors.

Author Contributions

Manisha Singh has drafted this manuscript with input and supervision of Benu Adhikari, Thilini Thrimawithana and Ravi Shukla. All authors provided critical feedback and contributed to the final version of this manuscript.

References

Ali, A.T., Hochfeld, W.E., Myburgh, R., Pepper, M.S., 2013. Adipocyte and adipogenesis. Eur. J. Cell Biol. 92 (6-7), 229–236. doi:10.1016/j.ejcb.2013.06.001.

Appeldoorn, M.M., Vincken, J.P., Gruppen, H., Hollman, P.C., 2009. Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. J. Nutr. 139 (8), 1469–1473. doi:10.3945/jn.109.106765.

Ashrafi, K., 2007. Obesity and the regulation of fat metabolism. WormBook 1–20. doi:10.1895/wormbook.1.130.1.

Azqueta, A., Collins, A., 2016. Polyphenols and DNA damage: a mixed blessing. Nutrients 8 (12). doi:10.3390/nu8120785.

Baboota, R.K., Bishnoi, M., Ambalam, P., Kondepudi, K.K., Sarma, S.M., Boparai, R.K., Podili, K., 2013. Functional food ingredients for the management of obesity and associated co-morbidities – a review. J. Funct. Foods. 5 (3), 997–1012. doi:10.1016/j.jff.2013.04.014.

Badshah, H., Ullah, I., Kim, S.E., Kim, T.H., Lee, H.Y., Kim, M.O., 2013. Anthocyanins attenuate body weight gain via modulating neuropeptide Y and GABAB1 receptor in rats hypothalamus. Neuropeptides 47 (5), 347–353. doi:10.1016/j.npep.2013.06.001.

Balaji, M., Ganjayi, M.S., Hanuma Kumar, G.E., Parim, B.N., Mopuri, R., Dasari, S., 2016. A review on possible therapeutic targets to contain obesity: the role of phytochemicals. Obes. Res. Clin. Pract. 10 (4), 363–380. doi:10.1016/j.orcp.2015.12.004.

Bays, H.E., 2004. Current and investigational antiobesity agents and obesity therapeutic treatment targets. Obes. Res. 12, 1197–1211.

Bhushani, J.A., Kurrey, N.K., Anandharamakrishnan, C., 2017. Nanoencapsulation of green tea catechins by electrospraying technique and its effect on controlled release and in-vitro permeability. J Food Eng. 199, 82–92. doi:10.1016/j.jfoodeng.2016.12.010.

Bhuyan, D.J., Basu, A., 2017. Phenolic compounds: potential health benefits and toxicity. In: Vuong, Q.V. (Ed.), Utilisation of Bioactive Compounds from Agricultural and Food Production Waste. CRC PressTaylor & Francis Group, pp. 27–59.

Birari, R.B., Bhutani, K.K., 2007. Pancreatic lipase inhibitors from natural sources: unexplored potential. Drug Discov. Today. 12 (19-20), 879–889. doi:10.1016/j.drudis.2007.07.024.

Bitou, N., Ninomiya, M., Tsujita, T., Okuda, H., 1999. Screening of lipase inhibitors from marine algae. Lipids 34 (5), 441–445. doi:10.1007/s11745-999-0383-7.

Błaszczyk, A., Sady, S., Sielicka, M., 2018. The stilbene profile in edible berries. Phytochem. Rev. 18 (1), 37–67. doi:10.1007/s11101-018-9580-2.

Boix-Castejon, M., Herranz-Lopez, M., Perez Gago, A., Olivares-Vicente, M., Caturla, N., Roche, E., Micol, V., 2018. Hibiscus and lemon verbena polyphenols modulate appetite-related biomarkers in overweight subjects: a randomized controlled trial. Food Funct 9 (6), 3173–3184. doi:10.1039/c8fo00367j.

Bonkovsky, H.L., 2006. Hepatotoxicity associated with supplements containing Chinese green tea (*Camellia sinensis*. Ann. Intern. Med 144 (1), 68–71.

Brey, C.W., Nelder, M.P., Hailemariam, T., Gaugler, R., Hashmi, S., 2009. Krüppel-like family of transcription factors: an emerging new frontier in fat biology. Int. J. Biol. Sci. 5 (6), 622–636.

Brglez Mojzer, E., Knez Hrncic, M., Skerget, M., Knez, Z., Bren, U., 2016. Polyphenols: extraction methods, antioxidative action. Bioavail. Anticarci. Effects. Molecules. 21 (7). doi:10.3390/molecules21070901.

Burkholder-Cooley, N., Rajaram, S., Haddad, E., Fraser, G.E., Jaceldo-Siegl, K., 2016. Comparison of polyphenol intakes according to distinct dietary patterns and food sources in the Adventist Health Study-2 cohort. Br. J. Nutr. 115 (12), 2162–2169. doi:10.1017/S0007114516001331.

Cannon, B., Nedergaard, J., 2004. Brown adipose tissue: function and physiological significance.. Physiol. Rev 84 (1), 277–359. doi:10.1152/physrev.00015.2003.

Cardona, F., Andres-Lacueva, C., Tulipani, S., Tinahones, F.J., Queipo-Ortuno, M.I., 2013. Benefits of polyphenols on gut microbiota and implications in human health. J. Nutr. Biochem. 24 (8), 1415–1422. doi:10.1016/j.jnutbio.2013.05.001.

Carpene, C., Pejenaute, H., Del Moral, R., Boulet, N., Hijona, E., Andrade, F., Villanueva-Millan, M.J., Aguirre, L., Arbones-Mainar, J.M., 2018. The dietary antioxidant piceatannol inhibits adipogenesis of human adipose mesenchymal stem cells and limits its glucose transport and lipogenic activities in adipocytes. Int. J. Mol. Sci. 19 (7). doi:10.3390/ijms19072081.

Chantre, P., Lairon, D., 2002. Recent findings of green tea extract AR25 (exolise) and its activity for the treatment of obesity. Phytomedicine 9 (1), 3–8.

Chen, I.J., Liu, C.Y., Chiu, J.P., Hsu, C.H., 2016. Therapeutic effect of high-dose green tea extract on weight reduction: a randomized, double-blind, placebo-controlled clinical trial. Clin. Nutr. 35 (3), 592–599. doi:10.1016/j.clnu.2015.05.003.

Cheng, M., Zhang, X., Miao, Y., Cao, J., Wu, Z., Weng, P., 2017. The modulatory effect of (-)-epigallocatechin 3-O-(3-O-methyl) gallate (EGCG3″Me) on intestinal microbiota of high fat diet-induced obesity mice model. Food Res Int 92, 9–16. doi:10.1016/j.foodres.2016.12.008.

Chiva-Blanch, G., Badimon, L., 2017. Effects of polyphenol intake on metabolic syndrome: current evidences from human trials. Oxid. Med. Cell. Longev. 2017, 1–18. doi:10.1155/2017/5812401.

Correa, T.A.F., Rogero, M.M., Hassimotto, N.M.A., Lajolo, F.M., 2019. The two-way polyphenols-microbiota interactions and their effects on obesity and related metabolic diseases. Front. Nutr. 6, 188. doi:10.3389/fnut.2019.00188.

Cory, H., Passarelli, S., Szeto, J., Tamez, M., Mattei, J., 2018. The role of polyphenols in human health and food systems: a mini-review. Front. Nutr. 5, 87. doi:10.3389/fnut.2018.00087.

D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., 2007. Polyphenols, dietary sources and bioavailability. Ann. Ist. Super. Sanita. 43 (4), 348–361.

D'Archivio, M., Filesi, C., Vari, R., Scazzocchio, B., Masella, R., 2010. Bioavailability of the polyphenols: status and controversies. Int. J. Mol. Sci. 11 (4), 1321–1342. doi:10.3390/ijms11041321.

Dahiya, D.K., Renuka, Puniya, M., Shandilya, U.K., Dhewa, T., Kumar, N., Kumar, S., Puniya, A.K., Shukla, P., 2017. Gut microbiota modulation and its relationship with obesity using prebiotic fibers and probiotics: a review. Front. Microbiol. 8, 563. doi:10.3389/fmicb.2017.00563.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15 (10), 7313–7352. doi:10.3390/molecules15107313.

Dal-Pan, A., Blanc, S., Aujard, F., 2010. Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity. BMC Physiol 10 (1), 11. doi:10.1186/1472-6793-10-11.

Dallas, C., Gerbi, A., Elbez, Y., Caillard, P., Zamaria, N., Cloarec, M., 2014. Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. Phytother. Res. 28 (2), 212–218. doi:10.1002/ptr.4981.

Dao, L., Friedman, M., 1992. Chlorogenic acid content of fresh and processed potatoes determined by ultraviolet spectrophotometry. J. Agric. Food Chem. 40 (11), 2152–2156.

Dima, C., Assadpour, E., Dima, S., Jafari, S.M., 2020. Nutraceutical nanodelivery; an insight into the bioaccessibility/bioavailability of different bioactive compounds loaded within nanocarriers. Crit Rev Food Sci Nutr 1–35. doi:10.1080/10408398.2020.1792409.

Dominguez-Avila, J.A., Wall-Medrano, A., Velderrain-Rodriguez, G.R., Chen, C.O., Salazar-Lopez, N.J., Robles-Sanchez, M., Gonzalez-Aguilar, G.A., 2017. Gastrointestinal interactions, absorption, splanchnic metabolism and pharmacokinetics of orally ingested phenolic compounds. Food Funct 8 (1), 15–38. doi:10.1039/c6fo01475e.

Duda-Chodak, A., Tarko, T., Satora, P., Sroka, P., 2015. Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. Eur. J. Nutr. 54 (3), 325–341. doi:10.1007/s00394-015-0852-y.

Duwaerts, C.C., Maher, J.J., 2019. Macronutrients and the adipose-liver axis in obesity and fatty liver. Cell. Mol. Gastroenterol. Hepatol. 7 (4), 749–761. doi:10.1016/j.jcmgh.2019.02.001.

Edwards, C.A., Havlik, J., Cong, W., Mullen, W., Preston, T., Morrison, D.J., Combet, E., 2017. Polyphenols and health: interactions between fibre, plant polyphenols and the gut microbiota. Nutr. Bull. 356–360.

Fang, C., Kim, H., Noratto, G., Sun, Y., Talcott, S.T., Mertens-Talcott, S.U., 2018. Gallotannin derivatives from mango (Mangifera indica L.) suppress adipogenesis and increase thermogenesis in 3T3-L1 adipocytes in part through the AMPK pathway. J. Funct. Foods. 46, 101–109. doi:10.1016/j.jff.2018.04.043.

Fang, Z., Bhandari, B., 2010. Encapsulation of polyphenols – a review. Trends Food Sci. Technol. 21 (10), 510–523. doi:10.1016/j.tifs.2010.08.003.

Filippatos, T.D., Derdemezis, C.S., Gazi, I.F., Nakou, E.S., Mikhailidis, D.P., Elisaf, M.S., 2008. Orlistat-associated adverse effects and drug interactions a critical review. Drug Saf 31 (1), 53–65.

Fraga, C.G., Croft, K.D., Kennedye, D.O., Tomás-Barberán, F.A., 2019. The effects of polyphenols and other bioactives on human health. Food Funct 10, 514–528. doi:10.1039/c8fo01997e.

Friedman, J.M., Halaas, J.L., 1998. Leptin and the regulation of body weight in mammals. Nature 395, 763. doi:10.1038/27376.

Funakoshi, T., Kanzaki, N., Otsuka, Y., Izumo, T., Shibata, H., Machida, S., 2018. Quercetin inhibits adipogenesis of muscle progenitor cells in vitro. Biochem. Biophys. 13, 39–44. doi:10.1016/j.bbrep.2017.12.003.

Ganjayi, M.S., Meriga, B., Hari, B., Oruganti, L., Dasari, S., Mopuri, R., 2017. PolyPhenolic rich fraction of Terminalia paniculata attenuates obesity through inhibition of pancreatic amylase, lipase and 3T3-L1 adipocyte differentiation. J. Nutr. Intermed. Metab. 10, 19–25. doi:10.1016/j.jnim.2017.11.003.

Gargouri, Y., Julien, R., Sugihara, A., Verger, R., Sarda, L., 1984. Inhibition of pancreatic and microbial lipases by proteins. Biochim. Biophys. Acta - Lipids Lipid Met. 795 (2), 326–331.

Geoffroy, P., Ressault, B., Marchioni, E., Miesch, M., 2011. Synthesis of Hoodigogenin A, aglycone of natural appetite suppressant glycosteroids extracted from Hoodia gordonii. Steroids 76 (7), 702–708. doi:10.1016/j.steroids.2011.03.014.

Gong, D.-W., He, Y., Karas, M., Reitman, M., 1997. Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, β3-adrenergic agonists, and leptin. J. Biol. Chem. 272 (39), 24129–24132.

Gonzalez-Castejon, M., Rodriguez-Casado, A., 2011. Dietary phytochemicals and their potential effects on obesity: a review. Pharmacol. Res. 64 (5), 438–455. doi:10.1016/j.phrs.2011.07.004.

Gonzalez, S., Fernandez, M., Cuervo, A., Lasheras, C., 2014. Dietary intake of polyphenols and major food sources in an institutionalised elderly population. J. Hum. Nutr. Diet. 27 (2), 176–183. doi:10.1111/jhn.12058.

Grosso, G., Stepaniak, U., Topor-Madry, R., Szafraniec, K., Pajak, A., 2014. Estimated dietary intake and major food sources of polyphenols in the Polish arm of the HAPIEE study. Nutrition 30 (11-12), 1398–1403. doi:10.1016/j.nut.2014.04.012.

Haslam, D., 2016. Weight management in obesity - past and present. Int J Clin Pract 70 (3), 206–217. doi:10.1111/ijcp.12771.

Herranz-Lopez, M., Olivares-Vicente, M., Boix-Castejon, M., Caturla, N., Roche, E., Micol, V., 2019. Differential effects of a combination of Hibiscus sabdariffa and Lippia citriodora polyphenols in overweight/obese subjects: a randomized controlled trial. Sci. Rep. 9 (1), 2999. doi:10.1038/s41598-019-39159-5.

Herranz-Lopez, M., Olivares-Vicente, M., Encinar, J.A., Barrajon-Catalan, E., Segura-Carretero, A., Joven, J., Micol, V., 2017. Multi-targeted molecular effects of hibiscus sabdariffa polyphenols: an opportunity for a global approach to obesity. Nutrients 9 (8). doi:10.3390/nu9080907.

Hollman, P. C. H., Trijp, J. M. P. v., Buysman, M. N. C. P., Gaag, M. S. v. d., Mengelers, M. J. B., Vries, J. H. M. d., Katan, M. B., 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man FEBS Letters. 418, 152-156.

Hsu, C.L., Yen, G.C., 2008. Phenolic compounds: evidence for inhibitory effects against obesity and their underlying molecular signaling mechanisms. Mol. Nutr. Food Res. 52 (1), 53–61. doi:10.1002/mnfr.200700393.

Ifie, I., Marshall, L.J., 2018. Food processing and its impact on phenolic constituents in food. Cogent Food Agric 4 (1), 1507782. doi:10.1080/23311932.2018.1507782.

Istek, N., Gurbuz, O., 2017. Investigation of the impact of blueberries on metabolic factors influencing health. J. Function. Foods 38, 298–307. doi:10.1016/j.jff.2017.09.039.

Jackson, V.M., Breen, D.M., Fortin, J.P., Liou, A., Kuzmiski, J.B., Loomis, A.K., Rives, M.L., Shah, B., Carpino, P.A., 2015. Latest approaches for the treatment of obesity. Expert Opin Drug Discov 10 (8), 825–839. doi:10.1517/17460441.2015.1044966.

Jamar, G., Santamarina, A.B., Flygare, A.C., Gagliardi, A., de Rosso, V.V., Dourado, V.Z., Pisani, L.P., 2020. Effects of the jucara fruit supplementation on metabolic parameters in individuals with obesity: a double-blind randomized controlled trial. J Nutr Biochem 83, 108430. doi:10.1016/j.jnutbio.2020.108430.

Jiao, X., Wang, Y., Lin, Y., Lang, Y., Li, E., Zhang, X., Zhang, Q., Feng, Y., Meng, X., Li, B., 2018. Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. J. Nutr. Biochem. 64, 88–100. doi:10.1016/j.jnutbio.2018.07.008.

Jimenez-Gomez, Y., Mattison, J.A., Pearson, K.J., Martin-Montalvo, A., Palacios, H.H., Sossong, A.M., Ward, T.M., Younts, C.M., Lewis, K., Allard, J.S., Longo, D.L., Belman, J.P., Malagon, M.M., Navas, P., Sanghvi, M., Moaddel, R., Tilmont, E.M., Herbert, R.L., Morrell, C.H., Egan, J.M., Baur, J.A., Ferrucci, L., Bogan, J.S., Bernier, M., de Cabo, R., 2013. Resveratrol improves adipose insulin signaling and reduces the inflammatory response in adipose tissue of rhesus monkeys on high-fat, high-sugar diet. Cell Metab 18 (4), 533–545. doi:10.1016/j.cmet.2013.09.004.

Kalupahana, N.S., Moustaid-Moussa, N., Claycombe, K.J., 2012. Immunity as a link between obesity and insulin resistance. Mol. Aspects Med. 33 (1), 26–34. doi:10.1016/j.mam.2011.10.011.

Kang, J.G., Park, C.Y., 2012. Anti-obesity drugs: a review about their effects and safety. Diabetes Metab 36 (1), 13–25. doi:10.4093/dmj.2012.36.1.13.

Karagianni, P., Talianidis, I., 2015. Transcription factor networks regulating hepatic fatty acid metabolism. Biochimica et Biophysica Acta. 1851 (1), 2–8. doi:10.1016/j.bbalip.2014.05.001.

Kazemipoor, M., Hamzah, S., Hajifaraji, M., Radzi, C.W., Cordell, G.A., 2016. Slimming and appetite-suppressing effects of caraway aqueous extract as a natural therapy in physically active women. Phytother. Res. 30 (6), 981–987. doi:10.1002/ptr.5603.

Khan, M.K., Ahmad, K., Hassan, S., Imran, M., Ahmad, N., Xu, C., 2018. Effect of novel technologies on polyphenols during food processing. Innov. Food Sci. Emerg. Technol. 45, 361–381. doi:10.1016/j.ifset.2017.12.006.

Kim, J.K., So, H., Youn, M.J., Kim, H.J., Kim, Y., Park, C., Kim, S.J., Ha, Y.A., Chai, K.Y., Kim, S.M., Kim, K.Y., Park, R., 2007. Hibiscus sabdariffa L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway. J. Ethnopharmacol 114 (2), 260–267. doi:10.1016/j.jep.2007.08.028.

Kim, K.-H., Kim, G.-N., Lee, K.W., 2013. Phytochemicals in the Prevention and treatment of obesity and its related cancers. In: Coulston, A.M., Boushey, C.J., Ferruzzi, M. (Eds.), Nutrition in the Prevention and Treatment of Disease. Academic Press, London, United Kingdom, pp. 391–406.

Kim, K.K., 2019. Understanding the mechanism of action and clinical implications of anti-obesity drugs recently approved in Korea. Korean J Fam Med 40 (2), 63–71. doi:10.4082/kjfm.19.0013.

Kim, N.H., Jegal, J., Kim, Y.N., Heo, J.D., Rho, J.R., Yang, M.H., Jeong, E.J., 2018. Chokeberry extract and its active polyphenols suppress adipogenesis in 3T3-L1 adipocytes and modulates fat accumulation and insulin resistance in diet-induced obese mice. Nutrients 10 (11). doi:10.3390/nu10111734.

Kiss, A., Takács, K., Nagy, A., Nagy-Gasztonyi, M., Cserhalmi, Z., Naár, Z., Halasi, T., Csáki, J., Némedi, E., 2019. In vivo and in vitro model studies on noodles prepared with antioxidant-rich pseudocereals. J. Food Measure. Character. 13 (4), 2696–2704. doi:10.1007/s11694-019-00190-9.

Klontz, K.C., Timbo, B.B., Street, D., 2006. Consumption of dietary supplements containing citrus aurantium (bitter orange)—2004 california behavioral risk factor surveillance survey (BRFSS). Ann. Pharmacother. 40 (10), 1747–1751. doi:10.1345/aph.1H196.

Kumar, S., Alagawadi, K.R., 2013. Anti-obesity effects of galangin, a pancreatic lipase inhibitor in cafeteria diet fed female rats. Pharm. Biol. 51 (5), 607–613. doi:10.3109/13880209.2012.757327.

Kumar Singh, A., Cabral, C., Kumar, R., Ganguly, R., Kumar Rana, H., Gupta, A., Rosaria Lauro, M., Carbone, C., Reis, F., Pandey, A.K., 2019. Beneficial effects of dietary polyphenols on gut microbiota and strategies to improve delivery efficiency. Nutrients 11 (9). doi:10.3390/nu11092216.

Kuriyan, R., Raj, T., Srinivas, S.K., Vaz, M., Rajendran, R., Kurpad, A.V., 2007. Effect of Caralluma fimbriata extract on appetite, food intake and anthropometry in adult Indian men and women. Appetite 48 (3), 338–344. doi:10.1016/j.appet.2006.09.013.

Lai, C.S., Ho, M.H., Tsai, M.L., Li, S., Badmaev, V., Ho, C.T., Pan, M.H., 2013. Suppression of adipogenesis and obesity in high-fat induced mouse model by hydroxylated polymethoxyflavones. J. Agric. Food Chem. 61 (43), 10320–10328. doi:10.1021/jf402257t.

Lambert, J.D., Sang, S., Yang, C.S., 2007. Possible controversy over dietary polyphenols: benefits vs risks. Chem. Res. Toxicol. 20, 583–585.

Laparra, J.M., Sanz, Y., 2010. Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol. Res. 61 (3), 219–225. doi:10.1016/j.phrs.2009.11.001.

Lee, M., Sorn, S.R., Park, Y., Park, H.K., 2016. Anthocyanin rich-black soybean testa improved visceral fat and plasma lipid profiles in overweight/obese korean adults: a randomized controlled trial. J Med Food 19 (11), 995–1003. doi:10.1089/jmf.2016.3762.

Lee, M.J., Wang, Z.Y., Li, H., Chen, L., Sun, Y., Gobbo, S., Balentine, D.A., Yang, C.S., 1995. Analysis of plasma and urinary tea polyphenols in human subjects. Cancer Epidemiol. Biomark. Prev. 4 (4), 393–399.

Lefterova, M.I., Lazar, M.A., 2009. New developments in adipogenesis. Trends Endocrinol. Metab. 20 (3), 107–114. doi:10.1016/j.tem.2008.11.005.

Li, S., Tan, H.Y., Wang, N., Cheung, F., Hong, M., Feng, Y., 2018. The potential and action mechanism of polyphenols in the treatment of liver diseases. Oxid. Med. Cell. Longev. 2018, 8394818. doi:10.1155/2018/8394818.

Liu, J., He, Z., Ma, N., Chen, Z.Y., 2020. Beneficial effects of dietary polyphenols on high-fat diet-induced obesity linking with modulation of gut microbiota. J Agric Food Chem 68 (1), 33–47. doi:10.1021/acs.jafc.9b06817.

Liu, P.K., Weng, Z.M., Ge, G.B., Li, H.L., Ding, L.L., Dai, Z.R., Hou, X.D., Leng, Y.H., Yu, Y., Hou, J., 2018. Biflavones from Ginkgo biloba as novel pancreatic lipase inhibitors: Inhibition potentials and mechanism. Int. J. Biol. Macromol. 118 (Pt B), 2216–2223. doi:10.1016/j.ijbiomac.2018.07.085.

Liu, S., Li, D., Huang, B., Chen, Y., Lu, X., Wang, Y., 2013. Inhibition of pancreatic lipase, alpha-glucosidase, alpha-amylase, and hypolipidemic effects of the total flavonoids from Nelumbo nucifera leaves. J Ethnopharmacol 149 (1), 263–269. doi:10.1016/j.jep.2013.06.034.

Lopez de Dicastillo, C., Pina, C., Garrido, L., Arancibia, C., Galotto, M.J., 2019. Enhancing thermal stability and bioaccesibility of acai fruit polyphenols through electrohydrodynamic encapsulation into zein electrosprayed particles. Antioxidants (Basel) 8 (10). doi:10.3390/antiox8100464.

Lowell, B.B., Spiegelman, B.M., 2000. Towards a molecular understanding of adaptive thermogenesis. Nature 404, 652. doi:10.1038/35007527.

Luna-Guevara, M.L., Luna-Guevara, J.J., Hernández-Carranza, P., Ruíz-Espinosa, H., Ochoa-Velasco, C.E., 2019. Phenolic compounds: a good choice against chronic degenerative diseases. Stud. Nat. Prod. Chem. 59, 79–108. doi:10.1016/b978-0-444-64179-3.00003-7.

Ma, I.T., Madura 2nd, J.A., 2015. Gastrointestinal complications after bariatric surgery. Gastroenterol. Hepatol. 11 (8), 526–535.

MacLean, D.B., Luo, L.G., 2004. Increased ATP content/production in the hypothalamus may be a signal for energy-sensing of satiety: studies of the anorectic mechanism of a plant steroidal glycoside. Brain Res 1020 (1-2), 1–11. doi:10.1016/j.brainres.2004.04.041.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79, 727–747.

Matos, M.J., Santana, L., Uriarte, E., Abreu, O.A., Molina, E., Yordi, E.G., 2015. Coumarins — an important class of phytochemicals. In: Rao, A.V., Rao, L.G. (Eds.), Phytochemicals - Isolation, Characterisation and Role in Human Health Eds.. InTech, Rijeka, Croatia.

Mayneris-Perxachs, J., Alcaide-Hidalgo, J.M., de la Hera, E., del Bas, J.M., Arola, L., Caimari, A., 2019. Supplementation with biscuits enriched with hesperidin and naringenin is associated with an improvement of the Metabolic Syndrome induced by a cafeteria diet in rats. J. Funct. Foods. 61. doi:10.1016/j.jff.2019.103504.

McDougall, G.J., Kulkarni, N.N., Stewart, D., 2009. Berry polyphenols inhibit pancreatic lipase activity in vitro. Food Chem 115 (1), 193–199. doi:10.1016/j.foodchem.2008.11.093.

Mele, L., Bidault, G., Mena, P., Crozier, A., Brighenti, F., Vidal-Puig, A., Del Rio, D., 2017. Dietary (Poly)phenols, brown adipose tissue activation, and energy expenditure: a narrative review. Adv. Nutr. 8 (5), 694–704. doi:10.3945/an.117.015792.

Mennen, L.I., Walker, R., Bennetau-Pelissero, C., Scalbert, A., 2005. Risks and safety of polyphenol consumption. Am J Clin Nutr 81 (1), 326S–329S. doi:10.1093/ajcn/81.1.326S.

Miglio, C., Chiavaro, E., Visconti, A., Fogliano, V., Pellegrini, N., 2008. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. J. Agric. Food Chem. 56 (1), 139–147. doi:10.1021/jf072304b.

Milder, I.E., Arts, I.C., van de Putte, B., Venema, D.P., Hollman, P.C., 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93 (3), 393–402. doi:10.1079/bjn20051371.

Min, S.Y., Yang, H., Seo, S.G., Shin, S.H., Chung, M.Y., Kim, J., Lee, S.J., Lee, H.J., Lee, K.W., 2013. Cocoa polyphenols suppress adipogenesis in vitro and obesity in vivo by targeting insulin receptor. Int. J. Obes. 37 (4), 584–592. doi:10.1038/ijo.2012.85.

Mohamed, G.A., Ibrahim, S.R.M., Elkhayat, E.S., El Dine, R.S., 2014. Natural anti-obesity agents.. Bull. Fac. Pharm. Cairo Univ 52 (2), 269–284. doi:10.1016/j.bfopcu.2014.05.001.

Moon, H.S., Lee, H.G., Choi, Y.J., Kim, T.G., Cho, C.S., 2007. Proposed mechanisms of (-)-epigallocatechin-3-gallate for anti-obesity. Chem-Biol Interact 167 (2), 85–98. doi:10.1016/j.cbi.2007.02.008.

Morales-de la Pena, M., Salvia-Trujillo, L., Rojas-Grau, M.A., Martin-Belloso, O., 2011. Changes on phenolic and carotenoid composition of high intensity pulsed electric field and thermally treated fruit juice-soymilk beverages during refrigerated storage. Food Chem 129 (3), 982–990. doi:10.1016/j.foodchem.2011.05.058.

Moumita, S., Das, B., Sundaray, A., Satpathi, S., Thangaraj, P., Marimuthu, S., Jayabalan, R., 2018. Study of soy-fortified green tea curd formulated using potential hypocholesterolemic and hypotensive probiotics isolated from locally made curd. Food Chem 268, 558–566. doi:10.1016/j.foodchem.2018.06.114.

Murakami, A., 2014. Dose-dependent functionality and toxicity of green tea polyphenols in experimental rodents. Arch. Biochem. Biophys. 557, 3–10. doi:10.1016/j.abb.2014.04.018.

Naczk, M., Shahidi, F., 2006. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. J. Pharm. Biomed. Anal. 41 (5), 1523–1542. doi:10.1016/j.jpba.2006.04.002.

Nyambe-Silavwe, H., Villa-Rodriguez, J.A., Ifie, I., Holmes, M., Aydin, E., Jensen, J.M., Williamson, G., 2015. Inhibition of human α-amylase by dietary polyphenols. J. Function. Foods 19, 723–732. doi:10.1016/j.jff.2015.10.003.

OECD. (2017). *Obesity Update*. Retrieved from http://www.oecd.org/health/obesity-update.htm

Olthof, M.R., Hollman, P.C.H., Katan, M.B., 2001. Chlorogenic acid and caffeic acid are absorbed in humans. J. Nutr. 131 (1), 66–71.

Oniki, K., Kawakami, T., Nakashima, A., Miyata, K., Watanabe, T., Fujikawa, H., Nakashima, R., Nasu, A., Eto, Y., Takahashi, N., Nohara, H., Suico, M.A., Kotani, S., Obata, Y., Sakamoto, Y., Seguchi, Y., Saruwatari, J., Imafuku, T., Watanabe, H., Maruyama, T., Kai, H., Shuto, T., 2020. Melinjo seed extract increases adiponectin multimerization in physiological and pathological conditions. Sci Rep 10 (1), 4313. doi:10.1038/s41598-020-61148-2.

Pandey, K.B., Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev. 2 (5), 270–278.

Parisi, O.I., Puoci, F., Restuccia, D., Farina, G., Iemma, F., Picci, N., 2014. Chapter 4 - polyphenols and their formulations: different strategies to overcome the drawbacks associated with their poor stability and bioavailability. In: Watson, R.R., Preedy, V.R., Zibadi, S. (Eds.), Polyphenols in Human Health and Disease. Academic Press, San Diego, pp. 29–45.

Park, S., Kang, S., Jeong, D.Y., Jeong, S.Y., Park, J.J., Yun, H.S., 2015. Cyanidin and malvidin in aqueous extracts of black carrots fermented with Aspergillus oryzae prevent the impairment of energy, lipid and glucose metabolism in estrogen-deficient rats by AMPK activation. Genes Nutr 10 (2), 455. doi:10.1007/s12263-015-0455-5.

Peng, C.H., Cheng, J.J., Yu, M.H., Chung, D.J., Huang, C.N., Wang, C.J., 2020. Solanum nigrum polyphenols reduce body weight and body fat by affecting adipocyte and lipid metabolism. Food Funct 11 (1), 483–492. doi:10.1039/c9fo02240f.

Rahim, A., Takahashi, Y., Yamaki, K., 2015. Mode of pancreatic lipase inhibition activity in vitro by some flavonoids and non-flavonoid polyphenols. Food Res. Int. 75, 289–294. doi:10.1016/j.foodres.2015.05.017.

Rasheed, N.O., Ahmed, L.A., Abdallah, D.M., El-Sayeh, B.M., 2017. Nephro-toxic effects of intraperitoneally injected EGCG in diabetic mice: involvement of oxidative stress, inflammation and apoptosis. Sci. Rep. 7, 40617. doi:10.1038/srep40617.

Renaud, J., Martinoli, M., 2019. Considerations for the use of polyphenols as therapies in neurodegenerative diseases. Int. J. Mol. Sci. 20, 1883. doi:10.3390/ijms20081883.

Ringman, J.M., Frautschy, S.A., Teng, E., Begum, A.N., Bardens, J., Beigi, M., Gylys, K.H., Badmaev, V., Heath, D.D., Apostolova, L.G., Porter, V., Vanek, Z., Marshall, G.A., Hellemann, G., Sugar, C., Masterman, D.L., Montine, T.J., Cummings, J.L., Cole, G.M., 2012. Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. Alzheimer's Res. Ther. 4 (5), 43. doi:10.1186/alzrt146.

Rodriguez-Perez, C., Segura-Carretero, A., Del Mar Contreras, M., 2017. Phenolic compounds as natural and multifunctional anti-obesity agents: a review. Crit. Rev.Food Sci. Nutr. 1–18. doi:10.1080/10408398.2017.1399859.

Roh, E., Song, D.K., Kim, M.S., 2016. Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism. Exp. Mol. Med. 48, e216. doi:10.1038/emm.2016.4.

Romo Vaquero, M., Yanez-Gascon, M.J., Garcia Villalba, R., Larrosa, M., Fromentin, E., Ibarra, A., Roller, M., Tomas-Barberan, F., Espin de Gea, J.C., Garcia-Conesa, M.T., 2012. Inhibition of gastric lipase as a mechanism for body weight and plasma lipids reduction in Zucker rats fed a rosemary extract rich in carnosic acid. PLoS One 7 (6), e39773. doi:10.1371/journal.pone.0039773.

Salvado, L., Palomer, X., Barroso, E., Vazquez-Carrera, M., 2015. Targeting endoplasmic reticulum stress in insulin resistance. Trends Endocrinol. Metab. 26 (8), 438–448. doi:10.1016/j.tem.2015.05.007.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. Journal of Nutrition 130 (8S Suppl), 2073s–2085s. doi:10.1093/jn/130.8.2073S.

Scheepens, A., Tan, K., Paxton, J.W., 2010. Improving the oral bioavailability of beneficial polyphenols through designed synergies. Genes Nutr 5 (1), 75–87. doi:10.1007/s12263-009-0148-z.

Schmid, B., Rippmann, J.F., Tadayyon, M., Hamilton, B.S., 2005. Inhibition of fatty acid synthase prevents preadipocyte differentiation. Biochem. Biophys. Res. Commun. 328 (4), 1073–1082. doi:10.1016/j.bbrc.2005.01.067.

Seregelj, V., Tumbas Saponjac, V., Levic, S., Kalusevic, A., Cetkovic, G., Canadanovic-Brunet, J., Nedovic, V., Stajcic, S., Vulic, J., Vidakovic, A., 2019. Application of encapsulated natural bioactive compounds from red pepper waste in yogurt. J Microencapsul 36 (8), 704–714. doi:10.1080/02652048.2019.1668488.

Shah, N., Greenberg, J.A., Leverson, G., Funk, L.M., 2016. Predictors of high cost after bariatric surgery: a single institution review. Surgery 160 (4), 877–884. doi:10.1016/j.surg.2016.06.038.

Sharma, K., Kang, S., Gong, D., Oh, S.-H., Park, E.-Y., Oak, M.-H., Yi, E., 2018. Combination of garcinia cambogia extract and pear pomace extract additively suppresses adipogenesis and enhances lipolysis in 3T3-L1 cells. Pharmacogn. Mag. 14 (54), 220–226. doi:10.4103/pm.pm_388_17.

Shi, D., Chen, C., Zhao, S., Ge, F., Liu, D., Song, H., 2014. Walnut polyphenols inhibit pancreatic lipase activity in vitro and have hypolipidemic effect on high-fat diet-induced obese mice. J. Food Nutr. Res. 2 (10), 757–763. doi:10.12691/jfnr-2-10-16.

Shoji, T., Akazome, Y., Kanda, T., Ikeda, M., 2004. The toxicology and safety of apple polyphenol extract. Food Chem. Toxicol. 42 (6), 959–967. doi:10.1016/j.fct.2004.02.008.

Singla, P., Bardoloi, A., Parkash, A.A., 2010. Metabolic effects of obesity: a review. World J. Diabetes. 1 (3), 76–88. doi:10.4239/wjd.v1.i3.76.

Smeriglio, A., Barreca, D., Bellocco, E., Trombetta, D., 2017. Proanthocyanidins and hydrolysable tannins: occurrence, dietary intake and pharmacological effects. Br. J. Pharmacol. 174 (11), 1244–1262. doi:10.1111/bph.13630.

Smith, B.R., Schauer, P., Nguyen, N.T., 2011. Surgical approaches to the treatment of obesity: bariatric surgery. Med. Clin. N. Am. 95 (5), 1009–1030. doi:10.1016/j.mcna.2011.06.010.

Spalletta, S., Flati, V., Toniato, E., Di Gregorio, J., Marino, A., Pierdomenico, L., Marchisio, M., D'Orazi, G., Cacciatore, I., Robuffo, I., 2018. Carvacrol reduces adipogenic differentiation by modulating autophagy and ChREBP expression. PLoS One 13 (11), e0206894. doi:10.1371/journal.pone.0206894.

Srivastava, G., Apovian, C.M., 2018. Current pharmacotherapy for obesity. Nat Rev Endocrinol 14 (1), 12–24. doi:10.1038/nrendo.2017.122.

Srivastava, R.A., Pinkosky, S.L., Filippov, S., Hanselman, J.C., Cramer, C.T., Newton, R.S., 2012. AMP-activated protein kinase: an emerging drug target to regulate imbalances in lipid and carbohydrate metabolism to treat cardio-metabolic diseases. J. Lipid Res. 53 (12), 2490–2514. doi:10.1194/jlr.R025882.

Sroka, Z., Cisowski, W., 2003. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. Food Chem. Toxicol. 41 (6), 753–758. doi:10.1016/s0278-6915(02)00329-0.

Taguchi, C., Fukushima, Y., Kishimoto, Y., Suzuki-Sugihara, N., Saita, E., Takahashi, Y., Kondo, K., 2015. Estimated dietary polyphenol intake and major food and beverage sources among elderly Japanese. Nutrients 7 (12), 10269–10281. doi:10.3390/nu7125530.

Takei, K., Nakagawa, Y., Wang, Y., Han, S.I., Satoh, A., Sekiya, M., Matsuzaka, T., Shimano, H., 2017. Effects of K-877, a novel selective PPARalpha modulator, on small intestine contribute to the amelioration of hyperlipidemia in low-density lipoprotein receptor knockout mice. J. Pharmacol. Sci. 133 (4), 214–222. doi:10.1016/j.jphs.2017.02.003.

Thakar, C.V., Kharat, V., Blanck, S., Leonard, A.C., 2007. Acute kidney injury after gastric bypass surgery. Clin J Am Soc Nephrol 2 (3), 426–430. doi:10.2215/CJN.03961106.

Tong, Q., Hotamisligil, G.S., 2001. Molecular mechanisms of adipocyte differentiation. Rev. Endocr. Metab. Dis. 2 (4), 349–355.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2 (12), 1231–1246. doi:10.3390/nu2121231.

Tsujita, T., Takaichi, H., Takaku, T., Sawai, T., Yoshida, N., Hiraki, J., 2007. Inhibition of lipase activities by basic polysaccharide. J. Lipid Res. 48 (2), 358–365. doi:10.1194/jlr.M600258-JLR200.

Tucci, S.A., Boyland, E.J., Halford, J.C., 2010. The role of lipid and carbohydrate digestive enzyme inhibitors in the management of obesity: a review of current and emerging therapeutic agents. Diabetes Metab Syndr Obes 3, 125–143. doi:10.2147/dmsott.s7005.

Urbatzka, R., Freitas, S., Palmeira, A., Almeida, T., Moreira, J., Azevedo, C., Afonso, C., Correia-da-Silva, M., Sousa, E., Pinto, M., Vasconcelos, V., 2018. Lipid reducing activity and toxicity profiles of a library of polyphenol derivatives. Eur. J. Med. Chem. 151, 272–284. doi:10.1016/j.ejmech.2018.03.036.

Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F., Gallo, A., Scalfi, L., Fogliano, V., 2007. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. J. Nutr. 137 (9), 2043–2048.

Wang, L., Zeng, B., Liu, Z., Liao, Z., Zhong, Q., Gu, L., Wei, H., Fang, X., 2018a. Green tea polyphenols modulate colonic microbiota diversity and lipid metabolism in high-fat diet treated HFA mice. J. Food Sci. 83 (3), 864–873. doi:10.1111/1750-3841.14058.

Wang, S., Moustaid-Moussa, N., Chen, L., Mo, H., Shastri, A., Su, R., Bapat, P., Kwun, I., Shen, C.-L., 2014. Novel insights of dietary polyphenols and obesity. J. Nutr. Biochem. 25 (1), 1–18. doi:10.1016/j.jnutbio.2013.09.001.

Wang, T., Xue, C., Zhang, T., Wang, Y., 2018b. The improvements of functional ingredients from marine foods in lipid metabolism. Trends Food Sci. Technol. 81, 74–89. doi:10.1016/j.tifs.2018.09.004.

Wong, S.K., Chin, K.Y., Suhaimi, F.H., Fairus, A., Ima-Nirwana, S., 2016. Animal models of metabolic syndrome: a review. Nutr Metab 13, 65. doi:10.1186/s12986-016-0123-9.

Wood Dos Santos, T., Cristina Pereira, Q., Teixeira, L., Gambero, A., J, A.V., Lima Ribeiro, M., 2018. Effects of Polyphenols on thermogenesis and mitochondrial biogenesis. Int. J. Mol. Sci. 19 (9). doi:10.3390/ijms19092757.

World Health Organization. (2020). Obesity and overweight. Retrieved from https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.

Xu, X., Wang, H.-J., Murphy, P.A., Cook, L., Hendrich, S., 1994. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J. Nutr. 124 (6), 825–832. doi:10.1093/jn/124.6.825, http://doi.org/%J The Journal of Nutrition.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B.B., Kadowaki, T., 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat. Med. 8 (11), 1288–1295. doi:10.1038/nm788.

You, Q., Chen, F., Wang, X., Luo, P.G., Jiang, Y., 2011. Inhibitory effects of muscadine anthocyanins on alpha-glucosidase and pancreatic lipase activities. J. Agric. Food Chem. 59 (17), 9506–9511. doi:10.1021/jf201452v.

You, Q., Chen, F., Wang, X., Luo, P.G., Jiang, Y., 2018. In vitro inhibition of lipase, α-amylase,α-glucosidase, andangiotensin-converting enzyme by defatted rice branextracts of red-pericarp rice mutant. Cereal Chem 95, 167–176.

Yu, J.H., Kim, M.S., 2012. Molecular mechanisms of appetite regulation. Diabetes Metab 36 (6), 391–398. doi:10.4093/dmj.2012.36.6.391.

Yun, J.W., 2010. Possible anti-obesity therapeutics from nature–a review. Phytochemistry 71 (14-15), 1625–1641. doi:10.1016/j.phytochem.2010.07.011.

Zhang, M., Yang, X.J., 2016. Effects of a high fat diet on intestinal microbiota and gastrointestinal diseases. World J Gastroenterol 22 (40), 8905–8909. doi:10.3748/wjg.v22.i40.8905.

Zhao, L., Zhang, Q., Ma, W., Tian, F., Shen, H., Zhou, M., 2017. A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. Food Funct 8 (12), 4644–4656. doi:10.1039/c7fo01383c.

Zhou, H., Li, H.M., Du, Y.M., Yan, R.A., Ou, S.Y., Chen, T.F., Wang, Y., Zhou, L.X., Fu, L., 2017. C-geranylated flavanones from YingDe black tea and their antioxidant and alpha-glucosidase inhibition activities. Food Chem 235, 227–233. doi:10.1016/j.foodchem.2017.05.034.

Zhou, J., Mao, L., Xu, P., Wang, Y., 2018. Effects of (-)-epigallocatechin gallate (EGCG) on energy expenditure and microglia-mediated hypothalamic inflammation in mice fed a high-fat diet. Nutrients 10 (11). doi:10.3390/nu10111681.

Zielinski, H., Kozlowska, H., Lewczuk, B., 2001. Bioactive compounds in the cereal grains before and after hydrothermal processing. Innov. Food Sci. Emerg. Technol. 2, 159–169.

CHAPTER 3

Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*

Future Foods 4 (2021) 100087

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo

Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*

Manisha Singh [a,*], Thilini Thrimawithana [b], Ravi Shukla [a,c], Benu Adhikari [a,*]

[a] School of Science, RMIT University, Melbourne, VIC 3083, Australia
[b] Discipline of Pharmacy, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC 3083, Australia
[c] Nanobiotechnology Research Laboratory and Center for Advanced Materials and Industrial Chemistry, RMIT University, Melbourne, VIC 3001, Australia

ARTICLE INFO

Keywords:
Hibiscus sabdariffa
Polyphenols
Delphinidin 3-O-sambubioside chloride
Cyanidin 3-O-sambubioside chloride
Potassium hydroxycitrate

ABSTRACT

Hibiscus sabdariffa calyces were used to extract the polyphenolic compounds with three different solvents (water, methanol, and ethanol). The augmentation of temperature from 23 °C to 90 °C caused an adverse effect to polyphenols through thermal degradation and reduced antioxidant activity. Methanol and ethanol were more effective solvents than water for extracting polyphenolic compounds. The storage stability study at -30 °C for ten weeks showed that the antioxidant activity of extracts significantly decreased after 6 weeks; however, total phenolic content did not change significantly. Delphinidin 3-O-sambubioside chloride and cyanidin 3-O-sambubioside chloride were found to be the most abundant polyphenolic compounds in all three extracts. Potassium hydroxycitrate (180.14 μg/mg) was extracted from *H. sabdariffa* calyces, which is the highest reported value so far. Therefore, *H. sabdariffa* serves as a potential source for extracting potassium hydroxycitrate and phenolic compounds which are important for food and pharmaceutical applications.

1. Introduction

Hibiscus sabdariffa, also known as rosella or roselle, is an herbaceous subshrub belonging to the Malvaceae family grown in tropical and subtropical parts of the world (Da-Costa-Rocha et al., 2014). It is cultivated during the rainy season and produces bright red blooms. The calyces of this plant are then processed and preserved either by freezing or drying. In the food industry, both frozen and dried calyces of roselle are used in making beverages (tea and wines), jams, ice cream, sauces, and syrups. The calyces of this plant are also traditionally used for the treatment of ailments such as cold, cough, hypercholesterolemia, hypertension and indigestion (Riaz and Chopra, 2018).

H. sabdariffa calyces from different regions contain upto 29.53–87% carbohydrate, 7.4–12.3% ash, 5.5–9.14% protein, and 0.47–1.32% fat on a dry weight basis (Jabeur et al., 2017, 2019; Salami and Afolayan, 2021). In addition, roselle calyces are rich in dietary fibres, organic acids, and bioactive compounds. More recently, the total phenolic compounds, flavonoids, and anthocyanins in roselle calyces are quantified as 10.44–19.75 mg of gallic acid equivalents/g, 5.8–42.57 mg of catechin equivalents/g, and 4.45–5.39 mg of cyanidin-3-glucoside/g of dried calyces, respectively (Morales-Luna et al., 2018). The aqueous and methanolic extracts of *H. sabdariffa* calyces are shown to contain delphinidin 3-O-sambubioside and cyanidin 3-O-sambubioside as the major phenolic compounds, followed by chlorogenic acid, hydroxycoumarin, gallic acid, catechin, and epicatechin. Hibiscus acid and hydroxycitric acid (HCA) are identified as two main organic acids in the aqueous and methanolic extract (Fernández-Arroyo et al., 2011; Morales-Luna et al., 2018). HCA is a derivative of citric acid containing a hydroxyl group at the second carbon which is commonly extracted from *Garcinia cambogia* (Yamada et al., 2007). Naturally, it is present either as a biologically active free acid or its lower active lactone form. However, the free acid form is unstable, and therefore, HCA is extracted or stabilized in the form of salts (Na^+, K^+ or Ca^{2+}) (Chuah et al., 2013). *H. sabdariffa* calyces also contain ascorbic, citric, malic, and tartaric acids, which contribute to their acidic flavour (Izquierdo-Vega et al., 2020). These bioactive compounds and organic acids attribute to the reported bioactive properties such as antioxidative, anti-inflammatory, antilipidemic and antidiabetic properties of roselle. Currently, extract from roselle calyces is considered as a potential therapeutic agent for treating chronic diseases such as diabetes (Wang et al., 2011), cardiovascular diseases (Asgary et al., 2016) and obesity (Amaya-Cruz et al., 2019; Kao et al., 2016). Given the rising consumer preference for natural product-based therapies, including functional foods and supplements, roselle calcyes have the potential of being used to manage aforementioned health conditions. Functional compounds extracted from roselle calcyes can be easily incorporated in many food matrices and deliver to people who have risk of developing the condition or to reduce disease progression.

* Corresponding authors.
E-mail addresses: manisha.singh@rmit.edu.au (M. Singh), benu.adhikari@rmit.edu.au (B. Adhikari).

https://doi.org/10.1016/j.fufo.2021.100087
Received 21 July 2021; Received in revised form 4 September 2021; Accepted 28 September 2021

The polyphenolic compounds from *H. sabdariffa* are extracted in the aqueous medium (Kao et al., 2016) and acidified ethanol (Borrás-Linares et al., 2015). Mohd-Esa et al. (2010) extracted bioactive compounds from different parts of roselle using water and methanol (80% v/v) and reported the latter to be more effective in terms of higher total phenolic yield and preservation of potency. Similarly, Sindi et al. (2014) compared the effectiveness of four different solvents including water, ethyl acetate, hexane, and methanol with or without formic acid for extracting bioactive compounds from roselle. The authors reported that the aqueous extraction with and without formic acid was better than other solvents for the recovery of phenolic compounds including anthocyanins.

Although hydroxycitric acid has been identified in various extracts of roselle calyces, its specific extraction is not commonly reported. Also, the effectiveness of solvents used for the extraction of polyphenolic compounds from *H. sabdariffa* is rarely compared. In addition, the stability of polyphenol-rich extracts obtained from the commonly used extraction method is not currently reported. Hence, this study aims to develop effective solvent extraction method for the extraction of polyphenolic compounds from *H. sabdariffa* and determine the storage stability of these extracts. Moreover, this study aims to develop a specific method for the extraction of potassium hydroxycitrate from roselle.

2. Materials and methodology

2.1. Plant material

Frozen calyces of *H. sabdariffa* were donated by Wild Hibiscus Flower Co., NSW, Australia and were stored at -20 °C until their use.

2.2. Chemicals

Methanol, ethanol, potassium hydroxide, folin-ciocalteu reagent, sodium carbonate, aluminium chloride hexahydrate, potassium acetate, 2, 2-Diphenyl-1-picrylhydrazyl free radical (DPPH), ferric chloride hexahydrate, hydrochloric acid, iron (II) sulfate, sodium acetate trihydrate, 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), acetonitrile (HPLC grade), sulphuric acid, formic acid (HPLC grade), dimethyl sulfoxide (DMSO) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were procured from Sigma-Aldrich (Castle Hill NSW, Australia). Standards used for this study: 4-hydroxybenzoic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), caffeic acid, catechin hydrate, *ρ*-coumaric acid, cyanidin chloride, delphinidin chloride, ferulic acid, gallic acid, potassium hydroxycitrate, quercetin, and rutin hydrate were also acquired from Sigma-Aldrich (Castle Hill NSW, Australia). Cyanidin-3-O-sambubioside chloride and delphinidin-3-O-sambubioside chloride were purchased from Extrasynthese (Genay Cedex, France). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), penicillin, streptomycin, trypsin–EDTA, and phosphate-buffered saline (PBS) were purchased from Thermo Fischer Scientific Pty. Ltd. (Scoresby, VIC, Australia). All of the above-mentioned chemicals were of analytical reagent grade except acetronitrile and formic acid which were HPLC grade, and were used as received. For all the experiments, water from the Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., NSW, Australia) was used.

2.3. Preparation of sample

Frozen calyces of *H. sabdariffa* were dried using a freeze drier (Labconco Triad 7400030, Missouri, USA) and powdered using a grinder (Breville, Sydney, Australia). The powder was sieved through 44 mesh size (353 μm) and stored in ziplock bags in a desiccator covered with aluminium foil at room temperature for further tests. The moisture content of powder was measured with a moisture analyzer (MB45 Ohaus, Nanikon, Switzerland) and was 3.11 ± 0.09% (w/w).

2.4. Extraction of bioactive compounds with water and organic solvents

Polyphenolic compounds from powdered calyces were extracted using water, methanol, and ethanol. Calyces powder (5 g) was mixed with 100 mL of purified water at four different temperatures (23 °C, 50 °C, 75 °C, and 90 °C). The mixtures were heated using a hot plate (MR HeiTec, Heidolph, Germany) and agitated using a magnetic stirrer at 500 rpm for 2 h to extract the polyphenols. Then the extract was centrifuged (Allegra 64R, Beckman Coulter, Australia) at 11,000 × g for 20 min, filtered with Whatman no. 1 and the supernatant was freeze-dried (Labconco Triad 7400030, Missouri, USA). Polyphenolic compounds were also extracted using three concentrations (50, 75, and 100% v/v) of methanol and ethanol at room temperature (23 °C) using a magnetic stirrer at 500 rpm for 2 h as mentioned above. It was then vacuum filtered using a Whatman no. 1 filter paper and then evaporated in a rotary vacuum concentrator to obtain dried extracts (Christ, Osterode am Harz, Germany). The dried extracts were weighed, and the yield was calculated. Finally, the samples were flushed with nitrogen and stored in amber-colored bottles at -30 °C for further tests.

2.5. Characterization of extracts

The stock solutions of extracts were prepared at the concentration of 1 mg/mL in the solvent used for their extraction. For total phenolic content (TPC) and 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH radical scavenging assay), the stock solution was diluted to 0.1 mg/mL in purified water whereas, for total flavonoid content (TFC) and ferric reducing antioxidant power (FRAP) assay, the 1 mg/mL solution was used.

2.5.1. Total phenolic content

The total phenolic content of the extract was determined by using the modified Folin-Ciocalteu method as mentioned by Ainsworth and Gillespie (2007). The assay was conducted by adding 100 μL of sample in a 48-well plate (Greiner Bio-One, Austria). To this, 200 μL of Folin-Ciocalteu reagent (10% v/v) was added and mixed thoroughly. Then 800 μL of 7.5% (w/v) sodium carbonate solution was added to the solution for neutralization. The mixture was allowed to react in dark at room temperature for 1 h with intermittent shaking. The absorbance was measured spectrophotometrically at 765 nm using a CLARIOstar® microplate reader (BMG Labtech, Germany). The total phenolic content (TPC) of the extracts was determined using gallic acid as the standard and is expressed as mg gallic acid equivalents (GAE)/g of dry extract. Six different concentrations of gallic acid ranging from 0.01 to 0.1 mg/mL were used to establish a standard curve and the R^2 value of the regression equation was 0.99.

2.5.2. Total flavonoid content

The total flavonoid content of the extracts was determined by a colorimetric method as described by Chang et al. (2002) with minor modifications. Sample (100 μL) was mixed with 300 μL of 95% (v/v) ethanol, 20 μL of 10% (w/v) aluminium chloride hexahydrate, 20 μL of 1 M potassium acetate and finally 560 μl of water was added in the 48-well plate. The mixture was incubated for 30 min at room temperature and the absorbance was read at 415 nm spectrophotometrically with the microplate reader mentioned above. Rutin was used as the standard to determine TFC values of the samples. A standard (calibration) curve (R^2 = 0.99) was established using six concentrations of rutin in 0.01–0.2 mg/mL range. The TFC values are presented as mg rutin equivalents (RE)/g of dry extract.

2.5.3. DPPH radical scavenging assay

The DPPH radical-scavenging activity of each extract was measured by following a method as reported by Mishra et al. (2012). For this, 300 μL of sample and 300 μL of DPPH solution (0.004% w/v in methanol) were poured into a 48-well plate. After incubating in dark

for 45 min, the absorbance of the solution was recorded at 515 nm with the microplate reader. The DPPH inhibition (%) was calculated using Eq. (1) given below.

$$\text{DPPH inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100\ \% \quad (1)$$

Trolox was used as a positive standard and calibration curve ($R^2 = 0.99$) was obtained from six concentrations of trolox ranging from 1 to 10 μg/mL. Finally, the results are expressed as mg Trolox equivalents (TE)/g of dry extract.

2.5.4. Ferric reducing antioxidant power assay

The FRAP analysis of the extract samples was carried out according to the procedure followed by Benzie and Strain (1996) and Wong et al. (2006) with minor modifications. TPTZ (10 mM) solution was prepared in 40 mM HCl. FRAP reagent was prepared by mixing acetate buffer (pH 3.6), TPTZ solution, and 20 mM iron (III) chloride solution in a 10:1:1 (v/v) ratio. This solution was freshly prepared and warmed to 37 °C before use. Then, FRAP solution (300 μL) was added to 20 μL of sample in the 48-well plate. After 10 min, the absorbance was measured at 593 nm using the microplate reader. Iron (II) sulfate was used as the standard for this assay. The standard curve ($R^2 = 0.99$) was generated by plotting six concentrations of iron (II) sulfate from 100 to 1000 μM and was used for the determination of FRAP values. The results are expressed as μmol Fe (II)/g of dry extract.

2.5.5. Assessing the stability of the extract

Storage stability of the extracts was determined by storing the nitrogen flushed samples at -30 °C and evaluating TPC, TFC, DPPH, and FRAP every week for 10 weeks. Samples were stored separately in amberedcolored bottles and were tested every week one sample at a time to avoid opening and closing.

2.6. Characterization of polyphenols in extracts

The polyphenolic profile of the extracts was quantified using Agilent 1290 Infinity Ultra-Performance Liquid Chromatography (UPLC) coupled to a diode array detector (DAD) system (Agilent Technologies, California, USA) (Morales-Luna et al., 2018). Three μL of sample diluted in the mobile phase was injected into ZORBAX Eclipse Plus Rapid Resolution HD C18 column (2.1 × 150 mm, 1.8 μm) at 25 °C. The mobile phase was comprised of acetonitrile and water in the ratio of 10:90 with 1% (v/v) formic acid as solvent A and acetonitrile with 0.1% (v/v) formic acid as solvent B. The flow rate was maintained at 0.2 mL/min throughout the experiment. The gradient elution was initiated with 95% A and held for 2 min which was decreased to 80% at 22 min and then to 60% at 27 min. The conditions were returned to 95% A at 32 min and then maintained at the isocratic condition for 8 min. Absorbance was measured at 280 nm, 320 nm, 365 nm, and 520 nm for hydroxybenzoic acids and flavanols, hydroxycinnamic acids, flavonols and anthocyanins, respectively. Commercial standards were used for method validation and sample quantification, and the results are expressed as milligram of each phenolic compound per gram of dry extract.

2.7. Validation of UPLC-DAD method

Standard solutions of 4-hydroxybenzoic acid, caffeic acid, catechin hydrate, coumaric acid, cyanidin chloride, cyanidin-3-O-sambubioside chloride, delphinidin-3-O-sambubioside chloride, delphinidin chloride, ferulic acid, quercetin, and rutin hydrate were prepared by dissolving 1 mg/mL of each in ethanol. Similarly, one mg/mL solutions of gallic acid was prepared using Milli-Q water. The above-mentioned standard solutions were diluted to 0.05, 0.1, 0.5, 1, 5, 10 and 50 ug/mL in mobile phase (A). They were injected into UPLC-DAD system to create a calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) were calculated for reliability and accuracy of the results. LOD and LOQ were calculated using Eqs. (2) and (3), respectively.

$$\boldsymbol{LOD\ (Limit\ of\ Detection) = \frac{3.3\sigma}{S}} \quad (2)$$

$$\boldsymbol{LOQ\ (Limit\ of\ Quantification) : \frac{10\sigma}{S}} \quad (3)$$

Where σ is the standard deviation of the y-intercept of the regression line and S is the slope of the calibration line.

2.8. Extraction of potassium hydroxycitrate

Hydroxycitric acid was extracted in the form of its potassium salt according to the method described by Majeed et al. (2004). *Hibiscus* calyces powder (5 g) was dissolved in 15 mL of methanol at 65 °C for 3 h. The extract was filtered through Whatman no 1 filter paper and the filtrate was collected. The residue was extracted twice with the same volume of methanol at the same temperature and time. All three extracts were combined and treated with 1 M methanolic potassium hydroxide at pH 10 for 3 h to precipitate potassium hydroxycitrate. The precipitated potassium hydroxycitrate was filtered and washed with methanol and finally dried in a vacuum oven (XLF020, Ezzi Vision, Australia) at 70 °C for 2 h. The dried hydroxycitrate was then stored at 4 °C in an airtight container. We also treated the methanolic extract with activated charcoal for 1 h to remove anthocyanins that contribute to the color of extract. Then it was filtered through Whatman no 1 filter paper and treated with 1 M of methanolic potassium hydroxide at pH 10 for 3 h to precipitate potassium hydroxycitrate. The precipitated potassium hydroxycitrate was filtered and washed with methanol and vacuum oven dried as mentioned above.

2.9. Characterization of potassium hydroxycitrate

2.9.1. Characterization using Fourier transform infrared spectroscopy (FTIR)

A Perkin Elmer Spectrum Two FTIR spectrometer (Norway, CT, USA) fitted with a Gladi ATR from Pike Technologies (Wisconsin, USA) was used to obtain transmittance spectra of standard potassium hydroxycitrate and samples obtained as described above. Measurements were carried out from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1} and averaged using 64 scans.

2.9.2. Quantification of potassium hydroxycitrate using high performance liquid chromatography with ultra violet detector (HPLC-UV)

The concentration of potassium hydroxycitrate in the extract/sample was determined using an HPLC (Agilent 1220 Infinity LC) coupled to UV/Vis detector system (Agilent Technologies, Palo, Alto, CA) according to Jayaprakasha and Sakariah (2002). The sample was dissolved in water at 1 mg/mL and 10 μL was injected into a reverse-phase column (Zorbax Eclipse XDB – C18, 4.6 × 250 mm, 5 μm) at 25 °C. Isocratic elution was achieved using 0.01 M H_2SO_4 as mobile phase at a flow rate of 1 mL/min. The detection wavelength for these tests was 210 nm.

2.9.3. Validation of HPLC-UV method

Standard stock solution (1 mg/mL) of potassium hydroxycitrate was prepared in water which was then diluted to 50 to 800 μg/mL. These standards were injected into the HPLC-UV system and the calibration curve was prepared. LOD, LOQ, and recovery were calculated for reliability and accuracy of the results. LOD and LOQ were calculated using Eqs. (2) and (3), respectively given above. To determine the recovery, 200 μg/mL of standard potassium hydroxycitrate was added to the sample, and recovery was calculated using Eq. (4).

$$\text{Recovery (\%)} = \frac{\text{Conc. of spiked sample} - \text{Conc. of unspiked sample}}{\text{Conc. of standard added}} \times 100\ \% \quad (4)$$

Table 1
Effect of extraction temperature and solvent concentration on yield, TPC, TFC, DPPH inhibition, and FRAP of aqueous extract.

Extraction parameter	Extract yield (%)	TPC(mg GAE/ g)	TFC(mg RE/ g)	DPPH inhibition (mg TE/ g)	FRAP(μmol Fe/ g)
Water at 23 °C	29.96 ± 1.64^{a}	37.36 ± 1.23^{a}	14.79 ± 0.81^{a}	77.32 ± 3.48^{a}	767.99 ± 59.21^{a}
Water at 50 °C	34.92 ± 0.28^{b}	25.45 ± 2.13^{b}	13.30 ± 0.49^{ab}	73.42 ± 2.23^{ab}	643.32 ± 17.46^{b}
Water at 75 °C	39.09 ± 2.39^{b}	18.47 ± 2.56^{c}	12.44 ± 0.81^{b}	70.45 ± 0.85^{b}	638.03 ± 42.88^{b}
Water at 90 °C	46.88 ± 1.62^{c}	11.49 ± 0.71^{d}	9.34 ± 0.32^{c}	68.40 ± 1.48^{b}	607.40 ± 34.91^{b}
Methanol (23 °C)					
50% aq.	38.42 ± 0.91^{a}	30.79 ± 1.88^{c}	17.35 ± 0.64^{b}	79.93 ± 0.32^{b}	1001.48 ± 58.82^{b}
75% aq.	37.95 ± 0.52^{a}	39.82 ± 2.56^{b}	19.81 ± 1.03^{a}	84.57 ± 0.97^{a}	1168.41 ± 73.27^{a}
100%	33.19 ± 0.42^{b}	51.72 ± 0.71^{a}	21.41 ± 0.49^{a}	85.13 ± 1.12^{a}	943.37 ± 73.27^{b}
Ethanol (23 °C)					
50% aq.	39.25 ± 0.28^{a}	35.71 ± 4.32^{b}	19.49 ± 0.92^{a}	80.86 ± 3.17^{a}	1037.40 ± 59.38^{a}
75% aq.	37.06 ± 0.18^{b}	35.30 ± 2.13^{b}	20.88 ± 1.47^{a}	83.09 ± 2.25^{a}	1115.58 ± 73.68^{a}
100%	15.01 ± 0.69^{c}	48.85 ± 1.88^{a}	21.73 ± 1.29^{a}	84.94 ± 2.81^{a}	1057.48 ± 47.58^{a}

The different letters in superscript within a column indicate statistically significant differences ($p < 0.05$) within the group.
TPC = Total phenolic content, TFC = Total flavonoid content, DPPH =2, 2-Diphenyl-1-picrylhydrazyl inhibition, FRAP = Ferric reducing antioxidant power, GAE = Gallic acid equivalents, RE= Rutin equivalents, TE = Trolox equivalents and Fe = Ferrous ion equivalents

2.10. Cytotoxicity assay

MTT assay was performed with 3T3-L1 preadipocytes to determine the cytotoxicity of *Hibiscus* extracts. 3T3-L1 cells have a fibroblast-like appearance that can be differentiated into mature adipocytes under appropriate treatment. The cells were maintained in DMEM supplemented with 10% bovine calf serum (BCS) and 1% penicillin-streptomycin. The 3T3-L1 cells were harvested and seeded in a 96-well plate (Corning, Sigma-Aldrich) at a concentration of 7000 cells per cm^2. The cells were incubated at 37 °C humidified incubator maintained at 5% CO_2 for 18 h. A stock solution of 100 mg/mL of each extract was prepared in DMSO whereas potassium hydroxycitrate was dissolved in water. They were serially diluted in media and cells were treated with concentrations of 10, 50, 100, 200, 500, and 1000 μg/mL for 48 h. Following the incubation, the cells were washed with PBS. Then, 100 μL of MTT reagent (0.5 mg/mL in serum-free media) was added to each well and again incubated at 37 °C for 4 h. After incubation, the reagent was replaced with 100 μL of DMSO to dissolve formazan crystals from live cells. The plate was gently agitated and placed in the dark for 30 min incubation at room temperature. Then absorbance of the resulting solution was measured at 570 nm using a microplate reader. The percent inhibition was expressed as the percentage of the viable cells compared to untreated control.

2.11. Statistical analysis

Each of the experiments was performed in triplicate and the results are exhibited as mean ± standard deviation. SPSS statistical software (SPSS 23.0, IBM, Armonk, NY, USA) was used to perform statistical analysis. One-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference tests and independent samples t-tests were applied to determine the significant difference (95% confidence, $p < 0.05$) between two mean values.

3. Result and discussion

3.1. Effect of temperature on the yield, phenolic content, flavonoid content and antioxidant activity

The effect of temperature on the yield and antioxidant properties of the aqueous extract of *H. sabdariffa* is provided in Table 1. As can be observed, the yield of extract increased significantly ($p < 0.05$) by almost 1.5 times when the temperature increased from 23 °C to 90 °C. However, the use of higher extraction temperature resulted in a reduction in the total phenolic content (TPC) as well as the extract's antioxidant properties (TPC, TFC, DPPH, and FRAP; Table 1). All the properties, except FRAP, remain unchanged ($p > 0.05$) when the temperature was increased from 23 °C to 50 °C. However, a further increase in temperature leads to a significant decrease. Kao et al. (2016) reported an extraction yield of 50% from *H. sabdariffa* in an aqueous medium at 95 °C for 2 h which is close to the ~47% yield in this study under similar condition. Thirty percent extraction yield was reported when calyces were extracted at 50 °C for 1 h in water (Alarcon-Aguilar et al., 2007) which is also close to the ~35% yield achieved in this study. The TPC and TFC values of roselle extracted in boiling water were reported to 14.24 mg GAE/g and 10.37 mg catechin equivalent/g, respectively, (Amaya-Cruz et al., 2019) which is comparable to the corresponding values (11.49 mg GAE/g and 9.34 mg RE/g, respectively) obtained in this study. The small differences between the reported values and our data can be due to variation in variety and geographical source of *H. sabdariffa.* It is reported that elevated temperatures weaken the cell wall of *H. Sabdariffa* calyces and make it easier for extracting solvents to interact with the cellular components (Rosello-Soto et al., 2019). Faster outward diffusion of cellular components at higher temperatures increases the extraction. However, it also leads to a greater degree of degradation of heat-sensitive compounds including polyphenols and other antioxidants (Rosello-Soto et al., 2019). Beside, higher temperatures can also hasten and increase the extraction of non-phenolic compounds such as dietary fibres and sugars and lowers the proportion of phenolic and flavonoid compounds in the final extract. These observations show that extraction of polyphenols from *Hibiscus* at lower temperature is better than at higher temperature and therefore, 23 °C was used for the subsequent studies.

3.2. Effect of solvent on the yield, phenolic content, flavonoid content and antioxidant activity

After noting that the TPC, TFC, DPPH, and FRAP of the extracts were negatively impacted by the higher temperature (Table 1), a room temperature (23 °C) extraction method in methanol and ethanol was used for polyphenols. The influence of three different concentrations (50%, 75%, and 100%) of methanol and ethanol on these antioxidant properties of polyphenols is presented in Table 1.

As can be observed, a higher concentration of both methanol and ethanol led to significantly ($p < 0.05$) lower extraction yield. The extraction yield in pure methanol (33.19%) and pure ethanol (15.01%) was substantially lower than their corresponding values at 50% methanol (38.42%) and 50% ethanol (39.25%). Interestingly, TPC and TFC values in the methanolic (51.72 mg GAE/g and 21.41 mg RE/g) and ethanolic extract (48.85 mg GAE/g and 21.73 mg RE/g) increased significantly ($p < 0.05$) when the concentration of methanol and ethanol increased above 50% (v/v). The highest TPC was achieved in 100% methanol extract whereas both pure methanol and ethanol assisted in the highest

Table 2.
Linearity of calibration curves, limit of detection (LOD) and limit of quantification (LOQ) of standard polyphenols as a function of concentration (x).

Standards	Standard calibration equation (y = ax + b)			LOD(μg/mL)	LOQ(μg/mL)
	a	b	R^2		
Hydroxybenzoic acids					
Gallic acid	122.71	82.42	0.997	0.29	0.88
4-Hydroxybenzoic acid	142.73	50.20	0.999	0.88	2.68
Hydroxycinnamic acids					
Caffeic acid	476.82	-72.47	0.999	0.30	0.92
Ferulic acid	511.50	-13.09	0.999	0.11	0.34
ρ-Coumaric acid	527.98	-45.06	0.999	0.14	0.42
Flavonols					
Catechin hydrate	61.42	6.77	0.999	0.30	0.90
Flavonols					
Rutin hydrate	144.91	42.64	0.999	0.76	2.31
Quercetin	492.48	117.40	0.999	0.67	2.04
Anthocyanins					
Cyanidin 3-O-sambubioside chloride	147.78	95.59	0.996	1.58	4.80
Cyanidin chloride	326.43	-178.12	0.999	1.05	3.17
Delphinidin 3-O-sambubioside chloride	146.04	224.34	0.990	3.45	10.45
Delphinidin chloride	15.34	-26.37	0.998	3.44	10.42

TFC values. The variation in the concentration of ethanol did not significantly affect the DPPH and FRAP values. In methanolic extracts, DPPH value increased significantly with the increase of methanol concentration. But FRAP value of methanolic extract initially increased at 75% and then decreased at 100%. Pimentel-Moral et al. (2018) observed a large variation in extraction yield (10.3% to 47.1%) from *H. sabdariffa* when microwave-assisted extraction was used as a function of ethanol concentration (6% to 84%). The TPC and TFC of 25 different varieties of *H. sabdariffa* in acidified ethanol extract were reported to be between 24–100 mg GAE/g and 4.19–22.6 mg quercetin equivalent/g, respectively, (Borrás-Linares et al., 2015) which agree to our results. Do et al. (2014) observed a similar pattern of decrease in extraction yield and increase in TPC and TFC with the increase in the concentration of ethanol and methanol in *Limnophila aromatic* extracts. The results presented in Table 1 shows that the combination of water and organic solvent leads to a higher extraction yield. This may be explained as a combination of solvents with a variation in the degree of polarity may facilitate the extraction of a broad range of compounds based on their solubility in different solvents (Do et al., 2014).

3.3. Storage stability of extract

Aqueous and alcoholic extracts of *H. sabdariffa* with the highest polyphenolic content and antioxidant activity were selected to study their storage stability (water at 23 ˚C, 100% methanol at 23 ˚C, and 100% ethanol at 23 ˚C). The stability of extract during storage at -30 ˚C was studied in terms of TPC, TFC, DPPH, and FRAP values, and the results are presented in Fig. 1. The TPC values were not significantly affected by storage at -30 ˚C for 10 weeks whereas TFC values decreased significantly after 8 weeks of storage. This indicates that the polyphenols and flavonoids from *H. sabdariffa* are stable up to 8 weeks when stored with nitrogen flushing. However, there was a significant decrease in DPPH and FRAP values after 6 weeks of storage indicating that the decline in antioxidant activity of extracts during storage may be due to the degradation/oxidation of phenolic compounds (Ali et al., 2018).

3.4. Validation of UPLC-DAD method for the quantification of polyphenols

The standard calibration curves for all the polyphenolic standards were generated based on the intensity (y) at different concentration (x) at specific wavelength. The calibration equation, LOD, LOQ and regression coefficient (R^2) values for each tested polyphenol is presented in Table 2. The regression line for all the standards showed high degree of linearity ($R^2 > 0.99$) in the concentration range (x) tested.

Lowest LOD and LOQ were attributed by ferulic acid whereas highest values by delphinidin chloride. Comparatively, the LOD and LOQ values for anthocyanins are higher than that for other polyphenols. Fathimah et al. (2020) reported LOD and LOQ values ranging from 0.219 to 0.256 μg/mL and 0.808 to 1.245 μg/mL for four different polyphenolic compounds from *H. sabdariffa* flowers quantified with UPLC-PDA method.

3.5. Quantification of polyphenols in the extracts

The main polyphenol compounds of *H. sabdariffa* extracts were determined using UPLC-DAD on 0-week and 10-week of storage, and the results are summarised in Table 3. The UPLC-DAD chromatogram of methanolic extract from *H. sabdariffa* was presented as representative chromatogram in Fig. 2. Delphinidin 3-O-sambubioside chloride (20.71–35.29 mg/g) and cyanidin 3-O-sambubioside chloride (7.27–11.07 mg/g) were found to be the dominant polyphenolic compounds in all three extracts. Morales-Luna et al. (2018) also reported the maximum concentration of delphinidin 3-O-sambubioside chloride and cyanidin 3-O-sambubioside chloride among the polyphenols in both aqueous and methanolic extract of roselle. As can be seen from the data, the methanolic extract had a higher concentration of these two anthocyanins followed by ethanolic and water extracts. These data also corroborate total phenolic content (Table 1) which showed a similar concentration of total polyphenolic content in aqueous, methanolic, and ethanolic extracts. Catechin hydrate was found to be the third most abundant polyphenolic compound in these three extracts. When stored at -30 ˚C, most of the polyphenolic compounds in the extract were only slightly reduced as compared to their respective initial value except *ρ*-coumaric acid and caffeic acid which faintly increased during the storage period (Table 3).

3.6. Characterization of potassium hydroxycitrate

3.6.1. Yield

The yield of potassium hydroxycitrate from untreated and activated charcoal treated method was 150.2 ± 7.10 mg/g and 33.2 ± 2.1 mg/g of dried calyces accounting for 15.02% and 3.33%, respectively of dried calyces. Treatment with activated charcoal was carried out to remove the color compounds, mostly anthocyanins, and to obtain the more concentrated and clear extract. The charcoal treatment substantially reduced the yield of potassium hydroxycitrate (15.02% to 3.33%); albeit it improved the purity of the extracted potassium hydroxycitrate.

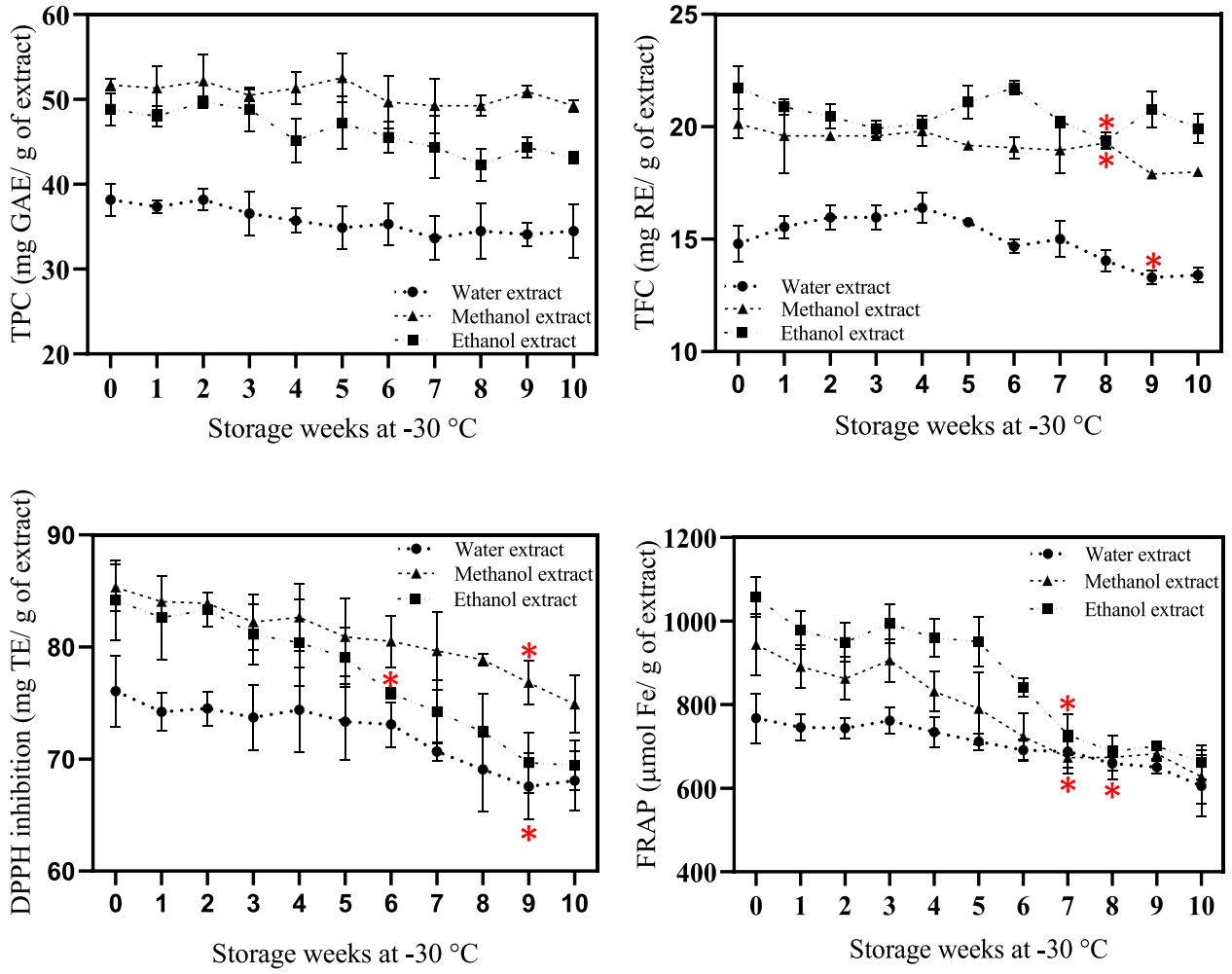


Fig. 1. Storage stability study of *H. sabdariffa* extracts at -30 °C in terms of total phenolic content (TPC), total flavonoid content (TFC), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) inhibition, Ferric reducing antioxidant power (FRAP) values. * indicates statistically significant ($p < 0.05$) decrease in the value as compared to the initial value.

Table 3.
The concentration of major polyphenolic compounds in aqueous, methanol and ethanol extracts of *Hibiscus sabdariffa.*

Compound (mg/ g)	Retention time (min)	Water Extract		Methanol Extract		Ethanol Extract	
		0 week	10 week	0 week	10 week	0 week	10 week
Hydroxybenzoic acids							
Gallic acid	2.21	ND	ND	ND	ND	0.81 ± 0.13	0.64 ± 0.05
4-Hydroxybenzoic acid	4.71	ND	ND	0.13 ± 0.06	0.16 ± 0.02	ND	ND
Hydroxycinnamic acids							
Caffeic acid	5.47	0.18 ± 0.00	0.19 ± 0.06	0.27 ± 0.01	0.36 ± 0.04	0.46 ± 0.09	0.31 ± 0.08
Ferulic acid	11.58	ND	ND	ND	ND	ND	ND
ρ-Coumaric acid	9.55	ND	ND	0.23 ± 0.04	0.30 ± 0.03	ND	ND
Flavanols							
Catechin hydrate	3.91	1.25 ± 0.21	0.821 ± 0.25	1.88 ± 0.24	1.65 ± 0.15	1.79 ± 0.15	1.65 ± 0.23
Flavonols							
Rutin hydrate	12.64	0.16 ± 0.07	0.12 ± 0.03	0.39 ± 0.04	0.35 ± 0.03	0.46 ± 0.04	0.27 ± 0.02
Quercetin	26.56	ND	ND	0.05 ± 0.02	0.02 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
Anthocyanins							
Cyanidin 3-O-sambubioside chloride	3.32	7.27 ± 0.28	7.01 ± 0.27	11.07 ± 0.84	10.31 ± 0.11	8.87 ± 0.37	6.16 ± 0.06
Cyanidin chloride	12.09	ND	ND	ND	ND	ND	ND
Delphinidin 3-O-sambubioside chloride	2.49	20.71 ± 0.83	19.519 ± 0.64	35.29 ± 2.69	32.52 ± 0.157	23.98 ± 1.12	16.66 ± 0.22
Delphinidin chloride	6.92	ND	ND	ND	ND	ND	ND

ND = Not determined

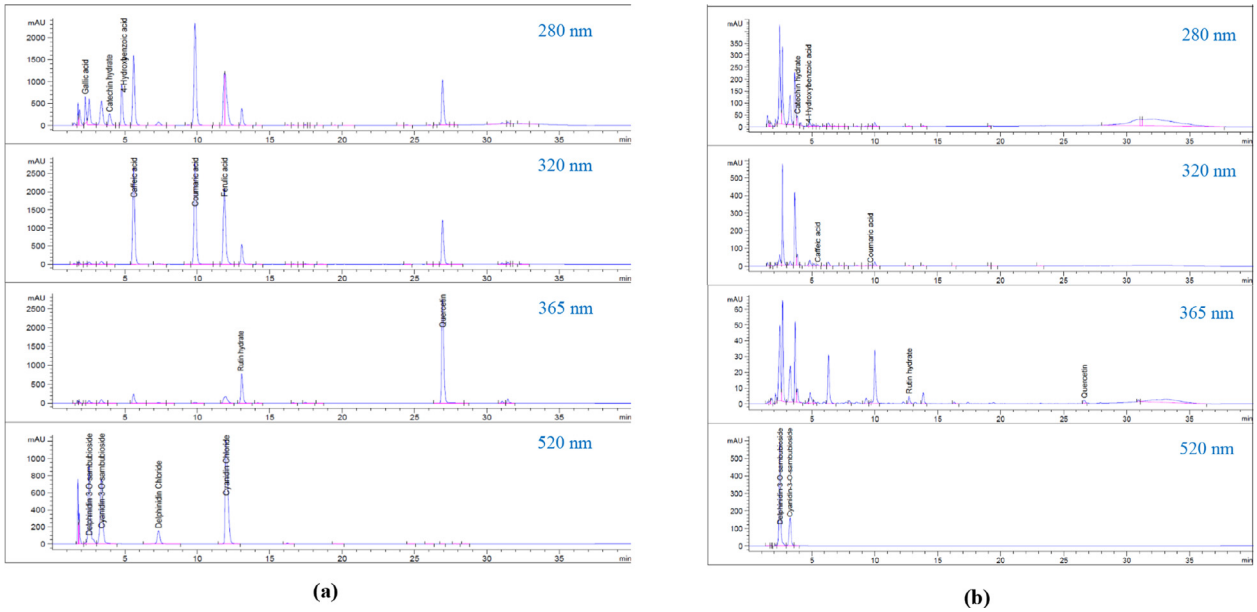


Fig. 2. UPLC-DAD chromatograms of (a) polyphenols used as the standard (50 μg/mL) and (b) methanolic extract from *H. sabdariffa* presented as representative sample.

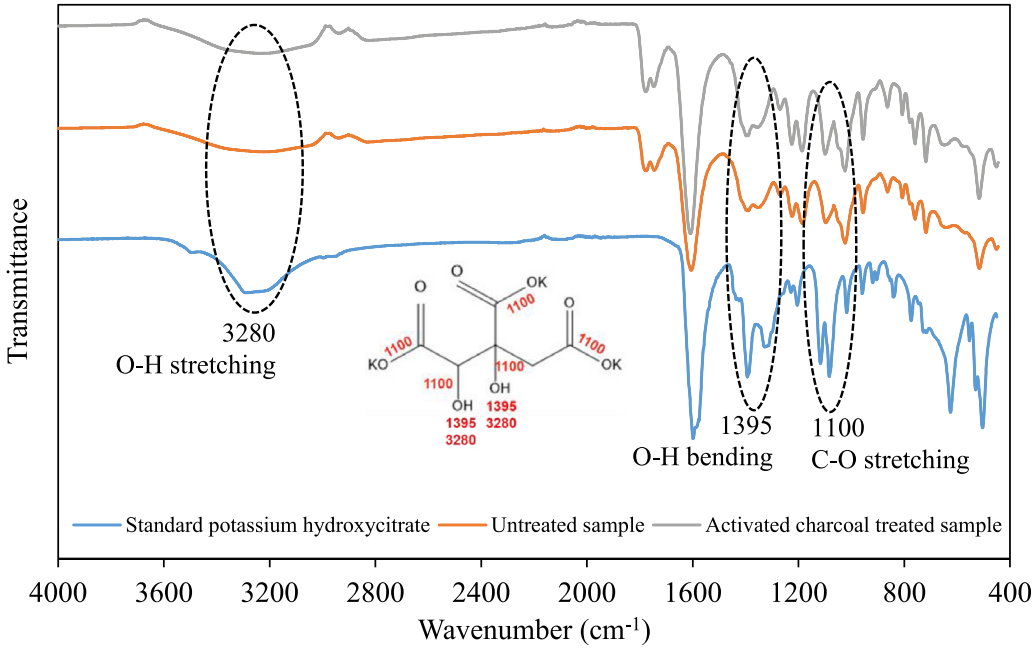


Fig. 3. FTIR spectra of standard potassium hydroxycitrate, untreated sample and activated charcoal treated sample.

3.6.2. Comparison of extracted and commercial potassium hydroxycitrate

The FTIR spectra of potassium hydroxycitrate extracted in this work and that of the commercially available standard are provided in Fig. 3. The spectra confirmed that the extracted potassium hydroxycitrate had similar bonds (C-O and O-H) as in the commercial one. All the characteristic bonds present in the standard appeared in the extracted sample albeit at different intensities indicating the presence of some impurities in the extracted sample. The key spectral peaks were observed at 3280 cm^{-1} for O-H stretching, 1395 cm^{-1} for O-H bending, and 1100 cm^{-1} for C-O stretching. The position of these functional groups in the chemical structure of potassium hydroxycitrate is also presented in Fig. 3. Hence, this confirms that the both samples contain potassium hydroxycitrate along with some impurities.

3.6.3. Validation of HPLC-UV method for potassium hydroxycitrate

The standard calibration equation of potassium hydroxycitrate as a function of its concentration yielded a straight line with a regression coefficient (R^2 >0.99) confirming a high degree of linearity. Recovery of potassium hydroxycitrate from the extract was 89.32% which signified a significantly higher recovery. The LOD and LOQ values of potassium hydroxycitrate were 2.34 μg/mL and 7.10 μg/mL, respectively. These values indicated that even though HPLC-UV can detect potassium hydroxycitrate to a concentration level of 2.34 μg/mL, the quantifiable concentration is 7.10 μg/mL or above. The LOD and LOQ values validate the HPLC-UV protocol used in this study for the quantification of potassium hydroxycitrate.

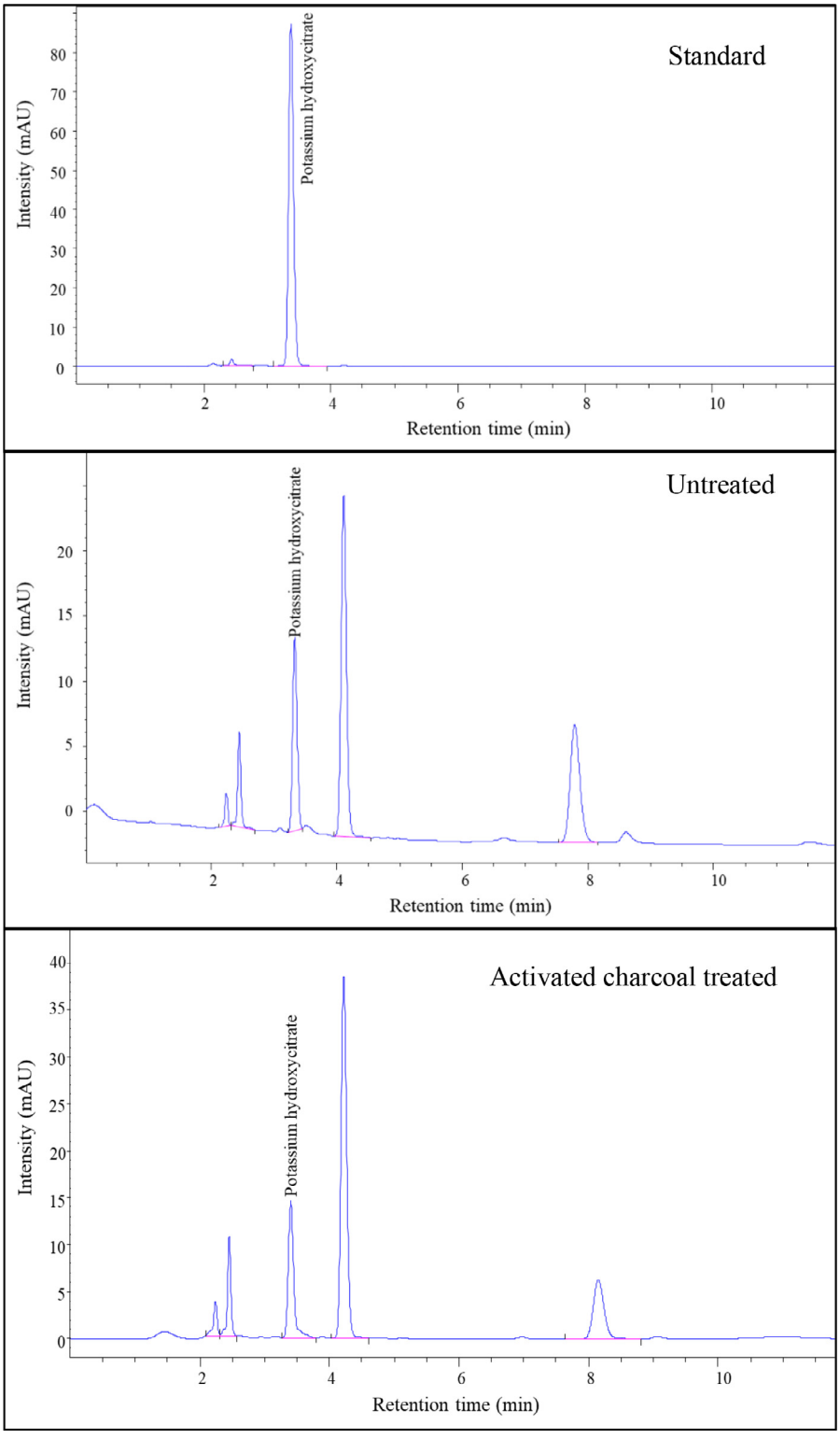


Fig. 4. HPLC-UV chromatogram of standard Potassium hydroxycitrate, untreated sample and charcoal treated sample in solution (1 mg/mL)

3.6.4. Quantification of potassium hydroxycitrate

In HPLC-UV analysis, the peak for potassium hydroxycitrate (1 mg/mL) (in both the standard and the sample) was observed at the retention time of 3.38 min (Fig. 4). Using the calibration curve described in previous section, the concentrations of potassium hydroxycitrate in untreated and activated charcoal treated samples were calculated as 119 µg/mg and 180.14 µg/mg of dry extract, respectively.

To the best of our knowledge there is no report on the extraction of potassium hydroxycitrate from *H. sabdariffa*. Fernández-Arroyo et al. (2011) quantified hydroxycitric acid in an aqueous extract of *H. sabdariffa* and reported to be 8.29 µg/mg of dry extract. Here, we need to consider the form of hydroxycitric acid reported in their study and our extract. The instability of free acid form might have caused a transformation in its lactone form leading to the lower con-

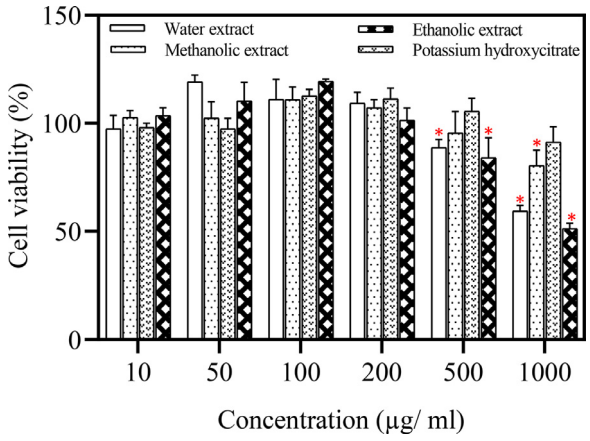


Fig. 5. Effect of concentrations of *H. sabdariffa* extracts and potassium hydroxycitrate on the viability of 3T3-L1 cells. * indicates statistically significant ($p < 0.05$) decrease in the value as compared to the control

centration in the extract. However, the precipitation method used in this study stabilizes the hydroxycitric acid into stable potassium salt form. We have achieved extraction at the concentration of 119 µg/mg and 180.14 µg/mg in untreated and charcoal treated extracts which are 14.4 and 21.7 times higher than the above-reported value. This substantially higher concentration of potassium hydroxycitrate, achieved in this study, can be attributed to the improved precipitation method specific for its extraction. In comparison, raw dried *Garcinia* fruits, from which hydroxycitric acid is commercially extracted, contain the acid in the concentration of 5.2% to 29.4%. *Garcinia* fruit extracts have been commercialised as herbal products with various concentrations of hydroxycitric acid ranging from 50 to 65% (500 to 650 µg/mg) (Seethapathy et al., 2018).

3.7. Cytotoxicity assay

The outcome of cytotoxicity assay of *Hibiscus* extracts and potassium hydroxycitrate is presented in Fig. 5. As can be observed, the extracts and potassium hydroxycitrate did not present any toxicity to 3T3-L1 cells up to the concentration of 200 µg/mL. When the concentration of extract and potassium hydroxycitrate was increased to 500 µg/mL, the percentage of cell viability decreased below 90% in the case of aqueous extract and ethanol extract whereas the viability was still above 90% in methanol extract and potassium hydroxycitrate. Statistical analaysis (*t*-test, $p < 0.05$) indicated that cell viability was significantly decreased after treatment with the concentration of 500 µg/mL for water and ethanol extract whereas 1000 µg/mL for the methanol extract as compared to the untreated control. Even at 1000 µg/mL, potassium hydroxycitrate did not have any significant adverse effect on the cell viability. Therefore, this assay concludes that aqueous and ethanol extract has a more toxic effects causing the death of cells than methanol extract on the 3T3-L1 cells. It is worth pointing out here that the methanol content was fully eliminated from methanolic extract through the evaporation to avoid adverse effect to the cells.

4. Conclusion

H. sabdariffa calyces are rich in polyphenolic compounds and hydroxycitric acid. This study used water, methanol, and ethanol as solvents to extract the polyphenolic compounds and methanol for potassium hydroxycitrate from these calyces. The results show that higher temperatures (50 °C and above) caused adverse effects to the polyphenols through thermal degradation and reduced their antioxidant activity. Methanol and ethanol extracted a much higher amount of polyphenols from *Hibiscus* calyces. The antioxidant activity of the extracts decreased significantly when stored at -30 °C after 6 weeks; however, total polyphenolic content did not change significantly. Delphinidin 3-O-sambubioside chloride and cyanidin 3-O-sambubioside chloride are two major polyphenols extracted in aqueous, methanolic, and ethanolic extracts of *H. Sabdariffa*. Potassium hydroxycitrate at a concentration of 180.1 µg/mg was also extracted from *H. sabdariffa* calyces which is the highest reported recovery of this compound.

Declaration of Competing Interest

There are no conflicts of interest to declare.

Acknowledgment

The first author acknowledges the scholarship support provided to her by RMIT University.

References

Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat. Protoc. 2 (4), 875–877. doi:10.1038/nprot.2007.102.

Alarcon-Aguilar, F.J., Zamilpa, A., Perez-Garcia, M.D., Almanza-Perez, J.C., Romero-Nunez, E., Campos-Sepulveda, E.A., et al., 2007. Effect of Hibiscus sabdariffa on obesity in MSG mice. J. Ethnopharmacol. 114 (1), 66–71. doi:10.1016/j.jep.2007.07.020.

Ali, A., Chong, C.H., Mah, S.H., Abdullah, L.C., Choong, T.S.Y., Chua, B.L., 2018. Impact of storage conditions on the stability of predominant phenolic constituents and antioxidant activity of dried piper betle extracts. Molecules 23 (2). doi:10.3390/molecules23020484.

Amaya-Cruz, D., Perez-Ramirez, I.F., Perez-Jimenez, J., Nava, G.M., Reynoso-Camacho, R., 2019. Comparison of the bioactive potential of roselle (Hibiscus sabdariffa L.) calyx and its by-product: phenolic characterization by UPLC-QTOF MS(E) and their anti-obesity effect *in vivo*. Food Res. Int. 126, 108589. doi:10.1016/j.foodres.2019.108589.

Asgary, S., Soltani, R., Zolghadr, M., Keshvari, M., Sarrafzadegan, N., 2016. Evaluation of the effects of roselle (Hibiscus sabdariffa L.) on oxidative stress and serum levels of lipids, insulin and hs-CRP in adult patients with metabolic syndrome: a double-blind placebo-controlled clinical trial. J. Complement. Integr. Med. 13 (2), 175–180. doi:10.1515/jcim-2015-0030.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239 (1), 70–76. doi:10.1006/abio.1996.0292.

Borrás-Linares, I., Fernández-Arroyo, S., Arráez-Roman, D., Palmeros-Suárez, P.A., Del Val-Díaz, R., Andrade-Gonzáles, I., et al., 2015. Characterization of phenolic compounds, anthocyanidin, antioxidant and antimicrobial activity of 25 varieties of Mexican Roselle (Hibiscus sabdariffa). Ind. Crops Prod. 69, 385–394. doi:10.1016/j.indcrop.2015.02.053.

Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C., 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10 (3), 178–182.

Chuah, L.O., Ho, W.Y., Beh, B.K., Yeap, S.K., 2013. Updates on antiobesity effect of garcinia origin (-)-HCA. Evid. Based Complement. Altern. Med. 2013, 751658. doi:10.1155/2013/751658.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., Heinrich, M., 2014. Hibiscus sabdariffa L. - a phytochemical and pharmacological review. Food Chem 165, 424–443. doi:10.1016/j.foodchem.2014.05.002.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., et al., 2014. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. J. Food Drug Anal. 22 (3), 296–302. doi:10.1016/j.jfda.2013.11.001.

Fathimah, R.N., Setyaningsih, W., Carrera, C., Palma, M., 2020. Microwave-assisted extraction of phenolics from Hibiscus sabdariffa flowers: method development and validation. Proceedings 70 (1). doi:10.3390/foods_2020-07681.

Fernández-Arroyo, S., Rodríguez-Medina, I.C., Beltrán-Debón, R., Pasini, F., Joven, J., Micol, V., et al., 2011. Quantification of the polyphenolic fraction and *in vitro* antioxidant and *in vivo* anti-hyperlipemic activities of Hibiscus sabdariffa aqueous extract. Food Res. Int. 44 (5), 1490–1495. doi:10.1016/j.foodres.2011.03.040.

Izquierdo-Vega, J.A., Arteaga-Badillo, D.A., Sanchez-Gutierrez, M., Morales-Gonzalez, J.A., Vargas-Mendoza, N., Gomez-Aldapa, C.A., et al., 2020. Organic acids from roselle (Hibiscus sabdariffa L.)-a brief review of its pharmacological effects. Biomedicines 8 (5). doi:10.3390/biomedicines8050100.

Jabeur, I., Pereira, E., Barros, L., Calhelha, R.C., Sokovic, M., Oliveira, M., et al., 2017. Hibiscus sabdariffa L. as a source of nutrients, bioactive compounds and colouring agents. Food Res. Int. 100 (Pt 1), 717–723. doi:10.1016/j.foodres.2017.07.073.

Jabeur, I., Pereira, E., Caleja, C., Calhelha, R.C., Sokovic, M., Catarino, L., et al., 2019. Exploring the chemical and bioactive properties of Hibiscus sabdariffa L. calyces from Guinea-Bissau (West Africa). Food Funct. 10 (4), 2234–2243. doi:10.1039/c9fo00287a.

Jayaprakasha, G.K., Sakariah, K.K., 2002. Determination of organic acids in leaves and rinds of Garcinia indica (Desr.) by LC. J. Pharm. Biomed. Anal. 28 (2), 379–384. doi:10.1016/s0731-7085(01)00623-9.

Kao, E.S., Yang, M.Y., Hung, C.H., Huang, C.N., Wang, C.J., 2016. Polyphenolic extract from Hibiscus sabdariffa reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. Food Funct. 7 (1), 171–182. doi:10.1039/c5fo00714c.

Majeed, M., Badmaev, V., Rajendran, R. (2004). United States Patent No. 6,770,782 B1.

Mishra, K., Ojha, H., Chaudhury, N.K., 2012. Estimation of antiradical properties of antioxidants using DPPH assay: a critical review and results. Food Chem. 130 (4), 1036–1043. doi:10.1016/j.foodchem.2011.07.127.

Mohd-Esa, N., Hern, F.S., Ismail, A., Yee, C.L., 2010. Antioxidant activity in different parts of roselle (Hibiscus sabdariffa L.) extracts and potential exploitation of the seeds. Food Chem. 122, 1055–1060. doi:10.1016/j.foodchem.2010.03.074.

Morales-Luna, E., Perez-Ramirez, I.F., Salgado, L.M., Castano-Tostado, E., Gomez-Aldapa, C.A., Reynoso-Camacho, R., 2018. The main beneficial effect of roselle (Hibiscus sabdariffa) on obesity is not only related to its anthocyanin content. J. Sci. Food Agric. doi:10.1002/jsfa.9220.

Pimentel-Moral, S., Borras-Linares, I., Lozano-Sanchez, J., Arraez-Roman, D., Martinez-Ferez, A., Segura-Carretero, A., 2018. Microwave-assisted extraction for Hibiscus sabdariffa bioactive compounds. J. Pharm. Biomed. Anal. 156, 313–322. doi:10.1016/j.jpba.2018.04.050.

Riaz, G., Chopra, R., 2018. A review on phytochemistry and therapeutic uses of Hibiscus sabdariffa L. Biomed. Pharmacother. 102, 575–586. doi:10.1016/j.biopha.2018.03.023.

Rosello-Soto, E., Marti-Quijal, F.J., Cilla, A., Munekata, P.E.S., Lorenzo, J.M., Remize, F., et al., 2019. Influence of temperature, solvent and pH on the selective extraction of phenolic compounds from tiger nuts by-products: triple-TOF-LC-MS-MS characterization. Molecules 24 (4). doi:10.3390/molecules24040797.

Salami, S.O., Afolayan, A.J., 2021. Evaluation of nutritional and elemental compositions of green and red cultivars of roselle: Hibiscus sabdariffa L. Sci. Rep. 11 (1), 1030. doi:10.1038/s41598-020-80433-8.

Seethapathy, G.S., Tadesse, M., Urumarudappa, S.K.J., S, V.G., Vasudeva, R., Malterud, K.E., et al., 2018. Authentication of Garcinia fruits and food supplements using DNA barcoding and NMR spectroscopy. Sci. Rep. 8 (1), 10561. doi:10.1038/s41598-018-28635-z.

Sindi, H.A., Marshall, L.J., Morgan, M.R., 2014. Comparative chemical and biochemical analysis of extracts of Hibiscus sabdariffa. Food Chem. 164, 23–29. doi:10.1016/j.foodchem.2014.04.097.

Wang, S.C., Lee, S.F., Wang, C.J., Lee, C.H., Lee, W.C., Lee, H.J., 2011. Aqueous extract from Hibiscus sabdariffa linnaeus ameliorate diabetic nephropathy via regulating oxidative status and Akt/Bad/14-3-3gamma in an experimental animal model. Evid. Based Complement. Alternat. Med. 2011, 938126. doi:10.1093/ecam/nep181.

Wong, S., Leong, L., Williamkoh, J., 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chem. 99 (4), 775–783. doi:10.1016/j.foodchem.2005.07.058.

Yamada, T., Hida, H., Yamada, Y., 2007. Chemistry, physiological properties, and microbial production of hydroxycitric acid. Appl. Microbiol. Biotechnol. 75 (5), 977–982. doi:10.1007/s00253-007-0962-4.

CHAPTER 4

Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of *Hibiscus sabdariffa*

Food Bioscience 50 (2022) 101992

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of *Hibiscus sabdariffa*

Manisha Singh [a,*], Thilini Thrimawithana [b], Ravi Shukla [a,c], Benu Adhikari [a,**]

[a] School of Science, RMIT University, Melbourne, VIC, 3083, Australia
[b] Discipline of Pharmacy, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC, 3083, Australia
[c] NanoBiotechnology Research Laboratory (NBRL) and Centre for Advanced Materials & Industrial Chemistry (CAMIC), RMIT University, Melbourne, VIC, 3001, Australia

ARTICLE INFO

Keywords:
Hibiscus sabdariffa
Obesity
Pancreatic lipase
α-Amylase
α-Glucosidase
Molecular docking

ABSTRACT

Obesity is a prominent global public health challenge. This study is aimed to explore the inhibition properties of organic and aqueous extracts of *Hibiscus sabdariffa* against the major enzymes associated with obesity (α-amylase, α-glucosidase, and pancreatic lipase). Extraction was carried out using water and organic solvents (methanol, ethanol and ethyl acetate) and inhibitory effect of these extracts on above-mentioned enzymes was carried out using *in vitro* method. All four extracts showed different yet significant potential to inhibit these enzymes. The organic extracts showed much stronger inhibitory effect on the enzymes than aqueous extract as the former were richer in polyphenols. The potassium hydroxycitrate, which is a salt of major organic acid of *H. sabdariffa*, did not hinhibit any of these enzymes. The combination of polyphenol-rich extract with potassium hydroxycitrate did not bring about additional inhibition indicating that inhibition potential of *H. sabdariffa* extract against the above-mentioned enzymes is due to the polyphenolic compounds. The molecular docking approach showed that the phenolic compounds of *H. Sabdariffa*, particularly catechin hydrate and rutin hydrate, have higher affnity to bind at the active sites of these enzymes, generate hydrogen bonds and thus inhibit their enzymatic activity.

1. Introduction

Obesity is a multifaceted chronic lifestyle disease that arises from imbalanced calorie intake and expenditure. In some cases, genetic susceptibility and environmental factors also contribute to obesity (Hruby & Hu, 2015). Obesity is considered a major risk factor for the development of metabolic disorders such as diabetes, hypertension, cardiovascular diseases, and other complications (Akil & Ahmad, 2011). The global burden of diseases, injuries, and risk factors study reported that high body mass index was one of the leading risks responsible for 4.72 million deaths in the world (Stanaway et al., 2018). In 2014, it imposed a huge global economic burden of the order of US $2.0 trillion which was equivalent to 2.8% of global gross domestic product (Dobbs et al., 2014).

A myriad of pharmacotherapeutic options like orlistat, lorcaserin, phentermine/topiramate, naltrexone/bupropion, semaglutide, and liraglutide are currently used for the treatment of obesity. However, the side effects of these drugs such as kidney and liver failures, high blood pressure, high pulse rate, palpitation, memory impairment, and paresthesia have limited their use (Kang & Park, 2012). Therefore, plant-derived medications, possessing anti-obesity functions, have gained attention as an adjunct or first line of protection in public health for contending with diseases and their complications (Unuofin et al., 2019). In addition, these plant-derived products are believed to have fewer or no side effects (Nagalingam, 2017).

Hibiscus sabdariffa belongs to the Malvaceae family and is a herbaceous subshrub that grows well in tropical and subtropical countries (Da-Costa-Rocha et al., 2014). Traditionally, many parts of this plant including the calyces have been used to cure conditions such as cough, hypercholesterolemia, indigestion, and high blood pressure (Riaz & Chopra, 2018). *H. sabdariffa* calyces contain phenolic compounds such as anthocyanins, chlorogenic acid, hydroxycoumarin, gallic acid, catechin, epicatechin, etc. It contains organic acids (hydroxycitric acid, ascorbic acid, citric acid, malic acid, and tartaric acid) which contribute to its acidic flavour (Izquierdo-Vega et al., 2020). These plant-derived compounds exhibit various bioactive properties such as anti-diabetic,

* Corresponding author. School of Science, RMIT University, Melbourne, VIC, 3083, Australia.
** Corresponding author. School of Science, RMIT University, Melbourne, VIC, 3083, Australia.
E-mail addresses: s3736496@student.rmit.edu.au (M. Singh), benu.adhikari@rmit.edu.au (B. Adhikari).

https://doi.org/10.1016/j.fbio.2022.101992
Received 4 July 2022; Received in revised form 18 August 2022; Accepted 19 August 2022
Available online 28 August 2022

anti-inflammatory, anti-lipidemic, and antioxidant activities (Ofosu et al., 2020). Therefore, extracts from calyces of *H. sabdariffa* can be considered a potential therapeutic to combat chronic diseases such as cardiovascular diseases (Asgary et al., 2016), diabetes mellitus (Wang et al., 2011), and obesity (Amaya-Cruz et al., 2019; Kao et al., 2016).

Obesity can be prevented through the inhibition of enzymes that interfere with the absorption of dietary fats and carbohydrates in the intestine. Notably, α-amylase and α-glucosidase are the key enzymes responsible for the hydrolysis of starch into simple sugars whereas lipases are responsible for the breakdown of fats into absorbable monoacylglycerols and free fatty acids. Inhibition of these enzymes hinders the digestion of dietary fats and carbohydrates leading to reduced calorie intake (Singh et al., 2020). Some classes of phytochemicals including polyphenols have been recognized as inhibitors of α-amylase, α-glucosidase (Rasouli et al., 2017), and pancreatic lipase (Jaradat et al., 2017; Spínola et al., 2020). It has also been shown that one of the lactone forms of hydroxycitric acid (also known as hibiscus acid) is able to inhibit α-amylase (Hansawasdi et al., 2000) and α-glucosidase (Hansawasdi et al., 2001). In addition, the enzyme inhibiting potential of potassium salt of hydroxycitric acid against α-amylase and pancreatic lipase has also been reported (Utami et al., 2019). However, to date, the effective concentration of different extracts from *H. sabdariffa is* not compared and the inhibition potential of potassium hydroxycitrate from *H. sabdariffa* is not reported. Therefore, this study is aimed at investigating the *in vitro* enzyme inhibition potential of different extracts of *H. sabdariffa.* Three different enzymes (α-amylase, α-glucosidase, and pancreatic lipase) responsible for metabolizing carbohydrates and lipids are selected for this study due to their key role in controlling obesity. In addition, molecular docking modelling is performed to determine the interaction between typical polyphenols of *H. sabdariffa* and the three major enzymes listed above.

2. Experimental

2.1. Plant material

Frozen calyces sample of *H. sabdariffa* used in this study was donated by Wild Hibiscus Flower Co. (NSW, Australia).

2.2. Reagents

α-Amylase from porcine pancreas (Type I-A), α-glucosidase from *Saccharomyces cerevisiae*, lipase from porcine pancreas (Type VI-S), acarbose, orlistat, and potassium hydroxycitrate tribasic monohydrate were purchased from Sigma-Aldrich (NSW, Australia). Methanol, ethanol, hexane, ethyl acetate, potassium hydroxide, activated charcoal, dimethyl sulfoxide (DMSO), sodium chloride, sodium phosphate monobasic dihydrate, sodium phosphate dibasic, starch, sodium hydroxide, potassium sodium tartrate tetrahydrate, 3,5-dinitro salicylic acid (DNSA), 4-nitrophenyl β-D-glucopyranoside (ρ-NPG) and 4-methylumbelliferyl oleate (4-MUO) were also obtained from Sigma-Aldrich (NSW, Australia). All of the aforementioned chemicals were of analytical grade. For all the experiments, deionised water obtained from the Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., NSW, Australia) was used.

2.3. Sample preparation and extraction of polyphenolic compounds with water and organic solvents

The sample preparation was initiated by freeze-drying the calyces of *H. sabdariffa* in a freeze drier (Labconco Triad 7400030, USA) set at −40 °C and 12 Pa vacuum pressure, and the dried calyces were crushed to a fine powder with a grinder (Breville, Sydney, Australia). This powder was then passed through a sieve with a pore size of 353 μm, stored in amber colored glass bottles and kept in a desiccator at room temperature (23 °C) until further tests. The desiccator was also covered with aluminum foil to protect the sample from the light. This powder had a moisture content of 3.11% ± 0.09% (w/w) as measured by a moisture analyser (MB45 Ohaus, Switzerland). The powdered calyces were used to extract polyphenolic compounds using different solvents including water, methanol, and ethanol. Five grams of powdered calyces were mixed with 100 mL of MilliQ water, methanol (100%), and ethanol (100%) at room temperature (23 °C) and agitated using a magnetic stirrer (MR HeiTec, Heidolph, Germany) at 500 rpm for 2 h. After this, the stirred mixture was centrifuged (Allegra 64R, Beckman Coulter, Australia) at 11,000×*g* for 20 min at room temperature (23 °C) and vacuum filtered with Whatman no. 1 filter paper. The collected supernatant from water extraction was freeze-dried where as the extracts from methanol and ethanol were concentrated to dryness using a rotary vacuum concentrator (John Morris, Australia) at 45 °C and 10 Pa. The dried extracts were flushed with nitrogen in amber-colored bottles and stored at −30 °C for further tests. The total phenolic content of the extracts was 37.36 ± 1.23, 51.72 ± 0.71, and 48.85 ± 1.88 mg gallic acid equivalent per g of dry extract, for water, methanol, and ethanol extracts, respectively as reported in our previous study (Singh et al., 2021). The polyphenols present in all three extracts were determined using ultra-performance liquid chromatography method with diode array detection (UPLC-DAD) and identified to be caffeic acid, catechin hydrate, cyanidin 3-O-sambubioside chloride, delphinidin 3-O-sambubioside chloride, and rutin hydrate. The 4-hydroxybenzoic acid, gallic acid, quercetin, and ρ-coumaric acid were only present in methanol and ethanol extract (Singh et al., 2021).

2.4. Partition extraction of polyphenolic compounds

For the partition extraction of polyphenols in ethyl acetate, the protocol from Peng et al. (2011) was followed. The yield of polyphenolic compounds from this method was reported to be comparable to or higher than the yield from aqueous extraction (Yang et al., 2010). Hence, this method was followed in this study. The powdered calyces (10 g) were extracted with methanol (100 mL) at 60 °C for 30 min with continuous agitation at 500 rpm. It was vacuum filtered through a Whatman No. 1 filter paper, and the residue was re-extracted twice more with methanol as described above. The supernatants were combined and evaporated to dryness in a rotary vacuum concentrator at 45 °C and 10 Pa. The residue obtained was dissolved in deionized water (20 mL) and extracted thrice with hexane (3 × 20 mL) in a separating funnel. The aqueous solution was re-extracted with ethyl acetate for an additional three times (3 × 20 mL) in a separating funnel. Finally, the ethyl acetate fraction was evaporated to dryness using a rotary vacuum concentrator (John Morris, Australia) at 45 °C and 10 Pa. This method yielded 0.59 ± 0.02 g of dry extract from 10.00 g of powdered calyces. The dried extract was stored at the same condition as mentioned in section 2.3 at −30 °C before further experiments. This extract had total phenolic content of 162.15 ± 6.40 mg gallic acid equivalent per g of dry extract as measured by the spectrophotometric Folin-Ciocalteu method (Singh et al., 2021).

2.5. Extraction of potassium hydroxycitrate

Potassium hydroxycitrate was extracted from the *H. sabdariffa* calyces in methanol according to Majeed et al. (2004) as reported in our earlier study (Singh et al., 2021). Hydroxycitric acid from *H. sabdariffa* was extracted as its potassium salt which is more stable as compared to the free acid. The powdered calyces (5g) were extracted with methanol (15 mL) at 65 °C in a hot plate using 500 rpm agitation for 3 h. The extract was then filtered through Whatman no 1 filter paper and the supernatant was collected separately. The calyces residue was extracted twice at the same extraction conditions with the same volume of methanol. Following this, all three extracts were pooled together and treated with activated charcoal for 1 h to eliminate the compounds (mostly anthocyanins) responsible for the color of the extract. The resulting mixture was filtered and the supernatant was treated with

methanolic potassium hydroxide (1 M) to precipitate potassium hydroxycitrate at pH 10 for 3 h. The precipitated potassium hydroxycitrate was vacuum filtered and washed with methanol to remove the residual potassium hydroxide. Finally, it was dried in a vacuum oven (Ezzi Vision, Australia) at 50 °C and 10 Pa.

2.6. Physicochemical analysis of extracts

All three extracts (100 mg) were dissolved in 10 mL of water to obtain a 1% (w/v) extract solution. This solution was vortexed and filtered through a 0.45 μm filter. The total soluble solid content was measured using a refractometer (Atago, Japan). The pH was determined using a pH meter (Mettler Toledo, Switzerland). The colour was analysed using a chroma meter (CR –400, Konica Minolta, Japan) and was expressed in terms of L* (lightness), a* (redness/greenness), and b* (yellowness/blueness). The chroma value (C*) and hue angle (h°) were then calculated by using a* and b* values as described in equations (1) and (2), respectively (Duangmal et al., 2008).

$$C^* = \sqrt{(a^{*2} + b^{*2})} \quad (1)$$

$$h^\circ = tan^{-1}(b^*/a^*) \quad (2)$$

Equation (2) was applied only when both a* and b* values were positive. For negative a* and positive/negative b*, it was corrected by adding 180; similarly, for positive a* and negative b*, 360 was added to the hue angle (Mclellan et al., 1995).

2.7. Preparation of sample for enzyme assays

The water extract and potassium hydroxycitrate were dissolved in water whereas DMSO was used to dissolve other organic extracts. The stock solutions of all the samples were prepared at 200 mg/mL concentration, vortexed to dissolve completely, and further diluted with the respective buffer used for the particular assay. Control experiments with only DMSO at different concentrations were performed for all three enzyme assays.

2.8. α-Amylase inhibition assay

The *in vitro* inhibition of α-amylase by polyphenol-rich extract and potassium hydroxycitrate from *H. sabdariffa* was performed according to Wickramaratne et al. (2016). α-Amylase solution (2 U/mL) was prepared with 20 mM phosphate buffer containing 0.006 M NaCl (pH 6.9). DNSA reagent was prepared by mixing sodium potassium tartrate tetrahydrate (12 g dissolved in 8 mL of 2 M NaOH) and 20 mL of 96 mM of DNSA. To initiate the assay, 50 μL of α-amylase solution together with 50 μL of the sample was incubated at 30 °C for 10 min. Next, the same volume (50 μL) of 1% (w/v) starch solution in water was added as substrate and re-incubated at 30 °C for 3 min. To this, DNSA reagent (50 μL) was added and kept in a water bath (85–90 °C) for 10 min to stop the reaction. The content was cooled and diluted by adding 1.25 mL of MilliQ water. It was transferred to a 48-well plate (Greiner Bio-One, Austria) and the absorbance was recorded at 540 nm using a CLARIOstar® microplate reader (BMG Labtech, Germany). Acarbose is a known α-amylase inhibitor used for the treatment of type 2 diabetes. Hence, acarbose (10–100 μg/mL) was used as the positive control in these studies. The red colour of extract and substrate are anticipated to interfere with the spectrophotometric readings. So, to avoid any error in the measurement, an appropriate blank correction was accounted for. For control which represents 100% enzyme activity, the sample was replaced with the buffer. A separate blank was prepared for control and each sample by substituting enzyme with the buffer. The inhibition of α-amylase was calculated using equation (3).

$$\alpha - Amylase\ inhibition\ (\%) = 1 - \frac{(A_{sample} - A_{sample\ blank})}{(A_{control} - A_{control\ blank})} \times 100 \quad (3)$$

where A_{sample} is the absorbance of sample; $A_{sample\ blank}$ is the absorbance of sample blank; $A_{control}$ is the absorbance of control and $A_{control\ blank}$ is the absorbance of control blank.

IC_{50} value representing the concentration of extract or positive control to inhibit 50% of enzyme activity was determined by logarithmic regression analysis on GraphPad Prism 8.0.0 (GraphPad, San Diego, CA, USA).

2.9. α-Glucosidase inhibition assay

The α-glucosidase inhibition assay described by Lankatillake et al. (2021) was adopted in these tests. α-Glucosidase was dissolved in 100 mM sodium phosphate buffer (pH 6.9) to achieve the enzyme concentration of 1 U/mL. For this assay, 30 μL of enzyme solution was added with 50 μL of sample in a 96-well plate and incubated for 10 min at 37 °C. This was followed by the addition of 20 μL of 5 mM ρ-NPG substrate solution and incubation at dark for 20 min at the same temperature. Finally, the absorbance of yellow coloured ρ-nitrophenol was measured at 405 nm using a microplate reader. Acarbose, a known inhibitor of α-glucosidase, was taken as the positive control. For control signifying 100% enzyme activity, the sample was replaced with the buffer. A separate blank was prepared for control and each sample by substituting enzyme with the buffer. The α-glucosidase inhibition was calculated using equation (3). The IC_{50} values of the extract against α-glucosidase were calculated as mentioned in preceding section.

2.10. Pancreatic lipase inhibition assay

The potential of extracts to inhibit pancreatic lipase was determined using an *in vitro* method described previously by Podsedek et al. (2014) with modifications. Briefly, 50 U/mL of lipase solution was prepared in 20 mM Tris-HCl containing 150 mM NaCl and 1.3 mM $CaCl_2$ (pH 7.4). Then, 25 μL of the extract was mixed with 25 μL of lipase solution in a 96 well plate and incubated for 5 min at 37 °C. Afterward, 50 μL of 4-MUO substrate solution (0.1 mM) was added and incubated at 37 °C for 20 min. The fluorescence of 4-methylumbelliferone released by lipase at an excitation wavelength of 360 nm and an emission wavelength of 460 nm was recorded with a microplate reader. The control, control blank, and sample blank was prepared in the same way as described in preceding two sections. The inhibition (%) was calculated by using equation (4).

$$Pancreatic\ lipase\ inhibition\ (\%) = 1 - \frac{(F_{sample} - F_{sample\ blank})}{(F_{control} - F_{control\ blank})} \times 100 \quad (4)$$

where F_{sample} is the fluorescence of sample; $F_{sample\ blank}$ is the fluorescence of sample blank; $F_{control}$ is the fluorescence of control and $F_{control\ blank}$ is the fluorescence of control blank.

The IC_{50} values of extract and positive control against pancreatic lipase were calculated as mentioned in section 2.8.

2.11. Combination of polyphenolic extract and potassium hydroxycitrate

The spectrophotometric and HPLC analysis suggested that the polyphenols and potassium hydroxycitrate were present in the ratio of 3:1 in the original *H. sabdariffa* powder (Singh et al., 2021). By considering this ratio, five different combinations of polyphenol-rich ethyl acetate extract and potassium hydroxycitrate (1:1, 2:1, 3:1, 4:1, and 5:1) were prepared and tested to determine the combined effect of phenolic compounds and potassium hydroxycitrate on α-amylase, α-glucosidase and lipase activity. The potential effect on enzyme activity due to the interaction between polyphenolic extract and potassium hydroxycitrate was then discussed.

2.12. Molecular docking

Molecular docking (modelling) was performed to determine the possible interaction between polyphenols present in *H. sabdariffa* extract and key enzymes (α-amylase, α-glucosidase, pancreatic lipase) associated with obesity. The three-dimensional structures of pancreatic α-amylase (PDB code: 1OSE) (Sui et al., 2016), α-glucosidase (PDB code: 3A4A) (Liu et al., 2020), and porcine pancreatic lipase (PDB code: 1ETH) (Zhang et al., 2018) were obtained from Protein Data Bank (https://www.rcsb.org/). Similarly, the molecular structures of acarbose, orlistat, and standard polyphenols were taken from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The docking of enzymes and ligands (acarbose, orlistat, caffeic acid, catechin hydrate, cyanidin 3-O-sambubioside chloride, delphinidin 3-O-sambubioside chloride, and rutin hydrate) was conducted using Autodock Vina (v 1.1.2) (Trott & Olson, 2010). Before docking, the ligands and water from the structure of the enzyme were removed then hydrogen (only polar) and charges (Kollmann charges) were added in Autodock Tools (v 1.5.7). Since the binding site was not known for the polyphenols, blind docking was undertaken with the exhaustiveness of 200. A total of nine models were used and the best docking was estimated with the lowest Autodock Vina docking score (kcal/mol) indicating stronger binding affinity (Xue et al., 2022). The visual analysis of the best docking was performed in PyMOL (v 2.0.4) and BIOVIA Discovery Studio Visualizer (v 21.1.0.20298).

2.13. Statistical analysis

All the experiments were conducted in triplicate unless stated otherwise. The results are presented as mean ± standard deviation. Statistical analysis was performed using SPSS statistical software (SPSS 23.0, IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) was performed to determine significant difference between any two mean values. Tukey's Honest Significant Difference post-hoc method was used at 95% confidence level ($p < 0.05$).

3. Results and discussion

3.1. Physicochemical properties of extracts

The total soluble solids, pH, and colour values of extracts of *H. sabdariffa* obtained using water, methanol, ethanol and ethyl acetate as solvent are presented in Table 1. The total soluble solid content in all the extracts was comparable (1% w/v; $p > 0.05$) except in the ethyl acetate extract (0.83% w/v). This could possibly be due to low polarity of ethyl acetate compared to water, methanol and ethanol. All of the extracts were highly acidic in nature with pH ranging from 1.87 to 2.51 and these pH values were significantly different ($p < 0.05$) from each other. The methanolic extract was more acidic followed by ethanolic and aqueous extracts. These low pH values could be attributed to the presence of organic acids. Morales-Luna et al. (2019) reported a relatively higher pH range (2.54–3.49) of aqueous and methanolic extracts from *H. sabdariffa*. This difference in the pH of extracts can be due to different origin and geographical location of the plant. L* value signifies the lightness of the extract and the value ranged from 73.00 to 83.02 in these extracts. Compared to other three extracts, ethyl acetate extract was significantly lighter ($p < 0.05$). The aqueous, methanol, and ethanol extracts had distinct red colour as indicated by the positive a* values (25.48, 20.67 and 22.13 respectively). The b* value indicates the yellowness and blueness of the sample. Methanolic and ethanolic extract had a negative b* value of −1.34 and −0.94, respectively. In contrast, aqueous extract and ethyl acetate extract had a positive b* value of 1.37 and 3.40, respectively. For better interpretation of the color, the chroma value (C*) and hue angle (h°) were also determined based on a* and b* values as presented in Table 1 (Mclellan et al., 1995). C* value represents the saturation or intensity of color whereas h° value represents the relative amount of the color expressed as 0°/360° for red, 90° for yellow, 180° for green, and 270° for blue (Kortei et al., 2015; Mclellan et al., 1995). The h° value of aqueous, methanol, and ethanol extracts was close to 0° or 360° (0.05°, 359.93° and 359.96°, respectively) indicating redness of the extract. The C* value of aqueous, methanol, and ethanol extracts was 25.51, 20.72 and 22.15, respectively, indicating that the intensity of redness was higher in aqueous extract compared to other two. In contrast, ethyl acetate extract had a faint shade with negative a* value (−1.15) and h° value of 178.76°, which indicates a green color. However, C* value of 3.59 implied the low intensity of this color in the extract.

3.2. Inhibition of α-amylase

The inhibitory potential of water, methanol, ethanol and ethyl acetate extracts of *H. sabdariffa* against α-amylase is shown in Fig. 1A. These extracts inhibited α-amylase activity in the concentration range of 1.0–7.5 mg/mL. Among the extracts, methanol and ethanol extracts showed a similar level of effectiveness in inhibiting α-amylase in this concentration range. Water extract had the least potent in inhibiting α-amylase (required a higher concentration for inhibition) which may be due to relatively low concentration of polyphenols in it. Previous studies have shown that the ability of aqueous plant extracts to inhibit α-amylase depends on the total phenolic content in them (Kalita et al., 2018; Moein et al., 2017). However, the enzyme inhibition potential of phenolic plant extracts can be equally impacted by the composition of polyphenols as well (Spínola et al., 2020). The concentration range of extracts required for α-amylase inhibition was significantly ($p < 0.05$) higher compared to the concentration (dose) of acarbose which was the positive control (<0.40 mg/mL). The IC_{50} values of all extracts obtained from *H. sabdariffa* against each enzyme are tabulated in Table 2. The strongest α-amylase inhibition was shown by ethyl acetate extract which had the lowest IC_{50} value (3.69 mg/mL). The higher total polyphenol content in ethyl acetate extract might have attributed to this better α-amylase inhibition. The IC_{50} values of methanolic extract and ethanolic extract were comparable (3.88 and 3.86 mg/mL) but signifcantly lower than that of water extract (5.74 mg/mL). These results corroborated the findings of Buchholz and Melzig (2016) who had reported that the methanol extract was more effective against inhibiting α-amylase than the aqueous extract. When the IC_{50} values of extracts were compared with the positive control, acarbose was able to achieve 50% inhibition at 0.06 mg/mL whereas *H. sabdariffa* extract needed a substantially higher concentration (3.69–5.74 mg/mL) for the same level of inhibition. Therefore, these findings show that although *H. sabdariffa* extracts are not as potent as the pharmacological agents, they are still able to inhibit the α-amylase enzyme when used as a food supplement. Ademiluyi and Oboh (2013) had reported an IC_{50} value of α-amylase as

Table 1
Physicochemical properties of *H. sabdariffa* extracts extracted in different solvents.

Extract	Total soluble solids (%)	pH	L*	a*	b*	Chroma (C*)	Hue angle (h°)
Water extract	1.07 ± 0.06^{a}	2.51 ± 0.01^{a}	74.53 ± 0.17^{b}	25.48 ± 0.34^{a}	1.37 ± 0.31^{b}	25.51 ± 0.35^{a}	0.05 ± 0.01^{c}
Methanol extract	1.03 ± 0.06^{a}	1.87 ± 0.01^{c}	73.00 ± 0.24^{b}	20.67 ± 1.17^{b}	-1.34 ± 0.44^{c}	20.72 ± 1.15^{b}	359.93 ± 0.02^{a}
Ethanol extract	1.03 ± 0.06^{a}	2.09 ± 0.01^{b}	73.19 ± 0.13^{b}	22.13 ± 0.55^{b}	-0.94 ± 0.24^{c}	22.15 ± 0.54^{b}	359.96 ± 0.01^{a}
Ethyl acetate extract	0.83 ± 0.12^{b}	2.00 ± 0.01^{b}	83.02 ± 0.85^{a}	-1.15 ± 0.15^{c}	3.40 ± 0.62^{a}	3.59 ± 0.62^{c}	178.76 ± 0.04^{b}

Different letters in superscript within a column indicate statistically significant difference ($p < 0.05$) between each value.

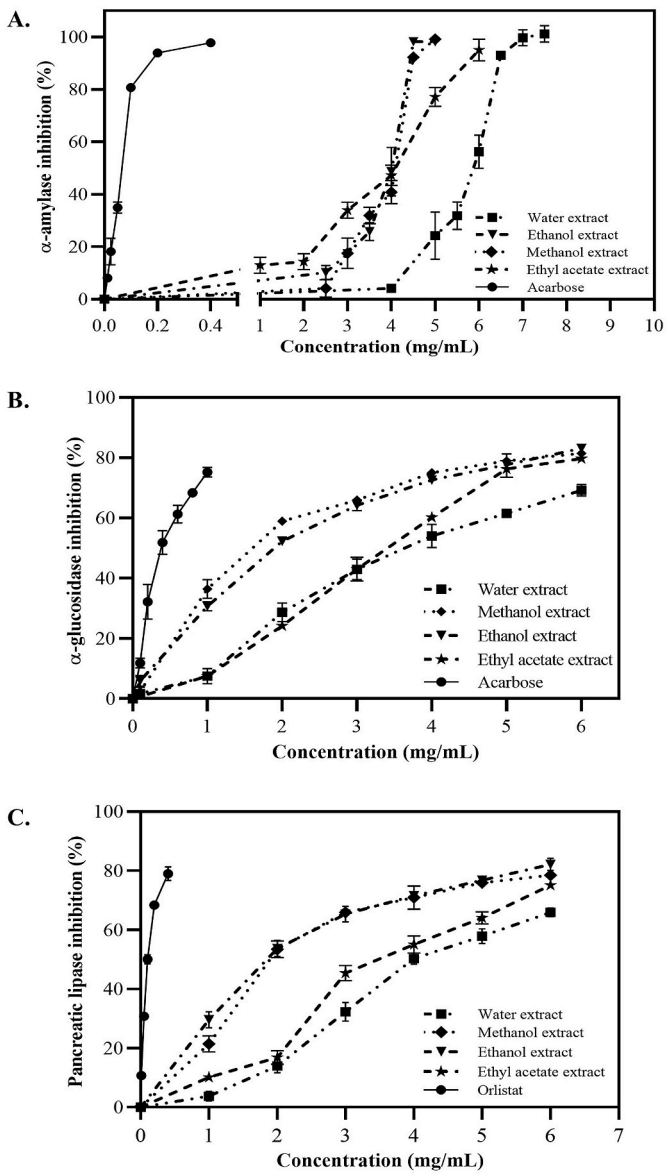


Fig. 1. Dose-dependent enzyme inhibition by *H. sabdariffa* extracts and positive controls (acarbose and orlistat) (A) α-amylase (B) α-glucosidase and (C) pancreatic lipase.

Table 2
Enzyme inhibition activity (IC_{50} values) of *H. sabdariffa* extracts.

Sample	Inhibitory activity, IC_{50} (mg/mL)		
	α-amylase inhibition	α-glucosidase inhibition	Pancreatic lipase inhibition
Acarbose (Positive control)	0.06	0.40	–
Orlistat (Positive control)	–	–	0.11
Water extract	5.74	3.65	4.25
Methanol extract	3.88	1.59	2.07
Ethanol extract	3.86	1.85	1.88
Ethyl acetate extract	3.69	3.28	3.61

low as 187.9 μg/mL in the case of aqueous extract of red variety of *H. sabdariffa*. This value is considerably lower than what has been achieved in this study. The discrepancy in these reported IC_{50} values could be attributed to variation in sample, extraction process, and methodology used.

Moein et al. (2022) have reported that the anthocyanin isolated from *Berberis integerrima* bunge fruits were capable of inhibiting α-amylase with IC_{50} value of 1.14 mg/mL. Similarly, the catehin-rich ethyl acetate fraction isolated from Qingzhuan tea showed inhibition against α-amylase with IC_{50} value of 4.6 mg/mL (Cheng et al., 2015). The *H. sabdariffa* extracts in our study also contained these phenolic compounds together with other polyphenolic compounds (e.g. caffeic acid and rutin hydrate) that might have contributed towards α-amylase inhibition. Neither potassium hydroxycitrate extracted from *H. sabdariffa* nor standard potassium hydroxycitrate showed any inhibition of the α-amylase activity when tested at the concentration range of 0.1–10.0 mg/mL.

3.3. Inhibition of α-glucosidase

Fig. 1B shows the α-glucosidase inhibition activity of *H. sabdariffa* extracts. The α-glucosidase inhibition was evident at 0.1–6.0 mg/mL concentrations of the extracts. Similar to α-amylase, α-glucosidase inhibition of methanol and ethanol extract was comparable and higher than that of the water extract. The water extract had the lowest inhibition after ethyl acetate extraction. Even the lowest tested concentration of all the extracts (0.01 mg/mL) showed an inhibitory effect on α-glucosidase. The IC_{50} value for methanol extract was lowest (1.59 mg/mL) followed by ethanol extract (1.85 mg/mL), ethyl acetate extract (3.28 mg/mL) and water extract (3.65 mg/mL) while that of acarbose was 0.40 mg/mL. Ifie et al. (2018) reported the IC_{50} value of around 6 mg/mL for aqueous extract of *H. sabdariffa* against α–glucosidase (rat intestinal maltase) compared to which our values are better. Ifie et al. (2016) reported that almost half of the α–glucosidase inhibition was contributed by cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside. Our earlier study (Singh et al., 2021) also showed that cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside were the major polyphenols present in aqueous, methanol, and ethanol extracts; however, these were not present in the ethyl acetate extract. Hence, it can be implied that these polyphenolic compounds could contribute towards inhibition of α–glucosidase. Also, the inhibitory potential of methanol extract was highest followed by ethanol extract which could be due to the higher concentration of cyanidin 3-O-sambubioside (11.07 and 8.87 mg/g) and delphinidin 3-O-sambubioside (35.29 and 23.98 mg/g) in methanol and ethanol extracts, respectively (Singh et al., 2021). No inihibition against α-glucosidase was presented by both extracted and standard potassium hydroxycitrate at 0.1–10.0 mg/mL.

3.4. Inhibition of pancreatic lipase

The inhibition of lipase activity by different extracts of *H. sabdariffa* is shown in Fig. 1C. Similar to the case in other two enzymes, the higher pancreatic lipase inhibition was shown by organic solvent extracts (methanol, ethanol, and ethyl acetate) than the aqueous extract. The ethanol extract had an IC_{50} value of 1.22 mg/mL which was the lowest concentration followed by methanol extract (1.32 mg/mL). The positive control, orlistat, presented an IC_{50} value of 0.11 mg/mL which is significantly ($p < 0.05$) lower compared to all extracts. Buchholz and Melzig (2016) reported that the IC_{50} values of methanolic extract of *H. sabdariffa* and orlistat against lipase were 35.8 μg/mL and as 0.19 ng/mL, respectively. The IC_{50} values of the methanolic extract and orlistat in this work was much higher than reported by Buchholz and Melzig (2016) which could be due to the variation in the sample itself, sample preparation and extraction method used. Our data as well as those referenced above indicate that orlistat is more potent than *H. sabdariffa* extract. However, when the effectiveness of methanolic extracts with orlistat is compared, our research has achieved better inhibition potential than reported in the literature. Similar to α-amylase and α-glucosidase, potassium hydroxycitrate from *H. sabdariffa* and commercial standard did not show any inhibition against pancreatic

lipase.

Among the three enzymes, the lowest IC_{50} value achieved by all of the *H. sabdariffa* extracts was observed in α-glucosidase activity and the highest was in α-amylase activity. This obsertvation indicates that *H. sabdariffa* extracts are stronger inhibitor of α-glucosidase as compared to pancreatic lipase and α-amylase. These extracts can be used to inhibit all the three enzymes if used at higher concentrations and thus would be effective as part of food supplement.

In all the abovementioned enzyme inhibition assays, the concentration of organic extracts remained in between 1 and 6 mg/mL which is equivalent to DMSO concentration of 0.5%–3.0% v/v in the extract. DMSO was used in these concentrations as a control and showed no effect on the activity of α-amylase, α-glucosidase and pancreatic lipase (Table S1). Even at 6.25% v/v DMSO, no intereference was observed in the α-amylase activity (Yilmazer-Musa et al., 2012). Similarly, Lankatillake et al. (2021) used 2% DMSO for reconstitution of extracts and reported that there was no observable effect on the activities of α-amylase, α-glucosidase and pancreatic lipase.

3.5. Effect of combination of polyphenolic extract and potassium hydroxycitrate on enzyme inhibition

Five different ratios (1:1, 2:1, 3:1, 4:1, and 5:1) of polyphenolic compounds (extracted in ethyl acetate) to standard potassium hydroxicitrate were tested against α-amylase, α-glucosidase, and pancreatic lipase activity and the results are tabulated in Table 3. The concentration of extract obtained from ethyl acetate extraction was kept constant at 5 mg/mL for all the combinations which exhibited about slightly above 50% inhibition potential. For this, the concentration of potassium hydroxycitrate was varied. As mentioned in section 3.2 to 3.4, the potassium hydroxycitrate (standard) did not inhibit any of the three enzymes. However, these combined formulaitons were used to assess whether the interaction between polyphenols and potassium hydroxycitrate could achive higher inhibition than achieved by polyphenol-rich extracts. In the case of α-amylase, all the combinations (ratios) resulted in antagonistic interaction and significantly reduced (by about 40%) the inhibition efficacy. The level of antagonistic interaction was different in the case of α-glucosidase inhibition. The α-glucosidase inhibition activity decreased when the polyphenolic extract and potassium hydroxycitrate were combined; however, only the 1:1 ratio showed a significant decline ($p < 0.05$). For pancreatic lipase, there was only a marginal and insignificant decrease in enzyme inhibition in the 1:1 and 2:1 ratios ($p > 0.05$) compared to the inhibition by ethyl acetate extract. The possible reason for this decrease in enzyme inhibition potential especially at the higher concentration of potassium hydroxycitrate can be due to the interaction between polyphenols and potassium hydroxycitrate which might have adversely affected the enzyme inhibitory potential of the former. Clouatre et al. (2004) reported that potassium and sodium salts of hydroxycitric acid can undergo chemical modifications in acidic condition and even bring about the changes in the product. Similar interactions are expected to occur in these formulations and the low pH of ethyl acetate extract is expected to be favouring such modifications. These results indicate that the combination of polyphenol-rich extract and potassium hydroxycitrate in fact lowers inhibitory effect of the former on these three tested enzymes.

Table 3
Enzyme inhibition by the combined formulation of polyphenol-rich ethyl acetate extract of *H. sabdariffa* and potassium hydroxycitrate.

Ratio of extract to potassium hydroxycitrate	α-amylase inhibition (%)	α-glucosidase inhibition (%)	pancreatic lipase inhibition (%)
Control (Only ethyl acetate extract)	77.12 ± 3.58^{a}	76.29 ± 2.74^{a}	64.08 ± 2.03^{a}
1:1	46.54 ± 1.53^{b}	69.62 ± 1.10^{b}	59.93 ± 0.75^{a}
2:1	45.60 ± 6.31^{b}	73.96 ± 0.57^{ab}	61.11 ± 1.07^{a}
3:1	45.23 ± 5.00^{b}	74.00 ± 2.67^{ab}	63.83 ± 1.26^{a}
4:1	41.30 ± 6.37^{b}	73.15 ± 0.66^{ab}	63.70 ± 3.62^{a}
5:1	44.11 ± 2.14^{b}	75.68 ± 1.14^{a}	64.15 ± 2.27^{a}

The different letters in superscript within a column indicate statistically significant differences ($p < 0.05$) between each values.

3.6. Bonding and binding affinity of phenolic compounds with tested digestive enzymes

The results presented in sections 3.2 to 3.4 showed that aqueous and organic extracts of *H. sabdariffa* could inhibit key enzymes associated with obesity. Therefore, *in silico* molecular docking modelling of five major polyphenols (caffeic acid, catechin hydrate, cyanidin 3-O-sambubioside chloride, delphinidin 3-O-sambubioside chloride, and rutin hydrate) that are present in both aqueous and organic extracts of *H. sabdariffa* extract was performed with α-amylase, α-glucosidase, and pancreatic lipase. The model with the lowest docking score (highest binding affinity) was selected as the best one to visualize their interactions. The docking scores of these compounds are presented in Table 4. Potential binding sites, nature, and length of bonds between the polyphenols and these enzymes were also identified. All of these are presented with the aid of three-dimensional (3D) and two-dimensional (2D) diagrams in Figs. 2–4.

Buisson et al. (1987) identified the active sites (regions) of porcine pancreatic α-amylase. The regions are surrounded by numerous charged side chains (Arg61, Asp165, Asp197, Lys200, Glu233, Asp236, and Asp300) and several aromatic or non-polar residues (Trp58, Trp59, Tyr62, His101, Pro163, Ile235, Tyr258, His299, His305, and Ala307). Among these residues, three carboxyl residues (Asp197, Asp300, and Glu233) create the active site (Larson et al., 2010; Qian et al., 1994). It was found, through the docking modelling, that all the above-mentioned polyphenols were able to bind to these active sites and form bonds with Tyr62, Asp197, Glu233, His299, Asp300, and His 305 (Fig. 2A and B). Besides, conventional hydrogen bonding and carbon-hydrogen bonding interactions as well as the interactions such as pi-anion or pi-cation, pi-sigma, pi-pi staked or pi-pi T-shaped, and pi-alkyl or alkyl were also present in the docked complexes. However, acarbose did not dock into this active site of α-amylase and formed five conventional hydrogen bonds at different binding sites with residues Thr6, Pro332, Arg398, Asp402 and Arg421. Caffeic acid, delphinidin 3-O-sambubioside chloride, and rutin hydrate formed four hydrogen bonds with the residues of α-amylase. The lowest and highest binding affinity with α-amylase were observed in the case of caffeic acid (−6.6 kcal/mol) and rutin hydrate (−9.1 kcal/mol), respectively. Acarbose (−7.7 kcal/mol) had a lower binding affinity than all of the polyphenols considered except caffeic acid. However, our results from *in vitro* α-amylase studies (section 3.2) suggested that acarbose has better inhibition activity (lower IC_{50} values) than the polyphenol-rich extract. This can be explained by the fact that molecular docking studies are performed under a theoretical ideal condition, and the docking prediction can differ from experimental

Table 4
Predicted docking score of compounds to α-amylase, α-glucosidase, and pancreatic lipase.

Compounds	Docking score (kcal/mol)		
	α-amylase	α-glucosidase	pancreatic lipase
Acarbose (control)	−7.7	−8.5	–
Caffeic acid	−6.6	−7.1	−6.7
Catechin hydrate	−9.0	−8.5	−9.7
Cyanidin 3-O-sambubioside chloride	−9.0	−9.1	−8.4
Delphinidin 3-O-sambubioside chloride	−8.8	−10.1	−8.6
Orlistat (control)	–	–	−7.2
Rutin hydrate	−9.1	−10.3	−9.0

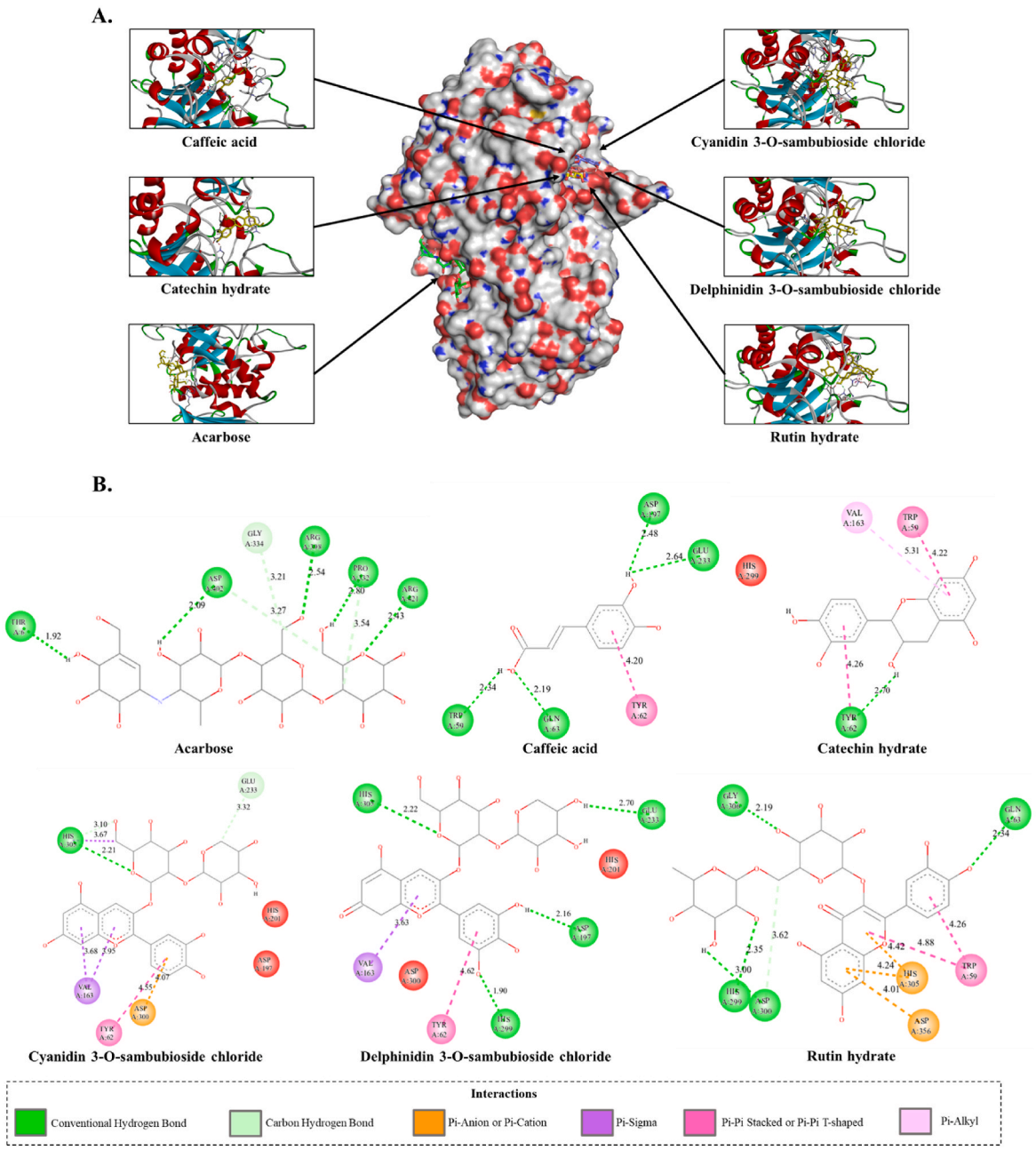


Fig. 2. (A) Superimposed 3D diagram with predicted binding poses of polyphenols and acarbose (control) docked into α-amylase (B) 2D diagram showing binding interactions and length of bonds (°A) of docked compounds with residues of α-amylase.

outcome (Sui et al., 2016). Also, the molecular docking was performed with pure compounds. While the polyphenol-rich extract of *H. sabdariffa* was comprised of mixture of compounds and the interactions between individual components might result in the alteration of enzyme inhibition potential. Baranowska et al. (2021) have reported that the interactions of various polyphenols in mixture can influence and modify the biological activity and may not resemble with or correspond with that of individual components. In addition, the concentration of individual polyphenols (except for delphinidin 3-O-sambubioside chloride) in the extracts was low in comparison to the acarbose concentration (IC_{50} of 0.06 mg/mL) used in the α-amylase inhibition studies. Moreover, the binding energy or docking score, which indicates the inhibition potential of compound is also affected by the chemical structure.

Molecular docking for α-glucosidase showed that all the polyphenols (except catechin hydrate) and acarbose could readily bind to the active site of this enzyme (Fig. 3A). The major amino acid residues of α-glucosidase that readily interact with the polyphenols were found to be Lys156, Tyr158, Ser240, Asp242, His280, Asp307, Thr310, Pro312, Leu313, Arg315, Glu411, and Asn415 (Fig. 3B). This model prediction agrees with the findings of Murugesu et al. (2019), who reported that quercetin, a known polyphenol that inhibits α-glucosidase, had hydrogen bonding interaction with Lys156, Thr310, Pro312, Leu313, Glu411, and Asn415 in addition to hydrophobic interaction with Arg315. Our model prediction indicated that acarbose formed nine conventional hydrogen bonds with Asp242, His280, Asp307, Thr310, Leu313, Arg315, Glu411, and Asn415 in the active site of α-glucosidase. Similar parttern of hydrogen bonding interactions between acarbose and α-glucosidase residue (Asp242, His280, Leu313, Arg315, and Asn415) were reported by Chen et al. (2022) and Liu et al. (2020). Delphinidin 3-O-sambubioside had seven hydrogen bonding interactions which might have contributed to the higher binding affinity (−10.1 kcal/mol). The docking score of rutin hydrate was predicted to be −10.3 kcal/mol,

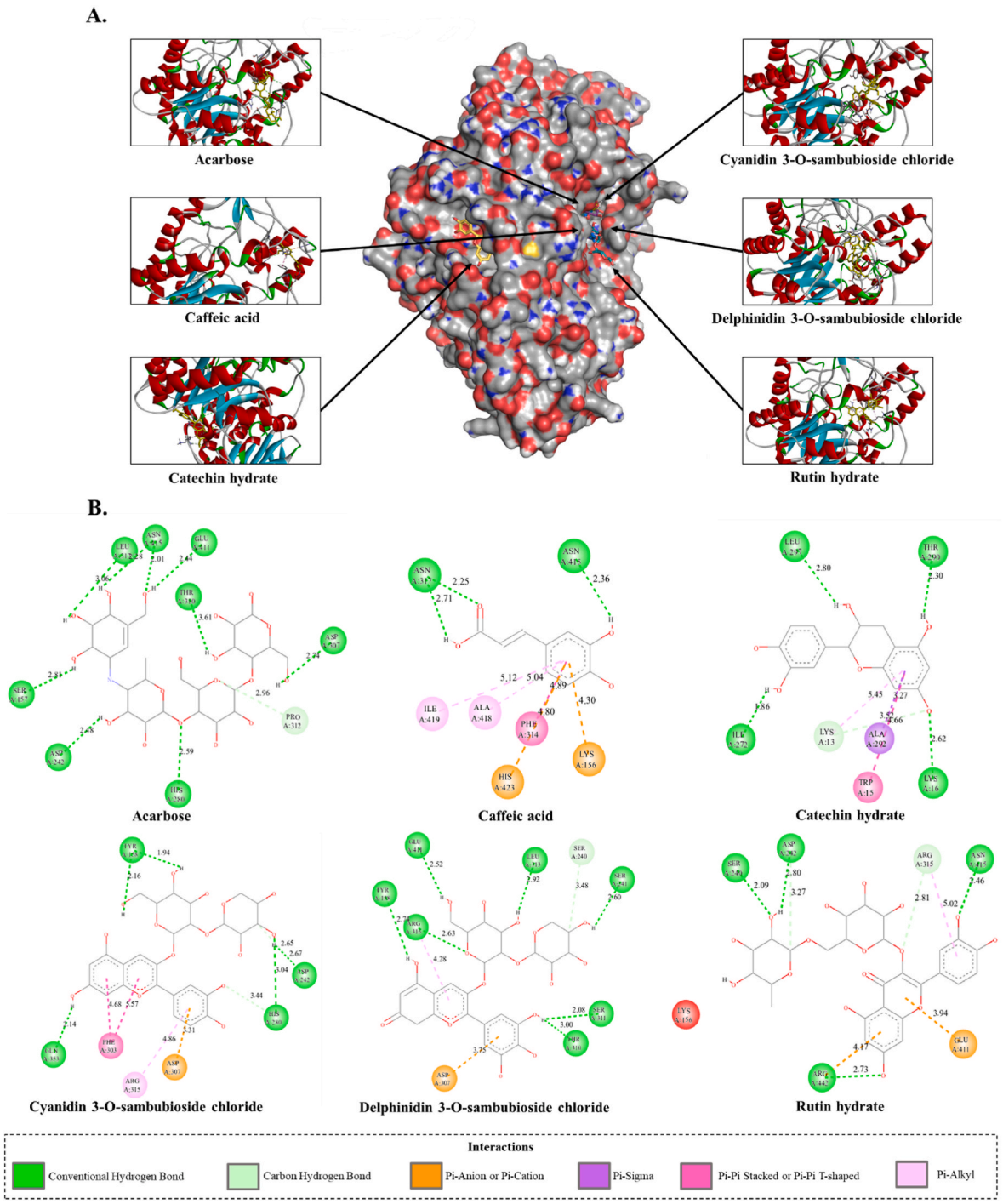


Fig. 3. (A) Superimposed 3D diagram with predicted binding poses of polyphenols and acarbose (control) docked into α-glucosidase (B) 2D diagram showing binding interactions and length of bonds (°A) of docked compounds with residues of α-glucosidase.

which was the highest binding affinity among all the tested phenolic compounds. It formed four conventional hydrogen bonds and three carbon-hydrogen bonds, two Pi-Anion or Pi-Cation, and one Pi-Alkyl bond with α-glucosidase. Caffeic acid formed only three hydrogen bonding interactions with α-glucosidase leading to lower binding affinity (−7.1 kcal/mol). Among the tested compounds, only caffeic acid had the lower binding affinity than acarbose (−8.5 kcal/mol) suggesting the superior stability of the other five polyphenol docked complexes.

The active site of porcine pancreatic lipase is known to be surrounded by Gly77, Phe78, Ile79, Asp80, Trp86, Tyr115, His152, Ser153, Leu154, Asp177, Pro181, His264, and Leu265. And the active site is specifically characterized by Ser153, Asp177, and His264 residues (Chen et al., 2020). The findings from this study indicated that orlistat (positive control), caffeic acid, and catechin hydrate can preferentially bind with the active sites of porcine pancreatic lipase as shown in Fig. 4A and B. These three compounds interacted and bonded with above-mentioned amino acid residues. Martinez-Gonzalez et al. (2017) showed that caffeic acid, coumaric acid, and quercetin interacted with the amino acid residues present at the active center as well as additional amino acid residues (Val26, Phe216, Arg257, and Ala261) present around the center which agrees with our modelling. However, the remaining three polyphenols (cyanidin 3-O-sambubioside chloride,

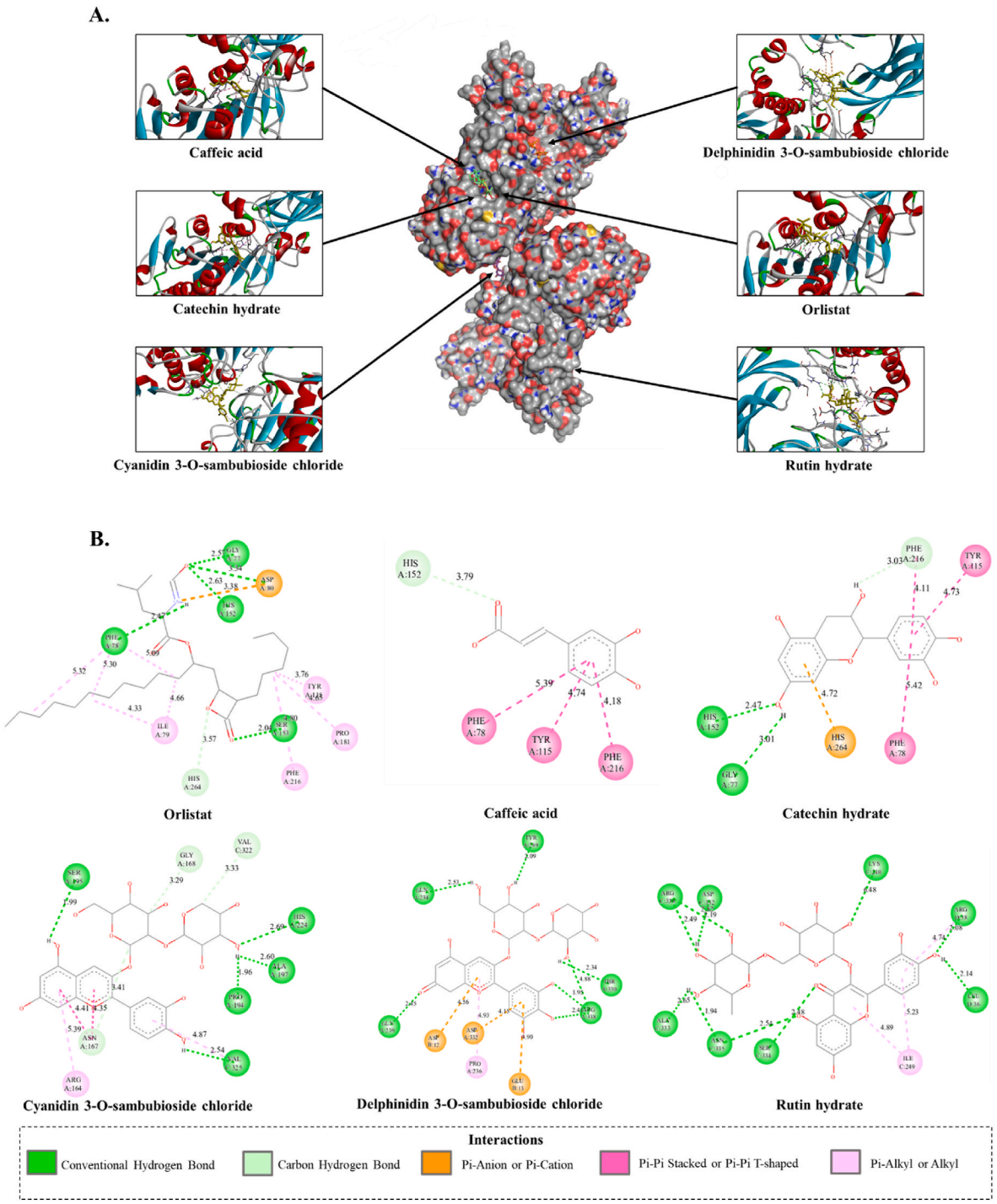


Fig. 4. (A) Superimposed 3D diagram with predicted binding poses of polyphenols and orlistat (control) docked into pancreatic lipase (B) 2D diagram showing binding interactions and length of bonds (°A) of docked compounds with residues of pancreatic lipase.

delphinidin 3-O-sambubioside chloride, and rutin hydrate) had a binding site away from the active site of porcine pancreatic lipase. The highest binding affinity in this case was predicted for catechin hydrate (−9.7 kcal/mol) followed by rutin hydrate (−9.0 kcal/mol). Catechin hydrate formed two hydrogen bond (Gly77, and His152) and three Pi-Pi Stacked or Pi-Pi T-shaped (Phe78, Tyr115 and Phe216) at the active site of porcine pancreatic lipase. In rutin hydrate-lipase complex, rutin interacted with Leu36, Arg38, Lys240, Asp332, Ala333, Ser334, Asn335 and Arg338 to form ten hydrogen bonds. The higher number of hydrogen bonds at shorter molecular space may have attributed to the predicted higher binding affinity of rutin hydrate. The positive control (orlistat) showed a lower binding affinity (−7.2 kcal/mol) than all the tested polyphenols except caffeic acid (−6.7 kcal/mol). Our prediction based on the docking score and binding affinity indicate that caffeic acid is the weakest inhibitor of α-amylase, α-glucosidase and pancreatic lipase.

Our findings on *in vitro* enzyme inhibition data showed all three organic extracts had lower IC_{50} vlaues (thus, higher potency) in comparision to the aqueous extract. Our earlier study (Singh et al., 2021) also showed that the concentration of caffeic acid, catechin hydrate, cyanidin 3-O-sambubioside chloride, delphinidin 3-O-sambubioside chloride, and rutin hydrate was higher in the organic extract. The

relative abundance and higher binding affinities of these polyphenols from molecular docking study indicate that the magnitude of enzyme inhibition is affected by the concentration of these polyphenols in the extract. Therefore, from the molecular docking analysis and *in vitro* enzyme tests, it can be stated that the natural polyphenols present in *H. sabdariffa* can contribute to the inhibition of α-amylase, α-glucosidase, and pancreatic lipase, and ultimately helps to slow down breakdown of starchy and fatty foods and thus reduce the prevalence of obesity.

4. Conclusion

This study examined the inhibitory effect of *H. sabdariffa* extracts against three key enzymes linked to obesity. All of the polyphenol-rich extracts showed significant inhibitory activity against α-amylase, α-glucosidase, and pancreatic lipase. Organic extracts were more effective than aqueous extract in inhibiting these enzymes likely due to higher polyphenol conent in the former. Potassium hydroxycitrate extracted from *H. sabdariffa* did not inhibit the activity of these three enzymes. The combination of polyphenols (ethyl acetate extract) and potassium hydroxycitrate did not achieve synergistic effects. Molecular docking studies of polyphenols demonstrated that rutin hydrate had the highest binding affinity for α-amylase and α-glucosidase while catechin hydrate had highest binding affinity for pancreatic lipase, indicating their superior enzyme inhibition properties. These results indicate that polyphenols in *H. sabdariffa* extracts possess great potential in inhibiting metabolic enzymes.

Author contributions

M.S planned and performed all the experiments, analyzed the data, and wrote the manuscript. B.A, T.T and R.S conceptualized and supervised the project and contributed in data analyses and finalization of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors wish to thank Lee Etherington from Wild Hibiscus Flower Co., NSW, Australia for donating *H. sabdariffa* calyces sample for this research. The first author also acknowledges the scholarship support provided to her by RMIT University.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fbio.2022.101992.

References

Ademiluyi, A. O., & Oboh, G. (2013). Aqueous extracts of roselle (*Hibiscus sabdariffa* Linn.) varieties inhibit α-amylase and α-glucosidase activities in vitro. *Journal of Medicinal Food, 16*(1), 88–93. https://doi.org/10.1089/jmf.2012.0004

Akil, L., & Ahmad, H. A. (2011). Relationships between obesity and cardiovascular diseases in four southern states and Colorado. *Journal of Health Care for the Poor and Underserved, 22*(4 Suppl), 61–72. https://doi.org/10.1353/hpu.2011.0166

Amaya-Cruz, D., Perez-Ramirez, I. F., Perez-Jimenez, J., Nava, G. M., & Reynoso-Camacho, R. (2019). Comparison of the bioactive potential of Roselle (*Hibiscus sabdariffa* L.) calyx and its by-product: Phenolic characterization by UPLC-QTOF MS (E) and their anti-obesity effect in vivo. *Food Research International, 126*, Article 108589. https://doi.org/10.1016/j.foodres.2019.108589

Asgary, S., Soltani, R., Zolghadr, M., Keshvari, M., & Sarrafzadegan, N. (2016). Evaluation of the effects of roselle (*Hibiscus sabdariffa* L.) on oxidative stress and serum levels of lipids, insulin and hs-CRP in adult patients with metabolic syndrome: A double-blind placebo-controlled clinical trial. *Journal of Complementary and Integrative Medicine, 13*(2), 175–180. https://doi.org/10.1515/jcim-2015-0030

Baranowska, M., Koziara, Z., Suliborska, K., Chrzanowski, W., Wormstone, M., Namiesnik, J., & Bartoszek, A. (2021). Interactions between polyphenolic antioxidants quercetin and naringenin dictate the distinctive redox-related chemical and biological behaviour of their mixtures. *Scientific Reports, 11*(1), Article 12282. https://doi.org/10.1038/s41598-021-89314-0

Buchholz, T., & Melzig, M. F. (2016). Medicinal plants traditionally used for treatment of obesity and diabetes mellitus - screening for pancreatic lipase and alpha-amylase inhibition. *Phytotherapy Research, 30*(2), 260–266. https://doi.org/10.1002/ptr.5525

Buisson, G., Duée, E., Haser, R., & Payan, F. (1987). Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 A resolution. Role of calcium in structure and activity. *The EMBO Journal, 6*(13), 3909–3916.

Chen, P., Dlamini, B. S., Chen, C., Kuo, Y., Shih, W., Lin, Y., Lee, C., & Chang, C. (2022). Structure related α-glucosidase inhibitory activity and molecular docking analyses of phenolic compounds from *Paeonia suffruticosa*. *Medicinal Chemistry Research, 31*(2), 293–306. https://doi.org/10.1007/s00044-021-02830-6

Cheng, Q., Cai, S., Ni, D., Wang, R., Zhou, F., Ji, B., & Chen, Y. (2015). In vitro antioxidant and pancreatic alpha-amylase inhibitory activity of isolated fractions from water extract of Qingzhuan tea. *Journal of Food Science and Technology, 52*(2), 928–935. https://doi.org/10.1007/s13197-013-1059-y

Chen, Z., Wu, Y., Hao, J., Liu, Y., He, K., Jiang, W., Xiong, M., Lv, Y., Cao, S., & Zhu, J. (2020). Molecular dynamic simulation of the porcine pancreatic lipase in non-aqueous organic solvents. *Frontiers in Bioengineering and Biotechnology, 8*, 676. https://doi.org/10.3389/fbioe.2020.00676

Clouatre, D. L., Clouatre, D. E., & Dunn, J. M. (2004). *Method for stable and controlled delivery of (-)-hydroxycitric acid*. US patent 20040101555A1.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., & Heinrich, M. (2014). *Hibiscus sabdariffa* L. - a phytochemical and pharmacological review. *Food Chemistry, 165*, 424–443. https://doi.org/10.1016/j.foodchem.2014.05.002

Dobbs, R., Sawers, C., Thompson, F., Manyika, J., Woetzel, J., Child, P., McKenna, S., & Spatharou, A. (2014). *Overcoming obesity: An initial economic analysis*. Discussion paper. from McKinsey Global Institute https://www.mckinsey.com/~/media/mckinsey/business%20functions/economic%20studies%20temp/our%20insights/how%20the%20world%20could%20better%20fight%20obesity/mgi_overcoming_obesity_full_report.ashx.

Duangmal, K., Saicheua, B., & Sueeprasan, S. (2008). Colour evaluation of freeze-dried roselle extract as a natural food colorant in a model system of a drink. *LWT - Food Science and Technology, 41*(8), 1437–1445. https://doi.org/10.1016/j.lwt.2007.08.014

Hansawasdi, C., Kawabata, J., & Kasai, T. (2000). Alpha-amylase inhibitors from roselle (*Hibiscus sabdariffa* Linn.) tea. *Bioscience, Biotechnology, and Biochemistry, 65*(5), 1041–1043.

Hansawasdi, C., Kawabata, J., & Kasai, T. (2001). Hibiscus acid as an inhibitor of starch digestion in the Caco-2 cell model system. *Bioscience, Biotechnology, and Biochemistry, 65*(9), 2087–2089.

Hruby, A., & Hu, F. B. (2015). The epidemiology of obesity: A big picture. *Pharmacoeconomics, 33*(7), 673–689. https://doi.org/10.1007/s40273-014-0243-x

Ifie, I., Ifie, B. E., Ibitoye, D. O., Marshall, L. J., & Williamson, G. (2018). Seasonal variation in *Hibiscus sabdariffa* (Roselle) calyx phytochemical profile, soluble solids and α-glucosidase inhibition. *Food Chemistry, 261*, 164–168. https://doi.org/10.1016/j.foodchem.2018.04.052

Ifie, I., Marshall, L. J., Ho, P., & Williamson, G. (2016). *Hibiscus sabdariffa* (Roselle) extracts and wine: Phytochemical profile, physicochemical properties, and carbohydrase inhibition. *Journal of Agricultural and Food Chemistry, 64*(24), 4921–4931. https://doi.org/10.1021/acs.jafc.6b01246

Izquierdo-Vega, J. A., Arteaga-Badillo, D. A., Sanchez-Gutierrez, M., Morales-Gonzalez, J. A., Vargas-Mendoza, N., Gomez-Aldapa, C. A., Castro-Rosas, J., Delgado-Olivares, L., Madrigal-Bujaidar, E., & Madrigal-Santillan, E. (2020). Organic acids from roselle (*Hibiscus sabdariffa* L.)-A brief review of its pharmacological effects. *Biomedicines, 8*(5), 100. https://doi.org/10.3390/biomedicines8050100

Jaradat, N., Zaid, A. N., Hussein, F., Zaqzouq, M., Aljammal, H., & Ayesh, O. (2017). Anti-lipase potential of the organic and aqueous extracts of ten traditional edible and medicinal plants in Palestine; a comparison study with orlistat. *Medicines, 4*(4), 89. https://doi.org/10.3390/medicines4040089

Kalita, D., Holm, D. G., LaBarbera, D. V., Petrash, J. M., & Jayanty, S. S. (2018). Inhibition of alpha-glucosidase, alpha-amylase, and aldose reductase by potato polyphenolic compounds. *PLoS One, 13*(1), Article e0191025. https://doi.org/10.1371/journal.pone.0191025

Kang, J. G., & Park, C. Y. (2012). Anti-obesity drugs: A review about their effects and safety. *Diabetes & Metabolism, 36*(1), 13–25. https://doi.org/10.4093/dmj.2012.36.1.13

Kao, E. S., Yang, M. Y., Hung, C. H., Huang, C. N., & Wang, C. J. (2016). Polyphenolic extract from *Hibiscus sabdariffa* reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. *Food & Function, 7*(1), 171–182. https://doi.org/10.1039/c5fo00714c

Kortei, N. K., Odamtten, G. T., Obodai, M., Appiah, V., & Akonor, P. T. (2015). Determination of color parameters of gamma irradiated fresh and dried mushrooms during storage. *Croatian Journal of Food Technology, Biotechnology and Nutrition, 10*(1–2), 66–71.

Lankatillake, C., Luo, S., Flavel, M., Lenon, G. B., Gill, H., Huynh, T., & Dias, D. A. (2021). Screening natural product extracts for potential enzyme inhibitors: Protocols, and the standardisation of the usage of blanks in alpha-amylase, alpha-glucosidase and lipase assays. *Plant Methods, 17*(1), 3. https://doi.org/10.1186/s13007-020-00702-5

Larson, S. B., Day, J. S., & McPherson, A. (2010). X-ray crystallographic analyses of pig pancreatic alpha-amylase with limit dextrin, oligosaccharide, and alpha-cyclodextrin. *Biochemistry, 49*(14), 3101–3115. https://doi.org/10.1021/bi902183w

Liu, Y., Zhan, L., Xu, C., Jiang, H., Zhu, C., Sun, L., Sun, C., & Li, X. (2020). α-Glucosidase inhibitors from Chinese bayberry (*Morella rubra* Sieb. Et Zucc.) fruit: Molecular docking and interaction mechanism of flavonols with different B-ring hydroxylations. *RSC Advances, 10*(49), 29347–29361. https://doi.org/10.1039/d0ra05015f

Majeed, M., Badmaev, V., & Rajendran, R. (2004). Process for the production of potassium hydroxy citric acid, and compositions containing the potassium hydroxy citric acid. *US Patent, 6*, 770–782. B1.

Martinez-Gonzalez, A. I., Alvarez-Parrilla, E., Diaz-Sanchez, A. G., de la Rosa, L. A., Nunez-Gastelum, J. A., Vazquez-Flores, A. A., & Gonzalez-Aguilar, G. A. (2017). In vitro inhibition of pancreatic lipase by polyphenols: A kinetic, fluorescence spectroscopy and molecular docking study. *Food Technology and Biotechnology, 55*(4), 519–530. https://doi.org/10.17113/ftb.55.04.17.5138

Mclellan, M. R., Lind, L. R., & Kime, R. W. (1995). Hue angle determinations and statistical analysis for multiquadrant hunter L,a,b data. *Journal of Food Quality, 18*(3), 235–240. https://doi.org/10.1111/j.1745-4557.1995.tb00377.x

Moein, S., Moein, M., & Javid, H. (2022). Inhibition of alpha-amylase and alpha-glucosidase of anthocyanin isolated from *Berberis integerrima* bunge fruits: A model of antidiabetic compounds. *Evidence-Based Complementary and Alternative Medicine, 2022*, Article 6529590. https://doi.org/10.1155/2022/6529590

Moein, S., Pimoradloo, E., Moein, M., & Vessal, M. (2017). Evaluation of antioxidant potentials and alpha-amylase inhibition of different fractions of Labiatae plants extracts: As a model of antidiabetic compounds properties. *BioMed Research International.* , Article 7319504. https://doi.org/10.1155/2017/7319504, 2017.

Morales-Luna, E., Perez-Ramirez, I. F., Salgado, L. M., Castano-Tostado, E., Gomez-Aldapa, C. A., & Reynoso-Camacho, R. (2019). The main beneficial effect of roselle (*Hibiscus sabdariffa*) on obesity is not only related to its anthocyanin content. *Journal of the Science of Food and Agriculture, 99*(2), 596–605. https://doi.org/10.1002/jsfa.9220

Murugesu, S., Ibrahim, Z., Ahmed, Q. U., Uzir, B. F., Nik Yusoff, N. I., Perumal, V., Abas, F., Shaari, K., & Khatib, A. (2019). Identification of alpha-glucosidase inhibitors from *Clinacanthus nutans* leaf extract using liquid chromatography-mass spectrometry-based metabolomics and protein-ligand interaction with molecular docking. *Journal of Pharmaceutical Analysis, 9*(2), 91–99. https://doi.org/10.1016/j.jpha.2018.11.001

Nagalingam, A. (2017). Drug delivery aspects of herbal medicines. In S. Arumugam, & K. Watanabe (Eds.), *Japanese Kampo medicines for the treatment of common diseases: Focus on inflammation* (pp. 143–164). Academic Press.

Ofosu, F. K., Daliri, E. B., Elahi, F., Chelliah, R., Lee, B., & Oh, D. (2020). New insights on the use of polyphenols as natural preservatives and their emerging safety concerns. *Frontiers in Sustainable Food Systems, 4*. https://doi.org/10.3389/fsufs.2020.525810

Peng, C. H., Chyau, C. C., Chan, K. C., Chan, T. H., Wang, C. J., & Huang, C. N. (2011). *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *Journal of Agricultural and Food Chemistry, 59*(18), 9901–9909. https://doi.org/10.1021/jf2022379

Podsedek, A., Majewska, I., Redzynia, M., Sosnowska, D., & Koziolkiewicz, M. (2014). In vitro inhibitory effect on digestive enzymes and antioxidant potential of commonly consumed fruits. *Journal of Agricultural and Food Chemistry, 62*(20), 4610–4617. https://doi.org/10.1021/jf5008264

Qian, M., Haser, R., Buisson, G., Duee, E., & Payan, F. (1994). The active center of a mammalian alpha-amylase. Structure of the complex of a pancreatic alpha-amylase with a carbohydrate inhibitor refined to 2.2-A resolution. *Biochemistry, 33*(20), 6284–6294. https://doi.org/10.1021/bi00186a031

Rasouli, H., Hosseini-Ghazvini, S. M., Adibi, H., & Khodarahmi, R. (2017). Differential alpha-amylase/alpha-glucosidase inhibitory activities of plant-derived phenolic compounds: A virtual screening perspective for the treatment of obesity and diabetes. *Food & Function, 8*(5), 1942–1954. https://doi.org/10.1039/c7fo00220c

Riaz, G., & Chopra, R. (2018). A review on phytochemistry and therapeutic uses of *Hibiscus sabdariffa* L. *Biomedicine & Pharmacotherapy, 102*, 575–586. https://doi.org/10.1016/j.biopha.2018.03.023

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. *Future Foods, 1–2*. https://doi.org/10.1016/j.fufo.2020.100002

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*. *Future Foods, 4*. https://doi.org/10.1016/j.fufo.2021.100087

Spínola, V., Llorent-Martínez, E. J., & Castilho, P. C. (2020). Inhibition of α-amylase, α-glucosidase and pancreatic lipase by phenolic compounds of *Rumex maderensis* (Madeira sorrel). Influence of simulated gastrointestinal digestion on hyperglycaemia-related damage linked with aldose reductase activity and protein glycation. *LWT - Food Science and Technology, 118*. https://doi.org/10.1016/j.lwt.2019.108727

Stanaway, J. D., Afshin, A., Gakidou, E., Lim, S. S., Abate, D., Abate, K. H., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, M., Abebe, Z., Abera, S. F., Abil, O. Z., Abraha, H. N., et al. (2018). Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990–2017: A systematic analysis for the global burden of disease study 2017. *The Lancet, 392*(10159), 1923–1994. https://doi.org/10.1016/s0140-6736(18)32225-6

Sui, X., Zhang, Y., & Zhou, W. (2016). In vitro and in silico studies of the inhibition activity of anthocyanins against porcine pancreatic α-amylase. *Journal of Functional Foods, 21*, 50–57. https://doi.org/10.1016/j.jff.2015.11.042

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry, 31*(2), 455–461. https://doi.org/10.1002/jcc.21334

Unuofin, J. O., Otunola, G. A., & Afolayan, A. J. (2019). Inhibition of key enzymes linked to obesity and cytotoxic activities of whole plant extracts of *Vernonia mesplilfolia* Less. *Processes, 7*(11). https://doi.org/10.3390/pr7110841

Utami, S., Endrini, S., Nafik, S., Lestari, I. M. T., Anindya, D., Bakar, E. A., Rozy, F., Said, F. F., Afifah, E., Arumwardana, S., Nufus, H., Rihibiha, D. D., Kusuma, H. S. W., Wibowo, S. H. B., & Widowati, W. (2019). In vitro antioxidant and anti-obesity activities of freeze-dried *Canarium sp., Averrhoa bilimbi* L. and *Malus domestica. The Indonesian Biomedical Journal, 11*(3), 225–337.

Wang, S. C., Lee, S. F., Wang, C. J., Lee, C. H., Lee, W. C., & Lee, H. J. (2011). Aqueous extract from *Hibiscus sabdariffa* Linnaeus ameliorate diabetic nephropathy via regulating oxidative status and Akt/Bad/14-3-3gamma in an experimental animal model. *Evidence-Based Complementary and Alternative Medicine.* , Article 938126. https://doi.org/10.1093/ecam/nep181, 2011.

Wickramaratne, M. N., Punchihewa, J. C., & Wickramaratne, D. B. (2016). In-vitro alpha amylase inhibitory activity of the leaf extracts of *Adenanthera pavonina. BMC Complementary Medicine and Therapies, 16*(1), 466. https://doi.org/10.1186/s12906-016-1452-y

Xue, Q., Liu, X., Russell, P., Li, J., Pan, W., Fu, J., & Zhang, A. (2022). Evaluation of the binding performance of flavonoids to estrogen receptor alpha by Autodock, Autodock Vina and Surflex-Dock. *Ecotoxicology and Environmental Safety, 233*, Article 113323. https://doi.org/10.1016/j.ecoenv.2022.113323

Yang, M. Y., Peng, C. H., Chan, K. C., Yang, Y. S., Huang, C. N., & Wang, C. J. (2010). The hypolipidemic effect of *Hibiscus sabdariffa* polyphenols via inhibiting lipogenesis and promoting hepatic lipid clearance. *Journal of Agricultural and Food Chemistry, 58*(2), 850–859. https://doi.org/10.1021/jf903209w

Yilmazer-Musa, M., Griffith, A. M., Michels, A. J., Schneider, E., & Frei, B. (2012). Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of alpha-amylase and alpha-glucosidase activity. *Journal of Agricultural and Food Chemistry, 60*(36), 8924–8929. https://doi.org/10.1021/jf301147n

Zhang, C., Ma, Y., Gao, F., Zhao, Y., Cai, S., & Pang, M. (2018). The free, esterified, and insoluble-bound phenolic profiles of *Rhus chinensis* Mill. fruits and their pancreatic lipase inhibitory activities with molecular docking analysis. *Journal of Functional Foods, 40*, 729–735. https://doi.org/10.1016/j.jff.2017.12.019

CHAPTER 5

Impact of phenolic extracts and potassium hydroxycitrate of *Hibiscus sabdariffa* on adipogenesis: A cellular study

International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

Original article

Impact of phenolic extracts and potassium hydroxycitrate of *Hibiscus sabdariffa* on adipogenesis: a cellular study

Manisha Singh,[1]* Thilini Thrimawithana,[2] Ravi Shukla,[1,3] Charles Stephen Brennan[1] & Benu Adhikari[1]*

1 School of Science, RMIT University, Melbourne VIC, 3083, Australia
2 Discipline of Pharmacy, RMIT University, School of Health and Biomedical Sciences, Melbourne VIC, 3083, Australia
3 NanoBiotechnology Research Laboratory (NBRL), Centre for Advanced Materials & Industrial Chemistry (CAMIC), RMIT University, Melbourne VIC, 3001, Australia

(Received 25 September 2022; Accepted in revised form 19 December 2022)

Summary Adipogenesis is a complex physiological process involving the formation of adipocytes and accumulation as adipose tissues. It is one of the contributors for the development of obesity. This study assessed the potential of phenolic extracts and potassium hydroxycitrate, obtained from *Hibiscus sabdariffa*, to inhibit adipogenesis. The phenolic extracts were obtained using organic solvents (methanol, ethanol and ethyl acetate) and water individually. Human adipose-derived stem cells (hADSCs) were selected to study the impact of these extracts on adipogenesis. Results showed that phenolic extracts were able to reduce lipid accumulation by about 95% in hADSCs, while potassium hydroxycitrate did not show any reduction. All the phenolic extracts downregulated the gene expression of two key adipogenic markers (PPAR-γ and aP2). Ethanol extracts exhibited the highest downregulation of PPAR-γ and aP2 by 3 and 10 times, respectively. There was no improvement in the anti-adipogenic potential when the phenolic extract was combined with potassium hydroxycitrate confirming that phenolic compounds were responsible for the inhibition of adipogenesis. These results indicate that phenolic extracts from *H. sabdariffa* have potential to regulate the expression of adipogenic genes and restrict the lipid accumulation in mature adipocytes. Thus, phenolic extracts can be used in formulations intended to manage obesity.

Keywords Adipogenesis, *Hibiscus sabdariffa*, human adipose-derived stem cells, lipid accumulation, obesity, phenolic extracts.

Introduction

Obesity is a complex and relapsing disorder associated with excessive accumulation of body fat due to an imbalance between energy intake and expenditure through metabolic, physical and other activities (Guo *et al.*, 2017). Changes in lifestyles have resulted in obesity becoming a growing issue in both adults and children worldwide. On average, about 60% of adults across 20 countries under Organisation for Economic Co-operation and Development (OECD) were categorised as overweight or obese in 2019 (OECD, 2021). Obesity can contribute to a variety of metabolic abnormalities such as cardiovascular diseases, dyslipidaemia, hypertension and type 2 diabetes. Obesity is currently managed with the use of lifestyle modifications and pharmacotherapy (Singh *et al.*, 2020). Fewer research is focused on the application food bioactive ingredients in the treatment and prevention of obesity (Liang *et al.*, 2021; An *et al.*, 2022). Among several anti-obesity mechanisms, research has largely been focused on treating or preventing obesity through the inhibition of adipogenesis (Jang *et al.*, 2016; Podsędek *et al.*, 2020; Guillemet *et al.*, 2022).

Adipocytes are considered vital components of fat tissues and play an important role in the regulation of energy and glucose homeostasis (Moseti *et al.*, 2016). However, excessive intake of energy, accompanied by low energy expenditure, can cause the expansion of adipocytes through the increase in size (hypertrophy) and/or number (hyperplasia or adipogenesis) leading to obesity. Environmental and genetic factors can also affect the formation and function of adipocytes (McPherson, 2007). Adipogenesis, also called adipogenic differentiation, is a complex process whereby preadipocytes differentiate to mature adipocytes (Eseberri *et al.*, 2019). Adipogenesis is characterised by

*Corresponding author: E-mail: benu.adhikari@rmit.edu.au (B. A.); s3736496@student.rmit.edu.au (M. S.)

doi:10.1111/ijfs.16269

changes in cell morphology, insulin sensitivity and gene transcription (Moseti *et al*., 2016). The differentiation of preadipocytes is initiated with the morphological transition from fibroblastic to spherical shape followed by the accumulation of triglyceride to form intracellular lipid droplets (Gregoire *et al*., 1998). The molecular regulation of adipogenesis commences with the activation of the transcription factors, CCAAT/enhancer binding proteins (C/EBP)-β and C/EBP-δ and sterol-regulatory element binding protein-1 (SREBP1). This leads to the transcription of C/EBP-α and peroxisome proliferative-activated receptor-γ (PPAR-γ), which is the master regulator of adipogenesis. The terminal stage of differentiation involves the transcription of late adipogenic transcriptional factors such as aP2 (fatty acid binding protein) and the accumulation of intracellular lipid droplets (Fink & Zachar, 2011).

In vitro models are frequently used to evaluate the efficacy of therapeutics on adipogenesis for the management of obesity. These include 3T3-L1, 3T3-F442A, C3H10T1/2, OP9, MEFs, human adipose-derived stem cells (hADSCs) and primary preadipocytes. Among these, 3T3-L1 cells (mouse cell line) have been extensively used as *in vitro* cell model for adipogenesis. However, the outcomes of these studies may be less informative or useful for the clinical application due to physiological and metabolic differences between the species (Ruiz-Ojeda *et al*., 2016). hADSCs are stem cells isolated from the adipose tissue that are capable of *in vitro* proliferation and can be differentiated into several cell lineages including adipogenic, chondrogenic, neural or osteogenic. The adipogenic differentiated hADSCs functionally and morphologically resemble the mature human adipocytes (Brannmark *et al*., 2014). This permits the differentiated hADSCs to be used as a more suitable *in vitro* adipocyte model to investigate the physiological and functional characteristics (Brannmark *et al*., 2014). Adipogenesis can be chemically induced in hADSCs using a mixture of dexamethasone, indomethacin, insulin and 3-isobutyl-1-methylxanthine (IBMX). The combination of dexamethasone and IBMX enhances the expression of PPAR-γ at the early stage of differentiation. Both of these differentiating agents also induce the expression of C/EBP-δ and C/EBP-β which are vital for adipogenesis (Scott *et al*., 2011).

Plants have been widely exploited for their anti-obesity potential, as these are considered safer alternatives to synthetic drugs (Li *et al*., 2016; Gomez-Zorita *et al*., 2017; Aranaz *et al*., 2019; Mladenova *et al*., 2021). In addition, consumers are increasingly aware of the benefits of functional or health-enhancing foods as studies are showing evidence of efficacy of bioactive ingredients from various plant sources (Ogundijo *et al*., 2022). *Hibiscus sabdariffa* is an herbaceous subshrub belonging to the Malvaceae family. Traditionally, the calyces from this plant are used for treating common cold, cough, hypercholesterolemia, hypertension and indigestion (Riaz & Chopra, 2018). Extracts from calyces of *H. sabdariffa* are explored as therapeutic agent for the treatment of chronic ailments such as diabetes mellitus (Wang *et al*., 2011), cardiovascular diseases (Asgary *et al*., 2016) and obesity (Kao *et al*., 2016; Amaya-Cruz *et al*., 2019). Studies have reported the inhibitory effect of phenolic compounds of *H. sabdariffa* (chlorogenic acid, caffeic acid, ellagic acid and gallic acid) and flavonoids (anthocyanins, catechins and quercetin) as well as organic acids (hydroxycitric acid and hibiscus acid) on obesity (Herranz-López *et al*., 2012; Kao *et al*., 2016; Amaya-Cruz *et al*., 2019). Studies have also shown an inhibitory effect of aqueous and ethyl acetate extracts of *H. sabdariffa* on adipogenesis (Kim *et al*., 2003; Kim *et al*., 2007; Kao *et al*., 2016; Janson *et al*., 2021) using 3T3-L1 cells. To the best of our knowledge, no studies has used hADSCs to assess the adipogenesis-suppressing effect of phenolic extract and potassium hydroxycitrate of *H. sabdariffa*, despite their closeness to human adipose cells. Hence, this study examined the potential of phenolic extracts and potassium hydroxycitrate from *H. sabdariffa* in inhibiting adipogenic differentiation in hADSCs. The phenolic compounds from *H. sabdariffa* were extracted in different solvents (water, methanol, ethanol and ethyl acetate) along with potassium hydroxycitrate in methanol. All of these extracts were comparatively tested on hADSCs to quantify their adipogenesis-suppressing effect. Adipogenic differentiation with subsequent lipid accumulation in the cells was measured. The expression level of the relevant genes (PPAR-γ and aP2) was also measured. The results showed the phenolic extracts of *H. sabdariffa* extracted in organic solvents were able to interfere with lipid accumulation during the formation of adipocytes. The outcome of this research is expected to be applicable in the management of obesity in humans.

Materials and methodology

Plant material

Frozen calyces of *H. sabdariffa* were generously gifted by Wild Hibiscus Flower Co. (NSW, Australia) and were stored at −20 °C upon receipt.

Chemicals

Methanol, ethanol, hexane, ethyl acetate, potassium hydroxide, activated charcoal and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (Castle Hill, NSW, Australia). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT), dexamethasone (DXM), 3-isobutyl-1-methylxanthine (IBMX), insulin, indomethacin, formaldehyde (36.5–38.0%), Nile red, oil red O solution (0.5% in isopropanol) were also purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin–streptomycin, phosphate-buffered saline (PBS, 7.4), 4,6-diamidino-2-phenylindole, dilactate (DAPI) were acquired from Thermo Fischer Scientific Pty. Ltd. (Scoresby, VIC, Australia). All the chemicals purchased were of analytical reagent grade and used as supplied without any further purification. Ultrapure water (Milli-Q grade water) from a Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., NSW, Australia) was used for all the experiments.

Extraction of phenolic compounds and potassium hydroxycitrate from *H. sabdariffa*

The frozen calyces of *H. sabdariffa* were prepared for extraction by freeze drying (Labconco Triad 7400030, USA) at −40 °C and 12 Pa. The calyces were then powdered in a grinder (Breville, Sydney, Australia) and sieved through 353 μm pore size. The powder was stored in amber-coloured glass bottles and kept in a desiccator covered with aluminium foil at room temperature (23 °C) until further tests.

The phenolic compounds were extracted from the powdered calyces in three different solvents (water, methanol and ethanol) following the protocol described in our previous studies (Singh *et al.*, 2021; Singh *et al.*, 2022). Briefly, powdered sample (5 g) was extracted at room temperature (23°C) with 100 mL of water, methanol (100%) or ethanol (100%) and was stirred (MR HeiTec, Heidolph, Germany) at 500 rpm for 2 h. The mixture was then centrifuged (Allegra 64R, Beckman Coulter, Australia) at 11 000 ***g*** for 20 min at room temperature (23°C) followed by vacuum filtration with Whatman no. 1 filter paper. The aqueous supernatant was freeze-dried at same condition as mentioned above while the organic supernatants were concentrated in a rotary vacuum concentrator (John Morris, Australia) at 45 °C and 10 Pa. The dried extracts were flushed with nitrogen and stored at −30 °C in an amber-coloured bottles until further evaluation. The total phenolic contents of water, methanol and ethanol extracts were previously reported to be 37.36 ± 1.23, 51.72 ± 0.71 and 48.85 ± 1.88 mg gallic acid equivalent per g of dry extract, respectively (Singh *et al.*, 2021).

Yang *et al.* (2010) have reported a partition extraction method that can extract phenolic compounds more efficiently than the aqueous extraction method. Therefore, this method was also used in this study to extract phenolic compounds by following the protocol as described in our previous study (Singh *et al.*, 2022) by Peng *et al.* (2011). The partition extraction was initiated by mixing 10 g of powdered calyces with 100 mL of methanol and the extraction was performed at 60 °C with agitation of 500 rpm for 30 min. The extract was vacuum filtered through a Whatman No. 1 filter paper, and the residue was re-extracted twice with methanol as described above. All the supernatants were pooled together, and the methanol was evaporated at 45 °C and 10 Pa with the rotary vacuum concentrator. The residue from this was solubilised in water (20 mL) and extracted thrice with hexane (3 × 20 mL) in a separating funnel. The aqueous fraction was again extracted with ethyl acetate (3 × 20 mL) in the separating funnel for three more times. Lastly, the ethyl acetate part was concentrated to dryness in the rotary vacuum concentrator. The dried extract was kept at −30 °C and at the same condition as mentioned above. The total phenolic content of this extract was 162.15 ± 6.40 mg gallic acid equivalent per g of dry extract as reported previously (Singh *et al.*, 2022).

The hydroxycitric acid was extracted in the form of potassium salt due to its better stability compared to the free acid form (Mena-García *et al.*, 2022) by following the method described by Majeed *et al.* (2004) which is also as reported in our previous study (Singh *et al.*, 2022). Five grams of powdered calyx was mixed with methanol (15 mL) and placed on a hot plate at 65 °C for 3 h with an agitation of 500 rpm. The mixture was filtered through Whatman no 1 filter paper, and the supernatant was collected. The residue was re-extracted twice using the same volume of methanol and using identical extraction conditions. All three supernatants were combined and treated with activated charcoal for 1 h to remove compounds like anthocyanins that contribute to the colour of the extract. This was filtered using Whatman no 1 filter paper, and the supernatant was treated with methanolic potassium hydroxide (1 M) for 3 h at constant pH of 10. The precipitated potassium hydroxycitrate was then vacuum filtered and washed with methanol. Finally, the residue was vacuum dried (Ezzi Vision, Australia) at 50 °C and 10 Pa in oven. The dried powder was stored at 4 °C in an airtight container.

Culturing human adipose-derived stem cells (hADSCs)

The hADSCs were obtained from ATCC (Manassas, VA, USA) and cultured in a growth medium containing DMEM (1 g L^{-1} glucose) supplemented with 20% FBS and 1% penicillin–streptomycin (100 U mL^{-1} and 100 μg mL^{-1}). Only early passages of hADSCs (passages 4–6) were used in these experiments. The cells were plated at a density of 4500 cells cm^{-2} and grown in an incubator at 37 °C and atmosphere of 95% air and 5% CO_2.

Determining cytotoxicity of DMSO, extracts and potassium hydroxycitrate on hADSCs

DMSO was used as a solvent to dissolve the extracts that are extracted in organic solvents as they were not soluble in water. Therefore, it was necessary to evaluate the toxicity of DMSO on hADSCs and determine the appropriate concentration that did not kill the cells. For this purpose, MTT assay was performed by following the method from Riss *et al.* (2016). The hADSCs were seeded in a 96-well plate (Corning, Sigma-Aldrich) at a concentration of 4500 cells per cm^2 in the growth medium with 20% FBS as mentioned above and incubated at 37 °C in a humidified incubator maintained at 5% CO_2 for 24 h. Following the incubation, eight different concentrations of DMSO (0.031, 0.063, 0.125, 0.250, 0.500, 1.000, 2.000 and 4.000% (v/v)) were tested on hADSCs for 72 h in a 37 °C humidified incubator. DMSO was serially diluted in the growth medium before the treatment. After incubation, the cells were washed with PBS, and media were replaced by 100 µL of MTT reagent (0.5 mg mL^{-1}) and incubated for another 4 h at 37 °C. Then, the MTT solution was substituted with of DMSO (100 µL) to solubilise formazan crystals. The plate was slightly agitated and incubated in the dark at room temperature for 30 min. Then absorbance of the resultant solution was recorded at 570 nm in a CLARIOstar® microplate reader (BMG Labtech, Ortenberg, Germany). The cell viability was calculated using the eqn (1).

$$\text{Cell viability } (\%) = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100. \quad (1)$$

The stock solution at 200 mg mL^{-1} was prepared in DMSO for all the organic solvent-based extracts of *H. sabdariffa* and in water for water extract and potassium hydroxycitrate. The cytotoxicity of *H. sabdariffa* calyces extracts and potassium hydroxycitrate was also performed through MTT assay as mentioned above. The stock solutions of extracts and potassium hydroxycitrate (200 mg mL^{-1}) were serially diluted in the growth media to obtain six different concentrations (63–2000 µg mL^{-1}) with a final DMSO concentration ranging from 0.03 to 1.00% (v/v) in the media. The total phenolic content at these concentrations was equivalent to 2.35–74.72 µg mL^{-1} for water extract, 3.26–103.44 µg mL^{-1} for methanol extract, 3.08–97.70 µg mL^{-1} for ethanol extract and 10.22–324.30 µg mL^{-1} for ethyl acetate extract. After incubating hADSCs for 24 h at 37 °C in a 96-well plate, they were treated with the above-mentioned six different concentrations of phenolic extracts and potassium hydroxycitrate, and the cells were incubated for 72 h in a 37 °C humidified incubator. After incubation, the MTT assay was performed as mentioned above, and the cell viability was calculated using eqn (1).

Adipogenic differentiation of human adipose-derived stem cells

The hADSCs were plated at a density of 4500 cells cm^{-2} in 8 well µ-slide (ibidi, Gräfelfing, Germany) and grown at 37 °C in a humidified incubator. Cells were grown to 100% confluency in the growth medium supplemented with 20% FBS and 1% penicillin–streptomycin and kept in a confluent state for 2 days. After 2-day of confluency, cells were incubated with adipogenesis-inducing medium comprising of DMEM (4.5 g L^{-1} glucose), 10% FBS, 1% penicillin–streptomycin (100 U mL^{-1} and 100 µg mL^{-1}), 1 µM DXM, 0.5 mM IBMX, 0.17 µM insulin and 0.2 mM indomethacin for 3 days (Qian *et al.*, 2010; Nielsen *et al.*, 2016). Following this, the cells were incubated in the adipogenesis maintenance medium (DMEM with 4.5 g L^{-1} glucose, 10% FBS, 1% penicillin–streptomycin and 0.17 µM insulin) for 18 days to progress differentiation and evaluate changes in response to *H. sabdariffa* extracts (Fig. 1).

Treatment of cells with *H. sabdariffa* extracts

The cells were grown to confluency and the differentiation was initiated with the adipogenesis-inducing medium as described previously. The stock solutions of extracts and potassium hydroxycitrate were serially diluted from 31 to 1000 µg mL^{-1} in the adipogenesis-inducing medium which gave the total phenolic content ranging from 1.16 to 162.15 µg mL^{-1}. Then, the cells were treated with these diluted extracts for the first 3 days of differentiation. Control experiments were also performed with only DMSO at respective concentrations in the extracts. After 3 days, it was replaced by adipogenesis-maintaining medium (DMEM, 10% FBS, 1% penicillin–streptomycin and 0.17 µM insulin) for up to the 21st day of differentiation.

Treatment with the combination of phenolic extract and potassium hydroxycitrate

The phenolic compounds-to-potassium hydroxycitrate ratio in the original *H. sabdariffa* powder was 3:1 (Singh *et al.*, 2021). Three different combinations (2:1, 3:1 and 4:1) of phenolic compound-rich ethyl acetate extract (due to its highest phenolic content) and standard potassium hydroxycitrate were tested on hADSCs to evaluate potential synergistic effect on adipogenic differentiation. The concentration of ethyl acetate

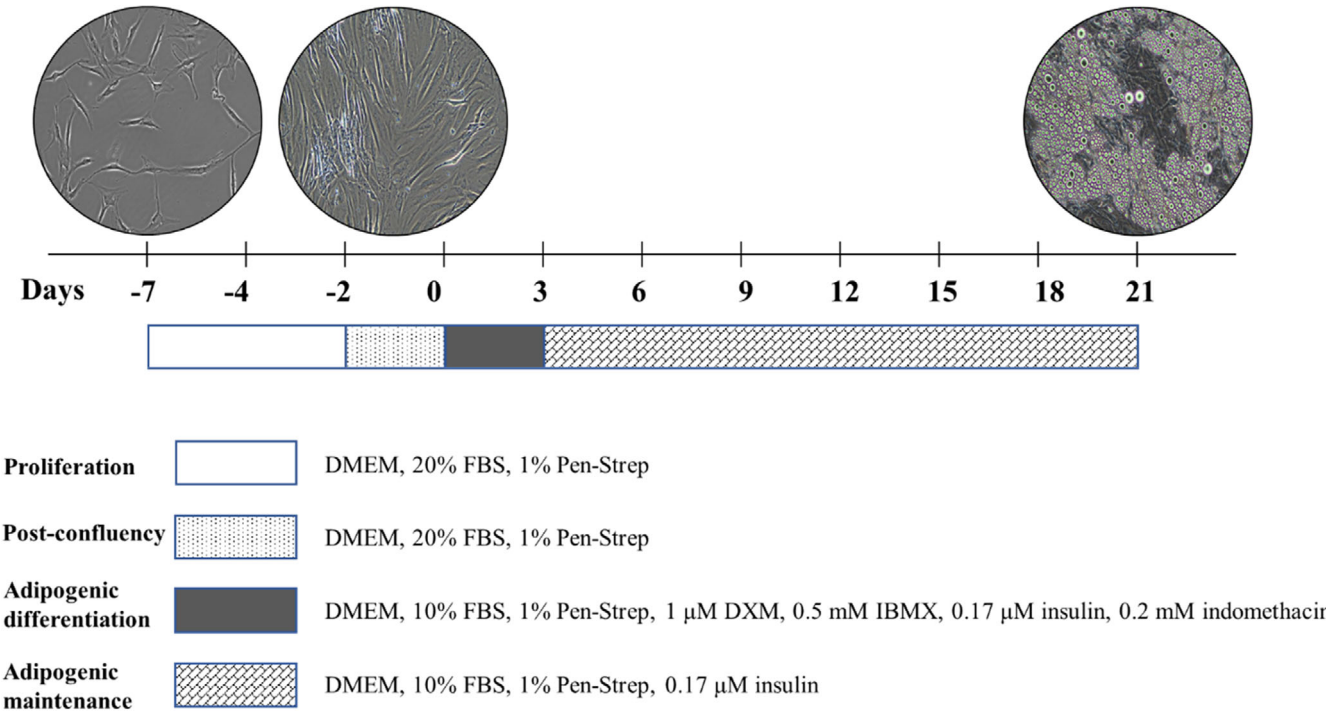


Figure 1 Adipogenic differentiation of human adipose-derived stem cells. The fibroblastic stem cells transformed into adipocytes with the accumulation of intracellular lipid after 21 days of differentiation in adipogenesis-inducing and adipogenesis-maintaining medium.

extract was kept constant (500 µg mL^{-1}) in all these combinations.

Measurement of lipid accumulation through oil red O staining method

Quantification of lipids with oil red O staining was performed according to Kraus *et al.* (2016). Oil red O solution (0.5% in isopropanol) was diluted with distilled water at a 3:2 ratio to prepare the fresh working solution. The solution was incubated for 20 min at room temperature and filtered through a 0.2 µm filter before use. The completely differentiated cells were washed twice with PBS and fixed with 4% formaldehyde for 30 min at room temperature. Subsequently, the cells were washed twice with PBS. Then, the cells were stained by adding 300 µL of oil red O working solution and incubated at room temperature for 10 min. Next, the cells were washed with water 4 times, air-dried and imaged with Eclipse TS 100 inverted microscope (Nikon Corporation, Tokyo, Japan) at 10× magnification. For the quantification of lipid accumulation, 200 µL of isopropanol was added to each well and kept in an orbital shaker with shaking for 15 min at room temperature. Finally, 100 µL of this solution was transferred to a 96-well plate (Corning, NY, USA), and the absorbance was noted at 510 nm on a CLARIOstar® microplate reader. The lipid accumulation was estimated by using eqn (2).

$$\text{Lipid accumulation } (\%) = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated (differentiated) cells}} \times 100. \quad (2)$$

Assessment of lipid accumulation through Nile red staining

The lipid accumulation was also measured by Nile red staining (Haselgrubler *et al.*, 2019) which is a hydrophobic fluorescent dye. Cells that were treated and differentiated for 21 days were fixed with 4% paraformaldehyde and washed with PBS as described in above section. The cells were stained with Nile red solution (50 µg mL^{-1} in PBS) for 30 min. Stained cells were rinsed twice with PBS and re-stained with DAPI (2 µg mL^{-1} in PBS) for 5 min. The stained cells were washed twice with PBS and were imaged on Nikon A1R laser on an ECLIPSE Ti-E inverted microscope (Minato, Tokyo, Japan). An excitation wavelength of 488 nm was used to induce fluorescence and an emission filter of 500–530 nm was used to observe and acquire the images of stained lipids. Five images were

taken from each well at 10× magnification, and Nile red-stained surface area was analysed in FIJI with Java 6 software (Madison, Wisconsin, USA) for the quantification of lipids.

RNA extraction and quantitative real-time qPCR

For gene expression analysis, cells were plated at a density of 4500 cells cm^{-2} in an 8-well μ-slide, and treated cells were allowed to differentiate for 21 days as described in previous section. Total cellular RNA was extracted using a TRIZOL® reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions and then quantified using NanoDrop™ One UV–Vis Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA (1 μg) was reverse transcribed using an LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Real-time PCR was performed with the Rotor-Gene Q real-time RT-qPCR System (Qiagen, MD, USA) using Luna® Universal qPCR Master Mix (New England Biolabs) under the following conditions: denaturation at 95 °C for 1 min, 40 cycles of amplification at 95 °C for 15 s and 60 °C for 30 s. After amplification, a melt curve was generated at 95 °C for 30 s and 60 °C for 30 s and ramped at 0.1 °C s^{-1}. The primers (IDT, Coralville, IA, USA) and their sequences for the genes of interest used in this study are given below (Fink & Zachar, 2011).

β-actin forward primer: 5′-TGACGGGGTCACCCACACTGTGCCCAT-3′.

β-actin reverse primer: 5′-CTAGAAGCATTTGCGGTGGACGATGGA-3′.

PPAR-γ forward primer: 5′-TCAGGTTTGGGCGGATGC-3′.

PPAR- γ reverse primer: 5′-TCAGCGGGAAGGACTTTATGTATG-3′.

aP2 forward primer: 5′-ATGGGATGGAAAATCAACCA-3′.

aP2 reverse primer: 5′-GTGGAAGTGACGCCTTTCAT-3′.

For relative gene expression level, β-actin was used as a housekeeping gene and was calculated using the comparative $2^{-\Delta\Delta Ct}$ method based on the difference between Ct values (Livak & Schmittgen, 2001).

Statistical analysis

All the experiments were performed in triplicate and the data are presented as mean ± SD. Data were analysed by using SPSS statistical software (SPSS 23.0, IBM, Armonk, NY, USA). A two-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference post hoc analysis was performed to determine statistically significant differences between experimental parameters. For all the tests, a significance level of 5% ($P < 0.05$) was applied.

Result and discussions

Cytotoxic effect of DMSO, phenolic extracts and potassium hydroxycitrate on hADSCs

DMSO is a polar organic solvent which is amphipathic in nature. It serves as a solvent for both polar and non-polar molecules (Tuncer *et al.*, 2018). In this study, DMSO was used to dissolve the organic solvent-based extracts, that is methanol, ethanol and ethyl acetate. However, DMSO may possess detrimental effects and can alter the morphology and viability of mesenchymal stem cells (Lee & Park, 2017). We found that DMSO did not induce toxicity up to the concentration of 1% (v/v) as the cell viability was comparable to that of the control (99.69%). When the DMSO concentration was increased to 2%, the cell viability decreased to 67.80% and to 37.50% at 4% of DMSO. Hence, as a vehicle or solvent, the maximum concentration of 1% of DMSO was selected for hADSCs for 72 h of treatment.

Sharma *et al.* (2015) reported the cytotoxic effect of DMSO on human umbilical cord mesenchymal stem cells. Treatment with 1% of DMSO for 48 h reduced the cell viability to just above 80.00%. With higher DMSO concentration, at 2% and 3%, the cell viability dropped to 57.75% and 36.37%, respectively. The cell viability for 1% DMSO in the study of Sharma *et al.* (2015) was considerably lower than in our current study on hADSCs; however, the viability loss at 2% DMSO was comparable. The disparity on cell viability reported in Sharma *et al.* (2015) and our study at 1% DMSO may be due to the different types of stem cells used. The cytotoxicity of DMSO is explained by its amphipathic nature allowing its affinity towards the plasma membrane. At higher concentrations and longer exposure time, it can form pores in the plasma membrane, reduces membrane selectivity and increases cell permeability leading to cell death (de Abreu Costa *et al.*, 2017).

As stated above, the maximum concentration of DMSO for treating hADSCs was maintained at 1%. Therefore, to keep the concentration of DMSO below this level, the highest concentration of methanol, ethanol and ethyl acetate extract that could be used was found to be 2000 μg mL^{-1}. The dose-dependent effect of *H. sabdariffa* extracts (water, methanol, ethanol and ethyl acetate) and potassium hydroxycitrate on hADSCs up to the concentration of 2000 μg mL^{-1} is presented in Fig. 2. Cell viability following treatment with the extracts remained above 90% for up to 1000 μg mL^{-1} in all the treatments. In the case of potassium hydroxycitrate, the cell viability was

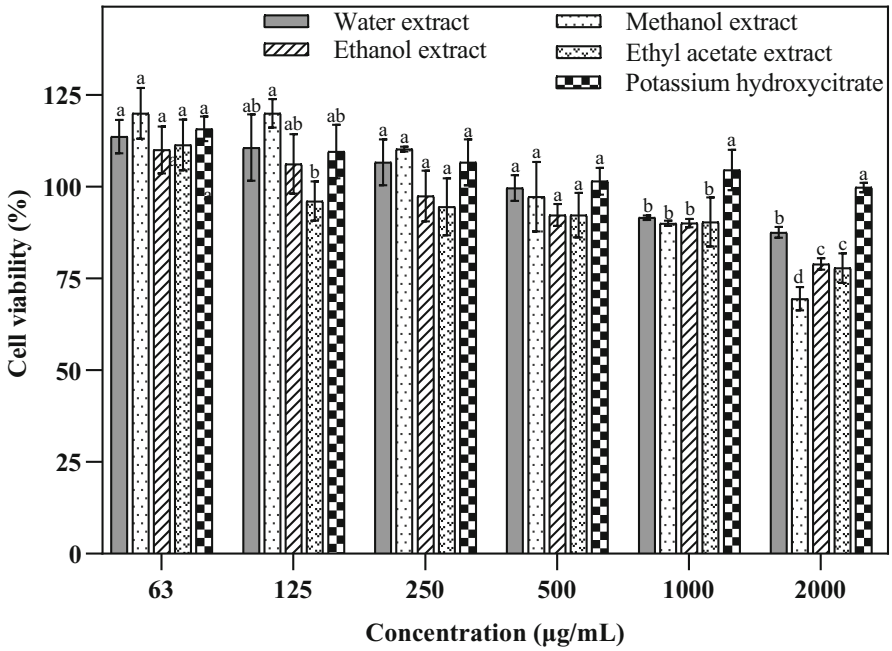


Figure 2 Effect of phenolic extracts and potassium hydroxycitrate of *Hibiscus sabdariffa* and their concentration on the cell viability of hADSCs in comparison with the untreated (control) cells. The different lower-case letters indicate statistically significant differences ($P < 0.05$) among the different samples at the same concentration.

comparable to that in the control (2000 µg mL^{-1}) which suggested its low toxicity on hADSCs. All four phenolic extracts reduced cell viability considerably at 2000 µg mL^{-1} concentration. The methanol extract was the most toxic ($P < 0.05$) to hADSCs followed by ethyl acetate, ethanol and water extract at a concentration of 2000 µg mL^{-1}. Previous study also showed that aqueous roselle extract did not impart toxicity to 3T3-L1 cells up to 1000 µg mL^{-1} concentration when treated for 9 days (Janson *et al.*, 2021).

The reduction in cell viability at higher concentration of extracts (>1000 µg mL^{-1}) is likely due to cellular toxicity of the phenolic compounds in the extracts. Ramarao *et al.* (2022) reported that *Moringa oleifera* leaf extract containing phenolic compounds reduced the cell viability to below 80% of human Wharton's Jelly mesenchymal stem cells due to cell cycle arrest and induction of apoptosis at a dose level of 437.43 µg mL^{-1}. The pH of the media (7.4) was lowered to 6.8–7.0 when the concentration of the extract (water, methanol, ethanol and ethyl acetate) added to the media was 2000 µg mL^{-1}. This variation in pH may also have impacted on the cell viability. Li *et al.* (2012) reported that human adipose-derived mesenchymal stem cells were sensitive to pH levels and the cell viability significantly decreased by nearly 40% when the pH was reduced from 7.4 to 6.8. Based on these reasons, extract concentration of 1000 µg mL^{-1} was selected as the highest concentration to be used for treating the hADSCs and to evaluate the effect of these extracts on adipogenesis.

Quantification of lipid accumulation through oil red O staining method

The hADSCs were treated with different concentrations of water, methanol, ethanol, ethyl acetate extracts and potassium hydroxycitrate during the induction phase of adipogenic differentiation. On the 21st day of differentiation, the cells were stained with oil red O as it is the lipophilic dye commonly used for staining neutral lipids such as triacylglycerols and cholesteryl esters (Fowler & Greenspan, 1985; Rogowski *et al.*, 2019). Figure 3 represents the oil red O stained hADSCs after treatment and differentiation for 21 days and the accumulated lipid is represented by the red droplets. The differentiated cells showed the highest accumulation of oil red O stain (indicating accumulation of lipids) whereas there was no stain in undifferentiated cells, highlighting the success of the differentiation process. The dose-dependent adipogenesis inhibition pattern by these extracts can be observed through the data presented in Fig. 3. At 1000 µg mL^{-1} concentration, all the phenolic extracts showed greater inhibition of adipogenic differentiation as indicated by the low number of lipid droplets. However, in the case

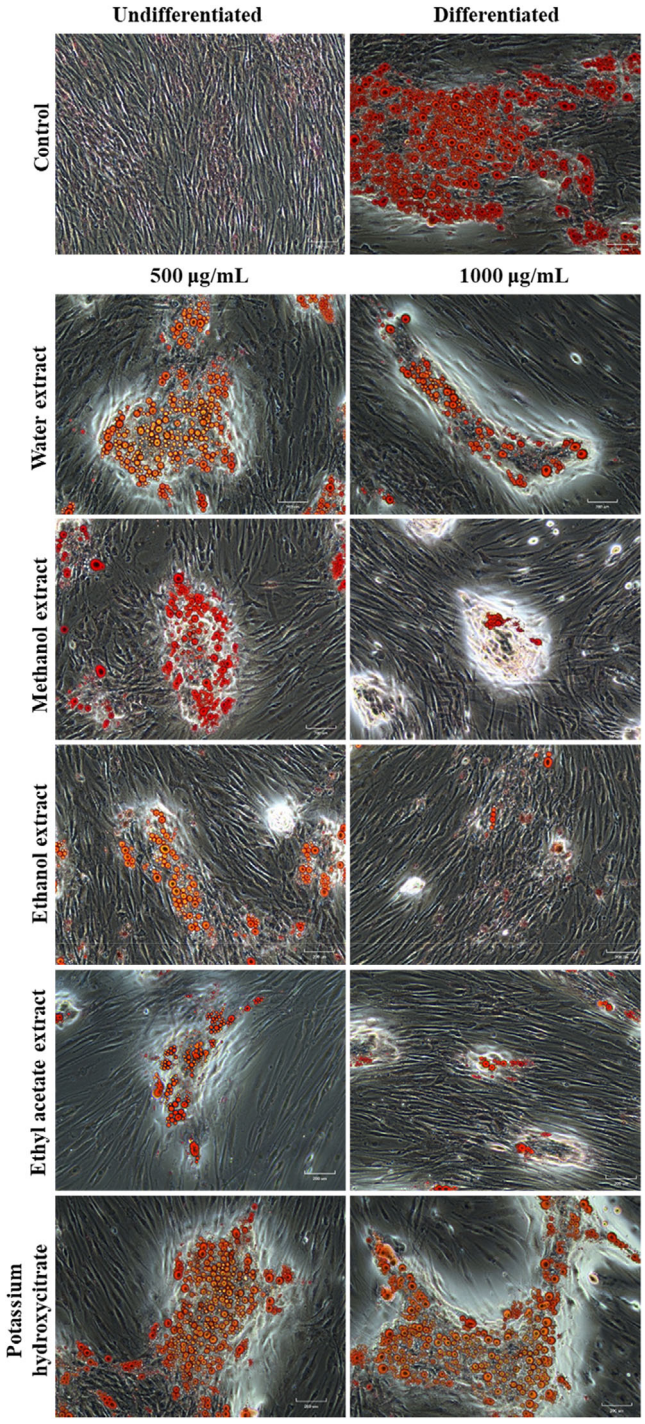


Figure 3 Representative microscopic images showing oil red O staining (lipid accumulation) of human adipose-derived stem cells after treatment with *Hibiscus sabdariffa* extracts and differentiating for 21 days. Red droplets represent the accumulation of lipid droplets. All the images were captured by light microscope at 10× magnification.

of potassium hydroxycitrate, there was no change in lipid accumulation as compared with the untreated differentiated cells.

The effect of phenolic extracts and potassium hydroxycitrate on lipid accumulation was also spectrophotometrically quantified using a microplate reader. A dose-dependent inhibition on the accumulation of lipids was observed in all these phenolic extracts (Fig. 4). Two-way ANOVA showed a significant ($P < 0.05$) effect of extract type, concentration of extract and their interaction on lipid accumulation. The lipid accumulation progressively decreased when the concentration of phenolic extracts was increased from 31 to 1000 µg mL^{-1}. A concentration of 250 µg mL^{-1} of methanol, ethanol and ethyl acetate extracts which is equivalent to total phenolic content of 12.93, 12.21 and 40.54 µg GAE mL^{-1} respectively was sufficient to reduce the lipid accumulation by more than 60% The highest inhibition was observed in the case of ethanol extract at 1000 µg mL^{-1} with the lowest lipid accumulation of only 6.31%. Kim *et al.* (2007) reported that aqueous *H. sabdariffa* extract reduced the lipid accumulation by about 30% at the same (1000 µg mL^{-1}) concentration in 3T3-L1 preadipocytes. In this study, the aqueous extract reduced lipid accumulation by 45% at the same concentration which is significantly higher than reported by these authors. This higher effectiveness may be due to the difference in cell types, the origin of the plant and the extraction method used. Potassium hydroxycitrate did not significantly alter the lipid accumulation in hADSCs ($P > 0.05$). This finding is not in agreement with that of Han *et al.* (2022) which showed significant reduction in lipid accumulation (oil red O staining) in 3T3-L1 cells treated with 178.65 µg mL^{-1} hydroxycitric acid from *Garcinia cambogia*. This could be attributed to different cell lines used. It also indicates that it is essential to assess the adipogenesis-suppressing effect of plant ingredients in cells close to that of human.

Quantification of lipid accumulation through Nile red staining method

The differentiated hADSCs were stained with Nile red for lipids and DAPI for the nucleic acid. The confocal images of stained cells are shown in Fig. 5. The green fluorescence represents the lipid droplets formed and accumulated after the adipogenic differentiation. All phenolic extracts obtained from *H. sabdariffa* were able to significantly ($P < 0.05$) reduce the lipid accumulation in hADSCs. Statistical analysis (two-way ANOVA) indicated significant ($P < 0.05$) differences in the lipid accumulation due to the type and concentration of extract (Fig. 6). The area covered by green

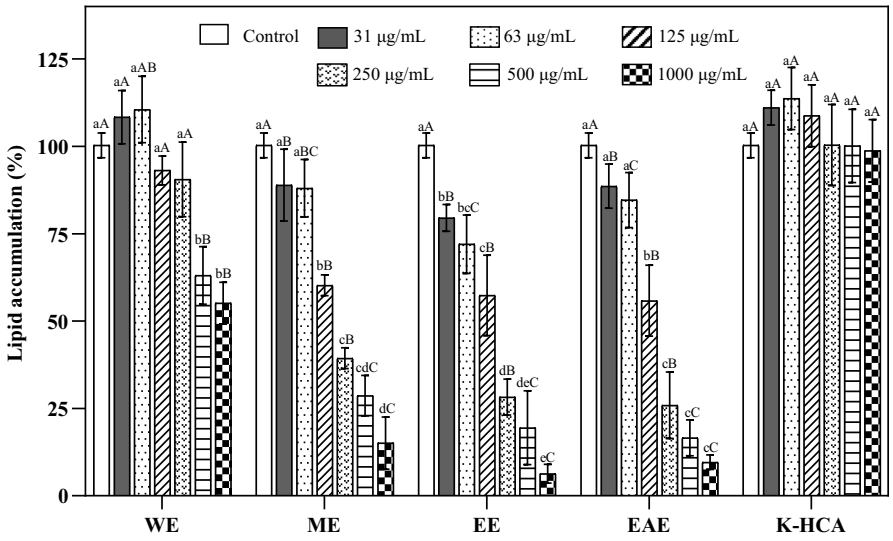


Figure 4 Effect of phenolic extract and potassium hydroxycitrate of *Hibiscus sabdariffa* on the lipid accumulation during adipogenesis quantified through oil red O staining. WE, water extract; ME, methanol extract; EE, ethanol extract; EAE, ethyl acetate extract; K-HCA, potassium hydroxycitrate. The different lower-case letters indicate statistically significant differences ($P < 0.05$) among the different concentration of same extract. The different capital letters indicate statistically significant differences ($P < 0.05$) between the different extract at same concentration.

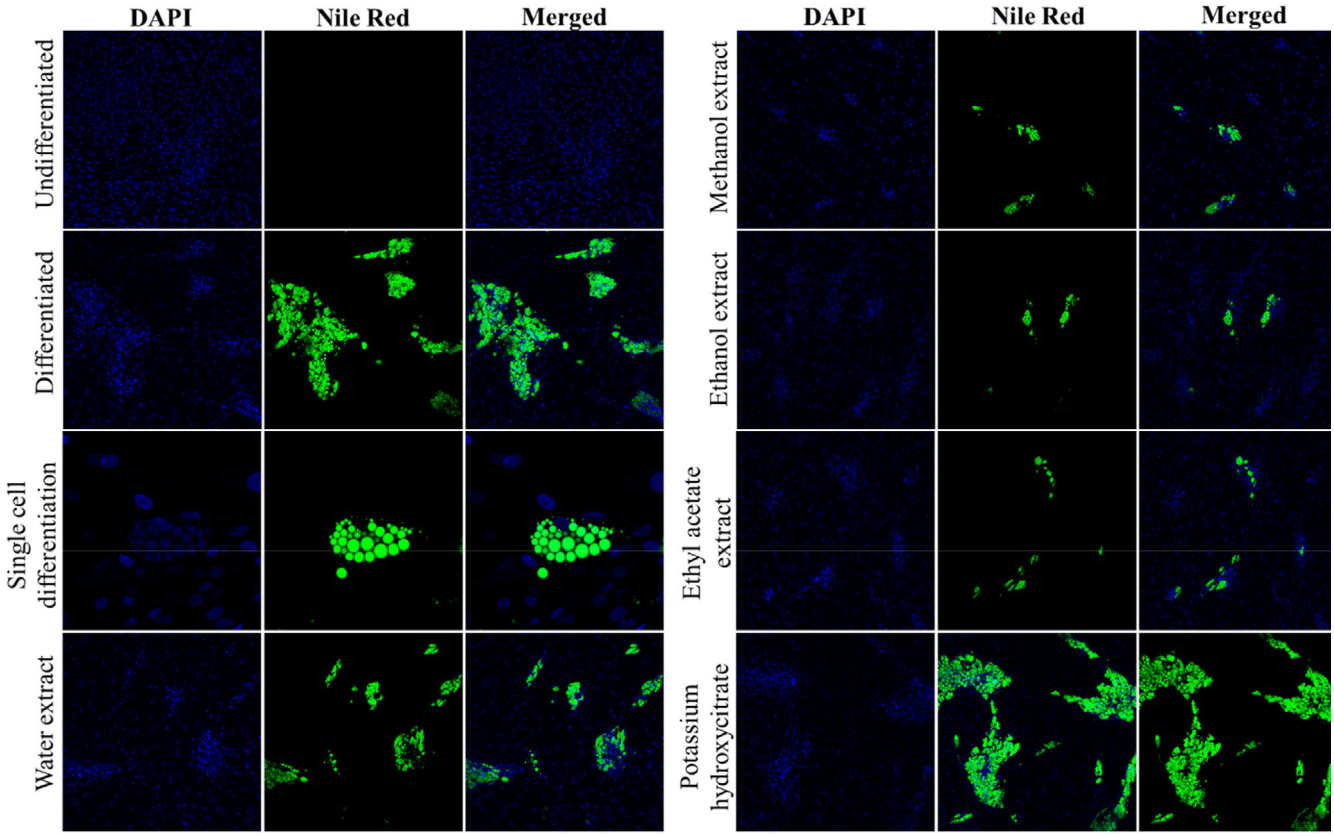


Figure 5 Representative confocal laser microscopic images of human adipose-derived stem cells after treatment with *Hibiscus sabdariffa* extracts at 1000 μg mL^{-1} and differentiating for 21 days. The cells are stained with Nile red for lipid droplets (green) and DAPI for the nucleus (blue). All the images were captured at 10× magnification except for single cell differentiation (at 60×).

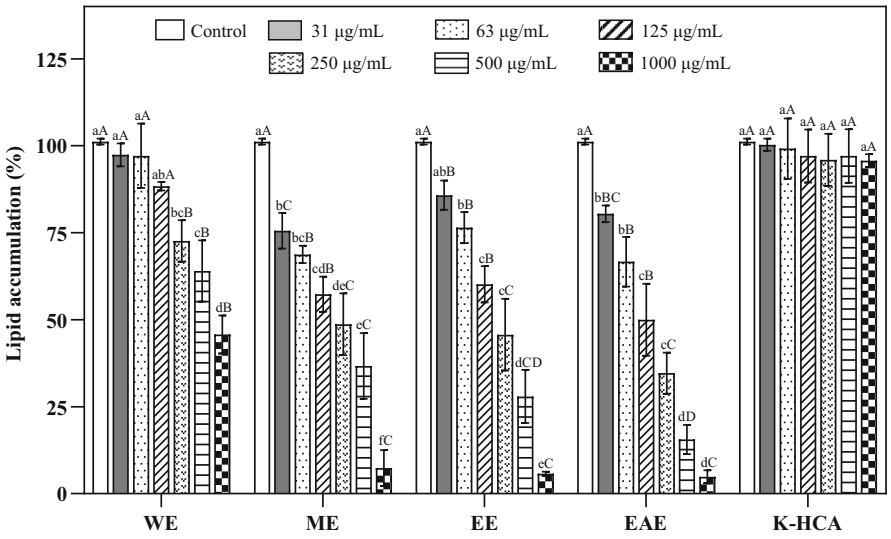


Figure 6 Effect of phenolic extract and potassium hydroxycitrate of *Hibiscus sabdariffa* on the lipid accumulation during adipogenesis quantified through Nile red staining. WE, water extract; ME, methanol extract; EE, ethanol extract, EAE, ethyl acetate extract; K-HCA, potassium hydroxycitrate. The different lower-case letters indicate statistically significant differences ($P < 0.05$) among the different concentration of same extract. The different capital letters indicate statistically significant differences ($P < 0.05$) between the different extract at same concentration.

fluorescence indicating lipid accumulation was highest in differentiated (untreated) cells. All the treated cells had reduced green fluorescence compared to the untreated (differentiated) cells. The fluorescence intensity decreased with the increase of concentration (500–1000 μg mL^{-1}) of extract used for the treatment for all the extracts confirming a reduction in lipid accumulation. A higher degree of reduction in lipid accumulation was observed in organic solvent-based extracts (methanol, ethanol and ethyl acetate extract). Compared with these organic solvent-based extracts, the water extract and potassium hydroxycitrate showed a higher accumulation of green fluorescence, indicating lower potency of these extracts compared to the organic extracts. For the vehicle control, the cells were treated with DMSO at the respective concentrations (0.02–0.50% v/v). The fluorescence intensity of the DMSO-treated sample was similar ($P > 0.05$) to untreated differentiated cells indicating minimal effects of DMSO on the adipogenic differentiation. These data indicated that polyphenolic extracts of *H. sabdariffa* interfered with the adipogenesis process and decreased lipid accumulation in hADSCs. In contrast, potassium hydroxycitrate isolated from *H. sabdariffa* did not affect the lipid accumulation in hADSCs. The results from oil red O staining described above corroborated with the findings obtained from Nile red staining as methanol and ethanol extracts were superior in reducing the accumulation of lipid in hADSCs. Also, the degree of lipid accumulation determined from both methods was comparable in most cases. Fowler & Greenspan (1985) also reported the pattern of staining by oil red O and Nile red in fatty liver of rabbit was similar.

Effect of *H. sabdariffa* extracts on mRNA expression of PPAR-γ and aP2

PPAR-γ and aP2 are the two key adipogenic markers that are expressed during the early and late stages of adipogenesis, respectively. The effect of extracts from *H. sabdariffa* on the relative gene expression level of PPAR-γ and aP2 in hADSCs is presented in Fig. 7. The phenolic extracts and potassium hydroxycitrate downregulated the expression of both PPAR-γ and aP2 at different levels at the end of differentiation. The downregulation of these gene expressions was dose-dependent as the fold change was higher at 1000 μg mL^{-1} than at 500 μg mL^{-1} in all extracts. The fold change in the expression of PPAR-γ was highest for ethanol extract followed by ethyl acetate, methanol and water extracts in comparison to the untreated differentiated cells (control). The ethanol extract was able to downregulate the PPAR-γ expression by 0.37-fold change at 1000 μg mL^{-1}. Potassium hydroxycitrate showed the minimum change in the expression of both aP2 and PPAR-γ. This is likely due to the involvement of these genes in different stages of adipogenesis. PPAR-γ is expressed during the early stage of adipogenesis whereas aP2 is expressed at the terminal stage. In this work, the cells were harvested on the 21st day of differentiation which was towards

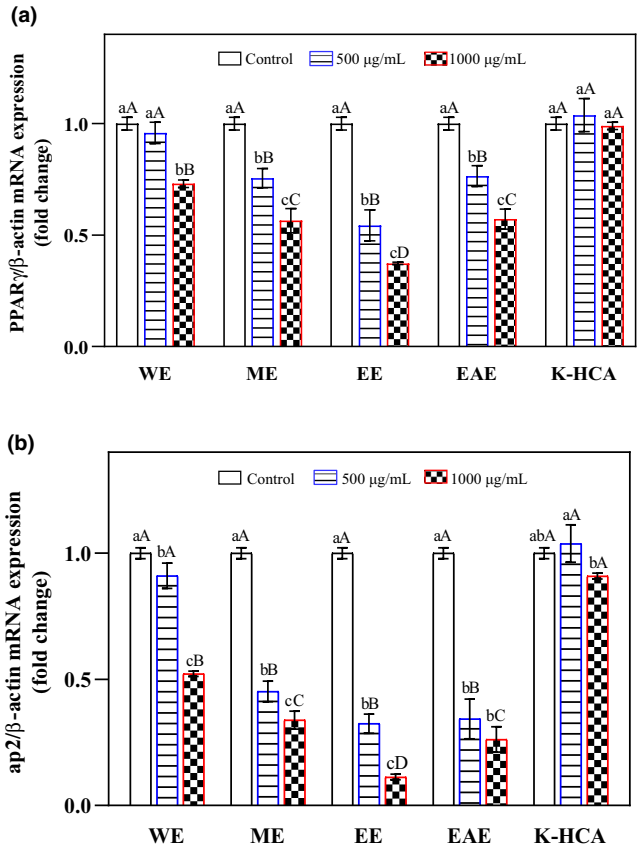


Figure 7 Effect of phenolic extract and potassium hydroxycitrate from *Hibiscus sabdariffa* on the expression of PPARγ (a) and aP2 (b) mRNA of hADSCs. Control: Differentiated (untreated), WE, water extract; ME, methanol extract; EE, ethanol extract; EAE, ethyl acetate extract; K-HCA, potassium hydroxycitrate. The different lower-case letters indicate statistically significant differences ($P < 0.05$) among the different concentration of same extract. The different capital letters indicate statistically significant differences ($P < 0.05$) between the different extract at same concentration.

the end of differentiation process which may be accounted for low fold change in the case of PPAR-γ. Janson *et al.* (2021) reported the potential of aqueous extract of *H. sabdariffa* in the suppression of adipogenic differentiation in 3T3-L1 preadipocytes by downregulating the gene expression of adipogenic markers (PPAR-γ, C/EBP-α and C/EBP-β). Likewise, Kim *et al.* (2003) and Kim *et al.* (2007) also reported the reduction of lipid accumulation by aqueous extract of *H. sabdariffa* in 3T3-L1 cells during adipogenesis by interfering the gene expression of PPAR-γ and C/EBP-α.

Our previous study identified anthocyanins; cyanidin 3-O-sambubioside chloride and delphinidin 3-O-sambubioside chloride, to be the major phenolic compounds present in *H. sabdariffa* extracts (Singh *et al.*, 2021). These anthocyanins, along with other phenolic compounds, may have accounted for this inhibition potential of *H. sabdariffa* extracts on adipogenic differentiation of hADSCs. Anthocyanins isolated from grape (Lee *et al.*, 2014) and fruit of *Vitis coignetiae Pulliat* (Han *et al.*, 2018) have been shown to downregulate the gene expression of aP2 and PPAR-γ in 3T3-L1 preadipocyte along with the significant reduction in lipid accumulation during adipogenesis. The results obtained from oil red O staining and Nile red staining were supported by the gene expression of adipogenic markers in hADSCs. These observations on the efficacy of phenolic extract from *H. sabdariffa to* inhibit the adipogenesis supported the notion that it can be used for preventing or managing obesity.

Combined effect of phenolic compounds and potassium hydroxycitrate

The hADSCs were treated with a combined formulations of ethyl acetate extract (due to the highest phenolic content) and potassium hydroxycitrate to determine potential synergistic effect on lipid accumulation during adipogenesis. Three different ratios of ethyl acetate extract and potassium hydroxycitrate (2:1, 3:1 and 4:1) were used. The result of this treatment is presented in Fig. 8. None of these combinations were able to further decrease lipid accumulation during adipogenesis. The lipid accumulation for all the ratios was comparable ($P > 0.05$) to that of ethyl acetate extract-treated cells at the same concentration (500 µg mL^{-1}). These findings showed that phenolic compounds including polyphenols in *H. sabdariffa* extract were the main compounds inhibiting adipogenesis and potassium hydroxycitrate had minimal influence on the adipogenesis process.

Conclusion

The findings of this study show that the phenolic extracts obtained from *H. sabdariffa* have the potential to interfere with adipogenesis in human adipose-derived stem cells. The accumulation of lipids at the end of adipogenic differentiation was significantly reduced by all the phenolic extracts tested. Quantification of expression of two key adipogenic markers (PPAR-γ and aP2) confirmed that phenolic extracts can downregulate these genes and interfere with adipogenesis leading to the reduction in formation of adipocytes and lipid accumulation. The strongest inhibition of adipogenesis in hADSCs was observed in ethanol extract as indicated by the highest downregulation of adipogenic genes and lowest accumulation of lipid. Potassium hydroxycitrate did not significantly affect adipogenesis. A synergistic effect of phenolic

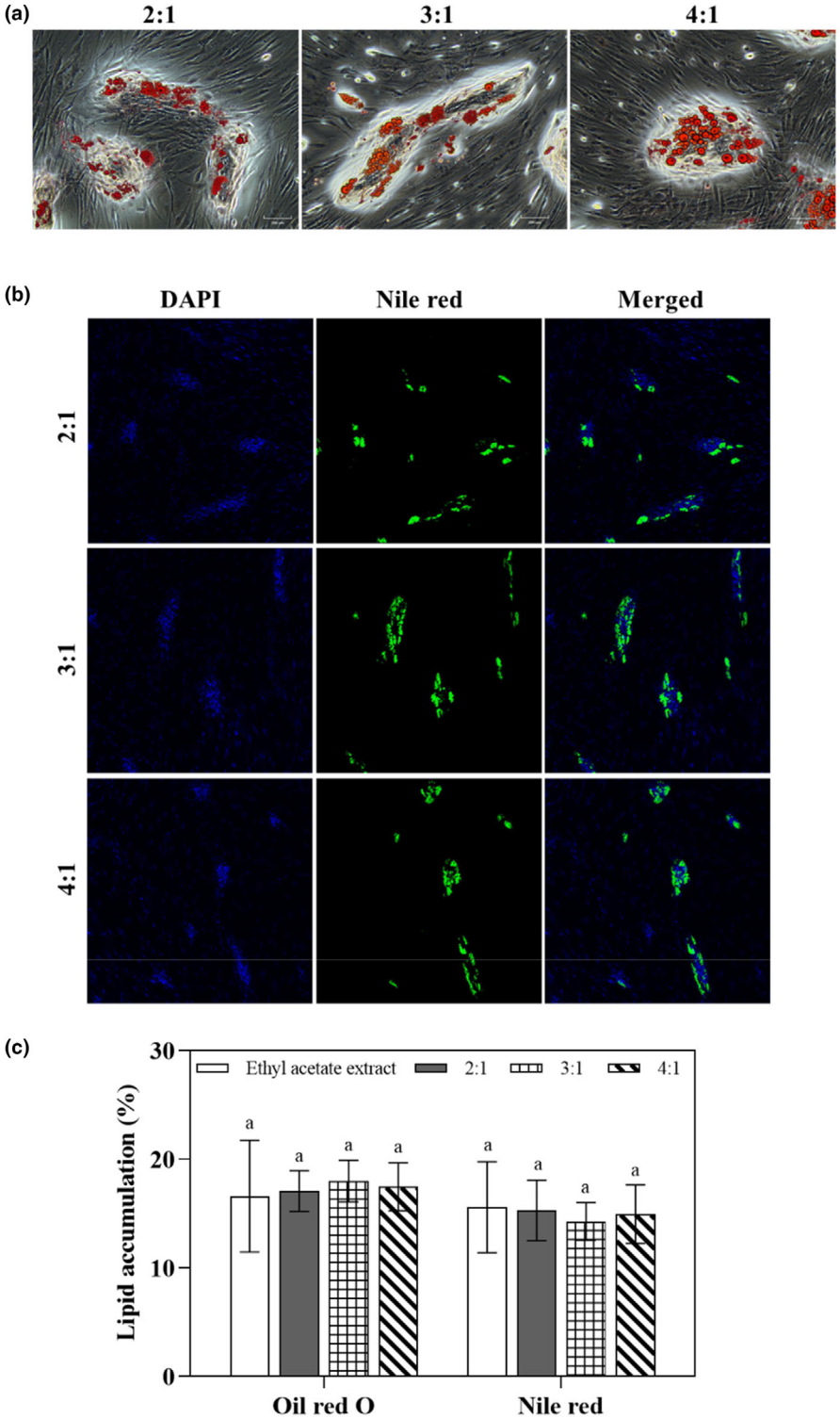


Figure 8 Effect of combination of ethyl acetate extract and potassium hydroxycitrate of *Hibiscus sabdariffa* on the lipid accumulation during adipogenesis (a) oil red O-stained images of treated cells at 10× magnification (b) Nile red stained images of treated cells at 10× magnification (c) Quantification of lipid accumulation by two different staining methods. The same letters indicate statistically no significant differences ($P > 0.05$) among the sample for each staining method.

compounds and potassium hydroxycitrate was not identified, confirming that phenolic compounds in *H. sabdariffa* are responsible for the anti-adipogenic effects. Therefore, the phenolic extracts from *H. sabdariffa* may be used as part of supplements to manage or treat obesity.

Acknowledgments

The authors gratefully acknowledge Lee Etherington (Wild Hibiscus Flower Co., NSW, Australia) for providing free samples of *H. sabdariffa* calyces. The first author acknowledges RMIT's scholarship support, which enabled the completion of this work. Open access publishing facilitated The University of Melbourne, Australia as part of the Wiley – The University of Melbourne, Australia agreement via the Council of Australian University Librarians.

Author contributions

Benu Adhikari: Conceptualization (equal); supervision (equal); validation (equal); writing – review and editing (equal). **Charles Stephen Brennan:** Writing – review and editing (equal). **Ravi Shukla:** Conceptualization (equal); supervision (equal); writing – review and editing (equal). **Manisha Singh:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal). **Thilini Thrimawithana:** Conceptualization (equal); supervision (equal); writing – review and editing (equal).

Ethical guidelines

Ethics approval was not required for this research.

Conflict of Interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

References

Amaya-Cruz, D., Perez-Ramirez, I.F., Perez-Jimenez, J., Nava, G.M. & Reynoso-Camacho, R. (2019). Comparison of the bioactive potential of Roselle (*Hibiscus sabdariffa* L.) calyx and its by-product: Phenolic characterization by UPLC-QTOF MS(E) and their anti-obesity effect in vivo. *Food Research International*, **126**, 108589.

An, M., Xu, Y.-N., Xiao, N. *et al.* (2022). Douchi ameliorates high-fat diet-induced hyperlipidaemia by regulation of intestinal microflora in rats. *International Journal of Food Science & Technology*, **57**, 2756–2769.

Aranaz, P., Navarro-Herrera, D., Zabala, M. *et al.* (2019). Phenolic compounds inhibit 3T3-L1 adipogenesis depending on the stage of differentiation and their binding affinity to PPARgamma. *Molecules*, **24**, 1045.

Asgary, S., Soltani, R., Zolghadr, M., Keshvari, M. & Sarrafzadegan, N. (2016). Evaluation of the effects of roselle (*Hibiscus sabdariffa* L.) on oxidative stress and serum levels of lipids, insulin and hs-CRP in adult patients with metabolic syndrome: A double-blind placebo-controlled clinical trial. *Journal of Complementary and Integrative Medicine*, **13**, 175–180.

Brannmark, C., Paul, A., Ribeiro, D. *et al.* (2014). Increased adipogenesis of human adipose-derived stem cells on polycaprolactone fiber matrices. *PLoS One*, **9**, e113620.

de Abreu Costa, L., Henrique Fernandes Ottoni, M., Dos Santos, M.G. *et al.* (2017). Dimethyl sulfoxide (DMSO) decreases cell proliferation and TNF-alpha, IFN-gamma, and IL-2 cytokines production in cultures of peripheral blood lymphocytes. *Molecules*, **22**, 1789.

Eseberri, I., Miranda, J., Lasa, A. *et al.* (2019). Effects of quercetin metabolites on triglyceride metabolism of 3T3-L1 preadipocytes and mature adipocytes. *International Journal of Molecular Science*, **20**, 264.

Fink, T. & Zachar, V. (2011). Adipogenic differentiation of human mesenchymal stem cells. In: *Mesenchymal Stem Cell Assays and Applications*. *Methods in Molecular Biology* (edited by M. Vemuri, L. Chase & M. Rao). Pp. 243–251, Vol. **698**. New Jersey: Humana Press.

This chapter provides the protocol for adipogenesis and transcriptional analysis of adipogenic genes for human adipose-derived stem cells which has been adapted in this study.

Fowler, S.D. & Greenspan, P. (1985). Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: Comparison with oil red O. *Journal of Histochemistry & Cytochemistry*, **33**, 833–836.

Gomez-Zorita, S., Lasa, A., Abendano, N. *et al.* (2017). Phenolic compounds apigenin, hesperidin and kaempferol reduce in vitro lipid accumulation in human adipocytes. *Journal of Translational Medicine*, **15**, 237.

Gregoire, F.M., Smas, C.M. & Sul, H.S. (1998). Understanding adipocyte differentiation. *Physiological Reviews*, **78**, 783–809.

Guillemet, D., Belles, C., Gomes, A. *et al.* (2022). Screening for anti-adipogenic, pro-lipolytic and thermogenic plant extracts by models associating intestinal epithelial cells with human adipose cells. *European Journal of Nutrition*, **61**, 2201–2215.

Guo, X., Cheng, M., Zhang, X., Cao, J., Wu, Z. & Weng, P. (2017). Green tea polyphenols reduce obesity in high-fat diet-induced mice by modulating intestinal microbiota composition. *International Journal of Food Science & Technology*, **52**, 1723–1730.

Han, J.H., Jang, K.W. & Myung, C.S. (2022). *Garcinia cambogia* attenuates adipogenesis by affecting CEBPB and SQSTM1/p62-mediated selective autophagic degradation of KLF3 through RPS6KA1 and STAT3 suppression. *Autophagy*, **18**, 518–539.

This study highlights the inhibitory effect of hydroxycitric acid from *Garcinia cambogia* on lipid accumulation in 3T3-L1 cells.

Han, M.H., Kim, H.J., Jeong, J.W., Park, C., Kim, B.W. & Choi, Y.H. (2018). Inhibition of adipocyte differentiation by anthocyanins isolated from the fruit of *Vitis coignetiae* pulliat is associated with the activation of AMPK signaling pathway. *Toxicological Research*, **34**, 13–21.

Haselgrubler, R., Lanzerstorfer, P., Rohrl, C. *et al.* (2019). Hypolipidemic effects of herbal extracts by reduction of adipocyte differentiation, intracellular neutral lipid content, lipolysis, fatty acid exchange and lipid droplet motility. *Scientific Reports*, **9**, 10492.

Herranz-López, M., Fernández-Arroyo, S., Pérez-Sanchez, A. *et al.* (2012). Synergism of plant-derived polyphenols in adipogenesis: Perspectives and implications. *Phytomedicine*, **19**, 253–261.

Jang, Y.S., Wang, Z., Lee, J.M., Lee, J.Y. & Lim, S.S. (2016). Screening of Korean natural products for anti-adipogenesis properties and isolation of Kaempferol-3-O-rutinoside as a potent anti-adipogenetic compound from *Solidago virgaurea*. *Molecules*, **21**, 226.

Janson, B., Prasomthong, J., Malakul, W., Boonsong, T. & Tunsophon, S. (2021). *Hibiscus sabdariffa* L. calyx extract prevents the adipogenesis of 3T3-L1 adipocytes, and obesity-related insulin resistance in high-fat diet-induced obese rats. *Biomedicine & Pharmacotherapy*, **138**, 111438.

Kao, E.-S., Yang, M.-Y., Hung, C.-H., Huang, C.-N. & Wang, C.-J. (2016). Polyphenolic extract from *Hibiscus sabdariffa* reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. *Food & Function*, **7**, 171–182.

Kim, J.K., So, H., Youn, M.J. *et al.* (2007). *Hibiscus sabdariffa* L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway. *Journal of Ethnopharmacology*, **114**, 260–267.

This research confirms the potential of *H. sabdariffa* water extract in inihibiting the lipid accumulation during adipocyte differentiation through oil red O staining in 3T3-L1 cells and helps to compare the findings of our study in hADSCs.

Kim, M.S., Kim, J.K., Kim, H.J. *et al.* (2003). Hibiscus extract inhibits the lipid droplet accumulation and adipogenic transcription factors expression of 3T3-L1 preadipocytes. *The Journal of Alternative and Complementary Medicine*, **9**, 499–504.

Kraus, N.A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B.J. & Kraus, D. (2016). Quantitative assessment of adipocyte differentiation in cell culture. *Adipocytes*, **5**, 351–358.

Lee, B., Lee, M., Lefevre, M. & Kim, H.R. (2014). Anthocyanins inhibit lipogenesis during adipocyte differentiation of 3T3-L1 preadipocytes. *Plant Foods for Human Nutrition*, **69**, 137–141.

Lee, H. & Park, J.B. (2017). Evaluation of the effects of dimethylsulphoxide on morphology, cellular viability, mRNA, and protein expression of stem cells culture in growth media. *Biomedical Reports*, **7**, 291–296.

Li, H., Liang, C., Tao, Y. *et al.* (2012). Acidic pH conditions mimicking degenerative intervertebral discs impair the survival and biological behavior of human adipose-derived mesenchymal stem cells. *Experimental Biology and Medicine (Maywood)*, **237**, 845–852.

Li, K.K., Liu, C.L., Shiu, H.T. *et al.* (2016). Cocoa tea (*Camellia ptilophylla*) water extract inhibits adipocyte differentiation in mouse 3T3-L1 preadipocytes. *Scientific Reports*, **6**, 20172.

Liang, J., Xu, R., Zong, K. *et al.* (2021). Structural analysis and anti-obesity effect of *Polygonatum cyrtonema* polysaccharide against obesity induced by high-fat diet in mice. *International Journal of Food Science & Technology*, **56**, 4473–4483.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, **25**, 402–408.

Majeed, M., Badmaev, V. & Rajendran, R. (2004). Process for the production of potassium hydroxy citric acid, and compositions containing the potassium hydroxy citric acid. US patent 6,770,782 B1.

McPherson, R. (2007). Genetic contributors to obesity. *Canadian Journal of Cardiology*, **23**(Suppl A), 23A–27A.

Mena-García, A., Sanz, M.L., Díez-Municio, M., Soria, A.C. & Ruiz-Matute, A.I. (2022). Development of a new method using gas chromatography coupled to mass spectrometry to determine (−)-hydroxycitric acid in *Garcinia gummi*-gutta food supplements. *Microchemical Journal*, **181**, 107811.

Mladenova, S.G., Vasileva, L.V., Savova, M.S. *et al.* (2021). Anti-adipogenic effect of *Alchemilla monticola* is mediated via PI3K/AKT signaling inhibition in human adipocytes. *Frontiers in Pharmacology*, **12**, 707507.

Moseti, D., Regassa, A. & Kim, W.K. (2016). Molecular regulation of adipogenesis and potential anti-adipogenic bioactive molecules. *International Journal of Molecular Science*, **17**, 124.

Nielsen, F.M., Riis, S.E., Andersen, J.I. *et al.* (2016). Discrete adipose-derived stem cell subpopulations may display differential functionality after in vitro expansion despite convergence to a common phenotype distribution. *Stem Cell Research & Therapy*, **7**, 177.

OECD (2021). *Health at a glance 2021: OECD indicators*. Paris: OECD Publishing.

Ogundijo, D.A., Tas, A.A. & Onarinde, B.A. (2022). Factors influencing the perception and decision-making process of consumers on the choice of healthier foods in the United Kingdom: A systematic review using narrative synthesis. *International Journal of Food Science & Technology*, **57**, 881–897.

Peng, C.H., Chyau, C.C., Chan, K.C., Chan, T.H., Wang, C.J. & Huang, C.N. (2011). *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *Journal of Agricultural and Food Chemistry*, **59**, 9901–9909.

Podsędek, A., Zakøos-Szyda, M., Polka, D. & Sosnowska, D. (2020). Effects of *Viburnum opulus* fruit extracts on adipogenesis of 3T3-L1 cells and lipase activity. *Journal of Functional Foods*, **73**, 104111.

Qian, S.W., Li, X., Zhang, Y.Y. *et al.* (2010). Characterization of adipocyte differentiation from human mesenchymal stem cells in bone marrow. *BMC Developmental Biology*, **10**, 47.

Ramarao, K.D.R., Somasundram, C., Razali, Z. *et al.* (2022). Antiproliferative effects of dried *Moringa oleifera* leaf extract on human Wharton's jelly mesenchymal stem cells. *PLoS One*, **17**, e0274814.

Riaz, G. & Chopra, R. (2018). A review on phytochemistry and therapeutic uses of *Hibiscus sabdariffa* L. *Biomedicine & Pharmacotherapy*, **102**, 575–586.

Riss, T.L., Moravec, R.A., Niles, A. *et al.* (2016). Cell viability assays. In: *Assay guidance manual* (edited by S. Markossian, A. Grossman, K. Brimacombe *et al.*). Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Rogowski, M., Bellusci, L., Sabatini, M. *et al.* (2019). Lipolytic effects of 3-iodothyronamine (T1AM) and a novel thyronamine-like analog SG-2 through the AMPK pathway. *International Journal of Molecular Science*, **20**, 4054.

Ruiz-Ojeda, F.J., Ruperez, A.I., Gomez-Llorente, C., Gil, A. & Aguilera, C.M. (2016). Cell models and their application for studying Adipogenic differentiation in relation to obesity: A review. *International Journal of Molecular Science*, **17**, 1040.

Scott, M.A., Nguyen, V.T., Levi, B. & James, A.W. (2011). Current methods of adipogenic differentiation of mesenchymal stem cells. *Stem Cells Development*, **20**, 1793–1804.

Sharma, S., Venkatesan, V., Prakhya, B.M. & Bhonde, R. (2015). Human mesenchymal stem cells as a novel platform for simultaneous evaluation of cytotoxicity and genotoxicity of pharmaceuticals. *Mutagenesis*, **30**, 391–399.

Singh, M., Thrimawithana, T., Shukla, R. & Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. *Future Foods*, **1-2**, 100002.

Singh, M., Thrimawithana, T., Shukla, R. & Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*. *Future Foods*, **4**, 100087.

Singh, M., Thrimawithana, T., Shukla, R. & Benu, A. (2022). Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of *Hibiscus sabdariffa*. *Food Bioscience*, **50**, 101992.

This study provides the methodology for the extraction of phenolic compounds and potassium hydroxycitrate from *H. sabdariffa*. It also gives the total phenolic content value in each extract to support the discussion in this study.

Tuncer, S., Gurbanov, R., Sheraj, I., Solel, E., Esenturk, O. & Banerjee, S. (2018). Low dose dimethyl sulfoxide driven gross molecular changes have the potential to interfere with various cellular processes. *Scientific Reports*, **8**, 14828.

Wang, S.C., Lee, S.F., Wang, C.J., Lee, C.H., Lee, W.C. & Lee, H.J. (2011). Aqueous extract from *Hibiscus sabdariffa Linnaeus ameliorate* diabetic nephropathy via regulating oxidative status and Akt/bad/14-3-3gamma in an experimental animal model. *Evidence-based Complementary and Alternative Medicine*, **2011**, 938126.

Yang, M.Y., Peng, C.H., Chan, K.C., Yang, Y.S., Huang, C.N. & Wang, C.J. (2010). The hypolipidemic effect of *Hibiscus sabdariffa* polyphenols via inhibiting lipogenesis and promoting hepatic lipid clearance. *Journal of Agricultural and Food Chemistry*, **58**, 850–859.

CHAPTER 6

Encapsulation of phenolic compounds-rich *Hibiscus sabdariffa* extracts in alginate-chitosan beads to preserve antioxidant property

Encapsulation of phenolic compounds-rich *Hibiscus sabdariffa* extracts in alginate-chitosan beads to preserve antioxidant property

Manisha Singh[a], Thilini Thrimawithana[b], Ravi Shukla[a,c], Benu Adhikari[a*]

[a] *School of Science, RMIT University, Melbourne, VIC 3083, Australia.*

[b] *Discipline of Pharmacy, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC 3083, Australia.*

[c] *Nanobiotechnology Research Laboratory and Centre for Advanced Materials & Industrial Chemistry, RMIT University, Melbourne, VIC 3001, Australia.*

*Corresponding author: School of Science, RMIT University, Melbourne, VIC 3083, Australia
Email address: benu.adhikari@rmit.edu.au (B. Adhikari), s3736496@student.rmit.edu.au (M. Singh)

Abstract

This study explored the encapsulation of the phenolic extracts of *Hibiscus sabdariffa* in alginate-chitosan beads through ionic gelation. Various combinations of alginate and chitosan were trialled to achieve highest encapsulation efficiency in terms of total phenolic content. The combination of 3% alginate, 0.2% chitosan and 1% extract gave highest encapsulation efficiency (91.9%). Freeze-dried capsule beads had rough and wrinkled surface with porous internal network structure. The oven-dried capsule beads had smooth surface and compact network. The release behaviour of the encapsulated phenolic compounds was investigated through an *in vitro* release study in simulated gastric (SGF) and intestinal (SIF) fluids. The alginate-chitosan matrix of the beads was able to restrict the release of phenolic compounds into SGF and deliver substantially higher (more than 50%) content in SIF. The antioxidant activity of phenolic compounds was preserved to a much higher level (81%) in the alginate-chitosan matrix. These microcapsule beads with well-preserved *H. sabdariffa* extract in the core can be used in food or pharmaceutical to impart their health benefits.

Key words: *Hibiscus sabdariffa,* encapsulation, alginate-chitosan beads, antioxidant activity, *in vitro* release

6.1 Introduction

Naturally derived phytochemicals such as phenolic compounds are emerging as health promoting ingredients due to their antioxidant properties (Shahidi and Ambigaipalan, 2015). Previous studies have revealed that phenolic compounds play key role in the prevention and treatment of chronic ailments such as cancer, cardiovascular diseases, neurodegenerative diseases and obesity (Dhakal et al., 2019; Sen and Chakraborty, 2011; Singh et al., 2020). Although phenolic compounds offer many health benefits, they are often unstable in their original state and can undergo rapid degradation. They are highly sensitive to oxygen, heat and light and their stability is also influenced by the storage period (Sun et al., 2017). Moreover, exposure to varying pH conditions in human gastrointestinal tract following ingestion can interfere with the antioxidant capacity of these compounds (Abdin et al., 2020). Consequently, it is important to formulate these bioactive phenolic compounds in a suitable delivery platform to optimise their bioactivity (Ballesteros et al., 2017).

Microencapsulation is a promising method that involves entrapping substances or active components into suitable wall materials in order to provide protective barrier, improve stability, achieve controlled release, and targeted delivery (Yang et al., 2020). A wide range of approaches including coacervation, emulsions, extrusion, fluidized bed coating, ionic gelation, application of liposomes are commonly used to encapsulate the bioactive compounds intended for food application (Mehta et al., 2022). Drying operation including spray drying, freeze drying, and oven drying is subsequently used, in many cases, to convert the microcapsules in solid/powder form intending to extend shelf life and to broaden the application of microcapsules as ingredients (Ravichandran et al., 2014). Ionic gelation is a simple and inexpensive method for encapsulating bioactive compound. This method is particularly suitable for heat sensitive bioactive compounds as it does not require high temperature and organic solvents (Kurozawa and Hubinger, 2017). It involves the formation of complexes when

a charged biopolymer is brought in contact with an ionized salt solution. Ionic gelation can be triggered either externally or internally. In external gelation, the biopolymer matrix containing the core material is extruded or dripped into anionic salt solution. In the case of internal gelation, mixture of biopolymer matrix, core material and anionic salt solution are extruded into acidified oil medium (Mehta et al., 2022; Naranjo-Duran et al., 2021).

Alginate, chitosan and pectin are the most widely used encapsulating materials for ionic gelation as they are food grade compounds (Kurozawa and Hubinger, 2017). Sodium alginate is a hydrophilic linear polysaccharide which is approved by US Food and Drug Administration and the European Food Safety Authority as a food additive (Stojanovic et al., 2012). It is a natural biopolymer extracted from brown seaweed and consist of α-L-guluronic and β-D mannuronic acid residues. In the presence of multivalent ions such as Ca^{2+}, sodium alginate can form a stable gel network (Zhang et al., 2021a). This alginate-Ca^{2+} hydrogel structures allows the controlled release of the active ingredient depending on the pH of the medium (Patel et al., 2016). However, the stability of alginate gels or beads are limited with the quick release of the active component due to exchange of ions in physiological conditions (Lee and Mooney, 2012). This erratic release can be overcome by coagulating or coating polyanionic calcium-alginate gel with a polycationic polymer like chitosan that tightens and stabilizes the surface. Chitosan is a readily available biopolymer extracted from chitin through a deacetylation process (Varma and Vasudevan, 2020). Previous studies have reported the formation of polyelectrolyte complexes by combining chitosan and alginate in presence of calcium chloride (Anal et al., 2003; Kulig et al., 2016)..

Hibiscus sabdariffa is a medicinal flowering plant belonging to Malvaceae family and is widely used in beverages and spices. The calyces of this plant are rich in phenolic compounds, especially anthocyanins (delphinidin 3-O-sambubioside chloride and cyanidin 3-O-sambubioside chloride) along with caffeic acid, catechin, chlorogenic acid, gallic acid, rutin,

and quercetin, all of which are highly antioxidative in nature (Morales-Luna et al., 2019; Singh et al., 2021). The therapeutical potential of *H. sabdariffa* calyces in prevention and treatment of diabetes mellitus, cardiovascular diseases and obesity is well reported in the literature (Amaya-Cruz et al., 2019; Asgary et al., 2016; Kao et al., 2016; Singh et al., 2022). However, for the commercial application in food and pharmaceutical, encapsulation of bioactive extracts from calyces of *H. sabdariffa* is desirable for the protection of their functional properties, improved stability and controlled and targeted release (Abdin et al., 2020). There is a dearth of literature that report the release behaviour of encapsulated bioactive extracts from *H. sabdariffa* through ionic gelation. Therefore, the aim of this study was to encapsulate of phenolic compound-rich extract of *H. sabdariffa* calyces in alginate-chitosan beads. The beads were then characterized for encapsulation efficiency, particle size, chemical interactions, morphology and surface properties. The release of the phenolic compounds was also determined through the consensus *in vitro* method in simulated oral, gastric and intestinal conditions. The effect of two drying methods; freeze drying and oven drying on morphology and *in vitro* release was also investigated. The results indicate that sodium alginate and chitosan can preserve the antioxidant activity of the phenolic extract from *H. sabdariffa* calyces and provide effective controlled release in the SIF.

6.2 Methodology

6.2.1 Plant material

The frozen calyces of *H. sabdariffa* were gifted by Wild Hibiscus Flower Co. (NSW, Australia) and were stored at -20°C until use.

6.2.2 Chemicals

Methanol, sodium alginate (W201502), chitosan (448877), calcium chloride, Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH), and sodium carbonate were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Alpha-amylase from human salivary (Type XIII-A), pepsin from porcine gastric mucosa, bile salts, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, magnesium chloride, ammonium carbonate, sodium hydroxide, hydrochloric acid and calcium chloride were also procured from Sigma Aldrich (Castle Hill, NSW, Australia). Pancreatin USP was acquired from MP Biomedicals (OH, USA). All of these chemicals were of analytical reagent grade and used as supplied. Ultrapure water obtained from the Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., NSW, Australia) was used for all the experiments.

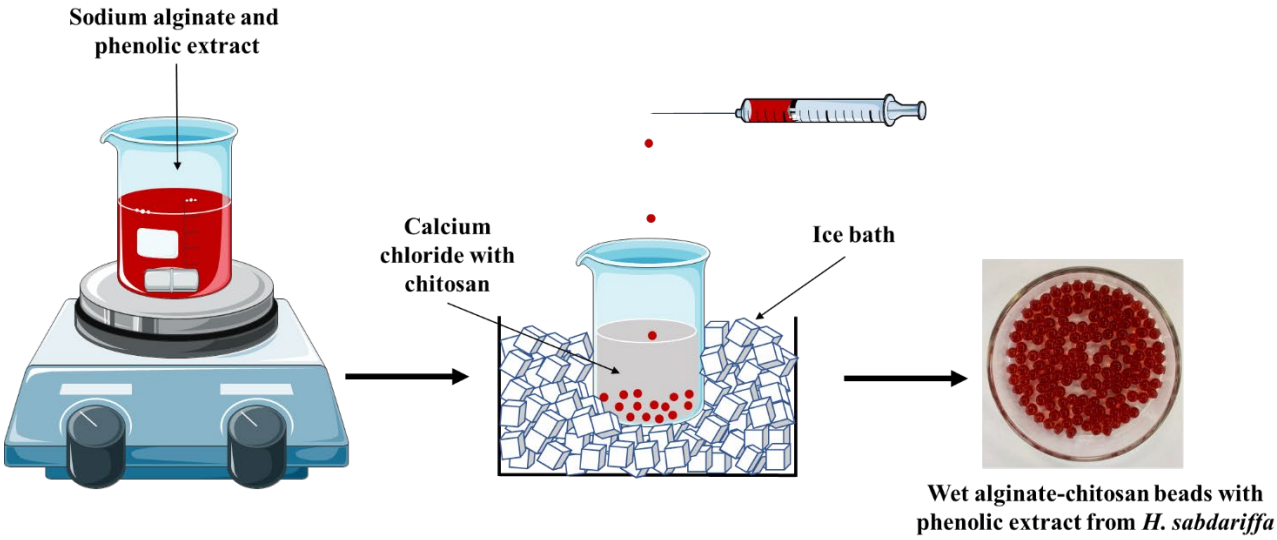
6.2.3 Preparation of plant material and extraction of phenolic compounds

H. sabdariffa calyces stored at -20°C were freeze dried (Labconco Triad 7400030, USA) at -40°C and 12 Pa. The dried calyces were powdered in a coffee grinder (Breville, Sydney, Australia), sieved through a pore size of 353 μm and stored at room temperature in amber coloured glass bottles inside a desiccator. The phenolic compounds from this powder (5g) were extracted by mixing it with methanol (100 mL) and agitating at 500 rpm for 2 h at room temperature (23°C) (Singh et al., 2021). Following this, the extract was centrifuged (Allegra 64R, Beckman Coulter, Australia) at 11000 ×*g* for 20 min and vacuum filtered with Whatman no. 1 filter paper. The filtrate was subjected to a rotary vacuum concentrator (John Morris, Australia) to evaporate the solvent and obtain dried extract. This extract was stored in amber-coloured at -30 °C bottles after flushing with nitrogen for further tests. Our previous study (Singh et al., 2021) revealed the presence of phenolic compounds such as 4-hydroxybenzoic acid, caffeic acid, coumaric acid, catechin hydrate, rutin hydrate, quercetin, cyanidin 3-O-

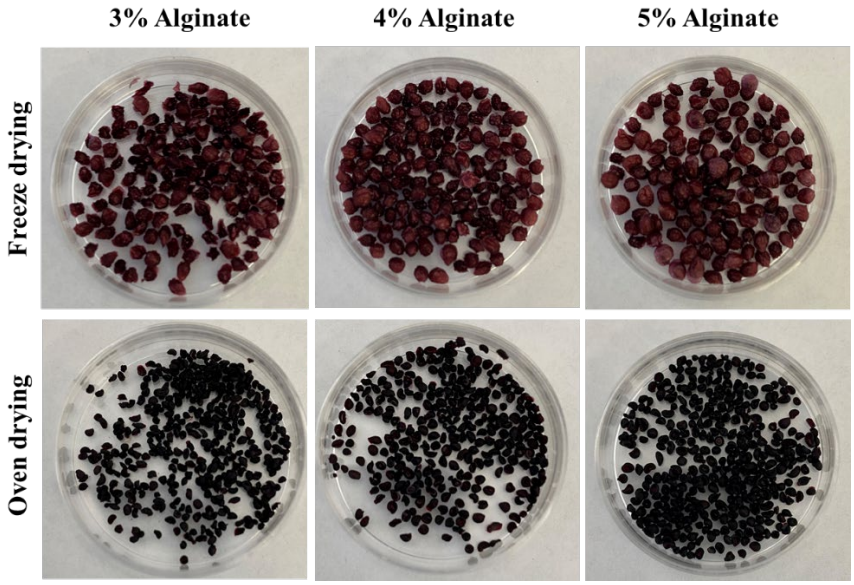
sambubioside chloride and delphinidin 3-O-sambubioside chloride in this methanolic extract with the total phenolic content of 51.72 ± 0.71 mg gallic acid equivalent per g of dry extract.

6.2.4 Encapsulation of phenolic extract in alginate-chitosan based micro beads

Different concentrations of sodium alginate (3, 4 and 5 % w/v) were prepared by dissolving the polymer in water at room temperature under continuous stirring at 250 rpm overnight. To this, phenolic extract (1, 2 and 3% % w/v) was added and agitated at 40°C for 30 min for homogenous mixing of extract-polymer solution. This mixture was then dropped by a syringe attached to stainless steel needle (23 gauge) into chilled (<4°C) $CaCl_2$ solution (1% w/v) containing chitosan (0.1, 0.2 and 0.3% w/v). The micro beads were kept for 5 min in ice cold $CaCl_2$ solution for hardening. The beads were filtered, washed using water and blotted gently to remove excess water from the surface (Fig. 1a). Finally, the beads were either freeze dried at -40°C and 12 Pa for 48 h or oven-dried (Thermoline Scientific, Australia) at 40°C for 6 h (Fig 1b). The dried beads were stored in an air-tight container and kept in a desiccator until further use.



(a)



(b)

Figure 1: (a) Process of encapsulation of phenolic compounds-rich *H.* sabdariffa extract in alginate-chitosan beads (b) Freeze-dried and oven-dried beads at different concentration of alginate, 0.2% chitosan and 1% extract

6.2.5 Characterization of beads

6.2.5.1 Determination of encapsulation efficiency

The encapsulation efficiency of the alginate-chitosan beads was determined by means of the percentage of total polyphenol content (TPC) encapsulated in the beads. This was indirectly calculated by measuring TPC in residual $CaCl_2$ solution (remained after removing the beads after gelling step) and subtracting it from TPC added into the sodium alginate solution before encapsulation step. The TPC was measured using Folin-Ciocalteu method as described in Singh et al. (2021). The sample (100 µL), 10% v/v Folin- Ciocalteu reagent (200µL) and 7.5% w/v sodium carbonate solution (800 µL) were mixed thoroughly in a 48-well plate (Greiner Bio-One, Austria). The absorbance was recorded at 765 nm using a CLARIOstar®microplate reader (BMG Labtech, Germany) after incubating this mixture in dark for 1 h at room temperature. The TPC was determined using gallic acid as the standard and is expressed as mg gallic acid equivalents (GAE). The encapsulation efficiency was calculated according to equation (1).

$$\text{Encapsulation efficiency (\%)} = \frac{TPC_i - TPC_g}{TPC_i} \times 100 \quad \text{(1)}$$

Where, TPC_i (mg) is the TPC of the sodium alginate solution before the encapsulation/gelling step and TPC_g (mg) is the TPC in the residual $CaCl_2$ solution as mentioned above.

6.2.5.2 Measurement of bead size

The size of the capsule beads was determined using a digital micrometre (Mitutoyo, USA). The diameter of 20 beads selected randomly was measured and the mean value was calculated.

6.2.5.3 Surface morphology of beads

The surface morphology of the capsule beads was acquired using a scanning electron microscope (SEM). The dry beads were attached to aluminium stubs with double sided

adhesive carbon tape and coated with 5 nm of iridium using a high vacuum sputter coater (Leica EM ACE600, USA). The beads were then examined in a FEI Quanta 200 ESEM (Hillsboro, Oregon, USA) and the images were captured. The images were captured at 35× and 350× magnification for freeze-dried beads whereas 50× and 350× magnification for oven-dried beads.

6.2.5.4 Chemical characterization of beads

Fourier transform infrared (FTIR) spectroscopy can be used to identify and quantify the functional group as each functional group absorbs radiation at a specific frequency of the infrared spectrum (George et al., 2015). The FTIR spectroscopic analysis was performed on the dry beads using Perkin Elmer Spectrum Two FTIR spectrometer (Norway, CT, USA) fitted with a Gladi ATR from Pike Technologies (Wisconsin, USA). The spectra were recorded in transmission mode at a resolution of 4 cm^{-1} over the range of 4000–400 cm^{-1} with 64 scans.

6.2.6 *In vitro* release test

The *in vitro* release test was performed in simulated human digestive conditions at 37°C by following a consensus method (Minekus et al., 2014; Pham et al., 2021). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared for oral, gastric, and intestinal stages, respectively, according to Minekus et al. (2014) as presented in Table 1.

Table 1: Preparation of simulated digestion fluids (stock solutions) for *in vitro* release study

Constituent	Stock concentration (M)	SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
		Volume of stock (mL)	Final concentration (mM)	Volume of stock (mL)	Final concentration (mM)	Volume of stock (mL)	Final concentration (mM)
KCl	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	1	6.8	13.6	12.5	25	42.5	85
NaCl	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	0.5	0.06	0.06	0.5	0.5	-	-

SSF = simulated salivary fluid, SGF = simulated gastric fluid (SGF), and SIF = simulated intestinal fluid. The final volume of each simulated fluid was made up to 500 mL with Milli-Q water and the respective pH was adjusted by adding HCl or NaOH.

i. Simulated oral digestion

The alginate micro beads (300 mg) were dispersed in 5 mL water. Then 3.5 mL SSF, 0.5 mL α-amylase (1500 U/mL in SSF), 25.0 µL 0.3 M $CaCl_2$ and 975.0 µL water was added to it. The mixture was agitated at 100 rpm for 2 min at pH 7.0.

ii. Simulated gastric digestion

Ten millilitres of oral bolus from (i) were mixed with 7.5 mL SGF, 1.6 mL porcine pepsin (25,000 U/mL in SGF), 5.0 µL of 0.3 M $CaCl_2$. The pH of this mixture was adjusted to 3.0 by adding 0.2 mL of HCl (1M). The final volume was made up to 20.0 mL by adding 695 µL water and stirred at 100 rpm for 2 h at 37°C.

iii. Simulated intestinal digestion

The gastric digesta (20 mL) was mixed with 11.0 mL of SIF solution, 5.0 mL of pancreatin (800 U/mL in SIF), 2.5 mL of 160 mM fresh bile salt, 40 µL of 0.3 M $CaCl_2$ solution and 150 µL of NaOH (1M) to adjust pH to 7.0. Finally, 1.31 mL of water was added to the mixture and agitated at 100 rpm for 2 h at 37°C.

The sample from gastric and intestinal digestion stages were collected at the interval of 10, 30, 60, 90, 120, 130, 150, 180, 210 and 240 min. The release of phenolic compounds from the beads undergoing digestion was determined in terms of total phenolic and antioxidant activity at each time point.

6.2.6.1 Determination of total phenolic content

The total phenolic content in the digesta was determined as described in section 6.2.5.1.

6.2.6.2 Measurement of antioxidant activity

The antioxidant activity of the samples was measured in terms of DPPH radical-scavenging activity according to Mishra et al. (2012). Briefly, 300 µL of digesta sample and 300 µL of DPPH solution (0.004% w/v in methanol) were added into a 48-well plate. After incubating in dark for 45 min, the absorbance of the solution was recorded at 515 nm with the microplate reader. The DPPH inhibition (%) was calculated using equation (2).

$$\text{DPPH inhibition (\%)} = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Trolox was used as a positive control and calibration curve was obtained from six concentrations of trolox ranging from 1 to 10 µg/mL. The results are expressed as mg Trolox equivalents (TE) released from the beads

6.2.7 Statistical analysis

Experiments were performed in triplicate and the results are exhibited as mean ± standard deviation. SPSS statistical software (SPSS 23.0, IBM, Armonk, NY, USA) was used to perform statistical analysis. One-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference tests and independent samples t-tests were applied to determine the significant difference (95% confidence, $p < 0.05$) between two mean values.

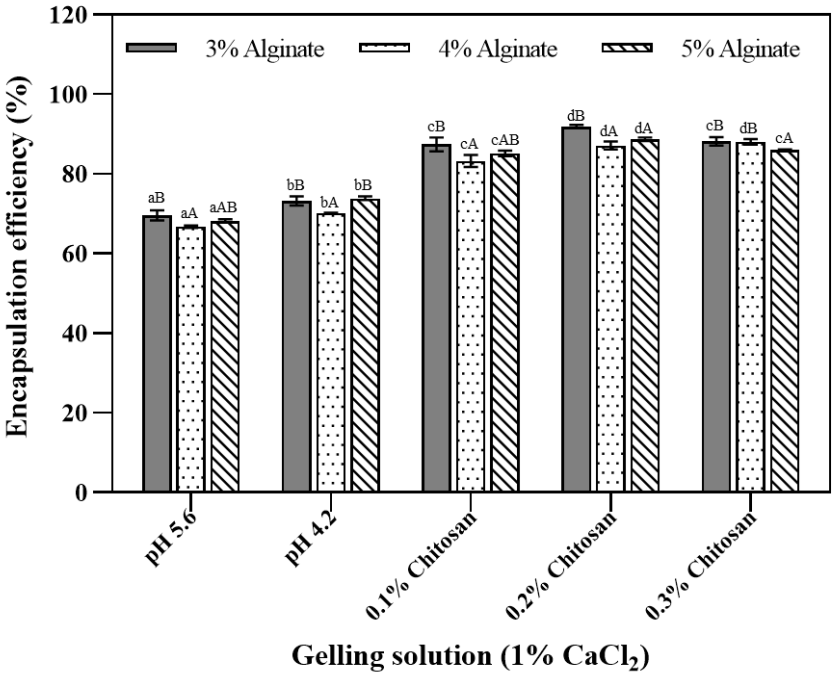
6.3 Result and Discussion

6.3.1 Encapsulation efficiency of alginate-chitosan beads

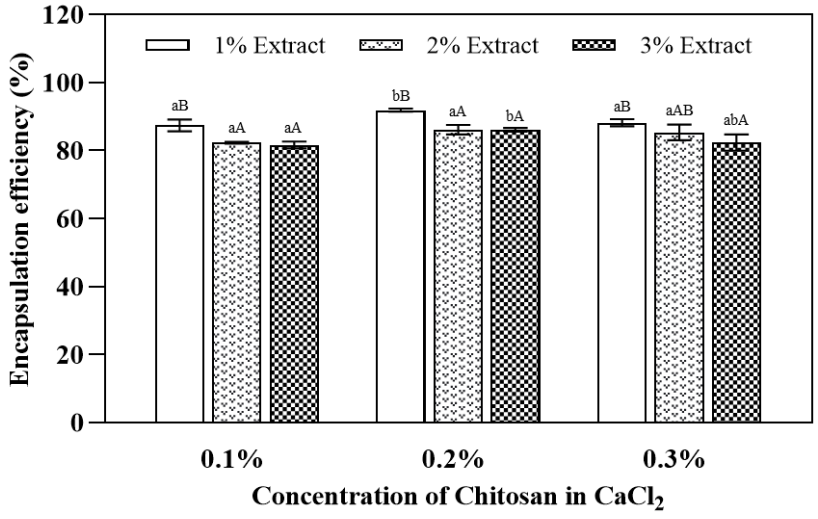
Different concentrations of sodium alginate, extract and chitosan trialled to encapsulate phenolic extract from *H. sabdariffa* calyces, and their respective encapsulation efficiency is presented in Figure 2. Figure 2a shows the effect of concentration of sodium alginate and chitosan on the encapsulation efficiency at constant 1% (w/v) extract. There was a significant improvement ($p<0.05$) in encapsulation efficiency with the addition of chitosan in gelling solution. Chitosan is insoluble in water and requires mildly acidic conditions for complete dissolution (Goñi et al., 2017). This resulted reduction in pH of $CaCl_2$ solution with chitosan from 5.6 to 4.2. Therefore, 1% (w/v) $CaCl_2$ solution at pH 4.2 was used to ascertain impact of this decrease of pH on encapsulation efficiency. A slight increase in encapsulation efficiency was observed when the pH was dropped to 4.2 but in the absence of chitosan. When the chitosan (0.1 to 0.3% w/v) was introduced, the encapsulation efficiency increased from 66.8 - 69.6% to 83.3 - 91.9%. The highest encapsulation efficiency (91.9%) was achieved for 3% alginate and 0.2% chitosan mixture with 1% extract. Interestingly, further increase in sodium alginate concentration from 3% to 5% negatively impacted on the encapsulation efficiency. In the case of chitosan, 0.2% was more suitable for encapsulating the phenolic compounds of *H. sabdariffa*. This finding is in good agreement with previous report by Takka and Gurel (2010) where they have reported alginate concentration of 3% and chitosan concentration of 0.25% to

be the optimum condition for encapsulation of bovine serum albumin in terms of encapsulation efficiency and *in vitro* release.

In order to optimise the extract concentration to be used in sodium alginate, three different concentrations, i.e., 1, 2 and 3%, were tested at the constant 3% alginate and three concentrations of chitosan (0.1, 0.2 and 0.3%). The effect of this varying extract concentration on encapsulation efficiency is shown in Figure 2b. The encapsulation efficiency decreased with the increase of extract concentration and 1% extract concentration achieved the highest encapsulation efficiency irrespective of the chitosan concentration used. Zam et al. (2014) and Najafi-Soulari et al. (2016) reported the optimum concentration of extract to be 1% for the encapsulation of pomegranate polyphenols and lemon balm extract, respectively, in alginate beads in terms of encapsulation efficiency. Khorshidian et al. (2019) observed similar effect of extract concentration on encapsulation efficiency where the extract concentration higher than 2% caused decline in the encapsulation efficiency. This may be due to the fact that phenolic extracts from *H. sabdariffa* are hydrophilic in nature and require water to be present in polymer matrix for them to get encapsulated. Moreover, increasing the concentration of extract could saturate the extract into the polymer matrix and increasing proportion unable to be encapsulated which leads to a lowered encapsulation efficiency (Hosseini et al., 2013; Najafi-Soulari et al., 2016).



(a)



(b)

Figure 2: Effect of different parameters on encapsulation efficiency of alginate-chitosan beads containing phenolic extract from *H. sabdariffa* (a) sodium alginate and chitosan concentration at 1% extract and (b) chitosan and phenolic extract concentration at 3% alginate and 1% $CaCl_2$.

Note: The different lower-case letters indicate statistically significant differences ($p<0.05$) among the different chitosan concentration at same alginate concentration (a and b). The different capital letters indicate statistically significant differences ($p<0.05$) between the alginate concentration (a) and extract concentration (b) at same chitosan concentration

6.3.2 Impact on bead size

The particle size data of dried alginate-chitosan beads is presented in Table 2. The difference in the diameter of freeze-dried and oven-dried beads was evident as freeze-dried beads were 2-3 times larger than the oven-dried ones. Freeze-dried beads had retained the original shape of beads. The increase of chitosan content in the $CaCl_2$ solution did not bring about any significant ($p>0.05$) changes on the particle size. The diameter of beads remains unaffected by the alginate concentration in case of freeze-dried sample. In the case of oven-dried sample, the bead size significantly increased ($p<0.05$) with the increasing concentration of sodium alginate. Pasparakis and Bouropoulos (2006) reported the average diameter of air-dried alginate beads from 21-gauge needle as 1.70 mm which is comparable to our findings. The particle size of beads can be altered by choosing different size of syringe needle (Rajmohan and Bellmer, 2019). For this reason, only one needle size (21-gauge with internal diameter of 0.337 mm) was used in this study to avoid additional variation.

Table 2: Mean diameter of freeze-dried and oven-dried alginate-chitosan beads.

Sample parameters (%)			Particle size (mm)	
Sodium alginate	**Chitosan**	**Extract**	**Freeze-dried beads**	**Oven-dried beads**
3	0.1	1	2.92 ± 0.08^{a}	0.99 ± 0.03^{a}
3	0.2	1	3.13 ± 0.15^{a}	1.03 ± 0.03^{a}
3	0.3	1	3.07 ± 0.19^{a}	1.01 ± 0.03^{a}
4	0.2	1	3.16 ± 0.11^{a}	1.30 ± 0.17^{b}
5	0.2	1	3.19 ± 0.09^{a}	1.57 ± 0.09^{c}

Note: The different letters in superscript within a column indicate statistically significant differences ($p < 0.05$) within the group.

6.3.3 Morphological characteristics of alginate-chitosan beads

SEM imaging permits the characterization of the shape, surface morphology and internal structure (when sectioned) of dried alginate-chitosan beads. The SEM images for freeze dried and oven-dried alginate-chitosan capsule beads are presented in Figure 3 and 4. As can be observed, the drying method had substantial effect on the surface and interior properties of the beads. Freeze dried beads more or less retained the initial shape and size whereas oven-dried beads experienced shrinkage. Freeze-dried beads were almost 2-3 times larger than oven-dried beads. Freeze-dried beads appeared to be more spherical with rough and wrinkled surface as also reported in the literature by Song et al. (2018) and Abdin et al. (2020). While oven-dried beads had much smoother surface with slightly oval shape. There were no cracks and pores on the bead's surface obtained from both drying methods. However, Abubakr et al. (2009) and Dalponte Dallabona et al. (2020) observed that oven drying had caused cracks at crosslinked polymer surfaces of alginate beads possibly because of collapsing of the polymer network structure during drying. The disparity in the findings of these studies from ours might be due to use of high drying temperature (70°C) and/or longer drying time (24 h) in the former. Few crystals on the surface of freeze-dried beads can be observed and these are likely to be crystal of sodium chloride formed due to the interaction of sodium ions from alginate and chloride ions in $CaCl_2$ solution. It can also be the residual calcium chloride from the gelling solution remained after drying (Najafi-Soulari et al., 2016). The surface of the beads became much smoother with the higher alginate concentrations.

The beads' cross-section revealed that freeze-dried beads had porous network with sheet-like layers (Fig. 5). This porous structure of freeze-dried beads was formed due to the removal of frozen water by sublimation. Freeze-dried alginate beads encapsulating *Syzygium cumini* seed polyphenols also presented comparable images with porous and fragile network (Abdin et al., 2020). In contrast, oven-dried beads had compact and rigid internal structure.

Rajmohan and Bellmer (2019) reported similar compact cross-sectional structure of spirulina-alginate beads prepared by ionic gelation. This may benefit in the sustained release of encapsulated phenolic extracts from the beads.

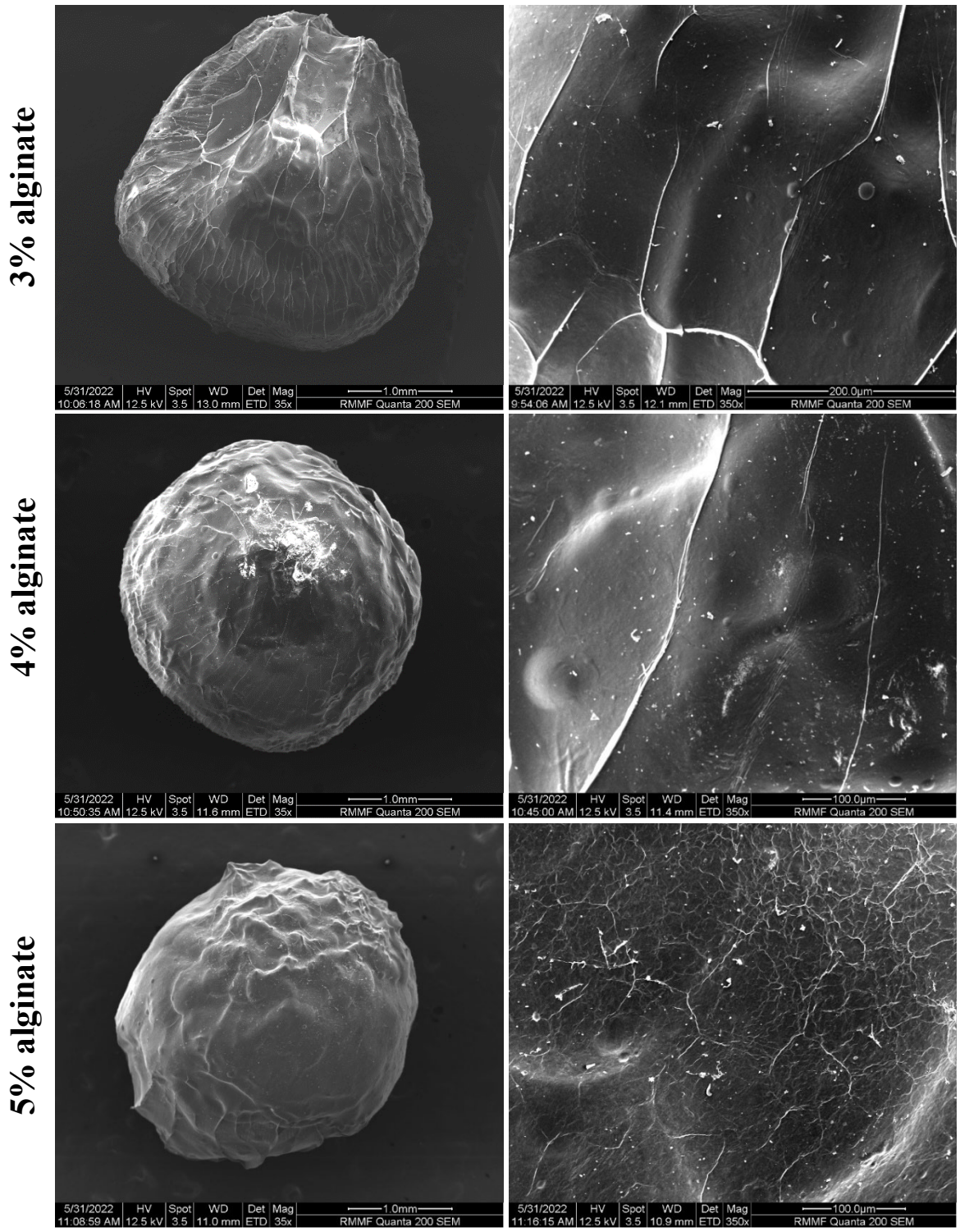


Figure 3: SEM micrographs of freeze-dried alginate-chitosan beads containing phenolic extract from *H. sabdariffa* calyces at 0.2% of chitosan and 1% of extract

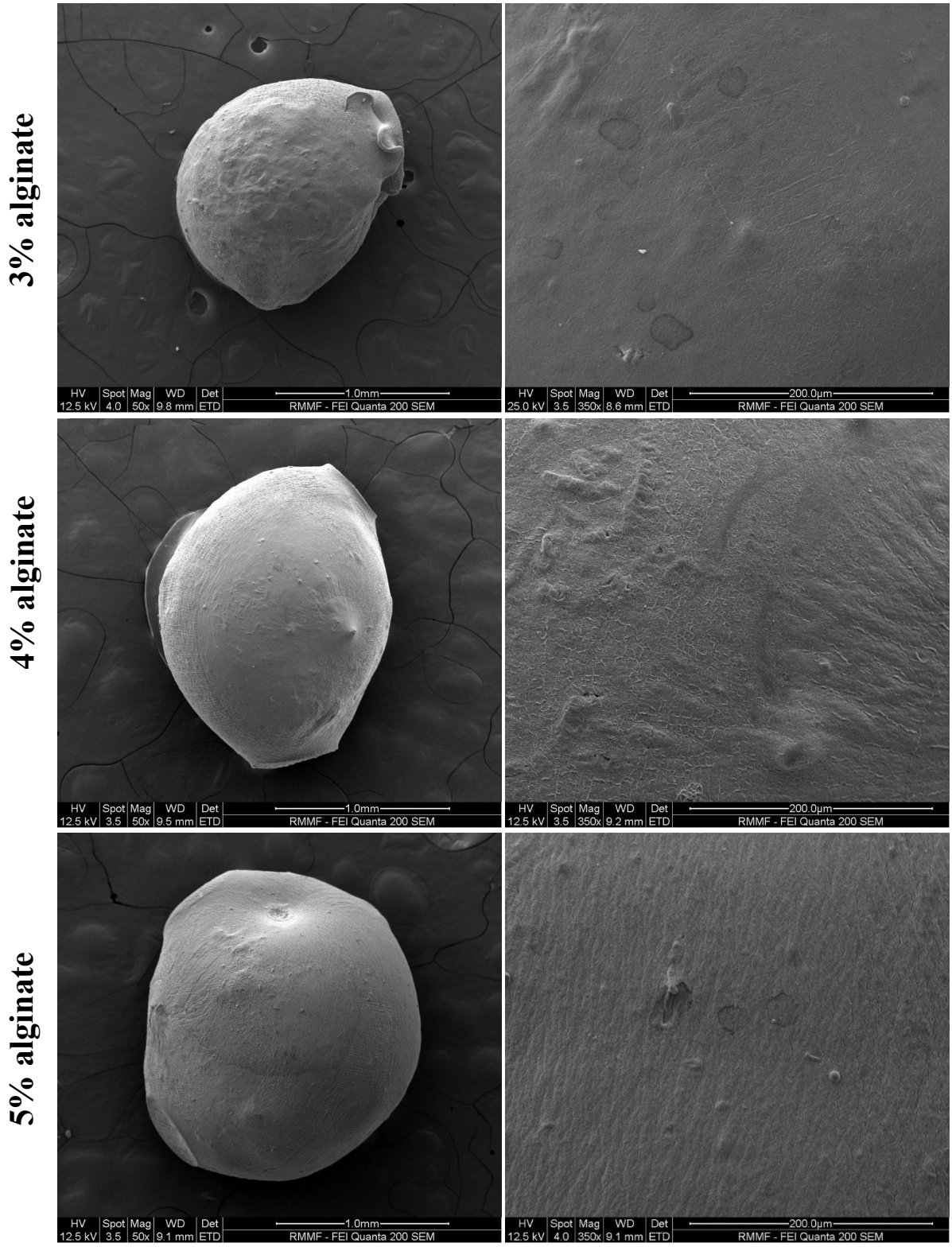


Figure 4: SEM micrographs of oven-dried alginate-chitosan beads containing phenolic extract from *H. sabdariffa* calyces at 0.2% of chitosan and 1% of extract

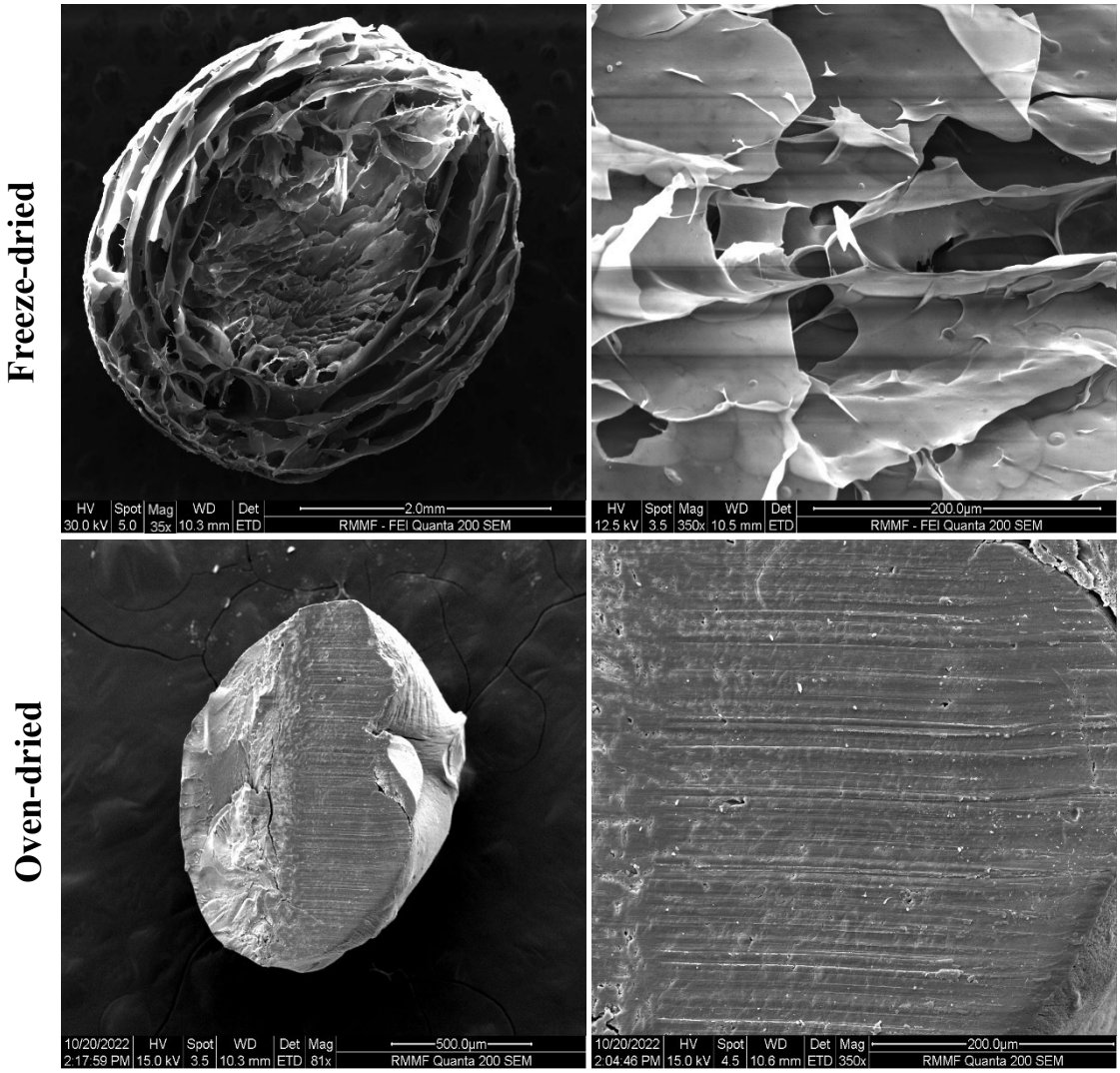


Figure 5: SEM cross sectional micrographs of freeze-dried and oven-dried alginate-chitosan beads containing phenolic extract from *H. sabdariffa* calyces at 3% alginate, 0.2% of chitosan and 1% of extract

6.3.4 Chemical characterization of beads through FTIR spectroscopy analysis

The FTIR spectra of alginate beads at different concentration of alginate, extract and chitosan are displayed in Figure 6 (a) and (b). The characteristic peaks of alginate beads can be seen at 1688–1600 cm^{-1} corresponding to symmetrical and asymmetrical stretching vibration for the carboxyl ion (COO–), indicating the presence of carboxylic acid, ester, or carbonyl groups. The bands at 1510–1360 cm^{-1} signifies the presence of C=C of the aromatic ring, and big peaks

between 1100 and 1000 cm^{-1} represents the C–O–C stretching vibrations. The distinct absorbance peak around 3350 cm^{-1} can be attributed to the absorbance of water. Similar peaks were reported by Dalponte Dallabona et al. (2020) for alginate beads encapsulating jaboticaba peel and propolis extracts. With the addition of extract, there was not significant changes in the spectra except for decrease of peak intensity within 3000 – 2850 cm^{-1} range. Similarly, the presence of chitosan in the gelling solution did not show any significant change in the FTIR spectra. This indicated that as the beads might have not taken enough chitosan to cause any functional changes.

The change of sodium alginate concentration in the matrix brought about three observable differences in the IR signal. As shown in Figure 6 (b), increasing alginate concentration increased the peak intensity at 1000, 1650 and 3000 – 2850 cm^{-1}. This is due to the increase of carboxyl ion (COO–) functional group of alginates with the increase in its mass concentration.

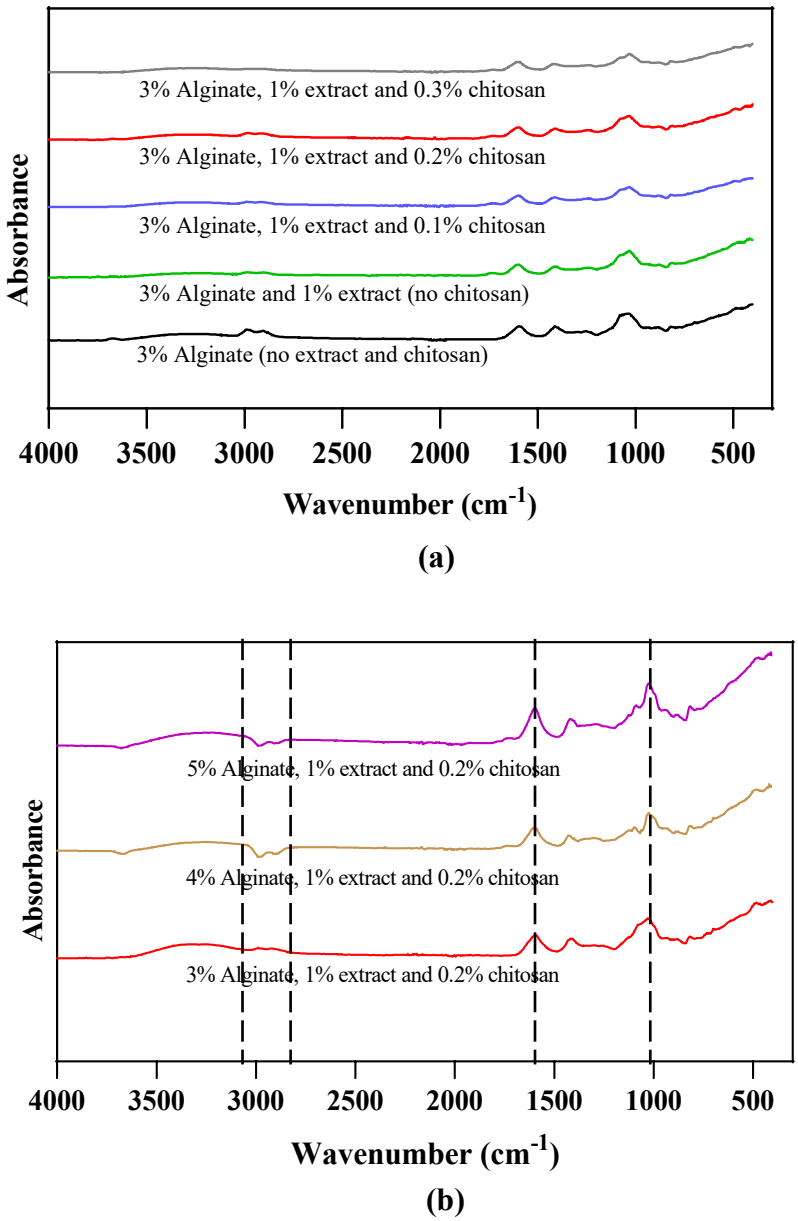


Figure 6: FTIR spectra of alginate-chitosan beads containing phenolic compounds from *H. sabdariffa* (a) at different chitosan concentration and (b) at different alginate concentration

6.3.5 *In vitro* release of phenolic compounds and their antioxidant activity from alginate-chitosan beads

6.3.5.1 Release of phenolic compounds

Most of the polyphenols are absorbed from intestine and provide protection against development of several chronic diseases (Zhang et al., 2021b). Therefore, one of the main aims

of encapsulating phenolic compounds is to control the release of phenolic compounds in the intestinal stage of digestion. Therefore, in this study the alginate-chitosan beads with highest encapsulation efficiency (3% sodium alginate, 0.2% chitosan and 1% extract) obtained from both freeze-drying and oven drying were used in the release studies. The *in vitro* release profile of phenolic compounds from these beads in SGF and SIF is shown in Figure 7a. Within the first 10 min in SGF, less than 10.0% phenolic compounds were released from both types of beads. The release abruptly increased to 25.7 – 32.5% at 30 min followed by slow release until 120 min in SGF (Fig. 7a). The release of the encapsulated phenolic compounds in SGF for 120 min was faster as well as higher from freeze-dried beads (46.4%) in comparison to oven-dried beads (37.9%). The higher release of phenolic compounds from freeze-dried beads can be explained by the porous network formed by freeze drying process (Fig. 5) allowing easier diffusion of phenolic compounds from the matrix to SGF. Alginate structure has the ability to become compact at low pH (<3.0) due to stabilization by intermolecular hydrogen bonding network which makes the beads fairly stable in SGF (pH 3) (Chuang et al., 2017) retaining more than 50% of the phenolic compounds. Patel et al. (2016) reported a 35–47 % release of ceftriaxone sodium from alginate beads in SGF maintained at pH 1.2 for the first 120 min which was comparable to our data. In the case of control sample (nonencapsulated phenolic extract), the phenolic content remained unchanged in SGF. This may be due to the fact that most the phenolic compounds are stable at acidic pH (Cao et al., 2021; Oancea and Drăghici, 2018; Zeng et al., 2017).

The remaining portion of the entrapped phenolic compounds (around 85% of total phenolic content) were burst released within 10 min in the SIF. Nearly 90% of these compounds were released after 120 min in SIF. The release of phenolic compounds was faster in SIF than in SGF. This phenomenon is due to the swelling and disintegration of alginate under neutral pH (Corstens et al., 2017). Compared to encapsulated, non-encapsulated phenolic

extract suffered 20% degradation of their initial phenolic compound upon 120 min in SIF which highlights the importance of encapsulation. Zeng et al. (2017)'s work also showed that unencapsulated catechins readily degraded at neutral pH.

6.3.5.2 Antioxidant activity of phenolic compounds released from beads

Antioxidant activity of the digesta was determined to investigate the bioactivity of phenolic compounds released in SGF and SIF as presented in Figure 7b. Although less than 10% phenolic compounds were released during the first 10 min in SGF, the antioxidant activity this digesta was found to be 30-36% of the total activity. The antioxidant activity increased slowly till 120 min in SGF to 40-45% due to continuous release of the phenolic compounds. Almost 10% of antioxidant activity was lost when the phenolic extract was subjected in SGF for digestion without encapsulation for 120 min. Exposure to acidic condition prevailing in simulated gastrointestinal conditions could reduce polyphenol's ability to scavenge free radicals (Ross et al. 2011). The antioxidant activity of freeze-dried beads was higher than that of oven-dried ones which could be attributed to the release of higher amount of phenolic compounds released from the former.

In SIF, the antioxidant activity of the released phenolic compounds was about 80% for both freeze-dried and oven-dried beads. The 20% shortfall in antioxidant activity may be due to 10% of phenolic compounds trapped within the matrix or being degraded in SIF. Although the released contents of phenolic compounds at the end 240 min of digestion (combined treatment in SGF and SIF) from encapsulated and un-encapsulated extracts were close, the antioxidant activity was significantly lower in case of non-encapsulated extract. This further highlights the advantage of encapsulating the phenolic extract of *H. sabdariffa* to preserve the bioactivity.

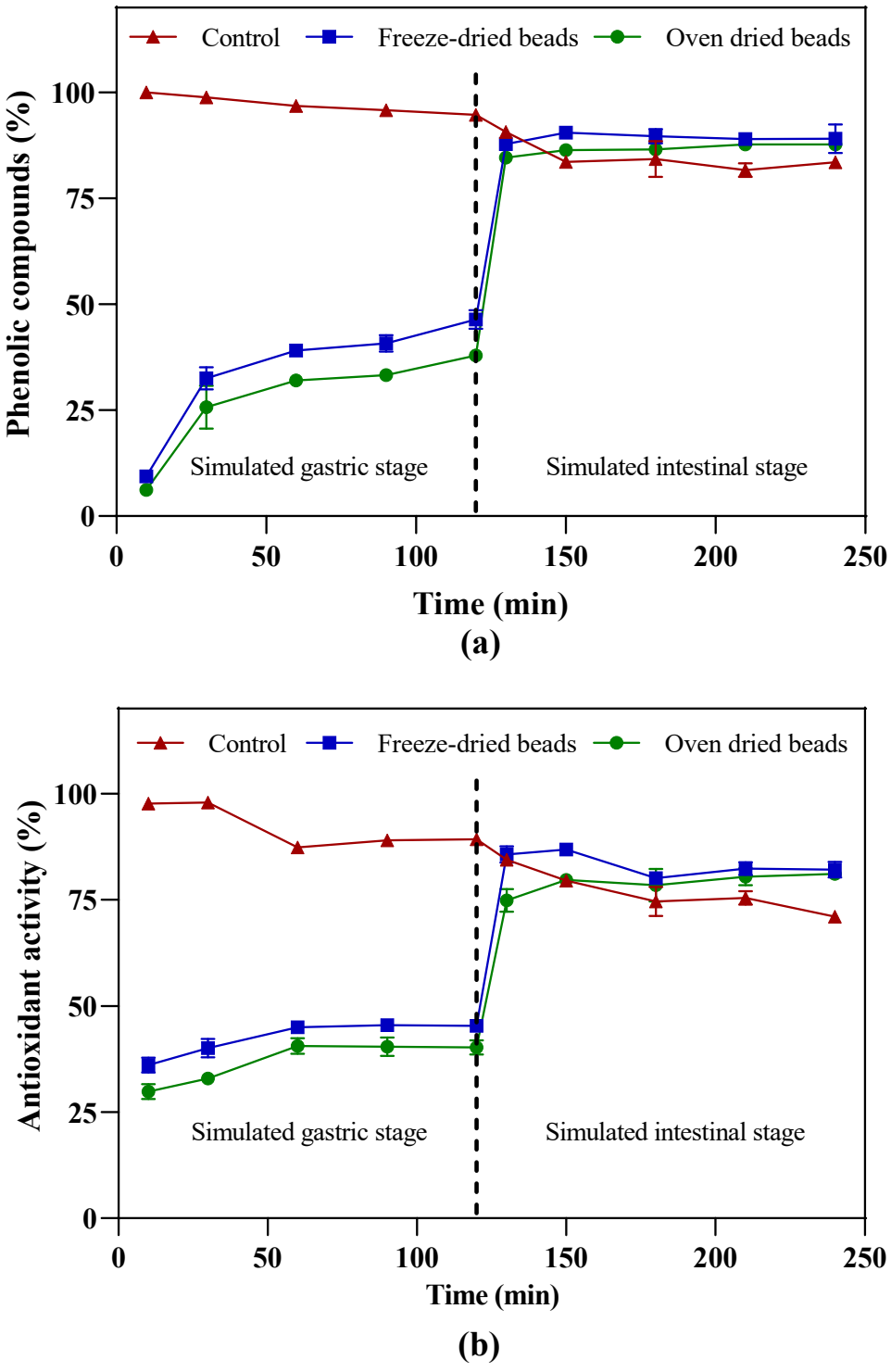


Figure 7: Release of (a) phenolic compounds and (b) antioxidant activity from freeze-dried and oven-dried alginate-chitosan beads encapsulating phenolic extract from *H. sabdariffa* calyces at 3% sodium alginate, 0.2% chitosan and 1% extract

6.4 Conclusion

Sodium alginate and chitosan can effectively encapsulate the phenolic compounds of *H. sabdariffa* through ionic gelation. The concentration of sodium alginate, chitosan and extract significantly affected the encapsulation efficiency. The combination of 3% sodium alginate, 0.2 % chitosan and 1% extract provided highest encapsulation efficiency of 91.9%. Freeze dried beads had rough surface and porous network whereas oven-dried beads had smooth surface with compact interior structure which affected on the release properties. The *in vitro* release data in SGF and SIF showed that encapsulation of *H. sabdariffa* extracts in alginate-chitosan combined matrix was able achieve controlled release of the phenolic compounds delivering most to intestinal stage. The encapsulation also retained the bioactivity (antioxidant activity) of phenolic compounds which was shown to be lost if they were not encapsulated. The capsules of phenolic compounds-rich *H. sabdariffa* developed in this study can be used as part of functional foods to manage chronic diseases where plant-based antioxidants are preferred.

CRediT authorship contribution statement

Manisha Singh: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft.

Thilini Thrimawithana: Supervision, Writing - review & editing.

Ravi Shukla: Supervision, Writing - review & editing.

Benu Adhikari: Conceptualization, Supervision, Validation, Writing - review & editing.

Acknowledgement

The authors gratefully acknowledge Lee Etherington (Wild Hibiscus Flower Co., NSW, Australia) for providing free samples of *H. sabdariffa* calyces. The first author acknowledges

RMIT's scholarship support, which enabled the completion of this work. The authors also acknowledge the facilities, and the scientific and technical assistance of the RMIT University's Microscopy & Microanalysis Facility.

Conflict of interest statement

The authors declare no conflict of interest.

Reference

Abdin, M., Salama, M.A., Riaz, A., Akhtar, H.M.S., & Elsanat, S.Y. (2020). Enhanced the entrapment and controlled release of Syzygium cumini seeds polyphenols by modifying the surface and internal organization of Alginate-based microcapsules. *Journal of Food Processing and Preservation,* 45(1). http://doi.org/10.1111/jfpp.15100

Abubakr, N., Lin, S., & Chen, X. (2009). Effects of Drying Methods on the Release Kinetics of Vitamin B12 in Calcium Alginate Beads. *Drying Technology,* 27, 1258-1265. http://doi.org/10.1080/07373930903267732

Amaya-Cruz, D., Perez-Ramirez, I.F., Perez-Jimenez, J., Nava, G.M., & Reynoso-Camacho, R. (2019). Comparison of the bioactive potential of Roselle (*Hibiscus sabdariffa* L.) calyx and its by-product: Phenolic characterization by UPLC-QTOF MS(E) and their anti-obesity effect in vivo. *Food Research International,* 126, 108589. http://doi.org/10.1016/j.foodres.2019.108589

Anal, A.K., Bhopatkar, D., Tokura, S., Tamura, H., & Stevens, W.F. (2003). Chitosan-alginate multilayer beads for gastric passage and controlled intestinal release of protein. *Drug Dev Ind Pharm,* 29(6), 713-724. http://doi.org/10.1081/ddc-120021320

Asgary, S., Soltani, R., Zolghadr, M., Keshvari, M., & Sarrafzadegan, N. (2016). Evaluation of the effects of roselle (*Hibiscus sabdariffa* L.) on oxidative stress and serum levels of lipids, insulin and hs-CRP in adult patients with metabolic syndrome: a double-blind placebo-controlled clinical trial. *Journal of Complementary and Integrative Medicine,* 13(2), 175-180. http://doi.org/10.1515/jcim-2015-0030

Ballesteros, L.F., Ramirez, M.J., Orrego, C.E., Teixeira, J.A., & Mussatto, S.I. (2017). Encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds by freeze-drying and spray-drying using different coating materials. *Food Chem,* 237, 623-631. http://doi.org/10.1016/j.foodchem.2017.05.142

Cao, H., Saroglu, O., Karadag, A., Diaconeasa, Z., Zoccatelli, G., Conte-Junior, C.A., Gonzalez-Aguilar, G.A., Ou, J., Bai, W., Zamarioli, C.M., de Freitas, L.A.P., Shpigelman, A., Campelo, P.H., Capanoglu, E., Hii, C.L., Jafari, S.M., Qi, Y., Liao, P., Wang, M., et al. (2021). Available technologies on improving the stability of polyphenols in food processing. *Food Frontiers,* 2(2), 109-139. http://doi.org/https://doi.org/10.1002/fft2.65

Chuang, J.-J., Huang, Y.-Y., Lo, S.-H., Hsu, T.-F., Huang, W.-Y., Huang, S.-L., & Lin, Y.-S. (2017). Effects of pH on the Shape of Alginate Particles and Its Release Behavior. *International Journal of Polymer Science,* 2017, 3902704. http://doi.org/10.1155/2017/3902704

Corstens, M.N., Berton-Carabin, C.C., Elichiry-Ortiz, P.T., Hol, K., Troost, F.J., Masclee, A.A.M., & Schroën, K. (2017). Emulsion-alginate beads designed to control in vitro intestinal lipolysis: Towards appetite control. *Journal of Functional Foods,* 34, 319-328. http://doi.org/https://doi.org/10.1016/j.jff.2017.05.003

Dalponte Dallabona, I., de Lima, G.G., Cestaro, B.I., Tasso, I.S., Paiva, T.S., Laureanti, E.J.G., Jorge, L.M.M., da Silva, B.J.G., Helm, C.V., Mathias, A.L., & Jorge, R.M.M. (2020). Development of alginate beads with encapsulated jabuticaba peel and propolis extracts to achieve a new natural colorant antioxidant additive. *Int J Biol Macromol,* 163, 1421-1432. http://doi.org/10.1016/j.ijbiomac.2020.07.256

Dhakal, S., Kushairi, N., Phan, C.W., Adhikari, B., Sabaratnam, V., & Macreadie, I. (2019). Dietary Polyphenols: A Multifactorial Strategy to Target Alzheimer's Disease. *Int J Mol Sci,* 20(20). http://doi.org/10.3390/ijms20205090

George, K.M., Ruthenburg, T.C., Smith, J., Yu, L., Zhang, Q., Anastasio, C., & Dillner, A.M. (2015). FT-IR quantification of the carbonyl functional group in aqueous-phase

secondary organic aerosol from phenols. *Atmospheric Environment,* 100, 230-237. http://doi.org/10.1016/j.atmosenv.2014.11.011

Goñi, M.G., Tomadoni, B., Roura, S.I., & Moreira, M.D.R. (2017). Lactic acid as potential substitute of acetic acid for dissolution of chitosan: preharvest application to Butterhead lettuce. *J Food Sci Technol,* 54(3), 620-626. http://doi.org/10.1007/s13197-016-2484-5

Hosseini, S.F., Zandi, M., Rezaei, M., & Farahmandghavi, F. (2013). Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study. *Carbohydrate Polymers,* 95(1), 50-56. http://doi.org/10.1016/j.carbpol.2013.02.031

Kao, E.S., Yang, M.Y., Hung, C.H., Huang, C.N., & Wang, C.J. (2016). Polyphenolic extract from *Hibiscus sabdariffa* reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. *Food & Function,* 7(1), 171-182. http://doi.org/10.1039/c5fo00714c

Khorshidian, N., Mahboubi, A., Kalantari, N., Hosseini, H., Yousefi, M., Arab, M., da Cruz, A.G., Mortazavian, A.M., & Mahdavi, F.S. (2019). Chitosan-Coated Alginate Microcapsules Loaded with Herbal galactagogue Extract: Formulation Optimization and Characterization. *Iran J Pharm Res,* 18(3), 1180-1195. http://doi.org/10.22037/ijpr.2019.1100776

Kulig, D., Zimoch-Korzycka, A., Jarmoluk, A., & Marycz, K. (2016). Study on Alginate¯Chitosan Complex Formed with Different Polymers Ratio. *Polymers (Basel),* 8(5). http://doi.org/10.3390/polym8050167

Kurozawa, L.E., & Hubinger, M.D. (2017). Hydrophilic food compounds encapsulation by ionic gelation. *Current Opinion in Food Science,* 15, 50-55. http://doi.org/https://doi.org/10.1016/j.cofs.2017.06.004

Lee, K.Y., & Mooney, D.J. (2012). Alginate: properties and biomedical applications. *Prog Polym Sci,* 37(1), 106-126. http://doi.org/10.1016/j.progpolymsci.2011.06.003

Mehta, N., Kumar, P., Verma, A.K., Umaraw, P., Kumar, Y., Malav, O.P., Sazili, A.Q., Domínguez, R., & Lorenzo, J.M. (2022). Microencapsulation as a Noble Technique for the Application of Bioactive Compounds in the Food Industry: A Comprehensive Review. *Applied Sciences,* 12(3). http://doi.org/10.3390/app12031424

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., et al. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food Funct,* 5(6), 1113-1124. http://doi.org/10.1039/c3fo60702j

Mishra, K., Ojha, H., & Chaudhury, N.K. (2012). Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chemistry,* 130(4), 1036-1043. http://doi.org/10.1016/j.foodchem.2011.07.127

Morales-Luna, E., Pérez-Ramírez, I.F., Salgado, L.M., Castaño-Tostado, E., Gómez-Aldapa, C.A., & Reynoso-Camacho, R. (2019). The main beneficial effect of roselle (Hibiscus sabdariffa) on obesity is not only related to its anthocyanin content. *J Sci Food Agric,* 99(2), 596-605. http://doi.org/10.1002/jsfa.9220

Najafi-Soulari, S., Shekarchizadeh, H., & Kadivar, M. (2016). Encapsulation optimization of lemon balm antioxidants in calcium alginate hydrogels. *Journal of Biomaterials Science, Polymer Edition,* 27(16), 1631-1644. http://doi.org/10.1080/09205063.2016.1226042

Naranjo-Duran, A.M., Quintero-Quiroz, J., Rojas-Camargo, J., & Ciro-Gomez, G.L. (2021). Modified-release of encapsulated bioactive compounds from annatto seeds produced

by optimized ionic gelation techniques. *Sci Rep,* 11(1), 1317. http://doi.org/10.1038/s41598-020-80119-1

Oancea, S., & Drăghici, O. (2018). pH and Thermal Stability of Anthocyanin-based Optimised Extracts of Romanian Red Onion Cultivars. *Czech Journal of Food Sciences,* 31, 283-291.

Pasparakis, G., & Bouropoulos, N. (2006). Swelling studies and in vitro release of verapamil from calcium alginate and calcium alginate-chitosan beads. *Int J Pharm,* 323(1-2), 34-42. http://doi.org/10.1016/j.ijpharm.2006.05.054

Patel, N., Lalwani, D., Gollmer, S., Injeti, E., Sari, Y., & Nesamony, J. (2016). Development and evaluation of a calcium alginate based oral ceftriaxone sodium formulation. *Prog Biomater,* 5, 117-133. http://doi.org/10.1007/s40204-016-0051-9

Pham, L.B., Wang, B., Zisu, B., Truong, T., & Adhikari, B. (2021). In-vitro digestion of flaxseed oil encapsulated in phenolic compound adducted flaxseed protein isolate-flaxseed gum complex coacervates. *Food Hydrocolloids,* 112, 106325. http://doi.org/https://doi.org/10.1016/j.foodhyd.2020.106325

Rajmohan, D., & Bellmer, D. (2019). Characterization of Spirulina-Alginate Beads Formed Using Ionic Gelation. *Int J Food Sci,* 2019, 7101279. http://doi.org/10.1155/2019/7101279

Ravichandran, K., Palaniraj, R., Saw, N.M., Gabr, A.M., Ahmed, A.R., Knorr, D., & Smetanska, I. (2014). Effects of different encapsulation agents and drying process on stability of betalains extract. *J Food Sci Technol,* 51(9), 2216-2221. http://doi.org/10.1007/s13197-012-0728-6

Sen, S., & Chakraborty, R., (2011). The Role of Antioxidants in Human Health *Oxidative Stress: Diagnostics, Prevention, and Therapy* (Vol. 1083). American Chemical Society. p. 1-37.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. *Journal of Functional Foods,* 18, 820-897. http://doi.org/10.1016/j.jff.2015.06.018

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. *Future Foods,* 1-2. http://doi.org/10.1016/j.fufo.2020.100002

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*. *Future Foods,* 4. http://doi.org/10.1016/j.fufo.2021.100087

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2022). Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of Hibiscus sabdariffa. *Food Bioscience,* 50, 101992. http://doi.org/https://doi.org/10.1016/j.fbio.2022.101992

Song, P., Wu, Y., Zhang, X., Yan, Z., Wang, M., & Xu, F. (2018). Preparation of Covalently Crosslinked Sodium Alginate/Hydroxypropyl Methylcellulose pH-Sensitive Microspheres for Controlled Drug Release. *BioResources,* 13(4), 8614-8628.

Stojanovic, R., Belscak-Cvitanovic, A., Manojlovic, V., Komes, D., Nedovic, V., & Bugarski, B. (2012). Encapsulation of thyme (Thymus serpyllum L.) aqueous extract in calcium alginate beads. *J Sci Food Agric,* 92(3), 685-696. http://doi.org/10.1002/jsfa.4632

Sun, H.-N., Mu, T.-H., & Xi, L.-S. (2017). Effect of pH, heat, and light treatments on the antioxidant activity of sweet potato leaf polyphenols. *International Journal of Food Properties,* 20(2), 318-332. http://doi.org/10.1080/10942912.2016.1160410

Takka, S., & Gurel, A. (2010). Evaluation of chitosan/alginate beads using experimental design: formulation and in vitro characterization. *AAPS PharmSciTech,* 11(1), 460-466. http://doi.org/10.1208/s12249-010-9406-z

Varma, R., & Vasudevan, S. (2020). Extraction, Characterization, and Antimicrobial Activity of Chitosan from Horse Mussel Modiolus modiolus. *ACS Omega,* 5(32), 20224-20230. http://doi.org/10.1021/acsomega.0c01903

Yang, M., Liang, Z., Wang, L., Qi, M., Luo, Z., & Li, L. (2020). Microencapsulation Delivery System in Food Industry—Challenge and the Way Forward. *Advances in Polymer Technology,* 2020, 7531810. http://doi.org/10.1155/2020/7531810

Zam, W., Bashour, G., Abdelwahed, W., & Khayata, W. (2014). Alginate-pomegranate peels' polyphenols beads: effects of formulation parameters on loading efficiency. *Brazilian Journal of Pharmaceutical Sciences,* 50(4), 741-748. http://doi.org/10.1590/s1984-82502014000400009

Zeng, L., Ma, M., Li, C., & Luo, L. (2017). Stability of tea polyphenols solution with different pH at different temperatures. *International Journal of Food Properties,* 20(1), 1-18. http://doi.org/10.1080/10942912.2014.983605

Zhang, H., Cheng, J., & Ao, Q. (2021a). Preparation of Alginate-Based Biomaterials and Their Applications in Biomedicine. *Mar Drugs,* 19(5). http://doi.org/10.3390/md19050264

Zhang, W., Qi, S., Xue, X., Al Naggar, Y., Wu, L., & Wang, K. (2021b). Understanding the Gastrointestinal Protective Effects of Polyphenols using Foodomics-Based Approaches. *Front Immunol,* 12, 671150. http://doi.org/10.3389/fimmu.2021.671150

CHAPTER 7

General Discussion, Conclusion and Recommendations

7.1 Introduction

The demand for natural supplements to manage weight loss is increasing as they are known to have less side effects (Batsis et al., 2021). Bioactive molecules extracted from plants, particularly phenolic compounds, are known to possess properties that can help manage obesity through various mechanisms (Gooda Sahib et al., 2012). This information triggered the idea to study the potential application of extract of *H. sabdariffa* as the source of natural compounds for the management of obesity. *H. sabdariffa* calyces have long been used as a herbal tea as they are rich in bioactive compounds such as phenolic acids, anthocyanins, flavonoids and polysaccharides (Nguyen & Chuyen, 2020).

This thesis aimed to evaluate the anti-obesity potential of phenolic compounds and hydroxycitric acid extracted from *H. sabdariffa* when used individually and combined including the mechanism involved. This thesis commenced by establishing most suitable solvent and optimum temperature for the extraction of phenolic compounds from *H. sabdariffa* (Chapter 3). This thesis also validated, for the first time, a method to extract hydroxycitric acid in the form of more stable potassium hydroxycitrate (Chapter 3). Mechanisms of anti-obesity activity was explored for both phenolic compound-rich extracts and potassium hydroxycitrate isolated from *H. sabdariffa*. *In vitro* enzyme inhibition assay was performed for three enzymes (α-amylase, α-glucosidase, and pancreatic lipase) associated with obesity (Chapter 4).

Inhibition of adipogenesis by phenolic compound-rich extracts and potassium hydroxycitrate was determined by using human adipose-derived stem cells (hADSCs) (Chapter 5). Adipogenesis was chemically induced in hADSCs and the effect on lipid accumulation and expression of key genes involved in adipogenesis was studied. This thesis also developed a ionic gelation-based and facile encapsulation method using food grade alginate and chitosan to enhance the stability and bioactivity of phenolic compounds extracted from *H. sabdariffa* (Chapter 6). These capsule beads were shown to deliver the phenolic compounds at the

intestinal stage of digestion which is likely to improve the stability of the active compounds. These capsule beads provided stability of phenolic compounds.

Therefore, this thesis provides scientific foundation for application of *H. sabdariffa* extracts in the management of obesity. It also provides underpinning mechanism through which these phenolic compounds impart the antiobesity effect and demonstrates the importance of encapsulation in improving the stability of phenolic compounds.

7.2 Main research findings and general discussion

The main research findings from this study and the general discussion on importance of these findings are presented below.

7.2.1 Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *H. sabdariffa*

Initially the process of extraction of phenolic compounds and potassium hydroxycitrate from the calyces of *H. sabdariffa* was optimised. The effect of temperature and solvent composition on the extraction yield, total phenolic content, total flavonoid content, and antioxidant activity of phenolic-rich extract from *H. sabdariffa* calyces were determined. The phenolic compounds were found to be temperature sensitive and were best extracted at room temperature. The organic solvents (methanol and ethanol) were more suitable for the extraction of phenolic compounds compared to water. Methanol extract (51.72 mg GAE/g of extract) contained highest phenolic content among all the extracts. Kim and Lee (2017) reported similar effect of higher polyphenol content with methanol in comparison to ethanol during the extraction of polyphenols from mulberry.

Twelve different phenolic compounds were identified in these extracts using UPLC-DAD and it was confirmed that delphinidn-3-O-sambubioside chloride and cyanidin 3-O-sambubioside chloride were the two major compounds in all of these extracts. The antioxidant

activity of the extracts was found to be closely related with the concentration of the phenolic compounds in them. The antioxidant activity of phenolic extract was stable for only five weeks during the storage at -30°C thus, demonstrating the need for encapsulation to improve storage stability. This study also presented an optimised method to extract hydroxycitric acid from *H. sabdariffa* in the form of potassium hydroxycitrate. The extracted potassium hydroxycitrate was characterized through FTIR and HPLC-UV method. The purity of the extracted potassium hydroxycitrate was 180.1 μg/mg which is, so far, the highest reported recovery of this compound.

7.2.2 Inhibition of enzymes associated with obesity by the phenol-rich extracts of *H. sabdariffa*

In vitro studies were used to determine the mechanisms through which phenolic compounds and potassium hydroxycitrate exerted the antiobesity functions. Inhibition of enzymes such as α-amylase, α-glucosidase and pancreatic lipase associated with obesity was evaluated. The results demonstrated that all the phenolic extracts of *H. sabdariffa* calyces inhibited these three enzymes in concentration (IC_{50}) dependent manner. The lowest IC_{50} values for α-amylase, α-glucosidase and pancreatic lipase was obtained in the case of ethyl acetate extract (3.69 mg/mL), methanol extract (1.59 mg/mL) and ethanol extract (1.88 mg/mL) respectively. These values were either higher or comparable with the findings from other studies on *H. sabdariffa* (Ademiluyi & Oboh, 2013; Buchholz & Melzig, 2016; Ifie et al., 2018) which may be due to the variation in the plant source, sample preparation, and extraction protocol used. Molecular docking confirmed the ability of these to bind with different binding affinities at the active sites of these enzyme and inhibit their enzymatic activity. Potassium hydroxycitrate did not present any inhibition against these three enzymes. Also, the combination of polyphenols and potassium hydroxycitrate did not produce any synergistic effect on this inhibition.

7.2.3 Phenolic extracts of *H. sabdariffa* help manage obesity through suppression of adipogenesis

The effect of *H. sabdariffa* extracts on adipogenesis or adipocyte differentiation in human adipose-derived stem cells was evaluated to determine antiobesity mechanisms by which these extracts impart clinical effects. This is the first study where the extracts of *H. sabdariffa* has been tested on human cell lines. The cytotoxicity of all the phenolic extracts and potassium hydroxycitrate on hADSCs was determined through MTT assay. The adipogenesis was chemically induced in hADSCs and lasted for 21 days. The effect of all *H. sabdariffa* extracts was tested during first 3 days of adipogenesis. All phenolic compound-rich extracts demonstrated significant inhibition of adipogenesis as measured by reduction in the lipid accumulation.

Kim et al (2007) reported that aqueous *H. sabdariffa* extract reduced the lipid accumulation by about 30% at the same (1000 μg/mL) concentration in 3T3-L1 preadipocytes. Current work achieved 45% decrease in accumulation. The variation in lipid accumulation may be contributed by different in cell lines, the origin of the plant and the extraction method used. Almost 95% reduction in lipid accumulation was observed in hADSCs with 1 mg/mL of ethanol and ethyl acetate extract. The superior inhibition of adipogenesis by organic (methanol, ethanol, and ethyl acetate) extracts as compared to aqueous extract can be due to higher polyphenolic content in the former. These extracts successfully downregulated two vital adipogenic genes (PPAR-γ and aP2) in hADSCs. Potassium hydroxycitrate did not interfere with the adipogenesis. In addition, synergism was not observed in the adipogenic inhibition when phenolic compounds and potassium hydroxycitrate were used together.

7.2.4 Encapsulation of *H. sabdariffa* extracts

In the final experimental chapter of the thesis, *H. sabdariffa* extract was encapsulated in alginate-chitosan matrix through ionic gelation. Alginate and chitosan were selected as shell materials as they are food grade and are known to support the sustained release in gastrointestinal conditions. The main objective of encapsulation was to preserve the bioactivity, improve stability, achieve targeted release in simulated human intestinal system. Different concentrations of alginate (3, 4 and 5%), chitosan (0.1, 0.2 and 0.3%) and extract concentration (1, 2 and 3%) were applied to achieve higher encapsulation efficiency. The combination of 3% alginate,0.2% chitosan and 1% extract provided highest encapsulation efficiency (91.9%) of phenolic compounds of *H. sabdariffa.* The encapsulated beads had a smooth surface with no observable cracks and pores on the surface. This encapsulating shell protected the antioxidant property of phenolic extract and exhibited control/targeted release in simulated gastrointestinal fluids.

7.3 Contribution made by this thesis to the body of knowledge

The thesis has made significant contributions to the body of knowledge relevant to the application of natural bioactive compounds for managing obesity. These contributions are listed below.

1. The literature component of the thesis synthesised the available knowledge on application of natural phenolic compounds as alternative ingredients in managing obesity including mechanism of action. The evidence on their effectiveness assessed through several *in vitro, in vivo,* and clinical trials were documented and the gap in knowledge was identified (Singh et al., 2020).
2. Extraction of phenolic compounds from *H. sabdariffa* was systematically studied in organic solvents and water and provides the basis for the selection of best solvent to achieve better

polyphenolic extraction. The storage stability study of phenolic compound-rich extract provides clear understanding on the sensitivity and oxidative degradation of polyphenols even at lower temperature (-30°C) (Singh et al., 2021).

3. There are limited reports on the extraction of hydroxycitric acid from *H. sabdariffa*. Therefore, this study was the first to detail the extraction of hydroxycitric acid in the form of more stable potassium hydroxycitrate from *H. sabdariffa* (Singh et al., 2021).
4. This study showed that phenolic compounds from *H. sabdariffa* can significantly inhibit the activity of key enzymes (α-amylase, α-glucosidase, and pancreatic lipase) associated with obesity. Binding of polyphenols on to the specific site of these enzymes, assessed through molecular docking studies was found to be the mechanism through which they interfered the enzymatic activity (Singh et al., 2022).
5. This study demonstrated, for the first time, that phenolic compounds of *H. sabdariffa* were able to inhibit adipogenesis or adipocyte differentiation in human adipose-derived stem cells. These phenolic compounds down regulated the gene expression of two important adipogenic markers. This finding is particularly important for making the case for the potential application of *H. sabdariffa* for managing obesity (Singh et al., 2023).
6. Alginate-chitosan composite shell material was developed which effectively (encapsulation efficiency = 91.9%) encapsulated phenolic compounds of *H. Sabdariffa*. Encapsulation of the phenolic extract in alginate-chitosan shell matrix was able to preserve the antioxidant activity and provide sustained release in simulated gastrointestinal fluids (Chapter 6).

7.4 Recommendations for future work

This thesis laid a foundation for the extract of *H. sabdariffa* to be used as a source of bioactive phenolic compounds for the management of obesity. Based on the findings and conclusions drawn from this research, following recommendations are made for future research.

1. A mixture of polyphenolic compounds from *H. sabdariffa* was studied to assess their anti-obesity effects. It will be interesting to understand the role of individual phenolic compound present in *H. sabdariffa* on the anti-obesity effect.
2. This thesis focused on inhibition of digestive enzymes and adipogenesis as mechanisms of action of phenolic compounds and potassium hydroxycitrate of *H. sabdariffa* to assess antiobesity effect. Studies on other mechanisms such as appetite suppression, regulation of lipid metabolism, stimulation of energy expenditure and modulation of gut microbiota as potential mechanisms of obesity management can significantly contribute to the existing body of knowledge.
3. It would be interesting to study a global gene expression profiling by RNA sequencing to assess the transcription changes triggered by this phenolic compound in adipose derived stem cells. Additionally, the underlining molecular mechanisms involved in repressing key adipogenic genes can also be examined.
4. Despite the promising current knowledge derived from human cell line studies, it would be important to undertake *in vivo* studies in human subjects to ascertain adipogenesis suppressing effect. Moreover, further clinical research is also desirable to determine optimum dose and safety aspects of these compounds in humans.
5. The anti-obesity potential of phenolic ingredient from *H. sabdariffa* can be developed as nutraceuticals and commercialized as supplements for the prevention and treatment of obesity.
6. Functional foods or beverages with low calories for obese people can be formulated by incorporating the encapsulated formulation from *H. sabdariffa*. Physicochemical characteristics and health benefits of such foods or beverages against obesity need to be studied.

References

Ademiluyi, A. O., & Oboh, G. (2013). Aqueous extracts of roselle (Hibiscus sabdariffa Linn.) varieties inhibit α-amylase and α-glucosidase activities in vitro. Journal of Medicinal Food, 16(1), 88-93. doi:10.1089/jmf.2012.0004

Batsis, J. A., Apolzan, J. W., Bagley, P. J., Blunt, H. B., Divan, V., Gill, S., . . . Kidambi, S. (2021). A Systematic Review of Dietary Supplements and Alternative Therapies for Weight Loss. Obesity (Silver Spring, Md.), 29(7), 1102-1113. doi:10.1002/oby.23110

Buchholz, T., & Melzig, M. F. (2016). Medicinal plants traditionally used for treatment of obesity and diabetes mellitus - Screening for pancreatic lipase and alpha-amylase inhibition. Phytotherapy Research, 30(2), 260-266. doi:10.1002/ptr.5525

Gooda Sahib, N., Saari, N., Ismail, A., Khatib, A., Mahomoodally, F., & Abdul Hamid, A. (2012). Plants' metabolites as potential antiobesity agents. ScientificWorldJournal, 2012, 436039. doi:10.1100/2012/436039

Ifie, I., Ifie, B. E., Ibitoye, D. O., Marshall, L. J., & Williamson, G. (2018). Seasonal variation in Hibiscus sabdariffa (Roselle) calyx phytochemical profile, soluble solids and α-glucosidase inhibition. Food Chemistry, 261, 164-168. doi:https://doi.org/10.1016/j.foodchem.2018.04.052

Kim, I., & Lee, J. (2017). Comparison of different extraction solvents and sonication times for characterization of antioxidant activity and polyphenol composition in mulberry (Morus alba L.). Applied Biological Chemistry, 60(5), 509-517. doi:10.1007/s13765-017-0303-y

Nguyen, Q. V., & Chuyen, H. V. (2020). Processing of Herbal Tea from Roselle (Hibiscus sabdariffa L.): Effects of Drying Temperature and Brewing Conditions on Total Soluble Solid, Phenolic Content, Antioxidant Capacity and Sensory Quality. Beverages, 6(1), 2. Retrieved from https://www.mdpi.com/2306-5710/6/1/2

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. Future Foods, 1-2. doi:10.1016/j.fufo.2020.100002

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from Hibiscus sabdariffa. Future Foods, 4. doi:10.1016/j.fufo.2021.100087

Singh, M., Thrimawithana, T., Shukla, R., & Adhikari, B. (2022). Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of Hibiscus sabdariffa. Food Bioscience, 50, 101992. doi:https://doi.org/10.1016/j.fbio.2022.101992

Singh, M., Thrimawithana, T., Shukla, R., Brennan, C. S., & Adhikari, B. (2023). Impact of phenolic extracts and potassium hydroxycitrate of Hibiscus sabdariffa on adipogenesis: a cellular study. International Journal of Food Science & Technology, 58(3), 1204-1218. doi:https://doi.org/10.1111/ijfs.16269

APPENDICES

Publications

The following are journal papers that form part of this thesis.

A list of Journal articles:

1. **Singh, M.,** Thrimawithana, T., Shukla, R. and Adhikari, B. (2020). Managing obesity through natural polyphenols: A review. Future Foods, 1, 100002
   (This published review paper is presented as **Chapter 2** in this thesis)

2. **Singh, M.,** Thrimawithana, T., Shukla, R. and Adhikari, B. (2021). Extraction and characterization of polyphenolic compounds and potassium hydroxycitrate from *Hibiscus sabdariffa*. Future Foods, 4, 100087
   (This published research paper is presented as **Chapter 3** in this thesis)

3. **Singh, M.,** Thrimawithana, T., Shukla, R. and Adhikari, B. (2022). Inhibition of enzymes associated with obesity by the polyphenol-rich extracts of *Hibiscus sabdariffa*. Food Bioscience, Volume 50, Part A, 101992
   (This published research paper is presented as **Chapter 4** in this thesis)

4. **Singh, M.,** Thrimawithana, T., Shukla, R. and Adhikari, B. (2023). Impact of phenolic extracts and potassium hydroxycitrate of *Hibiscus sabdariffa* on adipogenesis: A cellular study. International Journal of Food Science and Technology, 58: 1204-1218.
   (This published research paper is presented as **Chapter 5** in this thesis).

Manuscripts under preparation

5. **Singh, M.,** Thrimawithana, T., Shukla, R. and Adhikari, B. Encapsulation of phenolic compounds-rich *Hibiscus sabdariffa* extracts in alginate-chitosan beads to preserve antioxidant property
   (This manuscript is presented as **Chapter 6** in this thesis).