DESIGN AND DEVELOPMENT OF ORAL ANTIHYPERGLYCAEMIC FORMULATIONS FOR WATER-SOLUBLE PALM FRUIT EXTRACT

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DESIGN AND DEVELOPMENT OF ORAL ANTIHYPERGLYCAEMIC FORMULATIONS FOR WATER-SOLUBLE PALM FRUIT EXTRACT

by

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LIST OF SYMBOLS

° C	Temperature
R	Resolution
r^2	Correlation coefficient
N	Number of theoretical plates
T	Tailing factor

LIST OF ABBREVIATIONS

β-CD	Beta cyclodextrin
CD	Cyclodextrin
CSA	Caffeoyl-Shikimic acid
CS	Chitosan
DM	Diabetes mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
D-NC	Diabetic negative control
EMA	European Medicines Agency
FBG	Fasting blood glucose
FDA	Food and Drug Administration
FRAP	Ferric Reducing Ability of Plasma
FT-IR	Fourier-transformed infrared
GA	Gum Arabic
GAE	Gallic Acid Equivalent
GLI	Glibenclamide
p-HBA	p-Hydroxybenzoic acid
HED	Human equivalent dose
HFD	High-fat diet
HLB	Hydrophilic-Lipophilic Balance
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High Performance Liquid Chromatography
ICH	International Council for Harmonisation
IFD	International Diabetes Federation
IV	Intravenous

LE	Loading efficiency
LLE	Liquid-liquid extraction
LOD	Limits of detection
LOQ	Limits of quantification
MC	Mesoporous carriers
MPOB	Malaysian Palm Oil Board
Neu	Neusilin
OGTT	Oral glucose tolerance test
OPP	Oil palm phenolics
ORAC	Oxygen Radical Absorbance Capacity
PCA	Protocatechuic acid
PFJ	Palm fruit juice
POME	Palm oil mill effluent
RH	Relative humidity
RI	Refractive index
RSD	Relative standard deviation
SD	Sprague Dawley
SGF	Simulated gastric fluid
SPE	Solid-phase extraction
STZ	Streptozotocin
SR-WSPFE	Sugar-removed WSPFE
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TPC	Total phenolic content
WHO	World Health Organization
WSPFE	Water-soluble palm fruit extract

LIST OF APPENDICES

REKABENTUK DAN PEMBANGUNAN FORMULASI ORAL ANTIHIPERGLISEMIA UNTUK EKSTRAK TERLARUT-AIR BUAH SAWIT

ABSTRAK

Ekstrak terlarut-air buah sawit (WSPFE) didapati daripada sisa pengilangan minyak kelapa sawit di Malaysia. WSPFE mengandungi asid fenolik yang bernilai termasuk asid protocatechuic (PCA), asid p-hidroksibenzoik (p-HBA), dan tiga isomer asid caffeoyl-shikimic yang berbeza. WSPFE telah menunjukkan beberapa aktiviti terapeutik secara *in vitro* dan *in vivo*. Namun, kandungan gulanya yang tinggi mungkin akan menyebabkan kesan sampingan di dalam gastrousus, dan menimbulkan isu pematuhan penggunaan. Juga, kandungan fenolik yang lebih rendah berbanding dengan kandungan gula bermaksud kuantiti WSPFE yang perlu diambil adalah banyak untuk mencapai kesan terapeutik. Kaedah yang berlainan telah digunakan untuk menyingkirkan gula dan mengekstrak asid fenolik dalam WSPFE, iaitu penyarian cecair-cecair (LLE), pemendakan etanolik, hidrolisis beralkali, dan pengekstrakan fasa pepejal (SPE) menggunakan resin Amberlite XAD-2 dan Oasis HLB. Kaedah yang paling cekap bagi menyingkirkan gula dan mengekstrak fenolik adalah melalui SPE oleh Oasis HLB, diikuti dengan Amberlite XAD-2. Sampel WSPFE tersingkir-gula (SR-WSPFE) telah dikaji secara *in vitro* dengan menggunakan asai yang berlainan. Aktiviti antioksidan, perencatan alfa-amilase dan perencatan alfa-glukosidase yang paling tinggi telah ditunjukkan oleh SR-WSPFE yang diekstrak oleh Oasis HLB, diikuti dengan Amberlite XAD-2. Oleh itu, SR-WSPFE yang dihasilkan menggunakan Oasis HLB telah pilih untuk proses pembangunan produk yang seterusnya. Kajian pre-formulasi telah mendedahkan beberapa masalah bagi SR-WSPFE termasuk sifat

higroskopik, kandungan kelembapan yang tinggi, rasa yang pahit, dan sifat aliran yang lemah. Pembawa dan kaedah formulasi yang berlainan telah digunakan untuk mengatasi masalah SR-WSPFE. Gam Arabik dan kitosan telah digunakan untuk mengenkapsulasi ekstrak manakala β-siklodekstrin telah digunakan untuk menggabungkan SR-WSPFE ke dalam kompleks rangkuman. Penjerapan SR-WSPFE pada pembawa pepejal berliang-meso telah dilakukan dengan menggunakan Neusilin. Semua formulasi telah disediakan melalui pengeringan sembur pada nisbah pembawa-ekstrak yang berbeza. Formulasi SR-WSPFE-Neu, SR-WSPFE-β-CD, dan SR-WSPFE-GA pada nisbah 1:1 telah memperbaiki sifat-sifat tersebut, dan seterusnya dipilih untuk dinilai dalam kajian kestabilan tercepatkan selama enam bulan. SR-WSPFE-Neu dan SR-WSPFE-β-CD didapati stabil dan seterusnya dinilai bagi kesan antihiperglisemia secara *in vivo* pada tikus berbanding dengan metformin dan SR-WSPFE yang tidak terformulasi. Selepas pemberian setiap hari selama empat minggu, kedua-dua formulasi telah mengurangkan paras glukosa darah secara signifikan dengan lebih banyak berbanding dengan SR-WSPFE yang tidak terformulasi. Kedua-dua formulasi mencegah kenaikan paras glukosa darah selepas permuatan glukosa oral. Dapatan menunjukkan bahawa formulasi SR-WSPFE-β-CD dan SR-WSPFE-Neu telah meningkatkan penyampaian fenolik bioaktif, seterusnya menghasilkan kesan antihiperglisemia yang lebih baik.

DESIGN AND DEVELOPMENT OF ORAL ANTIHYPERGLYCAEMIC FORMULATIONS FOR WATER-SOLUBLE PALM FRUIT EXTRACT

ABSTRACT

Water-soluble palm fruit extract (WSPFE) is recovered from the palm oil production waste stream in Malaysia. WSPFE consists of valuable water-soluble phenolic acids including protocatechuic acid (PCA), p-hydroxybenzoic acid (p-HBA), and three isomers of caffeoyl-shikimic acid. WSPFE demonstrated several therapeutic activities *in vitro* and *in vivo*. However, the high sugar content of WSPFE may lead to gastrointestinal side effects, likely resulting in compliance issues. Also, the lower phenolic content compared to the sugar content means that a large quantity of WSPFE should be consumed to achieve the therapeutic effects. Different methods were used to remove the sugars and extract the phenolic acids in WSPFE, namely liquid-liquid extraction (LLE), ethanolic precipitation, alkaline hydrolysis, and solid phase extraction (SPE) by using Amberlite XAD-2 and Oasis HLB resins. The most efficient method for removing the sugars and extracting the phenolics was found to be through SPE by Oasis HLB, followed by Amberlite XAD-2. The sugar-removed WSPFE (SR-WSPFE) samples were examined *in vitro* using different assays. The highest antioxidant, alpha-amylase inhibition, and alpha-glucosidase inhibition activities were observed in SR-WSPFE that was extracted by Oasis HLB, followed by Amberlite XAD-2. Therefore, the SR-WSPFE obtained by using Oasis HLB was chosen for further product development process. Preformulation studies revealed some drawbacks of SR-WSPFE including hygroscopicity, high moisture content, bitter taste, and poor flow properties. Different carriers and formulation approaches were applied to overcome the problems of SR-WSPFE. Gum Arabic and chitosan were used to

encapsulate the extract whilst β-cyclodextrin was used to form an inclusion complex with the SR-WSPFE. Adsorption of SR-WSPFE onto a mesoporous solid carrier was carried out using Neusilin. All formulations were prepared by spray drying at different carrier-extract ratios. Sugar-removed water-soluble palm fruit extract-neusilin (SR-WSPFE-Neu), sugar-removed water-soluble palm fruit extract-beta cyclodextrin (SR-WSPFE-β-CD), and sugar-removed water-soluble palm fruit extract-gum Arabic (SR-WSPFE-GA) formulations at 1:1 ratio improved the properties, and thus, were chosen to be evaluated in an accelerated stability study for six months. SR-WSPFE-Neu and SR-WSPFE-β-CD were deemed stable and further evaluated for their *in vivo* antihyperglycaemic effects in rats in comparison to metformin and the unformulated SR-WSPFE. After four weeks of daily administration, both formulations significantly reduced the blood glucose levels more than the unformulated SR-WSPFE. Both formulations prevented the elevation of blood glucose level after oral glucose loading. The results suggest that formulations of SR-WSPFE-β-CD and SR-WSPFE-Neu enhanced the delivery of the bioactive phenolics, leading to a more efficacious antihyperglycaemic effect.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Natural and herbal drugs

Many plants and herbal extracts are used to alleviate health issues. Human used plants for therapeutic reasons since ancient times. There is an increase in the popularity of medicinal plant products currently for their therapeutic effect. The mechanism of action of the medicinal plants might be unclear for the public, but they believe in the ability of plants to treat different diseases. However, scientists have studied and understood the human body functions, and they can now explain the treating potentials of plant extracts (Gibbons, 2003; Özaslan & Oguzkan, 2018).

Plant extracts are biologically active chemical mixtures. Plant extracts contain different compounds and are derived from several parts of plants. Single plant extract can exert many therapeutic effects as it contains different bioactive compounds. The bioactive compounds in plants can work synergistically, additively or individually. Several effective drugs in the pharmaceutical market were derived and isolated from the plants like aspirin, morphine, and digoxin (Armendáriz-Barragán et al., 2016). Also, thousands of polyphenols were identified in plants where the main classes include phenols, flavonoids, phenolic acids, stilbenes, tannins, and lignans (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010; Kelly, Kelly, & O'Mahony, 2019). Those polyphenols were reported to have antioxidant potentials and could act as reducing agents, singlet oxygen quenchers, and hydrogen donors. Many diseases like cancer, diabetes, and cardiovascular diseases are associated with free radicals. (Lobo, Patil, Phatak, & Chandra, 2010; Losada-Barreiro & Bravo-Díaz, 2017).

There are many side effects and severe adverse reactions related to synthetic drug consumption. Thus, patients do not comply with these drugs in many cases to avoid adverse reactions. However, synthetic drugs are still the first choice of physicians to treat human diseases. Herbal drugs were traditionally used in different nations and related to their beliefs and ideologies in some places. They are cheap and cost-effective in comparison with chemical drugs. Plant extracts have exhibited synergistic effects when administered with synthetic drugs (Parasuraman, 2018).

Plant herbal medicines and extracts have disadvantages as well. Herbal extract products required less safety and quality evidence to be approved and reach the market. Thus, consumers may get low-quality or unsafe herbal medications. One of the main drawbacks of plant extracts manufacturing for therapeutic reasons is the batch-to-batch variation that results in dose estimating problems. (Parasuraman, 2018; Tang, Zhu, Li, & Cao, 2011).

1.2 Palm oil tree

The palm oil tree is a tropical monocotyledon tree (*Elaeis guineensis*). Originally, *Elaeis guineensis* was from West Africa, but it was brought to Asia in the 19th century. Two types of oil can be yielded from the oil palm fruit. Palm oil is extracted from the pulp of palm fruit, while the kernel palm oil can be derived from the kernel seed of the fruit. Currently, Malaysia, Indonesia, Thailand, and Nigeria are the main producers of palm oil (Sundram, Sambanthamurthi, & Tan, 2003).

Palm oil is used worldwide in a wide variety of industrial and food products, particularly as cooking oil because it is stable at high temperatures. Moreover, this oil is trans-fat free. It is the most produced vegetable oil in the world (Imoisi, Ilori, Agho, & Ekhator, 2015). Saturated and unsaturated fatty acids are the main components of

palm oil. For example, palmitic acid (C16:0) 44%, oleic acid (C18:1) 40%, linoleic acid (C18:2) 10% and stearic acid (C18:0) 5%. Crude oil contains TG metabolites such as diacylglycerol and monoacylglycerol. In addition, palm oil contains phenols, carotenoids (β-carotene), vitamin E (tocopherol and tocotrienol), sterols, and phenolic acids (Atawodi, Yusufu, Atawodi, Asuku, & Yakubu, 2011; Mba, Dumont, & Ngadi, 2015).

1.2.1 Palm oil by-product

The food and agriculture industries are responsible for billions of tons of by-product waste every year. Disposing of these by-products costs a high amount of money and affects the environment negatively. A few decades ago, food and edible oil wastes were discarded, polluting the environment or used as animal feed. However, these wastes are rich in valuable bioactive compounds including proteins, polysaccharides, polyphenols. Bioactive compounds in the waste streams could be recovered and purified (Sambanthamurthi, Sundram, & Ai, 2008).

Palm by-products contain enormous quantities of active compounds that could be used commercially in foods, nutraceuticals, and pharmaceuticals. For instance, oil palm extraction methods produce huge volumes of the waste stream of vegetation liquor that contain valuable hydrophilic phenolics. Malaysian Palm Oil Board (MPOB) has developed novel methods to recover these phenolics from the palm oil mill effluent (POME) (Leow, Fairus, & Sambanthamurthi, 2021). Several scientific studies related to WSPFE were reported recently due to its health-improving effects (Tan, Sambanthamurthi, Sundram, & Wahid, 2007; Leow, Fairus, & Sambanthamurthi, 2021).

1.2.2 Water-soluble palm fruit extract (WSPFE)

Around 85 million tons of phenolic-rich waste liquor streams are available every year globally (Sambanthamurthi, Tan, Sundram, Abeywardena, et al., 2011). Valuable phenolic acid compounds exist in this waste stream. Phenolic acids have exhibited many therapeutic effects. Therefore, recovering these phenolics from oil palm by-products would help in the development of new products with therapeutic effects. Many extraction methods were used to recover those phenolics (Sambanthamurthi, Tan, Sundram, Abeywardena, et al., 2011). Green technology with no solvents was used by Malaysian Palm Oil Board (MPOB) to recover the phenolics from palm production waste stream. Different terms were used to refer to the recovered phenolics including oil palm phenolics (OPP), palm fruit juice (PFJ), and water-soluble palm fruit extract (WSPFE). However, recently, WSPFE is the preferred term (Leow, Fairus, & Sambanthamurthi, 2021).

1.2.3 Identification and quantification of bioactive compounds in WSPFE

Natural plant extracts, natural products, and by-products are rich sources of bioactive compounds that have many pharmacologic effects. Phytochemicals in natural products have been used as therapeutic agents for the treatment of many diseases. Therefore, bioactive compounds should be identified and quantified precisely in the batches. Also, the biological effects of novel compounds should be screened using different assays. Several analytical techniques could be used to analyse and quantify the bioactive compounds in natural products. Nuclear Magnetic Resonance (NMR), liquid chromatography, gas chromatography, thin-layer chromatography, and spectrophotometry are among the main methods that have been used frequently to analyse the bioactive components of products. High Performance Liquid Chromatography (HPLC), for example, is one of the most used techniques to verify

and quantify the bioactive compounds in different products (Cieśla & Moaddel, 2016; Fu, Luo, Qin, & Yang, 2019).

Using HPLC, mass spectrometry (MS), and NMR, several phenolic acids were separated and identified in the WSPFE. Reversed-phase analytical HPLC-UV using C-18 column was utilized to analyse the phenolic acids in WSPFE. HPLC chromatogram in Figure 1.1 shows that protocatechuic acid (PCA), p-hydroxybenzoic acid (p-HBA) and three different isomers of caffeoyl-shikimic acid (3-O-CSA, 4-O-CSA and 5-O-CSA) are the major phenolics detected in WSPFE. (Sambanthamurthi, Yewai Tan, et al., 2011). Phenolic acids spread widely through the plant kingdom, and they have been linked to many different roles in the plant such as protein synthesis and enzyme activity. They are called phenolic acids or phenol-carboxylic acids because they contain phenolic ring with one carboxylic acid function. Tea, coffee, grains, fruits, vegetables, and spices contain high amounts of these phenolics (Saxena, Saxena and Pradhan, 2012; Goleniowski et al., 2013).

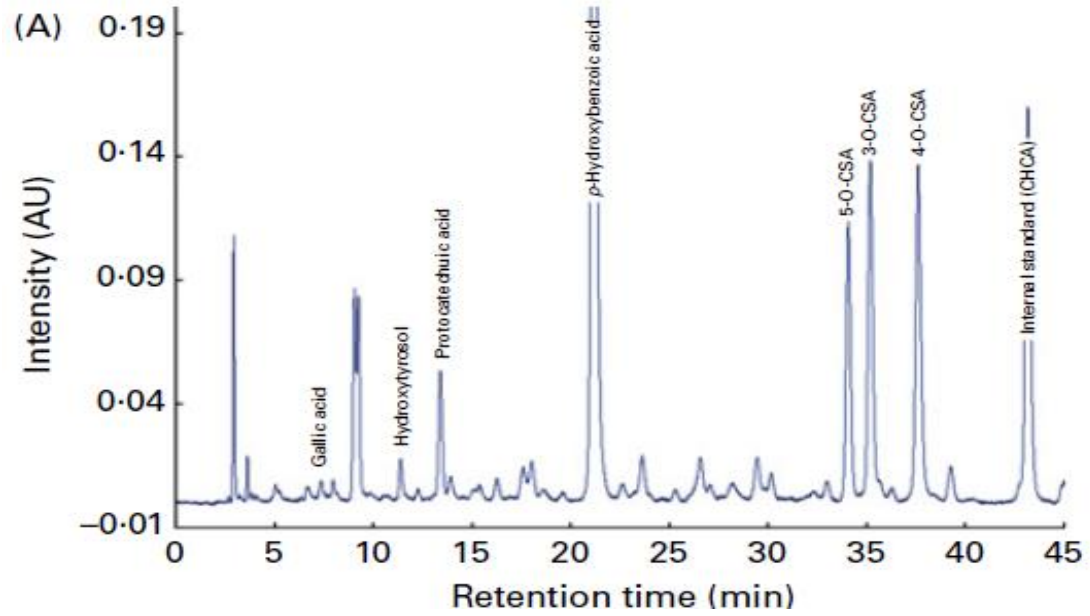

Figure 1.1 Reversed-phase HPLC-UV chromatogram of WSPFE showing the peaks of PCA, p-HBA, 3-O-CSA, 4-O-CSA and 5-O-CSA (Sambanthamurthi, Tan, Sundram, Abeywardena, et al., 2011)

1.2.4 Antioxidant effects of WSPFE

Phenolics in WSPFE are free radical scavengers and their scavenging activity is measured using 2,2-diphenyl-1 picrylhydrazyl (DPPH) reagent. According to R. Sambanthamurthi *et al*. WSPFE has shown strong scavenging ability by scavenging 50% of the DPPH in less than 30s at the different concentrations as illustrated in Figure 1.2. WSPFE's ability to scavenge free radicals and donate hydrogen atoms explain its high antioxidant effects. The antioxidant activity depends on the degree of hydroxylation of the phenolics. For example, caffeoyl-shikimic acid has four hydroxyl groups which indicate high antioxidant effects. The presence of several phenolic acids in the same extract could lead to synergistic antioxidant effects (Sambanthamurthi, Tan, Sundram, Abeywardena, et al., 2011).

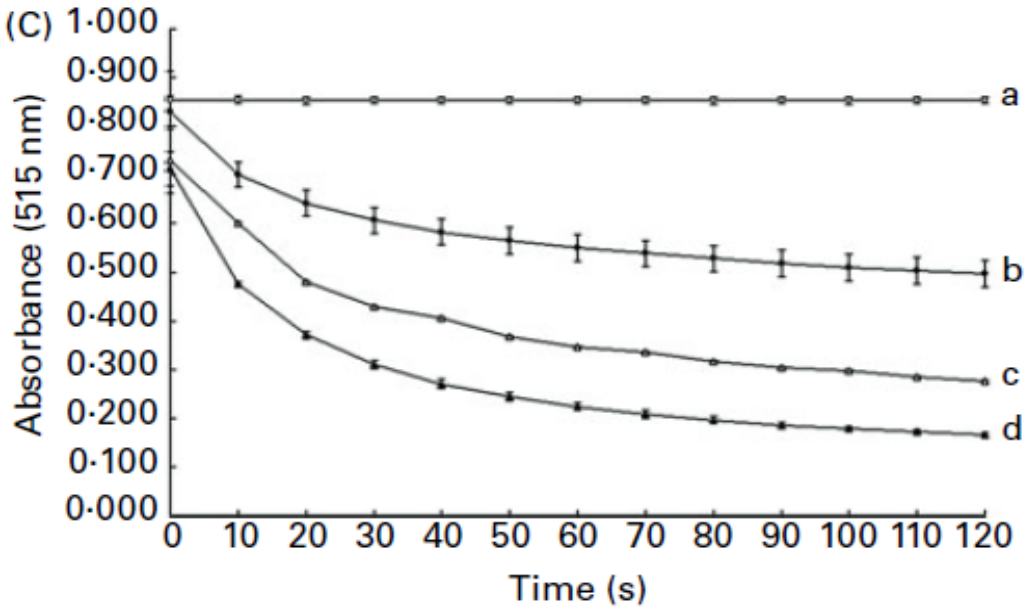

Figure 1.2 Antioxidant activity of WSPFE at 100 mg/l gallic acid equivalents (GAE) (b), 200 mg/l GAE (c) and 300 mg/l GAE (d) comparing to the blank (a) (Sambanthamurthi, Tan, Sundram, Abeywardena, et al., 2011)

1.2.5 Anticancer activity of WSPFE

WSPFE caused a considerable reduction in the tumour weight and volume in the BALB/c mice comparing to the control group that received water. Functional studies on the mice tumours revealed that WSPFE has expressed many genes including genes involved in the cell cycle. Thus, WSPFE may inhibit tumour growth *in vivo* by inducing a G1/S phase arrest in the cell cycle. Interestingly, WSPFE regulatory roles might be used to sensitize the tumour cells toward chemotherapy. Thus, WSPFE could be used in combination with some anticancer agents (Sambanthamurthi et al., 2011). Figure 1.3 shows the difference in tumour size between treatment and control groups.

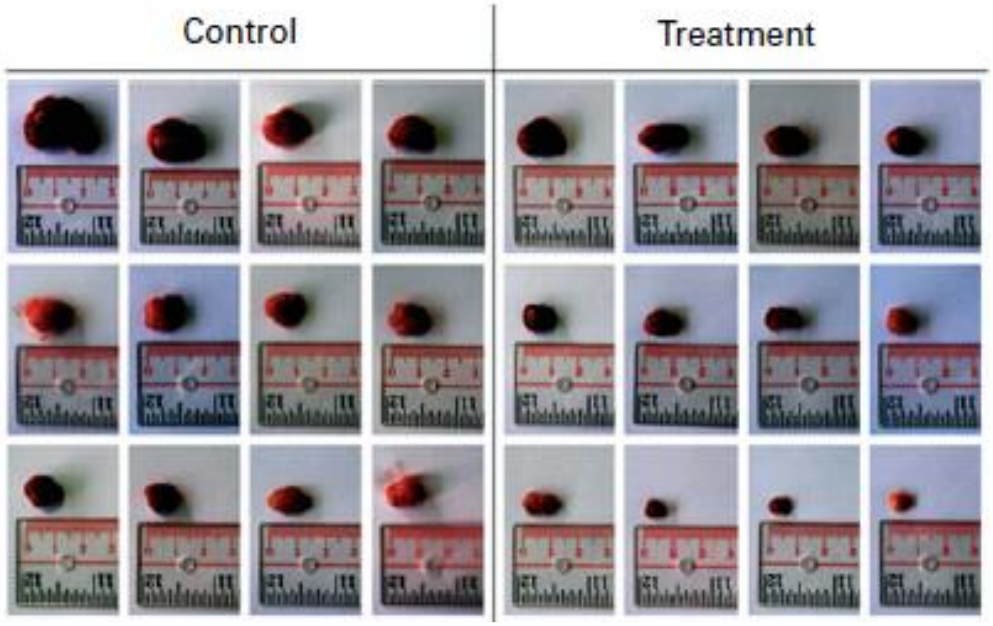

Figure 1.3 Tumour sizes in control and WSPFE treatment group (Sambanthamurthi et al., 2011)

1.2.6 Antihypertension and anti-atherosclerosis effects of WSPFE

Providing NO-deficient rats with WSPFE at two different concentrations has led to a reduction in their blood pressure (Sambanthamurthi, Tan, Sundram, Abeywardena et al., 2011). WSPFE may have effects directly on the endothelial NO-synthase. Besides, they could scavenge the ROS and decrease the oxidative stress associated with L-NAME resulting in reduced blood pressure. WSPFE has reduced the

blood pressure in spontaneously hypertensive rats (SHR) as well (Abeywardena, Sambanthamurthi, & Sundram, 2014; Sambanthamurthi et al., 2011).

Three groups of rabbits were fed with an atherogenic diet for 100 days including fats, sugars, proteins, and cholesterol. Distilled water was provided to the control group, and crude WSPFE was administered at 500mg/I GAE and 1000mg/I GAE to the other two groups. In comparison with the extensive fatty and fibrous plaques developed in the control animals, WSPFE caused a significant reduction in fatty plaques and fatty streaks. Lesion-free areas were more prevalent in the WSPFE administered groups compared to the control groups. Overall, WSPFE exhibited promising protection against atherosclerosis (Sambanthamurthi et al., 2011).

1.2.7 Neuroprotective effects of WSPFE

Comparing to the control group, mice fed with crude WSPFE showed an enhancement in cognitive function, spatial learning, and a significant improvement in motor function. The microarray analysis illustrated that WSPFE upregulated many neurotrophic genes in the brain. For example, genes involved in calcium ion binding, calmodulin binding, potassium ion transport, and transmembrane receptor protein tyrosine phosphatase activity were upregulated in the WSPFE treatment group. WSPFE also upregulated genes involved in nervous system development, neurotransmitter transport, striated muscle contraction, synaptic transmission, and synaptogenesis (Leow et al., 2013).

1.2.8 Antidiabetic effects of WSPFE

Crude WSPFE at different concentrations was provided to Nile rats (*Arvicanthis niloticus*). Crude WSPFE was administered to male Nile rats as a drink or mixed into the diet. Five experiments were carried out over 36 weeks. In all experiments, antilipemic and antihyperglycaemic effects were exhibited in direct

relation to phenolic concentrations in the WSPFE. WSPFE assisted in delaying the diabetes onset and reversing advanced diabetes with no side effects observed. Results suggest that WSPFE lowered the blood glucose *in vivo* due to slowing the glucose absorption rate, reducing insulin resistance, and increasing the insulin secretion (Bolsinger, Pronczuk, Sambanthamurthi, & Hayes, 2014). After administration of WSPFE, a hepatic gene expression study on Nile male rats was carried out via microarrays. The results suggested that the antihyperglycaemic effect of WSPFE is not related to insulin secretion (Sen Leow, Bolsinger, Pronczuk, Hayes, & Sambanthamurthi, 2016).

1.3 Removing the interferences and extracting the phenolic content

Polyphenols are extracted from different sources via different methods to be used as food additives, antioxidants, and health-promoting nutraceuticals. However, interferences like sugars, fats, proteins, and chlorophyll exist in the plants and their crude extracts in high amounts, where the phenolic components might be low. Thus, many of these products and extracts have to be consumed multiple times in high quantity to give an adequate pharmacologic effect. Interference compounds in plant extracts could be removed by an extra extraction process. This extraction process helps in retaining the bioactive compounds and removing the unwanted components (Dai & Mumper, 2010; Trikas, Papi, Kyriakidis, & Zachariadis, 2017). Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are the most used techniques for the extraction and isolation of phenolics from different samples (Dai & Mumper, 2010).

Liquid-liquid extraction is the conventional extraction method of polyphenols from plants and liquid samples. The main advantages of this method are the low-cost

and the simple procedures. It applies the partitioning of the compounds between two immiscible solvents. The solvent system of LLE consists of water and organic solvents. Ethanol, methanol, ethyl acetate, propanol, and acetone are among the solvents used in the LLE in combination with water at different ratios. However, large volumes of hazardous solvents are required to partition the phenolic compounds with low recovery rates and selectivity comparing to the new methods. LLE requires a long time of several extraction processes with large sample volumes relatively (Ajila et al., 2011).

Solid-phase extraction is used to clean, isolate, and purify samples as an alternative method to the LLE. Fewer solvents and less sample volume are required in the SPE comparing to the LLE. SPE facilitates adsorbing analytes onto different adsorbents (stationary phase) before the elution process that includes eluting the analytes by a liquid or gas (mobile phase). Adsorbents with high affinity to the desired compounds are used to retain these compounds from the sample. (Buszewski & Szultka, 2012; Poole, 2003). SPE offers using simple, flexible, inexpensive, and manual sample processing, although automation sampling is available at high levels. Several solid sorbents have been used to recover phenolic acids from natural products like honey including Amberlite XAD, Bond Elute C-18 cartridge, and Oasis HLB cartridges (Michalkiewicz, Biesaga, & Pyrzynska, 2008 Tomás-Lorente, García-Viguera, Ferreres, & Tomás-Barberán, 1992; Yung An et al., 2016).).

Amberlite XAD-2 is a polystyrene non-ionic resin that has been demonstrated to be a versatile chromatographic packing. Besides, Amberlite XAD-2 is characterized by low cost, compatibility with all solvents, and strong adsorbent properties. Total removal of sugars and interfering substances can be achieved using Amberlite XAD-2 by simple washing with water and without prior extraction (Baum, Saetre, &

Cantwell, 1980; Pyrzynska & Biesaga, 2009). Oasis hydrophilic-lipophilic balance (HLB) is a second-generation microporous copolymer produced from a balanced ratio of lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone. Reproducible isolation of acidic, basic and neutral compounds, whether polar or non-polar can be achieved by Oasis HLB sorbent with high recovery (Dias & Poole, 2002; Ziaková & Brandšteterová, 2002). Phenolic acids like p-hydroxybenzoic, protocatechuic, gallic, vanillic, syringic, and ferulic acids in different natural products were adsorbed and extracted by Oasis HLB (Klejdus & Kubá, 2000; Pyrzynska & Biesaga, 2009; Ziaková & Brandšteterová, 2002).

Ethanol precipitation was used previously to purify water extracts by precipitating the sugars using different ratios of ethanol. Although ethanol precipitation is not a new method, the research on this technology is scarce. Phenolic acids and flavonoids were successfully extracted and concentrated by ethanol precipitation in Chinese sweet tea leaves (Gong, Wang, Li, & Qu, 2013; Koh, Chou, & Liu, 2009). This technology works by changing the solubility of strongly polar and macromolecules components after adding ethanol to the water extract leading to precipitation. The main advantages of this technology can be summarized as the low cost of extraction, easy scaling up, and safety of the separation solvent. Also, this method can be utilized at low temperatures to maintain heat-sensitive compounds. However, total removal of sugars by ethanol precipitation has not been reported and only partial removal of sugars was achieved in the previous studies (Gong et al., 2013; Tai, Shen, Luo, Qu, & Gong, 2020; L. Zhang, Yan, Gong, Yu, & Qu, 2013).

Alkaline hydrolysis is the most commonly used method for bound phenolics extraction. It was employed extensively to extract phenolic acids from food and plants.

Alkaline hydrolysis is usually applied to liberate the phenolics from bound complexes. Bound phenolics were successfully released from the complexes by employing different ratios of NaOH at different temperatures (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Ideia, Sousa-ferreira, & Castilho, 2020; Yao et al., 2021). Thus, it was hypothesized that applying NaOH hydrolysis on plant extract and natural products might separate the phenolics from the sugars and other interfering compounds.

1.4 Diabetes Mellitus

Diabetes mellitus is a consequence of insulin secretion and action deficiencies leading to chronic metabolic disorders. It is characterized by an elevation in blood glucose level known as hyperglycaemia. Hyperglycaemia leads to the dysfunction of different body organs including the liver, pancreas, heart, eyes, and kidney. Patients with chronic hyperglycaemia are at high risk of several infections. Diabetes increases the risk of mortality and morbidity in patients comparing to non-diabetic people (American Diabetes Association, 2014). The number of diabetic patients has been increasing rapidly over the last decades. Diabetes cases has risen from 151 million in 2000 to 382 million in 2013, according to the International Diabetes Federation (IDF). The prevalence of diabetes will continue to increase worldwide in the next few decades, and it is expected to affect 700 million persons by 2045 (Ogurtsova et al., 2017).

1.4.1 Impact of diabetes on world economy

Diabetes affects human health and increases mortality rates. It also has a significant impact on the economy. The total cost of diabetes is estimated to be over one trillion US dollars globally, according to Christian Bommer *et al*. This estimation

is based on epidemiological and economic data obtained from 184 countries (Bommer et al., 2017). Most of these costs are direct medical costs coming from the diagnosis and treatment of the disease. Indirect costs of diabetes also affect the economy by reducing productivity. This huge burden of diabetes costs makes it one of the most expensive diseases in the world that impacts the high and low-income countries (Bommer et al., 2017; Zhang & Gregg, 2017).

1.4.2 Diabetes in Malaysia

Malaysia is in Southeast Asia, and it is one of the western pacific region countries. Since 2018, the diabetes prevalence among adults in Malaysia has been ranked the highest in the west pacific region. According to the international diabetes federation (IFD), 16.8% of Malaysian adults have diabetes. Diabetes is one of the most concerning issues in Malaysia due to the rapid increase in total cases number (Hussein et al., 2015; Nazaimoon, Mohamud, & Khir, 2013). As mentioned previously, the cost of diabetes management is expensive. In 2010, US$ 600 million was the estimated annual cost of diabetes treatment and management in Malaysia. Besides, the complications of diabetes are related to early deaths. For these reasons, well-organized approaches were developed by the Malaysian government to prevent, treat and control diabetes in the country (Ganasegeran et al., 2020).

1.4.3 Diabetes Mellitus classifications

Many ways could be used to classify diabetes, but the main two types include type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is characterized by insulin secretion deficiency, while insulin resistance and insufficient insulin secretion response are the main features of T2DM (Kaul, Tarr, Ahmad, Kohner, & Chibber, 2013). Gestational diabetes mellitus is another type of diabetes that identifies pregnant women who develop diabetes during their pregnancy period having

an intolerance to glucose at any level. Other types of diabetes follow under the "other specific types" category that includes diabetes of different causes such as defects in β-cells, defects in insulin actions, diseases of the exocrine pancreas, and diabetes caused by drugs and other chemicals (Baynest, 2015).

1.4.3(a) T1DM

T1DM is known as insulin-dependent or juvenile diabetes. In this type of diabetes, an autoimmune process destroys the beta cells resulting in insulin deficiency. Usually, insulin, anti-glutamic acid decarboxylase, or islet cell antibodies exist as markers of T1DM. Only 5-10 % of diabetic patients are diagnosed with T1DM. Persons who suffer from T1DM are more susceptible to autoimmune diseases like myasthenia gravis, pernicious anaemia and autoimmune hepatitis. A minor group of patients could develop T1DM called idiopathic diabetes which has unknown causes. All patients suffering from T1DM should be treated with insulin to control their blood glucose level (American Diabetes Association, 2014; Baynest, 2015).

1.4.3(b) T2DM

T2DM was known previously as insulin-independent diabetes or adult-onset diabetes. T2DM accounts for 90-95% of the diabetes patients in the world. Patients with T2DM have insulin resistance and relative insulin deficiency (Alam, Asghar, Azmi, & Malik, 2014; Persaud, Hauge-evans, & Jones, 2014). Individuals with this type of diabetes do not have to take insulin as a treatment to survive at the earlier stages. However, T2DM patients may have to use insulin injections at the advanced stages of the disease. There is no autoimmune destruction of the beta cells in T2DM, and the specific causes are not fully clear. Many predisposing factors may result in insulin resistance have been reported including obesity and overweight, excess growth hormone, excess use of corticosteroids, pregnancy, mutations of the insulin receptor,

hemochromatosis, lipodystrophy, and polycystic ovary disease (Stevanovic, 2019). People may live with T2DM for many years without noticing because hyperglycaemia may develop slowly at the early stage of the disease. Thus, T2DM might be asymptomatic at the earlier stages (American Diabetes Association, 2016).

1.4.4 Management and treatment of diabetes

1.4.4(a) T1DM treatment

For many years, diabetes organizations were updating the procedures and approaches for diabetes treatment. For T1DM patients, insulin injections are required daily to keep the metabolism of sugars, fats, and proteins as normal as possible. Insulin was developed in many forms including regular insulin that lasts 3-8 hours and the slowly absorbed insulin that lasts 10-48 hours. The American Diabetes Association has recommended two injections (basal and prandial) of insulin daily for most patients. Moreover, the FDA-approved agent pramlintide could be used for T1DM management in adults. Pramlintide is injected subcutaneously before each major meal. It delays gastric emptying and improves satiety (American Diabetes Association, 2021).

1.4.4(b) T2DM treatment

Weight reduction, calorie restriction, and exercise are usually used to reduce insulin resistance with pharmacologic treatments. Several oral antidiabetic agents were developed to manage the blood glucose and reduce the complications of type 2 diabetes (Marín-Peñalver, Martín-Timón, Sevillano-Collantes, & Cañizo-Gómez, 2016). Table 1.1 shows the available oral antidiabetic drugs that are used to control the blood glucose in patients with T2DM. Oral antidiabetic drugs are the main dosage form for T2DM management (Chan & Abrahamson, 2003; Thrasher, 2017).

Table 1.1 Oral antidiabetic agents (American Diabetes Association, 2021; Sterrett, Bragg, & Weart, 2016; Thrasher, 2017)

Antidiabetic groups	**Main drugs in the group**	**Mechanism of action**	**Common adverse effects**
Biguanides	Metformin	Inhibit glucogenesis and increase hepatic uptake by activating the adenosine monophosphate activated protein kinase in the liver	long-term intake might result in vitamin B12 and folic acid deficiency
Incretin mimetics	Exenatide Liraglutide Sitagliptin Saxagliptin	reduce the secretion of glucagon and increase the release of glucose dependent insulin	Gastrointestinal side effects like diarrhea and nausea
SGLT-2 inhibitors	Canagliflozin Dapagliflozin Empagliflozin Ertugliflozin	Inhibit the reabsorption process of glucose in the proximal renal tubules	modest reduction in the blood pressure and patients weight
Sulfonylureas 1st generation	Tolbutamide, Tolazamide and Chlorpropamide	Stimulate insulin secretion from the pancreatic β-cells. Block the KTAP channels and reduce the hepatic glucose production.	Hypoglycaemia, hunger, weight gain
Sulfonylureas 2nd generation	Glyburide Glimepiride Glipizide	Similar mechanism as 1st generation with higher potency	Hypoglycaemia, hunger, weight gain
Meglitinide	Repaglinide and Nateglinide	Short and fast insulin secretion enhancing effects	Hypoglycaemia

Table 1.1 (Continued)

Antidiabetic groups	Main drugs in the group	Mechanism of action	Common adverse effects
Thiazolidinedione	Rosiglitazone Pioglitazone	Increase the glucose uptake in muscular tissues, adipose tissues, and liver. Reduce the free fatty acid accumulation, decrease the inflammatory cytokines.	Heart failure and hepatotoxicity
Alpha glucosidase inhibitors	Acarbose Miglitol	Inhibit the intestinal alpha glucosidase enzyme that is responsible for sugars digestion.	Gastrointestinal side effects like diarrhea

1.5 *In vitro* evaluation of antidiabetic effects of natural products

1.5.1 Alpha-glucosidase and alpha-amylase enzymes inhibition assays

Reduction of glucose absorption is one of the therapeutic approaches to decrease postprandial plasma glucose levels. Inhibition of glucose hydrolysing enzymes leads to delay and reduction in the absorption of sugars into the bloodstream. Alpha-glucosidase is an enzyme that hydrolyses the oligo and disaccharides into monosaccharides. Moreover, alpha-amylase is the enzyme responsible for collapsing starch into simple sugars. Many bioactive natural compounds can inhibit both α-glucosidase and α-amylase. *In vitro* inhibition assays of these enzymes are widely used for earlier evaluation of the hypoglycaemic abilities of natural products (Taslimi & Gulçin, 2017; Tundis, Loizzo, & Menichini, 2010).

1.5.2 Antioxidant effects

Evidence from the literature suggests that oxidative stress might have a role in the pathogenesis of diabetes. Oxidative stress may reduce insulin secretion or increase insulin resistance. Therefore, natural antioxidants in food and crude plant extracts may have therapeutic effects for the treatment or alleviation of diabetes. Besides, there might be a correlation between the antioxidant abilities and the hypoglycaemic effects of natural compounds (Opara, 2004; Schaffer, Azuma, & Mozaffari, 2009; Shoba & Krishnakumari, 2018). Several *in vitro* tests could be used to evaluate the antioxidant effects of natural products including:

- DPPH free radicals scavenging assay.
- TEAC / ABTS Assay [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assay.
- ORAC (Oxygen Radical Absorbance Capacity) assay.
- FRAP (Ferric Reducing Ability of Plasma) assay.
- TRAP (Total Radical Trapping Antioxidant Parameter).
- PCL (Photochemiluminescence) assay (Schlesier, Harwat, Böhm, & Bitsch, 2002; Sudhakar Singh & Singh, 2008).

1.5.3 Glucose uptake by cell lines

In vitro glucose uptake into human and animal cells is commonly used to examine the antidiabetic effect of different compounds. This test is usually performed to evaluate the ability of compounds to enhance the insulin activity on glucose uptake into different types of cells. Glucose uptake could be measured using different tissue cultures like muscle, adipose, neuronal and hepatic cells. Radiolabelled glucose derivatives were used previously to measure cellular glucose uptake. However, new

fluorescent and colorimetric methods have been developed recently to quantify the glucose uptake into cultured cells (Henderson, 1964; Yamamoto et al., 2011).

1.6 *In vivo* evaluation of antidiabetic effects of natural compounds

Although *in vitro* assays give an initial evaluation of the pharmacologic effects of compounds, animal studies must be carried out to screen the effects of these compounds in the body systems. *In vivo* studies give a better understanding of the drug's effects on the body and vice versa. The absorption, distribution, metabolism, excretion, and toxicity of compounds could be examined on different animal models (Eddouks, Chattopadhyay, & Zeggwagh, 2012).

Pharmacologic, nutrition, genetic and surgical manipulations could be used to develop diabetes in animals, as shown in Table 1.2. Although big animals are still used, rodents are preferred in diabetic studies. Rodents are widely used currently because of their availability and ability to express diabetes in humans. Also, rodents exhibit the fast onset of diabetes, and they are inexpensive comparing to other animals like dogs and pigs (Manish Pal Singh, 2015). Animal studies should be carried out under the supervision of experts and with the approval of Animal Ethics Committees in charge. After induction of diabetes in animals, drugs and natural products are administered to the animals for a determined duration. Blood glucose concentrations should be measured several times during the experiment to evaluate the antihyperglycaemic effects of the compounds. Body and blood parameters must be recorded as well to understand the pharmacologic effects of the tested compounds (Fröde & Medeiros, 2008).

Table 1.2 Advantages and disadvantages of animal models used in diabetes (Fröde & Medeiros, 2008; Hasan, Ahmed, Mat Soad, & Tunna, 2018)

Diabetes animal model	**Advantages**	**Disadvantages**
Spontaneous diabetic animals	1- Small sample size required and low variability in the results. 2- Diabetes developed spontaneously and similar to the human T2DM.	1- Requires insulin in advanced stages for survival. 2- Limited availability and expensive. 3- Advanced maintenance is required.
Diet induced diabetic animals	1- No toxicity on the other organs. 2- Ability to develop diabesity like the human syndrome.	1- Requires dietary feeding for a long time. 2- Unsuitable for evaluating of antidiabetic effects on circulating blood glucose.
Chemical induced diabetic animals	1- High selectivity for pancreatic β-cells. 2- Low mortality and ketosis relatively. 3- Easier and cheaper.	1- Chemical toxicity on other organs. 2- Less stable diabetes and variation in the results.
Surgical induced diabetic animals	1- Reduced islet β-cells mass that resembles human T2DM. 2- Cytotoxicity of chemical induction is avoided.	1- High rate of mortality. 2- Requires complicated techniques and post-surgery procedures.
Transgenic diabetic animals	1- Used for investigations on specific genes and mutations.	1- Complex to be developed and expensive for normal experiments.

1.6.1 Chemical induction of diabetes in animals

Chemical/pharmacological induction of diabetes in animals is one of the most frequently used methods. Chemical initiation of diabetes is easier and cheaper than other methods, and it demonstrates lower mortality rates. Alloxan and streptozotocin (STZ) are the main chemical agents used to induce diabetes in animals. They could be administered parenterally, intravenously, intraperitoneally, or subcutaneously. Determination of the dose of these compounds depends mainly on the route of administration, animal species, and the nutritional status of the animals. Depending on the administered dose, administration of STZ and alloxan can develop both types of diabetes and glucose intolerance syndromes (Hasan et al., 2018).

Alloxan and STZ induce diabetes in animals via different mechanisms. Alloxan administration forms superoxide radicals and the redox cycle. Pancreatic β-cells are usually destroyed because of the elevation in cytosolic calcium and the dismutation of hydrogen peroxide. The dose of alloxan should be determined carefully to avoid overdosing toxicity. STZ acts by entering the β-cells via GLUT-2 that leads to alkylation of the DNA. Also, poly ADP-ribosylation is activated by STZ, which results in a reduction in the cellular ATP and NAD^+. Pancreatic β-cells are destroyed by necrosis as a result of STZ effects (Hasan et al., 2018; Wu & Huan, 2008).

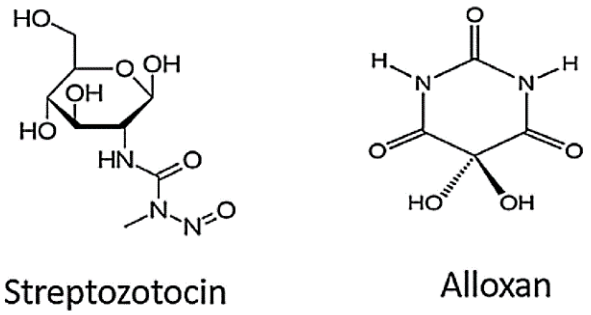

Figure 1.4 Structures of streptozotocin and alloxan (Hasan et al., 2018)

1.7 Drug design and development

Novel drug development stages take 10 to 12 years usually to be accomplished and to reach the market. Nowadays, the cost of developing a new drug from the initial trials to the marketing stage could be more than $1 billion dollars. Regulatory agencies like the Food and Drug Administration (FDA) and European Medicines Agency (EMA) have set many requirements for new drug approval (Chen et al., 2018). Drug development may fail due to many reasons like dosing, efficacy, safety, pharmacokinetics, and marketing. In pharmacokinetics, poor bioavailability is the main reason for drug development process delay or failure. A successful product should be accepted by the physicians and patients as well (Ciociola, Cohen, & Kulkarni, 2014).

1.7.1 Drugs bioavailability

Bioavailability refers to the amount of administered compound that reaches the blood circulation unchanged. Parenteral administration of drugs exhibits very high bioavailability including 100% bioavailability of intravenous injection. Absolute bioavailability measures the bioavailability of oral drug products compared to intravenous (IV) injection of the same drug. The relative bioavailability compares the bioavailability of two oral formulations of the same drug (El & Naggar, 2015; Paul, 2019).

Orally administered drugs must have high bioavailability to exert their pharmacologic effects in the target tissues. However, many drugs do not have the required physicochemical and pharmacokinetics properties leading to poor bioavailability. Thus, some of these drugs may have to be administered by other routes that may result in a compliance issue. Also, orally administered drugs with poor bioavailability should be used in high doses that increase the risk of toxicity, side

effects, costs, and patients' non-compliance (Fasinu, Pillay, Ndesendo, Toit, & Choonara, 2011).

The physiological state of the human and animal bodies has significant effects on the bioavailability of drugs. For example, gastric emptying, gastrointestinal pH, luminal enzymes intestinal mobility, blood flow rate, and first-pass metabolism are among the physiological factors that could affect the bioavailability of orally consumed drugs. Thus, drug molecules should be stable at different absorption sites and pH ranges in the body before reaching the bloodstream (Hurst, Loi, Brodfuehrer, & El-Kattan, 2007). Food intake could affect the bioavailability of drugs by altering physiological parameters like gastric pH and gastric emptying. Also, gastric emptying, gastric pH, intestinal surface area, and epithelial permeability vary between healthy individuals and patients (Karalis, Macheras, Van Peer, & Shah, 2008).

In addition, bioavailability is influenced by the physicochemical properties of the drugs. Physicochemical properties of drugs include solubility, aqueous dissolution rate, and gastrointestinal permeability. Aqueous solubility and gastrointestinal permeability play a vital role in the bioavailability of compounds. Poor aqueous solubility represents the main problem of newly developed drugs that leads to weak and variable bioavailability. Pre-formulation studies like solubility and partition coefficient are usually used to predict the bioavailability *in vitro* (Hurst et al., 2007; Martinez & Amidon, 2002).

1.7.2 Formulation development and dosage forms

Active pharmaceutical ingredients (API) and drug substances are mixed and developed in pharmaceutical dosage forms. They are formulated with one or more non-medicinal substances like excipients that exhibit different functions. For example, excipients could be used as diluents, glidants, colouring agents, flavours, solubilizers,

preservatives, emulsifiers, and adsorbents. Pre-formulation studies are usually performed to examine the physical, chemical, and biological properties of compounds. Pre-formulation tests are necessary to design the dosage form of compounds with suitable excipients. Formulation of active pharmaceutical compounds with non-medicinal excipients should lead to a safe, effective, stable dosage form. Pharmaceutical dosage forms are important for many reasons including:

- Protection of the active compounds from the impact of humidity.
- Protection of the orally administered compounds from the gastric acid.
- Removing the salty, bitter and unpleasant tastes of the drugs.
- To apply the drugs on different local sites (topical delivery by creams, ointments and gels).
- To administer the drugs directly into the blood stream.
- To control the release rate of drugs (controlled-release dosage forms) (Augsburger & Hoag, 2017).

Route of administration and dosage form selection plays a significant role in the drug development process. Many factors should be taken into consideration while choosing the route of administration and dosage form to develop a successful drug product. Those factors are included in three main criteria, which are product efficacy, patient's access, and safety. The main factors that affect dosage form selection are patient's health condition, patient's age, stability during shelf-life and using time, dose size or volume, physical appearance and frequency of administration, and cost effectiveness (Sam, Ernest, Walsh, & Williams, 2012). The oral route of administration is the most preferred route and the first choice for physicians and patients. Oral drugs can be easily administered by patients without medical supplies and special knowledge resulting in better compliance. Also, orally given drugs are