Optimization of extraction of polyphenols and antioxidants from *Sonneratia apetala* (Buch.-Ham.) fruits

A Thesis Submitted

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Submitted by

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Examination Roll No: MS-140701

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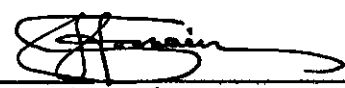

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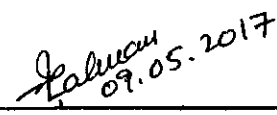

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NOVEMBER, 2016

DEDICATED TO MY

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Title

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DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action will be taken in any case of infringement regarding the declaration.

Tahmina Pervin ..... 09.05.2017 .....

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ABSTRACT

Consumers are increasingly aware of diet related health problems and therefore demanding natural ingredients which are expected to be safe and health-promoting. Health benefits of a diet rich in fruits and vegetables are attributed in part to their contents of polyphenols and other antioxidant components. Costal dwellers in several countries largely used the fruit of *Sonneratia apetala* (Buch.-Ham.) as foods and in treating various diseases. Reportedly, the fruit is a very rich source of health promoting bioactive components such as polyphenols, flavonoids, antioxidants etc. Therefore, the present research was aimed to optimize the extraction of polyphenols and antioxidants from the *S. apetala* fruits. To optimize the extraction of total polyphenols (TP), flavonoids (TF), DPPH radical scavengers (DRS) and total antioxidants (TA), different solvents (acetone, ethanol, methanol, distilled water or their mixtures), extraction temperatures (10, 30, 40, 55 or 70 °C), pH (1, 2, 3, 4, 5, 7 or 9), preextraction heating (30, 60, 90, 120, 150 or 180 °C for 0.5, 1, 2 or 4 h) and extraction times (0, 0.5, 1, 2, 5, 10, 20, 40, 60 or 120 h) were applied. The results showed that solvents, extraction temperatures, pH, preextraction heating and extraction times, all have significant effects on extraction optimization. Among the tested solvents, methanol and ethanol extracted the higher amount of TP, TF, DRS and TA at 30 °C. But at 30 °C, methanol : ethanol (1:1) mixture showed the best extraction efficiencies than ethanol, methanol or other solvent mixtures. When the effect of pH was tested, the increase in pH (from 1 to 3) led to higher yields of TP, TF, DRS and TA, followed by gradual decrease as the pH values increased. It was found that increase in heating temperatures from 30 to 90 °C led to higher yields of TP, TF, DRS and TA. But the extraction efficiencies decreased gradually as the heating temperatures increased; while the best heating time found was 1 h. The extraction of TP, TF, DRS and TA also increased significantly with the increase in extraction time (0 to 20 h). However, further increase in extraction time reduced extraction efficiencies. Thus, the optimum condition for extraction of polyphenols and antioxidants from the fruits was found to be preheating at 90 °C for 1 h, methanol : ethanol (1:1) mixture as extraction solvent, pH 3, 30 °C as the extraction temperature and 20 h as the extraction time. At this condition, optimum amount of TP, TF, DRS and TA were extracted, and the values were 53.8 ± 0.7 mg gallic acid equivalents (GAE), 9.2 ± 0.1 mg (+)-catechin equivalents (CE), 89.6 ± 0.8 mg ascorbic acid equivalents (AAE) and 36.2 ± 0.4 mg AAE per gram powder, respectively. Therefore, the study would help to improve extraction yield of phenolics from the fruits and its utilization in the preparation of functional foods and dietary supplements.

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Chapter One

INTRODUCTION

INTRODUCTION

Fruits and vegetables contain a lot of different phytochemicals that are plant derived bioactive components comprising beneficial effects on human health. These phytochemicals play an important role in preventing or treating some diseases caused by oxidative stress (Kaur and Kapoor, 2001). Various epidemiological studies have suggested that consumption of fruits and vegetables containing phytochemicals with potential antioxidant properties is associated with reducing the risk of human diseases, such as atherosclerosis, arthritis, cardiovascular diseases and cancers (Kris-Etherton et al., 2002), diabetes, neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Di Matteo and Esposito, 2003), along with inflammation and other aging disorders (Ames et al., 1993). Within the antioxidant compounds, polyphenols are widely distributed in the plant kingdom. Polyphenols are a group of aromatic secondary metabolites mainly existing in the plant's bark, roots, leaves and fruits. These compounds are known to induce higher antioxidant activity due to their redox properties along with metal chelation, hydrogen donation and singlet oxygen quenching (Rice-Evans et al., 1995; Rice-Evans et al., 1997). Beside antioxidant capacity, polyphenols have been reported to possess multiple biological effects such as antimicrobial (Bendini et al., 2006; Chu et al., 2000), anticancer, anti-allergic and cardiovascular protective activities (Hertog et al., 1995), due to which they have received much attention in the field of pharmaceutical, biochemical, nutraceuticals, cosmetic industry, and so on (Amaral et al., 2008).

Phenolic compounds have also been used in the food industry as natural colorants and preservatives (Dillard and German, 2000). As some synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) etc. may exhibit toxicity (Jayaprakasha et al., 2003), require high manufacturing costs and have lower efficiency than natural antioxidants (Soong and Barlow, 2004), there is a need to identify and possibly to

devise more economical ways to obtain effective natural antioxidants with potential to be incorporated into foods. However, many antioxidative phenolic compounds in plants are most frequently present as a covalently bound form with insoluble polymer (Robbins, 2003). To obtain these compounds from the vacuolar structures where they are found, either through rupturing plant tissue or through a process of diffusion, it is necessary to find an effective extraction and processing method (Seok-Moon et al., 2004).

Extraction is the first and most important stage in isolation, identification and use of phenolic compounds from plant materials and there is no single and standard extraction method for all samples. Polyphenols extraction is mainly complex for the following two reasons: (1) their extremely different chemical structure and interactions with other food components are not fully known, which is vital information for selecting solvents and extraction conditions, (2) polyphenols are susceptible to oxidation, thermal and alkaline degradation (Tura and Robards, 2002). Hence, specific processes must be designed and process parameters should be optimized for extraction of polyphenolic compounds.

In case of any sample, extraction yield is influenced by the chemical nature of the compounds (simple and complex phenolics), the extraction method employed (extraction by solvents, solid-phase extraction and supercritical extraction) (Goli et al., 2005), the storage time and conditions, and the presence of interfering substances (Naczk and Shahidi, 2004). Independent of new or old techniques, the extraction method must enable complete extraction of the compounds of interest and avoid their chemical modification (Zuo et al., 2002). Though new extraction techniques have been developed, classic extraction governs many laboratories owing to its simplicity and low monetary expenditure. Moreover, by selecting suitable solvents and applying possibly effective conditions of extraction (different durations and applied temperature), the efficiency of the process can be extensively regulated here. Among the typical extraction methods, solvent extraction is the mostly used one that is

designed to separate soluble phenolic compounds by diffusion from a solid matrix (plant tissue) using a liquid matrix (solvent). This process is widely employed for phenolic extraction from various vegetable materials (Liyana-Pathirana and Shahidi, 2005; Li et al., 2006). Many factors contribute to the efficiency of the solvent extraction process: type of solvent, pH, temperature, number of extraction steps, solvent/solid ratio, particle size of the solid matrix (Cacace and Mazza, 2003b) etc. But the role of each factor in the extraction process is not always understandable due to the different chemical characteristics of the solvent and the diverse structure and composition of the natural products. Thus, it is important to develop an optimal extraction method for quantification and identification of the natural antioxidants and polyphenol compounds for specific sample (Pinelo et al., 2005b). There are many examples of the extraction optimization of polyphenols and antioxidants from different fruits, leaves and woody plants (Dapkevicius et al., 1998; Lee et al., 2000). For instance, Al-Farsi and Lee (2008) optimized extraction of phenolics and dietary fibre from date seeds, where phenolic contents increased from 14.48 to 36.26 g/100 g seed concentrate. Tabart et al. (2007) found that at optimized condition the phenolics and antioxidants extraction from black currant leaves and buds increased significantly. Similarly, optimization of extraction conditions (acetone concentration 48.49%, extraction time 59.25 minutes and extraction temperature 40.88 °C) yielded 4661.17 mg gallic acid equivalent (GAE)/100 g dry weight (DW) of phenolic compounds from neem leaves (Hismath et al., 2011). Likewise, Bouterfas et al. (2014) reported that the maximum total phenolics (293.34 ± 14.60 mg GAE/g DW) were obtained at the optimum extraction conditions (60% aqueous methanol at 25°C for 180 min) from white horehound leaves.

Mangroves are carbon rich ecosystems that lie at the interface between land and sea in the tropical and subtropical regions, providing a wide array of resources and services, such as protection against natural calamities, habitats for wildlife and fisheries, support

socioeconomic activities and maintain ecological balance (Donato et al., 2011; Kathiresan and Bingham, 2001; Lee et al., 2014). There are about 39.3 million acres of mangrove forests in the warm coastlines of tropical oceans all over the world. Among these the Sundarbans' (latitude 21°31' - 22°30' North and longitude 88°10' - 89°51' East) is the largest single block of tidal halophytic mangrove forest in the world (Ghosh et al., 2002). It is situated along the coast of the Bay of Bengal and covers partly Bangladesh (59% of the forest, 6014 km^2) and India (Chaudhuri and Choudhury, 1994), being one of the most diverse forests harboring more than 334 plant species of 245 genera (Sarker et al., 2010). All mangrove plants flourish in extremely abiotic and biotic stressful environment of high salinity, high and low tides of water, high temperature and moisture, strong winds, muddy anaerobic soil and insects and microorganisms, respectively. To thrive in such hostile surroundings (Silva et al., 2011), alterations in their physiological and morphological processes have occurred resulting in the synthesis of novel chemical compounds (phytochemicals) as a defensive stress response (Edreva et al., 2008).

Mangrove plants contain many phytochemicals like alkaloids, phenolics, flavonoids, tannins etc. having antimicrobial (Abeysinghe, 2010), toxicological, pharmacological and ecological importance (Kokpal et al., 1990) and antioxidative, anti-HIV, anti-proliferative and anti-estrogenic activities (Field, 1995). In fact, many mangrove plant species and their extracts have been used in folk or traditional medicine as cures for various ailments and for other commercial purposes (Abeysinghe et al., 2006; Bandaranayake, 1998). These chemical compounds derived from the natural sources can play very significant roles in the new drug discovery process (Bandaranayake, 2002). Therefore, it is worth to screen mangrove plants for the presence of new natural beneficial compounds. The fruits of *Sonneratia apetala* (Buch.-Ham.), belonging to the Family Lythraceae, grow abundantly in the Sundarbans' mangrove forest. They are also called Mangrove apple and locally known as Keora (Das and

Ghose, 2003). The neighboring people of the Sundarbans' comprehensively consume the fruits in cooked and other preparations without being aware of the effects of the fruit on their physiological health. Local people used half-ripe and ripe fruits to treat coughs and intestinal parasites, respectively. According to Bandaranayake (1998), leaves of *S. apetala* have antihepatitis activity. Hossain et al. (2013) reported that the fruits, especially the seeds of *S. apetala* (Buch.-Ham.) have antioxidative, antidiabetic and antibacterial activities. Reports (Hossain et al., 2013; Hossain et al., 2016) showed that the seeds contained very high amount of polyphenols (300 ± 8.2 mg GAE/g extract) and flavonoids (30.6 ± 0.7 mg CE/g extract), which were near to that of *Phyllanthus emblica* fruit (339 mg GAE/g extract) (Hossain et al., 2008). As per another finding, various extracts of both the leaf and bark of *S. apetala* have high antioxidant, cytotoxic, antidiabetic and antibacterial activities (Patra et al., 2015). Pramanick et al. (2014) found that jelly prepared from *S. apetala* fruit pulp and fresh pulp contain high amount of vitamin C (important antioxidant: 42.71 mg/100 gm and 423.71 mg/100 gm, respectively), major and trace elements compared to other citrus fruits. Again, the fruit of *S. apetala* was non-toxic to the rodents during acute and chronic toxicity studies, since the acute dose did not show any mortality or toxic symptoms (Halder et al., 2015). Finally, as *S. apetala* fruits have high content of polyphenols and antioxidants to be consumed, no toxicity, high adaptability and seed production capacity, hence it might be the cheapest source of polyphenols. So, if it is possible to optimize the conditions for polyphenols extraction from *S. apetala* fruits, they can be extensively used in the food, cosmetics, pharmaceuticals and other industries.

However, despite some literatures published in relation to *S. apetala* phenolic compounds and their antioxidant activity, the optimization of extraction of polyphenols and antioxidants of *S. apetala* fruits was not reported yet. The potential use of *S. apetala* fruits as a source of

antioxidant phytochemicals calls for an optimization of the extraction process and recovery of these valuable compounds with analytical purposes.

Therefore, the resent study have been undertaken to find out the optimum conditions for the extraction of polyphenols and antioxidants from *S. apetala* fruits.

Objectives of the Study

The objectives of the research work are-

- To know the solvent(s) and temperature(s) that extract the highest amounts of polyphenols and antioxidants from *S. apetala* (Buch.-Ham.) fruits
- To detect the suitable pH and extraction time at which maximum amounts of polyphenols and antioxidants are extracted
- To determine the effect(s) of pre-extraction heat treatment on extraction
- To optimize the conditions for obtaining optimum amounts of polyphenols and antioxidants from the fruits

Chapter Two

REVIEW OF LITERATURE

LITERATURE REVIEW

Oxygen is an obligatory element for life. Living systems have evolved to survive in the presence of molecular oxygen that has double-edged properties. In one hand, it is essential for life and in other hand, it can also exacerbate the damage within the cell by oxidation (Shinde et al., 2006). Hence oxidation is not only an essential biological process for energy production in many living organisms but also the most important route for producing free radicals in living systems.

A free radical is a compound with one or more unpaired electrons in its outer orbital (Jesberger and Richardson, 1991) having a tendency to steal back an electron from neighboring molecules (Sreejayan and Rao, 1997), thus forming a new free radical. Consecutively, the newly formed radical then returns to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be "thousands of events long" (Goldfarb, 1999).

Free radicals produced in *in vivo* include superoxide, the hydroxyl radical, nitric oxide, oxygen-centered organic radicals such as peroxyl and alkoxyl radicals, and sulfur-centered thiol radicals. Other non-radicals include hydrogen peroxide, peroxynitrite, and hypochlorous acid, which are not radical species, but are actually or potentially damaging oxidants. These radical and non-radical species are collectively called reactive oxygen species (ROS) (Benzie and Strain, 2005).

Biological systems are persistently exposed to ROS generated both exogenously and endogenously. Accumulation of ROS can lead to damaging effects on DNA, proteins and lipids (Jing, 2006) that are central to the pathogenesis of chronic diseases including atherosclerosis, cancer, carcinogenesis, cardiovascular dysfunction, diabetes, drug toxicity, inflammation, reperfusion injury, and neurodegenerative diseases (Slater, 1991; Valko et al.,

2006). Deleterious effects of ROS are balanced by activities of natural antioxidants. Oxidative stress results when the balance between the productions of ROS exceeds the antioxidant capability of the target cell (Ahmad et al., 2009; Roy et al., 2009).

It is well known that all aerobic organisms possess antioxidant mechanisms to protect against the oxidative damages and various types of enzymes for the removal or repair of the damaged molecules (Auroma, 1998). Even so, these natural mechanisms can be ineffective sometimes and hence, dietary intake of antioxidants is essential (Tanizawa, 1992). The fact that synthetic antioxidants (i.e. butylated hydroxytoluene and butylated hydroxyanisole), commonly used in processed foods, possesses some side effects thus limiting their use as antioxidant agents (Branien, 1975). Therefore, research to find new sources of natural antioxidants is essential.

An antioxidant is a molecule proficient of inhibiting the oxidation of other molecules. The probable mechanisms of action of antioxidants were first ascertained when it was documented that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized (Moreau and Dufraisse, 1922) and being non-reactive in oxidized state. The human body is normally well outfitted with an array of 'antioxidative' strategies to protect against the injurious effects of free radicals, which is complex and consists of various intracellular and extracellular, endogenous and exogenous, and aqueous and lipid-soluble components that act in concert to prevent ROS. Antioxidants may counteract in three ways: increase the levels of enzymes that degrade free radicals, amplify the levels of proteins such as transferrin that can bind the metals stimulating the production of free radicals and chemical antioxidants such as vitamin C and E which act as direct free radical scavengers (Rahimi et al., 2005).

Fruits and vegetables are good sources of natural antioxidants. Diets rich in fruits and vegetables have long been linked with abridged risk of chronic disease, particularly

cardiovascular disease, cancers and diabetes (Faller and Fialho, 2009). This quality has been somewhat accredited to the presence of natural compounds possessing antioxidant properties (Podsedek, 2007), among which polyphenols are of the utmost interest.

Polyphenols are compounds possessing single or multiple aromatic rings with one or more hydroxyl groups. They are the most profuse secondary metabolites of plants, with above 8,000 phenolic structures presently known (Wollgast and Anklam, 2000), assorting from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Polyphenols include the following subclasses: flavonoids, phenolic acids, stibenges, lignans, tannins, oxidized polyphenols etc. (George et al., 2005). These are generally involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to plants' colors. They are ubiquitous in all plant organs and are therefore widespread constituents of human foods (cereals, chocolate, fruits, legumes, olive, vegetables etc.) and beverages (coffee, beer, tea, wine etc.).

In spite of their broad allocation, the health effects of dietary polyphenols have come to the consideration of nutritionists only recently. Researchers and food manufacturers have become more fascinated in polyphenols due to their potent antioxidant properties, abundance in the diet, and their convincing effects in avoiding various oxidative stress associated diseases (Manach et al., 2004). The defensive effects of these secondary plant metabolites in terms of cardiovascular, cancer and neurodegenerative diseases are figured out from epidemiologic data as well as *in vitro* and *in vivo* findings (Arts and Hollman, 2005; Rasmussen et al., 2005) and results in respective nutritional recommendations. Moreover, polyphenols were found to modulate the activity of a wide range of enzyme and cell receptors in signal transduction pathway (Hou et al., 2004). Thus, polyphenols also have other specific biological actions in preventing and/or treating diseases additionally with antioxidant properties.

But before being used, polyphenols must be extracted from plant materials as this is the first step in the utilization of phytochemicals in different sectors. Polyphenols can be extracted from fresh, frozen or dried plant samples. Though polyphenols share the common phenolic characteristics, these phytochemicals diverge significantly in their physicochemical properties owing to the structural diversity. Due to the chemical complexity and the recurrent occurrence of polyphenols in plants, extraction, separation, identification and analysis of polyphenols remain as complicated as ever. So, it is virtually impractical to develop a protocol for all polyphenols; however there are some universal approaches to these important aspects of polyphenol research.

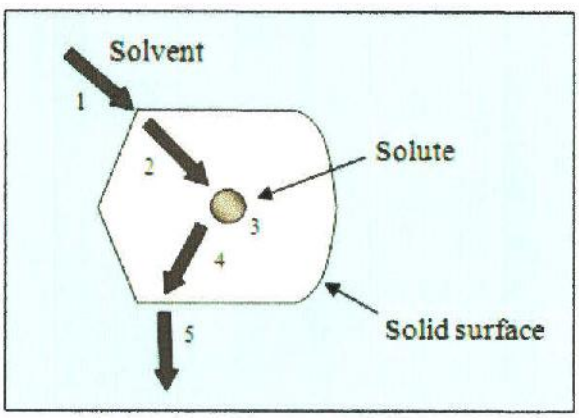

Figure 2.1: Mechanism of solid-liquid extraction (Ferhat et al., 2007).

Extraction of solutes from plant materials is a mass transfer process involving five consequent steps (Figure 2.1): (1) transfer of the solvent to the solid surface; (2) diffusion of the solvent into the solid phase; (3) dissolution of the solute in the solvent; (4) diffusion of the dissolved solute to the solid surface; and (5) transfer of the solute in the solution by diffusion or convection. Prior to extraction, samples are often dried, frozen or lyophilized so as to avoid degradation of native polyphenols, since elevated moisture or water content assists enzymatic activities (Stalikas, 2007). Many different extraction methods are available for different types of samples (Stalikas, 2007; Stalikas, 2010). Among them solvent extractions are the most generally used procedures to prepare extracts from plant materials

because of their user-friendliness, efficiency and extensive applicability. It is usually known that the yield of chemical extraction depends on the type of solvents with diverse polarities, extraction time and temperature, p^H, in addition to the chemical composition and physical individuality of the samples. Again the solubility of phenolics is administered by the chemical nature of the plant sample and the polarity of the used solvents. So choosing the appropriate solvent affects the amount and rate of extracted polyphenols (Xu and Chang, 2007). For this reason, type of solvent used for extraction has been the most investigated factor. Due to the phenolic nature, polyphenols are relatively hydrophilic, therefore free polyphenols, including aglycones, glycosides and oligomers, are extracted using water, polar organic solvents such as acetone, ethanol, ethyl acetate, methanol, and their combinations often with or without different proportions of water. For the most part, methanol has been found to be more competent in extraction of lower molecular weight polyphenols, while the higher molecular weight flavanols are better extracted with aqueous acetone (Metivier et al., 1980). Ethanol is another good quality solvent for polyphenol extraction, also being safe for human consumption (Shi et al., 2005) and it efficiently extracts flavonoids and their glycosides, catecols and tannins from raw plant materials (Bazykina et al., 2002), although solubility of these compounds can be improved using a mixed solvent over a limited compositional range (Cacace and Mazza, 2003a).

In order to influence the recovery of phenolic compounds from plant materials as well as to minimize energy cost of the process, it is also important to optimize the extraction time and temperature. Extraction time and temperature reflects the contradictory actions of solubilization and analyte degradation by oxidation (Robards, 2003). It is granted by many authors that an increase in the working temperature favors extraction by boosting both the solubility of solute and the diffusion coefficient (Spigno and De Faveri, 2007). Besides, at higher temperature the viscosity and the surface tension of the solvents decrease thus helping

the solvents to reach the sample matrices and eventually improving the extraction rate. However, many phenolic compounds are easily hydrolyzed and oxidized, which is influenced by long extraction times and high temperatures. Hence beyond a certain value phenolic compounds can be denatured and thus decreases the yield of phenolics in the extracts (Pinelo et al., 2005b; Yilmaz and Toledo, 2006).

Another important parameter of extraction optimization is the pH of the extraction solvent. In case of polyphenols extraction, acidic conditions are most favored since they are usually more stable in low pH and have less susceptibility towards oxidation. In acidic conditions polyphenols stay neutral as the hydroxyl groups are shielded by protonation, being readily extracted into organic solvents. This is done using weak acid or low concentrations of a strong acid. High acid concentration can cause hydrolysis of glycosides or acylglycosides and thus may give different pictures of native polyphenol profiles (Tsao, 2010).

Effect of heat treatment before extraction is also an essential factor for extraction optimization of polyphenols. It is reported that in plant foods, one-third of the phenolic compounds are phenolic acids, presented in free and bound forms. These bound phenolics may be linked to various plant components through ester, ether or acetal bonds. So, some processing methods like heat treatment may be employed to liberate them to enhance the antioxidant capacity. For example, Seok-Moon et al. (2004) reported that in case of citrus peel, heat treatment may liberate some low molecular weight phenolic compounds and therefore increase the antioxidant capacity.

The extraction of phenolic compounds from plant materials may also be influenced by other factors such as solvent-to-solid ratio and the particle size of the sample (Cacace and Mazza, 2003b). But each factor work in a diverse way in extraction of the polyphenols when different raw materials are used (Jiang et al., 2007). For these above mentioned reasons, there is a need

to optimize the extraction process for proper quantification of the polyphenols from a specific sample. As per examples, optimization of polyphenols extraction had been done in case of black current leaves and buds (Tabart et al., 2007), date seeds (Al-Farsi and Lee, 2008), *Ficus virens* leaves (Chen et al., 2013), mashua tubers (Chirinos et al., 2007), pomegranate peel (Wissam et al., 2012), Syrian and Chinese sumac fruits (Kossah et al., 2010) etc., where optimum conditions were different in case of every sample for maximum polyphenols and antioxidants extraction.

According to Shahidi and Naczk (2004) as these bioactive substances are often used in preparation of functional foods, as food additives and preservatives, in nutraceuticals, pharmaceuticals and cosmetic industries etc., the extraction and purification of polyphenols and antioxidants from miscellaneous sources is desired. Owing to the increased distresses over the possible toxicological effects of synthetic food additives (Namiki, 1990), plus consumer preference for natural products, there is an increased interest towards the search for natural substances (Amakura et al., 2002) especially polyphenols, from natural organic sources such as fruits, vegetables, cereals, herbs etc.

Mangroves are collection of plants growing in saline coastal habitats around the world and are the only woody halophytes dominated ecosystem situated at the confluence of land and sea, occupying a harsh environment, being daily subject to tidal changes in temperature, water and salt-exposure and varying degree of anoxia (Alongi, 2002). There may be no other group of plants with such highly developed morphological and physiological adaptations to extreme conditions. In these hard climatic situations plants generate ROS, which cause cellular damage and many plant disorders. Therefore these plants produce increased amounts of secondary metabolites, known as phytochemicals, in response to stressful conditions to protect cellular structure against oxidation (Chanwitheesuk et al., 2005). Maisuthisakal et al. (2007) reported that in hard environmental circumstances, production of the phytochemicals

is augmented in the plant tissues. For these reasons mangroves are rich sources of secondary metabolites of various types having wide range of therapeutic possibilities (Bandaranayake, 1998); comprising steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and phenolics (Bobbarala and Vadlpudi, 2009). Like other terrestrial plants, many mangrove plants have ethnopharmacological relevance and have also been exploited by the local people in search of remedies for various ailments. However, though several mangroves are extensively used in traditional medicine, only some of them were tested for biological activities. Vadlapudi et al. (2009) reported that due to the potentiality of scavenging free radicals, mangrove plants can be a vital source of antioxidant phytochemicals. An intensive study found that *Thespesia populneoides*, *Sonneratia apetala*, *Acaryochloris marina*, and *Rhizophora mucronata* from mangrove forest are good sources of natural antioxidants (Vadlapudi and Naidu, 2009). Another mangrove plant *Excoecaria agallocha* possesses a promising antioxidant property, anti-histamine-release and antifungal activity (Hossain et al., 2009; Patra et al., 2009).

In the Ganga-Brahmaputra estuary of lower Bengal distributed both in Bangladesh and India, the largest single chunk of tropical deltaic formation - the Sundarban mangroves, is found. The name Sundarbans', literally meaning "beautiful forest" is believed to be derived from Sundari or Sundri (*Heritiera fomes*), one of the most abundant tree species found in this forest (Naskar, 2004). The Sundarbans' is bestowed with the highest floral diversity in the form of mangroves harboring more than 334 plant species of 245 genera (Sarker et al., 2010) and attracts a lot of attention and curiosity in being novel in more than a single way. As a mangrove plant *S. apetala* (Buch.-Ham) has reported antioxidant and antimicrobial activities. According to the findings of Banerjee et al. (2008) leaves, stem bark and root of *S. apetala* have potential antioxidant activity. Hossain et al. (2013) and Hossain et al. (2016) reported that the fruits (especially seeds) had high amount of polyphenols, flavonoids and antioxidants, which is comparable to other edible fruits, along with antidiabetic and

antibacterial activities. Recently, antibacterial, antioxidant, antidiabetic and anticancer activities of leaf and bark extracts of *S. apetala* were also reported by Patra et al. (2015). Additionally, analgesic, antidiarrhoeal, antihelminthic and cytotoxic activities of ethanol extract of the fruit had also been published (Shefa et al., 2014). In accordance to another finding it had been reported by Halder et al. (2015) that in Hepato-toxicity study no mortality of mice or toxic symptoms were found even in the acute toxicity tests. Hence it was proved that *S. apetala* is non-toxic as an edible fruit.

Although there are few published papers regarding the beneficial effects of *S. apetala*, there is no report about the influence of variables, such as solvent, temperature, time, pH, heat etc., on the extraction of phenolic compounds from the fruits. Since, a large number of fruits of *S. apetala* are grown and consumed by the coastal people' each year without any toxicity, have high polyphenols and antioxidants, economically less expensive, it might be the cheapest source of polyphenols that could be utilized in the food, cosmetics, pharmaceuticals industries and so on. Thus it is of utmost importance to study the influence of these parameters on the optimization of the extraction of polyphenols and antioxidants from the fruits.

PLANT PREVIEW

Name of the sample plant: *Sonneratia apetala* Buchanan- Hamilton.

Used part of the plant as sample: Fruit

Local name: Keora (Bangladesh).

English name: Mangrove apple

Other local name:

Berembang (Malaya), Holzapfel mangrove (German), Kandal, Chipi (Marathi), Khirwa (Oriya), Kyalanki (Telugu), Mangrovenapfel, Pagatpat (the Philippines), Maram (Tamil).

Figure 3.1: Fruits of *S. apetala* (Buch-Ham.).

Systemic classification

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Myrtales

Family : Lythraceae

Genus : *Sonneratia*

Species : *S. apetala*

[IUCN Red List (April, 2011)]

Geographic distribution

It is widely distributed in Bangladesh, Myanmar, India (including the Andaman and Nicobar islands), Sri Lanka and some coastal regions of Asia, Africa and Australia; also introduced into Fujian and Guangdong provinces, China.

Description of the plant

Sonneratia apetala (Buch.-Ham.), family-Lythraceae, is a native plant growing profusely in the Sundarbans' mangrove forest; being named *Sonneratia* in honor of French naturalist, Pierre Sonnerat (1749-1814).

Plant: It grows as tree or shrub and also as creeping, glabrous, succulent herb, by the side of seaward fringes and intertidal areas, which is one of the woody mangrove species having high adaptability and seed production rate. The height ranges from 15 to 20 m, diameter at breast height (DBH) varies between 20 and 30 cm (Kairo et al., 2009), with thin, light brown, irregularly fissured bark.

Leaves: The leaves are simple, opposite, entire, leathery, narrow and light green in color with average length of 10-13 cm.

Flowers: According to Tomlinson (1986), flowers are comparatively smaller, cream colored (owing to the cream colored mass of stamens), possessing 4 calyx lobes with no petals (hence called apatala). The style contains 2-3 cm long curved, white colored stigma, compressing like umbrella or mushroom, while the upper portion is reddish color.

Fruits: There are two peak fruiting seasons, from September to November and April to June. Fruits are rounding, berrylike, leathery, indehiscent, with a star-like crown (calyx) (Figure 3.1); about 2×2.5 cm in diameter (Hossain et al., 2013).

Seeds: Seeds are typically U-shaped or sickle-shaped, 8-9.5 mm, numerous; embedded in foul-smelling pulp, irregularly angular or falcate; whereas the seed coat is thickened, roughened and somewhat corky.

General and medicinal uses

The fruits of *S. apetala* are eaten in some area of Bangladesh and Africa, as well as processed to produce sour sauce, chatney and marketed in many regions. Stem is used for paper pulp, matches and leaves mainly as fodder. Though the wood corrodes metal (probably because of the timber's high mineral content), the timber is used for building boats, piling and posts for bridges and houses due to the resistance to shipworm and pests. The pneumatophores are used to make floats for fishing nets.

S. apetala also has great medicinal value for a long time. It is reported that fruits broadly used for dysentery, sprain, swelling and bruises, in treatment of eye troubles (such as cataract) and open sores in children ears and also in heart troubles (Bandaranayake, 1998), while leaves to treat hepatitis (Bandaranayake, 1995). Ripe fruits are used to expel intestinal parasites, whereas half ripe fruits for coughs. The wall of an old fruit is given as a vermifuge. Fermented fruit juice is said to be useful in arresting haemorrhage. The fruits, specially the seeds have antioxidative, antidiabetic and antibacterial (Hossain et al., 2013) and the leaves and bark also have anticancer activities along with antibacterial, antioxidant, antidiabetic activities (Patra et al., 2015).

Reported antioxidant activity

The antioxidant activity of leaves, stem bark and root of *S. apetala* was reported by Banerjee et al. (2008). In accordance with the report, the amount of total phenolic content of leaves, stem bark and roots were 47.52 ± 2.22, 42.68 ± 2.75, and 42.75 ± 1.67 mg GAE/g of dry

material respectively; the reducing power were 5.71 ± 0.24, 7.06 ± 0.07, and 6.87 ± 0.10 mg AAE/g of dry material respectively and in case of DPPH radical scavenging assay, IC_{50} values for leaves, stem bark, and roots were 163.49 ± 6.32, 193.09 ± 14.35, and 183.04 ± 1.74 respectively.

According to Hossain et al. (2013), seeds of *S. apetala* contained very high amount of polyphenols (300 ± 8.2 mg GAE/g extract), flavonoids (30.6 ± 0.7 mg CE/g extract), anthocyanins (2.3 ± 0.03 μmol/g extract), vitamin C (4.0 ± 0.08 mg/g extract) and total antioxidant capacity.

In another report, Hossain et al. (2016) found that methanol fraction of seed (MS) showed the highest polyphenols content (221.9 mg GAE/g fraction), DPPH (IC_{50} = 2.1 μg/mL) and NO (IC_{50} = 490.8 μg/mL) free radical scavenging activity; also very strong reducing power (OD = 1.67 at 100 μg/mL), Fe^{2+} chelating and total antioxidant capacity.

Patra et al. (2015) also reported that acetone, ethanol, methanol and aqueous extracts of leaf and bark possess strong antioxidant properties. Here total phenol content ranged from 3.18 ± 0.01 to 8.63 ± 0.02 (% dry weight) and total antioxidant capacity from 52.30 ± 6.48 to 271.52 ± 3.85 (mg/g dry weight). This report also contained data for DPPH free radical scavenging assay, nitric oxide scavenging assay, ABTS scavenging assay, metal chelating assay and reducing power.

Reported antimicrobial activity

Being a mangrove plant, *S. apetala* also has antimicrobial activity. According to Bobbarala et al. (2009), the hexane, chloroform and methanol extracts of *S. apetala* plant had inhibitory effects on the various test microorganisms (including clinical multiple antibiotic resistant bacteria and phytopathogens), where methanolic extract showed the best activity. Likewise,

Jaimini et al. (2011) found chloroform and methanolic extracts of *S. apetala* leaf noticeably more active than the hexane extract against both gram-negative and gram-positive bacterial strains. Similar results were also reported by Hossain et al. (2013), according to whom methanolextract (300µg/disc) of *S. apetala* seed inhibited the growth of all tested gram-positive and gram-negative bacteria, while pericarp extract showed no inhibition of *E. coli*, *E. faecalis*, *Pseudomonas* sp., *S. flexneri* and *S. epidermidis*. Resembling the previous findings Panda et al. (2012) reported that methanolic extracts of *S. apetala* arial parts had species specific activity in inhibiting the growth of bacteria and fungi. Teja and Ravishankar (2013) also proposed that the ethanolic extract of *S. apetala* roots had antibacterial and antifungal activities.

Other reported activities

Besides antioxidant and antimicrobial activities, other potential activities of *S. apetala* have also been published. In one report Patra et al. (2015) postulated that the leaf and bark extracts of *S. apetala* had antidiabetic and anticancer activities along with antioxidant and antibacterial potentials. On the other hand, Shefa et al. (2014) reported the analgesic, antidiarrhoeal, antihelminthic and cytotoxic activities of ethanol extract of the *S. apetala* fruits. Thatoi et al. (2016) synthesized silver and zinc oxide nanoparticles using aqueous leaf extract of *S. apetala* and they found that the synthesized nanoparticles possess strong biological activities in terms of antioxidant, anti-inflammatory, antidiabetic and antibacterial potentials. Again, phytochemical constituents (Teja and Ravishankar, 2013); food safety evaluation in mice and rat (hepato-toxicity study) (Halder et al., 2015); nutrient compositions and common phenolics of *S. apetala* (Hossain et al., 2016) had also been documented.

MATERIALS AND METHODS

4.1 Instruments and glasswares used in the experiments

Beaker, conical flask, digital balance (OHAUS, PA104 Corp. USA), electric grinder, incubator (WiseCube), measuring cylinder, micropipette (Gilson, France), one and two drum vials, pH meter (pH Tester H196107, Hanna Instruments, USA), petri dish, spectrophotometer (Hitachi, U-2910), test tubes, funnel, vortex machine, water distiller, water bath (Thermostatic water bath, Shanghai, China), Whatman No. 1 filter paper etc.

4.2 Chemicals and reagents used in the experiments

Acetone (Sigma-Aldrich, France), aluminum chloride ($AlCl_3$. $6H_2O$), ammonium molybdate, DPPH (2, 2-diphenyl-1-picrylhydrazyl) (Wako Pure Chemical Industry Ltd., Osaka, Japan), ethanol (Merck, Germany), Folin-Ciocalteu's phenol reagent, gallic acid (GA) (Sigma-Aldrich Co.), L-ascorbic acid (AA) (Sigma-Aldrich Co.), metaphosphoric acid (HPO_3), methanol (Merck, Germany), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), sulfuric acid etc.

4.3 Collection of sample

The fruits of *S. apetala* (Buch.-Ham.) were collected from the world's largest mangrove forest, the Sundarbans', Khulna in August, 2015.

4.4 Preparation of sample

First the collected fruits were washed with tap water for removing dirt and undesirable materials. Then after wiping excess water they were cut into small pieces with a sharp knife and shed dried until complete evaporation of water. Afterward they were ground into powder

using a mechanical grinder. Finally the powdered sample was stored in air tight containers at room temperature (25-30 °C).

4.5 Detection of suitable solvent(s) and temperature(s) for extraction

Five gram (5 g) of fruit powder was taken in an air tight bottle and 100 mL of solvent [acetone, ethanol, methanol, distilled water (DW) or their mixtures] was added followed by vigorous shaking for 5 minutes. It was extracted for 20 hours at different temperatures (10, 30, 40, 55 or 70 °C) with frequent shaking. The mixture was then filtered through Whatman No. 1 filter paper and the filtrate was concentrated by a rotary evaporator, followed by volume adjustment. Finally extracts were stored in the refrigerator at 4 °C and subsequently used for further experiments.

4.6 Identification of appropriate pH for extraction

To identify the appropriate p^H, fruit powder was extracted at different pH (1, 2, 3, 4, 5, 7 or 9) for 20 hours at 30 °C by using methanol : ethanol (1:1) as the extraction solvent. Then after filtering, concentrating and volume adjustment, the extracts were stored at 4 °C for conducting experiments.

4.7 Effects of preextraction heating on extraction

For evaluating the effects of heat treatment before extraction, 5 g powder of the fruit was placed in a Pyrex Petri dish as a single layer and heated in an electric oven at 30, 60, 90, 120, 150 or 180 °C for 0.5, 1, 2 or 4 hours. After heating, the powder was allowed to cool to ambient temperature and then extracted with methanol : ethanol (1:1) as the extraction solvent, at pH 3 for 20 hours at 30 °C. Similar procedure was done to store the extracts in the refrigerator including filtering, concentrating and volume adjustment.

4.8 Optimization of extraction

To optimize the extraction of polyphenols and antioxidants from the fruits of *S. apetala*, fruit powder was first preheated at 90 °C for 1 hour; then cooled, followed by addition of methanol : ethanol (1:1) mixture as the extraction solvent and finally extracted at pH 3 with 30 °C as the extraction temperature for different time durations, comprising 0, 0.5, 1, 2, 5, 10, 20, 40, 60 or 120 hours. Afterward, the extracts were filtered, concentrated and volume adjusted. For performing further tests, extracts were saved in the refrigerator at 4 °C.

4.9 Determination of total phenolic content

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu method (Ough and Amerine, 1988), where gallic acid (GA) was used as the standard and results were expressed as mg gallic acid equivalents (GAE)/g of dry powder. In the experiment, one milliliter (1 mL) of diluted extract was mixed with 1 mL of 50% Folin-Ciocalteu's reagent and vortexed for 5 s. Then, 1 mL of 10% (w/v) sodium carbonate aqueous solution was added followed by mixing. The mixture was incubated at room temperature for 1 h and subsequently spectrophotometric measurement was made at 700 nm. Each experiment was conducted for three times.

4.10 Determination of total flavonoid content

Total flavonoid content was determined by a colorimetric method according to Zhishen et al. (1999), with (+)-catechin as the standard at different concentrations (20-40 µg/mL). Briefly, test tube containing 1.5 mL diluted sample was mixed with 75 µL of 5% (w/v) $NaNO_2$ solution by vortex machine. After 6 minutes, 150 µl of 10% (w/v) $AlCl_3.6H_2O$ solution was added and the mixture was allowed to stand for a further 5 minutes before 0.5 mL of 1 M NaOH was added. The mixture was finally brought to 2.5 mL with distilled water and

vortexed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. The results were expressed as mg of (+)-catechin equivalents (CE) per gram of dry powder from triplicate results.

4.11 Antioxidant assay

4.11.1 Determination of DPPH free radical scavenging activity

DPPH free radical scavenging activity was determined according to the method of Blios (1958). First 1.5 mL diluted extract was taken in the test tube and 0.5 mL of 0.5 M acetic acid buffer solution (pH 5.5) was added with vigorous shaking. Then after the addition of 1 mL of 0.2 mM DPPH in ethanol and proper mixing, it was incubated at room temperature for 40 minutes in the dark. Blank was prepared similarly, except that 1.5 mL 50% ethanol was used instead of sample. The amount of DPPH remaining was determined by measuring absorbance at 517 nm against 100% ethanol. Mean values were obtained from triplicates. % DPPH radical scavenging activity was calculated by using the following equation and results were expressed as mg ascorbic acid equivalent (AAE)/g powder.

$$\% \text{ DPPH radical scavenging activity} = \frac{(\text{control OD} - \text{sample OD}) \times 100}{\text{control OD}}$$

4.11.2 Determination of total antioxidant capacity

Determination of total antioxidant capacity was done according to the method of Prieto et al. (1999). Antioxidants can reduce Mo (VI) to Mo (V) and produce the green phosphate / Mo (V) compounds at acidic pH, which have an absorption peak at 695 nm. Based on this principle, the tubes containing 0.5 mL diluted extract and 2.5 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were vortexed and

incubated at 90 °C for 90 min. After cooling at room temperature, the absorbance was taken at 695 nm against a blank, where the composition of the blank was 0.5 mL diluted solvent with 2.5 mL reagent solution. The antioxidant capacity was expressed as mg ascorbic acid equivalent (AAE)/g dry powder by conducting each experiment for three times.

4.12 Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS), version 16.0. All data were expressed as means ± SD (standard deviation) of at least three replicates. Differences between variables were tested for significance by using one-way analysis of variance (ANOVA). Differences between means were considered to be significant at $p < 0.05$.

Chapter Five

RESULTS

RESULTS

5.1 Effects of solvent(s) and temperature(s) on extraction

In our experiment, acetone, ethanol, methanol, and distilled water were used as solvents to extract phenolic and antioxidant components from the *S. apetala* fruits. The fruit powder with each solvent was extracted at different temperatures (10, 30, 40, 55 or 70 °C) for 20 h. The amounts of total polyphenols (TP), flavonoids (TF), DPPH radical scavengers (DRS) and total antioxidants (TA) of the extracts were measured to find out the best extraction solvent(s) and temperature(s) (Table 5.1). Table 5.1 showed that among the tested solvents, methanol, and ethanol showed the similar results and they extracted the higher amounts of polyphenols, flavonoids, DPPH radical scavengers and total antioxidants at 30 °C. The highest amounts of TP extracted with ethanol and methanol were 44.1 ± 0.3 and 45.4 ± 0.4 mg GAE/g powder, respectively. Similar to TP, the highest amounts of TF, DRS and TA were also found at 30 °C while extracted with ethanol, and methanol.

As the highest amounts of TP, TF, DRS and TA from the fruits were extracted at 30 °C with ethanol, and methanol; the fruit powders were further extracted with different mixtures of solvent at 30 °C for 20 h. As shown in Table 5.1, methanol : ethanol (1:1) mixture showed significantly the highest amounts of TP (50.0 ± 0.5 mg GAE/g powder), TF (8.3 ± 0.2 mg CE/g powder), DRS (74.4 ± 0.7 mg AAE/g powder) and TA (27.4 ± 0.4 mg AAE/g powder). Followed by methanol : ethanol (1:1), the average extraction efficiencies of the other solvent mixtures for TP, TF, DRS and TA were: methanol : distilled water (1:1) = ethanol : distilled water (1:1) > methanol : ethanol : distilled water (2:2:1) > methanol : distilled water (4:1) = ethanol : distilled water (4:1). So from the above findings, as methanol : ethanol (1:1) mixture showed the best extraction efficiencies than ethanol, methanol or other solvent mixtures at 30 °C, it was selected as the most excellent extraction solvent and 30 °C as the suitable temperature for the fruits.

5.2 Effects of pH on extraction

To know the effects of pH on the extraction of polyphenols, flavonoids, DPPH radical scavengers and total antioxidants, powder of *S. apetala* fruits were extracted with the best solvent [methanol : ethanol (1:1) mixture] and temperature (30 °C) at various pH values, ranging 1, 2, 3, 4, 5, 7 and 9, for 20 h. Figure 5.1 showed that the increase in pH (from 1 to 3) led to higher yields of total phenolics, flavonoids, DPPH radical scavengers and total antioxidants. But the extraction efficiencies decreased gradually as the pH values increased. Statistical analysis clearly indicated that the pH significantly affected the yields of TP, TF, DRS and TA of the extracts. At pH 3, the highest amounts of TP, TF, DRS and TA were 50.2 ± 0.6 mg GAE/g powder, 8.3 ± 0.1 mg CE/g powder, 83.2 ± 1.4 mg AAE/g powder and 33.5 ± 0.1 mg AAE/g powder, respectively. Thus pH 3 was preferred as the appropriate one for extracting polyphenols and antioxidants from the fruits.

5.3 Effects of preextraction heating on extraction

Since numerous treatments like heating before extraction could liberate the bound polyphenols by cleaving esterified bond, the effects of preextraction heat treatment with different heating times and temperatures for extraction optimization were also determined. The effects of preextraction heat treatment at different heating temperatures (30, 60, 90, 120, 150 or 180 °C) for 1 h on TP, TF, DRS and TA were represented in Figure 5.2, where after heating of the fruit powders, extraction was done with methanol : ethanol (1:1) mixture, at pH 3 and 30 °C for 20 h. Figure 5.2 showed that the TP of the extracts significantly increased from 40.7 ± 1.6 to 53.8 ± 0.7 mg GAE/g powder after being heated from 30 to 90 °C. However, further heating caused a gradual decrease. Similar effects were found in case of TF, DRS and TA of the extracts. Nevertheless, 1 h preheating at 90 °C or 120 °C gave comparatively similar results for TP (53.8 ± 0.7 or 51.9 ± 0.5 mg GAE/g powder), TF (9.2 ±

Table 5.1: Effects of different solvents and temperatures on the extraction of total polyphenols, flavonoids, DPPH radical scavengers and total antioxidants from the fruits of *Sonneratia apetala* (Buch.-Ham.).

Solvent	Tempera-ture (°C)	Total polyphenols (mg GAE/g powder)	Flavonoids (mg CE/g powder)	DPPH scavengers		Total antioxidants (mg AAE/g powder)
				(mg AAE /g powder)	(%DPPH scavenging /100 µg powder)	
Acetone	10	10.3 ± 0.4^{a}	2.8 ± 0.1^{a}	24.6 ± 0.2^{a}	15.2 ± 0.1^{a}	5.2 ± 0.3^{a}
	30	16.5 ± 0.4^{b}	3.8 ± 0.4^{b}	27.5 ± 0.6^{a}	17.0 ± 0.3^{a}	6.5 ± 0.3^{b}
	40	22.1 ± 0.8^{c}	4.7 ± 0.3^{c}	41.0 ± 0.5^{b}	25.4 ± 0.3^{b}	10.6 ± 0.5^{c}
	55	23.5 ± 0.9^{c}	4.9 ± 0.1^{c}	51.3 ± 0.2^{c}	31.8 ± 0.2^{c}	12.5 ± 0.6^{c}
Methanol	10	39.0 ± 0.6^{a}	8.9 ± 0.5^{a}	70.9 ± 0.8^{a}	43.9 ± 0.5^{a}	21.7 ± 0.9^{a}
	30	45.4 ± 0.4^{b}	11.1 ± 0.4^{b}	73.7 ± 0.3^{a}	45.6 ± 0.2^{a}	23.2 ± 0.5^{a}
	40	35.0 ± 1.3^{c}	9.6 ± 0.5^{a}	49.3 ± 0.8^{b}	30.5 ± 0.5^{b}	16.0 ± 0.4^{b}
	55	35.2 ± 2.0^{c}	9.6 ± 0.1^{a}	55.7 ± 0.8^{b}	34.5 ± 0.5^{b}	17.5 ± 1.5^{b}
Ethanol	10	38.5 ± 1.7^{a}	8.9 ± 0.1^{a}	70.9 ± 0.4^{a}	43.9 ± 0.3^{a}	21.5 ± 0.9^{a}
	30	44.1 ± 0.3^{b}	10.2 ± 0.1^{b}	73.2 ± 0.9^{b}	45.4 ± 0.6^{b}	23.8 ± 1.0^{b}
	40	42.5 ± 1.4^{c}	9.7 ± 0.5^{a}	64.0 ± 1.0^{c}	39.6 ± 0.7^{c}	20.1 ± 2.2^{b}
	55	40.5 ± 1.3^{c}	7.6 ± 0.2^{c}	64.4 ± 0.1^{c}	39.9 ± 0.04^{c}	17.7 ± 0.2^{c}
	70	16.0 ± 0.2^{d}	6.6 ± 0.5^{c}	62.2 ± 1.6^{d}	20.4 ± 0.5^{d}	16.6 ± 0.2^{c}
DW	10	23.3 ± 1.3^{a}	3.5 ± 0.1^{a}	53.5 ± 0.9^{a}	9.8 ± 0.2^{a}	15.4 ± 1.5^{a}
	30	25.0 ± 0.7^{a}	4.2 ± 0.04^{b}	56.7 ± 0.3^{b}	10.4 ± 0.1^{b}	16.1 ± 1.9^{a}
	40	23.1 ± 1.3^{a}	4.5 ± 0.5^{b}	54.1 ± 0.1^{a}	9.9 ± 0.01^{a}	15.6 ± 1.8^{a}
	55	24.3 ± 0.7^{a}	4.0 ± 0.1^{ab}	55.4 ± 0.3^{b}	10.2 ± 0.1^{b}	15.2 ± 1.1^{a}
	70	22.7 ± 0.3^{a}	4.2 ± 0.3^{b}	47.6 ± 1.1^{c}	8.8 ± 0.2^{c}	9.2 ± 0.3^{b}
M : E (1:1)	30	50.0 ± 0.5^{a}	8.3 ± 0.2^{a}	74.4 ± 0.7^{a}	48.7 ± 0.5^{a}	27.4 ± 0.4^{a}
M : DW (1:1)	30	48.6 ± 0.6^{b}	5.3 ± 0.5^{b}	61.8 ± 0.7^{b}	40.4 ± 0.5^{b}	25.5 ± 0.1^{b}
M : DW (4:1)	30	41.2 ± 0.7^{c}	4.7 ± 0.1^{c}	68.4 ± 0.8^{c}	44.7 ± 0.5^{c}	24.5 ± 0.2^{c}
E : DW (1:1)	30	48.7 ± 0.5^{b}	5.0 ± 0.4^{b}	63.8 ± 1.7^{d}	46.6 ± 1.1^{d}	25.6 ± 0.1^{b}
E : DW (4:1)	30	39.2 ± 1.5^{c}	4.5 ± 0.3^{c}	65.4 ± 0.1^{b}	40.2 ± 0.1^{b}	23.1 ± 0.4^{d}
M : E : DW (2:2:1)	30	44.3 ± 0.2^{d}	5.4 ± 0.1^{b}	69.2 ± 0.5^{c}	45.2 ± 0.3^{c}	24.5 ± 0.3^{c}

Here, M - Methanol, E - Ethanol, DW - Distilled water.

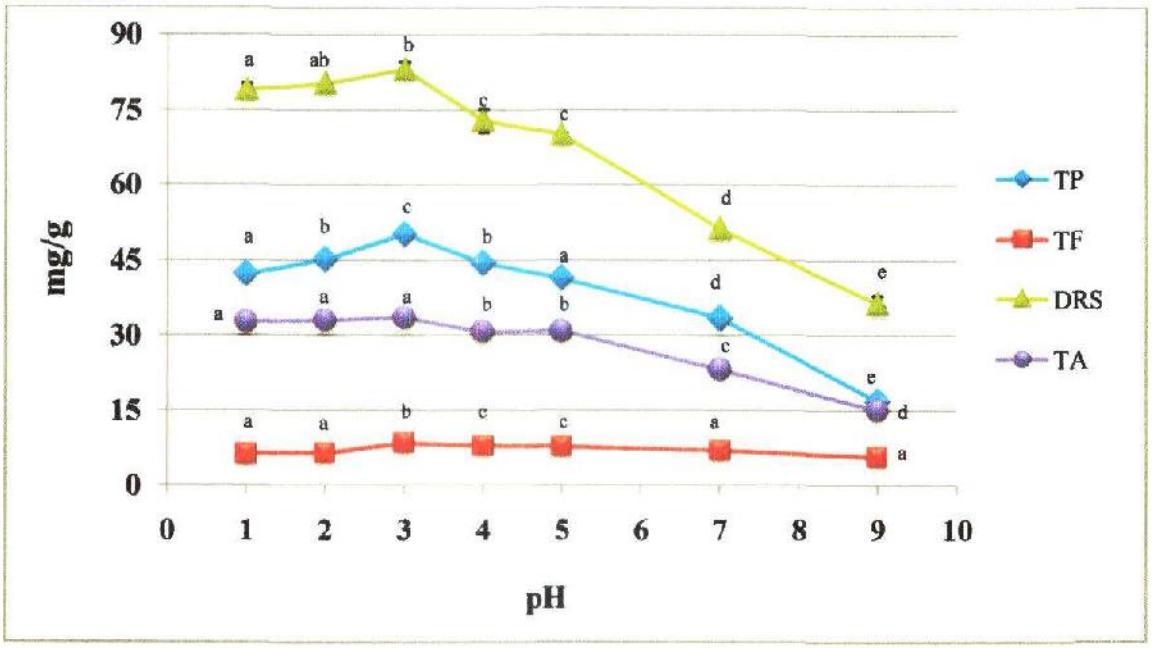

Figure 5.1: Total polyphenols (TP, mg GAE/g powder), flavonoids (TF, mg CE/g powder), DPPH radical scavengers (DRS, mg AAE/g powder) and total antioxidants (TA, mg AAE/g powder) of the fruit of *S. apetala* extracted with different pH at 30 °C for 20 h. Here, GAE- gallic acid equivalents, CE- catechin equivalents and AAE- ascorbic acid equivalents.

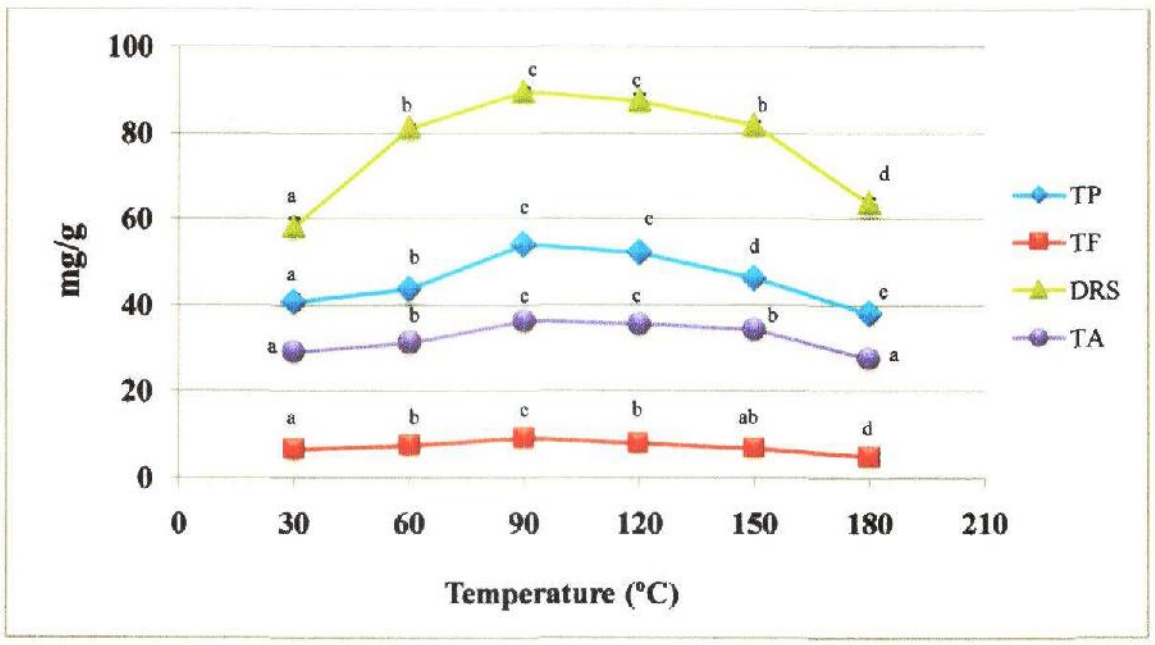

Figure 5.2: Effects of various preextraction heating temperatures for 1 h on the extraction of total polyphenos (TP, mg GAE/g), flavonoids (TF, mg CE/g), DPPH radical scavengers (DRS, mg AAE/g) and total antioxidants (TA, mg AAE/g) from the powder of *S. apetala* fruits.

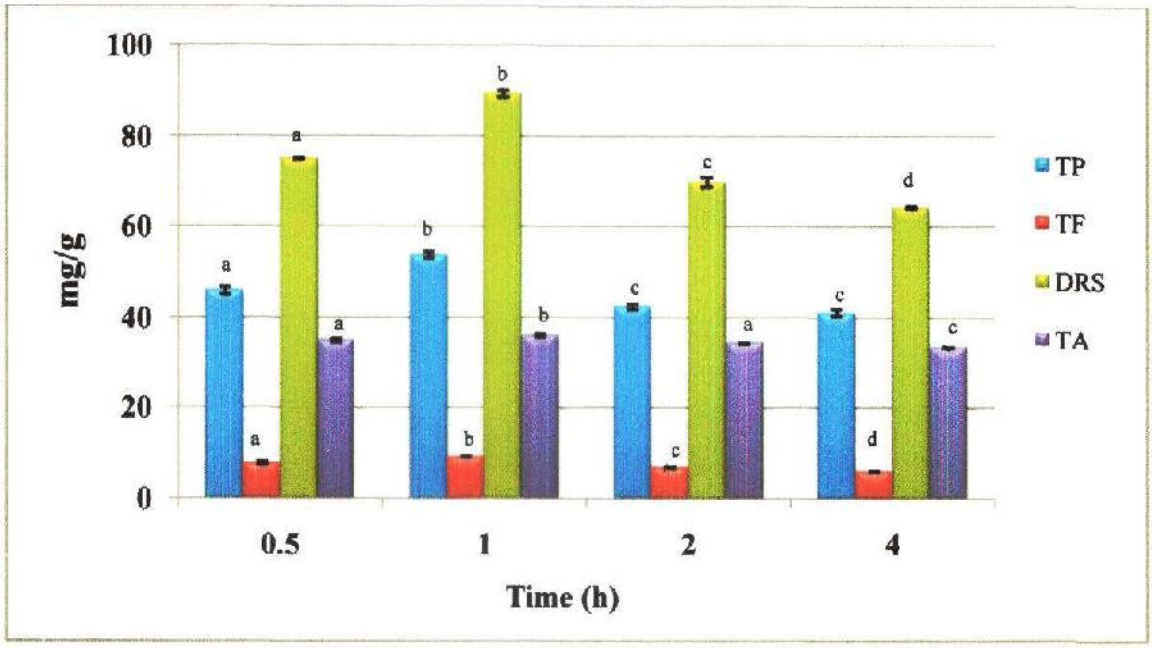

Figure 5.3: Effects of the different preextraction heating times on the extraction of total polyphenols (TP, mg GAE/g), flavonoids (TF, mg CE/g), DPPH radical scavengers (DRS, mg AAE/g) and total antioxidants (TA, mg AAE/g) from the powder of *S. apetala* fruits at 90 °C.

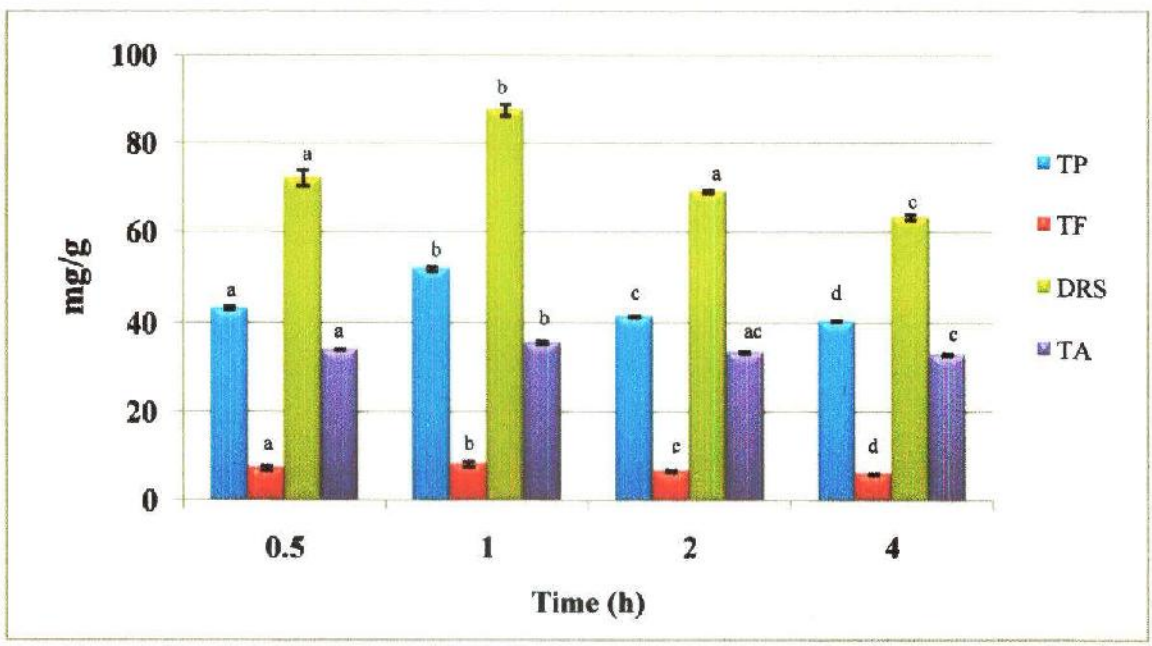

Figure 5.4: Effects of the different preextraction heating times on the extraction of total polyphenols (TP, mg GAE/g), flavonoids (TF, mg CE/g), DPPH radical scavengers (DRS, mg AAE/g) and total antioxidants (TA, mg AAE/g) from the powder of *S. apetala* fruits at 120 °C.

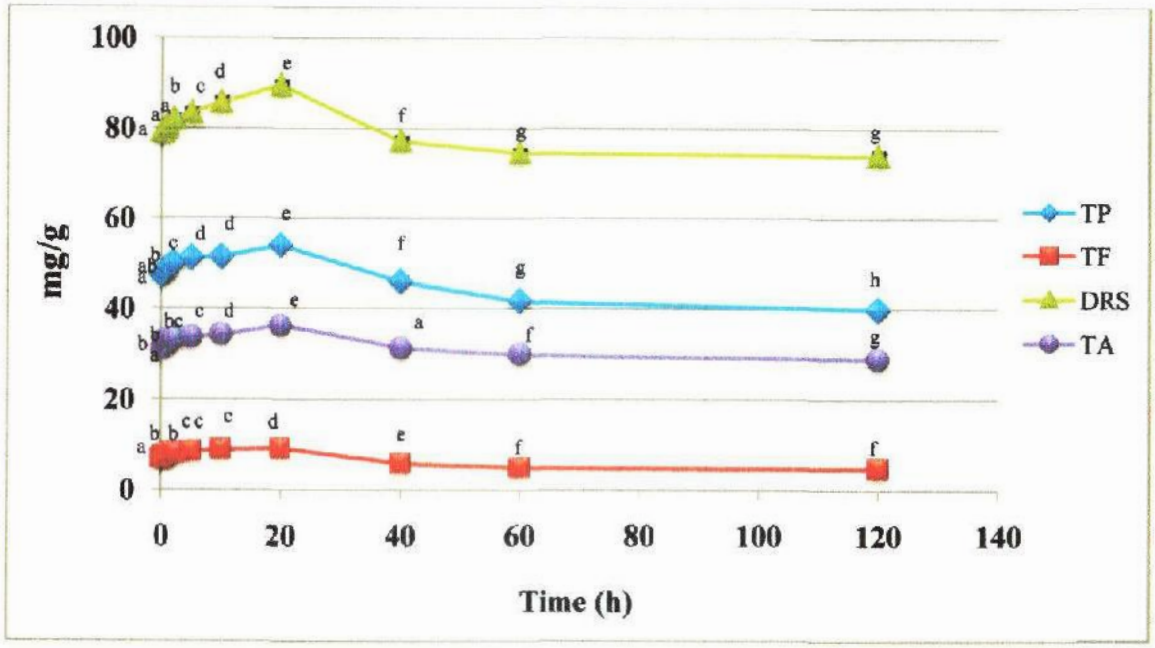

Figure 5.5: Effects of several extraction times on optimizing the extraction of total polyphenols (TP; mg GAE/g), flavonoids (TF; mg CE/g), DPPH radical scavengers (DRS; mg AAE/g) and total antioxidants (TA; mg AAE/g) from the powder of *S. apetala* fruits at 90 °C.

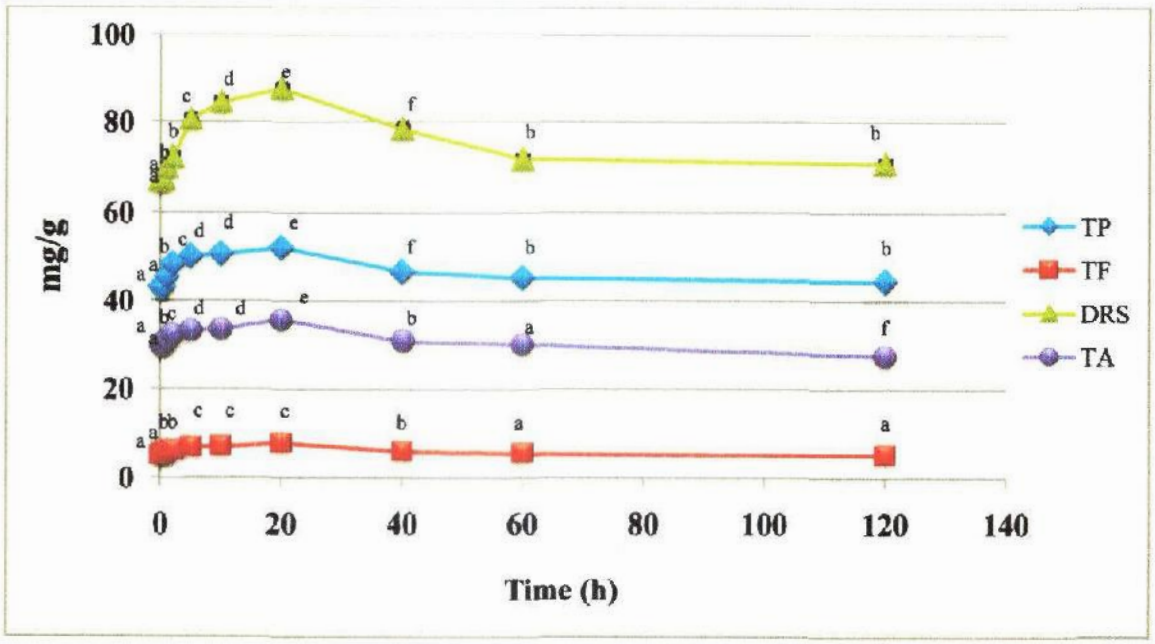

Figure 5.6: Effects of several extraction times on optimizing the extraction of total polyphenols (TP; mg GAE/g), flavonoids (TF; mg CE/g), DPPH radical scavengers (DRS; mg AAE/g) and total antioxidants (TA; mg AAE/g) from the powder of *S. apetala* fruits at 120 °C.

Chapter Six

DISCUSSION

DISCUSSION

Fruits and vegetables that have medicinal activity containing bioactive compounds are known to possess a variety of antioxidant properties (Bandaranayake, 1998), which act as natural protective agents against the oxidative stress generated in the human body. Among these bioactive antioxidant compounds, polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites that are an integral part of the human diet and it is estimated that we consume about 1 g of polyphenols per day (Scalbert and Williamson, 2000). To evaluate these polyphenols and antioxidant activities of natural compounds in food or biological systems, several methods are being used. One important method of antioxidant determination is the Folin–Ciocalteu assay that has been used many years for measuring the total polyphenol content in natural products, whose basic mechanism is an oxidation/reduction reaction (Prior et al., 2005). DPPH radical scavenging activity is another common method, where the free radical (DPPH$^{\bullet}$) is used to assess *in vitro* antioxidant activity. The reduction of this radical by hydrogen-donating antioxidant is monitored through the decrease of their optical density at long wavelength, while the results indicated that the extracts by hydrogen and/or electron donation, might prevent reactive radical species from reaching biomolecules such as lipoproteins, DNA, amino acids, proteins and sugars in susceptible biological and food systems (Halliwell et al., 1995). Total flavonoid content and total antioxidant capacity determination are other methods that are also used to measure flavonoids and total antioxidants, respectively. But before evaluating antioxidant assays, polyphenols, flavonoids and antioxidants need to be extracted with different solvents. Choosing the appropriate solvent affects the amount and rate of extracted polyphenols (Xu and Chang, 2007). According to Michiels et al. (2012), the properties of extracting solvents significantly affected the measured total phenolics content (± 25% variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables. The solubility of phenolics is also

governed by the polarity of the used solvents- higher the polarity, better the solubility of phenolic compounds (Naczk and Shahidi, 2006). Due to the phenolic nature, polyphenols are relatively hydrophilic; therefore they are extracted using water, polar organic solvents such as acetone, ethanol, ethyl acetate, methanol etc. Predominantly, methanol and ethanol have been found to be more competent in extraction of polyphenols and flavonoids (Bazykina et al., 2002; Metivier et al., 1980). So in the present study all the above mentioned *in vitro* methods were used and the results indicated that the extracts obtained from the fruits of *S. apetala* have remarkable polyphenols and antioxidant activities, the extent of which largely depends on the different extraction conditions. There are several principal factors that contribute to the efficiency of extraction, including type of solvent, volume of solvent, pH, temperature, number of extraction, particle size in the sample, heating etc. (Cacace and Mazza, 2003b). Some of these parameters were tested in this work for optimizing extraction of polyphenol and antioxidants from the *S. apetala* fruits.

Choosing extraction solvents is crucial for the complex food samples, as the amount and type of phenolic compounds being extracted will be determined by this. For a long time, different organic solvents, predominantly acetone, ethanol and methanol are most commonly employed in phenolics extraction from plant materials (Naczk and Shahidi, 2004), while different solvents showed distinguishing results for specific samples and phenolic substances. Our results showed that methanol, and ethanol were significantly effective for extracting total polyphenols (TP), flavonoids (TF), DPPH radical scavengers (DRS) and total antioxidants (TA) from the fruits compared to the other tested solvents. This was in accordance to other findings where methanol (Jakopic et al., 2009; Shi et al., 2005; Tsao and Deng, 2004), and ethanol (Lapornik et al., 2005; Spigno et al., 2007) had been found as the best solvents. The reason behind this is the polarity of the used solvent and solubility of phenolic compounds in them (Turkmen et al., 2006). We found that acetone showed lower extraction efficiencies

than methanol and ethanol due to it's less polarity. It is also well known that excessively high polar solvents like water do not give good extraction (Liu et al., 2000). In our experiment, water was found as less effective solvent, which is quite illustrative in comparison to the findings of Al-Farsi and Lee (2008). Chirinos et al. (2007) reported that water alone could yield highly impure extracts (containing organic acids, sugars, soluble proteins etc.), thus interfering the phenolic identification and quantification. Another reason behind our findings might be the enzyme polyphenol oxidase (PPO), which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive (Zhang et al., 2001).

Sometimes pure solvents might be incapable for the extraction of the targeted compounds, so the fruit powders were further extracted with different mixture of solvents. Among the different solvent mixtures, the best proved solvent for extracting polyphenols and antioxidants from the fruits was methanol : ethanol (1:1) mixture (Table 5.1), which indicated that our sample might have moderately polar profile. Similarly, another report also found that solvent mixture gave similar or better results in respect to one solvent (Chirinos et al., 2007). The use of water with other organic solvents also creates moderately polar medium thus ensuring better extraction (Lapornik et al., 2005). Like other findings aqueous methanol, aqueous ethanol and their mixtures also showed better results (Tabart et al., 2007) than single solvent.

As increase in temperature impacts the solubility, mass-transfer rate, and stability of phenolic compounds (Spigno et al., 2007) during extraction, the effects of temperature(s) on extraction efficiency was also investigated. However, it should be taken into account that some important biological active substances, such as total polyphenols (Jokic et al., 2010) and some flavonoids (Biesaga and Pyrzynska, 2013) are damaged at high extraction temperatures. In this study, ethanol, and methanol extracted the higher amounts of TP at 30 °C; while further increase in temperatures reduced TP contents. The effects of temperature on TF, DRS

and TA showed a similar trend like TP. The best solvent, methanol : ethanol (1:1) mixture also showed the highest TP (50.0 ± 0.5 mg GAE/g powder), TF (8.3 ± 0.2 mg CE/g powder), DRS (74.4 ± 0.7 mg AAE/g powder) and TA (27.4 ± 0.4mg AAE/g powder) at 30 °C (Table 1). Similar to our finding, many authors reported that extraction efficiencies of polyphenols and antioxidants were better at low temperatures. For instance, Bouterfas et al. (2014) found a maximum of 25 °C for the extraction of TP (293.34 ± 14.60 mg GAE/g dry weight) and TF (79.52 ± 0.55 mg CE/g dry weigh) from white horehound leaves. Kua et al. (2015) reported that the highest amount of free phenolics (500.9 µg GAE/g of dry weight) was extracted at 25 °C extraction temperature. Another report mentioned that 40 °C temperature was effective for the extraction of major polyphenols (sinensetin and rosmarinic acid) from *Orthosiphon stamineus* leaf (Akowuah and Zhari, 2010). According to Cacace and Mazza (2003a), 30-35 °C were the most optimal temperatures for anthocyanins extraction from ribes. Similarly, Herodez et al. (2003) indicated 20 °C and 0 °C for the highest yields of ethanolic extraction of carnosic acid; ursolic acid and oleanolic acid, respectively, from balm leaves.

Unlike our sample, many authors reported that polyphenols and antioxidants extraction increased at very high extraction temperatures (50 °C or more) (Chen et al., 2013; Spigno et al., 2007; Wissam et al., 2012). However, it should be noticed that though increasing extraction temperature have positive effects on extraction, exceeding certain values might promote possible concurrent decomposition of phenolic compounds that were already mobilized at lower temperatures. At elevated temperatures, remaining phenolic compounds in the plant matrix could even be broken down (Bouterfas et al., 2014). Moreover, PPO enzyme is more active at higher temperatures (> 30 °C), which might be the cause of degrading polyphenols and antioxidants by oxidation (Mizobutsi et al., 2010). So, from the results it can be confirmed that in case of our fruits, mild temperature (30 °C) was able to soften the plant tissues, to weaken the cell wall integrity, and thus favored the release of bound phenolic

compounds. And after 30 °C, increase in temperature caused degradation of polyphenols and antioxidants due to thermal breakdown, oxidation etc.

Like other conditions, pH of the extraction solvent is an indispensable parameter for extraction optimization. In our experiments, when pH was combined along with the best solvent and temperature, the extraction efficiency increased significantly. As the pH increased from 1 to 3, the extraction of polyphenols and antioxidants also increased in parallel, after which it started to decrease. Our results showed that the maximum yield of TP, TF, DRS and TA were achieved at pH 3, where the values were 50.2 ± 0.6 mg GAE/g powder, 8.3 ± 0.1 mg CE/g powder, 83.2 ± 1.4 mg AAE/g powder and 33.5 ± 0.1 mg AAE/g powder, respectively. Our findings were fairly clarifying with others. For example, Tabart et al. (2007) found that maximum yields of total phenolics (> 140 mg chlorogenic acid equivalents/g dry weight) and antioxidant activities from black currant leaves were obtained at pH 3. Related results were also found by Chirinos et al. (2007) (pH 2.11; 22.0 mg GAE/g dry matte), Ruenroengklin et al. (2008) (pH 4; 110 mg GAE/g dry weight) and Wissam et al. (2012) (pH 3.5; 18 % in dry weight), according to whom extraction efficiency increased at acidic pH. The increased extraction yield from the fruits under low pH conditions could be due to the inhibition of the enzymatic oxidation of phenolics. PPO is the main enzyme involved in the oxidation of phenolic compounds whose activity is pH dependent (Dicko et al., 2006). The reaction, called enzymatic browning, occurs readily when the pH is between 5 and 7, while the activity is irreversibly inhibited at pH < 3. Another reason for increased extraction yield might be that in acidic conditions polyphenols stay neutral as the hydroxyl groups are shielded by protonation, thus maintain stability. On the other hand, extraction efficiencies decreased at basic conditions as phenolic compounds easily converted to non-antioxidative quinones (Brooks et al., 1972) or to other metabolic intermediates (Leffler, 1993).

It has been found that the amount of polyphenols present in the plant foods mainly contribute to the antioxidant activities of it (Velioglu et al., 1998) and many of them are most frequently present as a covalently bound form with insoluble polymer. Since heat treatment before extraction could liberate them, the effect of different preextraction heating temperatures and times must be considered on the polyphenols and antioxidants extraction from the fruits. In our experiments, different heating temperatures, ranging 30, 60, 90, 120, 150 and 180 °C, for 1 h and times, ranging 0.5, 1, 2 and 4 h (at 90 and 120 °C) were evaluated. It was found that increase in heating temperatures from 30 to 90 °C led to significantly higher yields of total phenolics, flavonoids, DPPH radical scavengers and total antioxidants. But the extraction efficiencies decreased gradually as the heating temperatures increased. The maximum values of TP (53.8 ± 0.7 mg GAE/g powder), TF (9.2 ± 0.1 mg CE/g powder), DRS (89.6 ± 0.8 mg AAE/g powder) and TA (36.2 ± 0.4 mg AAE/g powder) were found after heating at 90 °C for 1 h (Figure 2), though heating at 90 and 120 °C for 1 h showed no significant differences between them. Similar result was found by Kim et al. (2006) who heated grape seeds at four different temperatures and found that thermal treatment increased the antioxidant activity of the extracts up to a certain value. Choi et al. (2006), after investigating the influence of heat treatment on shiitake mushroom, revealed that phenolic contents and antioxidant activity in the extracts increased as the heating temperature and time increased. They found maximum polyphenols (54.6 mg GAE/100 g wet weight) at 120 °C for 30 min heating and flavonoids (2.5 mg CE/100 g wet weight) at 100 °C for 30 min heating. According to Xu et al. (2007), the free fraction of phenolic acids increased prior to heating at 90 °C for 30 min in case of citrus peel extracts. Similarly, Yen and Hung (2000) found that the TP content of Hsian-tsao increased from 185 to 273 mg/g extracts with an increase in heating time from 0.5 to 2 h in boiling water (100 °C). The reasons behind the increase of polyphenols and antioxidants in the fruits after heating might be many, such as: (1) heat treatment might produce changes in the

extractability due to the disruption of the cell wall thus bound polyphenolic and flavonoid compounds may be released more easily (Jeong et al., 2004); (2) deactivation of endogenous oxidative enzymes at very high heating temperatures (> 80 °C), thus preventing loss of polyphenols by enzymatic oxidation (Nicoli et al., 1999); (3) formation of Maillard Reaction Products (MRPs), having great antioxidant activity (Nicoli et al., 1997), often exerting in a chain-breaking and DPPH type mechanism (Manzocco et al., 2001) etc. In contrast, reduction in polyphenols and antioxidants might be due to the formation of MRPs with pro-oxidant activities (Nicoli et al., 1999) and thermal degradation of the extracted compounds (Larrauri et al., 1997), at low heating temperatures and very high heating temperatures (> 100 °C), respectively.

Another critical factor in optimizing extraction of the polyphenols and antioxidants is the extraction time. Depending on the phenolic compounds present in samples, this might be as short as few minutes or long up to 24 hours or more according to various literatures (Hismath et al., 2011). In this study, the extraction time ranged from 0 to 120 h and showed a significant effect on the extraction. The extraction efficiency increased with increasing time, from 0 to 20 h. The highest values of TP (53.8 ± 0.7 mg GAE/g powder), TF (9.2 ± 0.1 mg CE/g powder), DRS (89.6 ± 0.8 mg AAE/g powder) and TA (36.2 ± 0.4 mg AAE/g powder) were recorded at 20 h (90 °C, 1 h preheat). Our results agreed with the findings of Lapornik et al. (2005), according to which the yield of polyphenols in grape alcohol extracts significantly increased from 12 to 24 h at room temperature. Another study that is in accordance to ours stated that the best extraction time of TP was estimated to 18 hours for black tea, *Camellia sinensis* (Turkmen et al., 2006). Similarly, many researchers found that as the extraction time increased, extraction efficiency also increased (Pinelo et al., 2005a; Spigno and De Faveri, 2007). But in case of our fruits, beyond 20 h, the extraction efficiency started to decline with further increase in extraction time and reached a plateau phase at 60 h to 120 h. This

surveillance might be well explained by Fick's second law of diffusion, according to which final equilibrium will be achieved between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time during extraction. Therefore, an extremely elevated extraction time beyond this certain period might not be useful to extract more phenolic antioxidants (Silva et al., 2007). Moreover, prolonged extraction time might lead to loss of polyphenols due to oxidation and the products might be polymerized into insoluble compounds (Chirinos et al., 2007; Naczk and Shahidi, 2006). Thus, from a recovery point of view it would be more convenient to work at lower temperature for a certain long time, as at a very small extraction time the compounds is not completed extracted as well as at enormously long extraction time phenolic antioxidants become degraded.

Finally, the optimum conditions for the extraction of polyphenols and antioxidants from the fruits of *S. apetala* were found to be preextraction heating at 90 °C for 1 h, methanol : ethanol (1:1) mixture as the best solvent, pH 3, extraction temperature 30 °C and extraction time 20 h. Under these optimized extraction conditions, the TP was 53.8 ± 0.7 mg GAE/g powder. Our results were comparatively higher than the earlier observations of Chirinos et al. (2007) in mashua tubers, Hismath et al. (2011) in neem and Bucic-Kojic et al. (2011) in fig fruits, according to which the TP at optimum conditions were 18.7 mg GAE/g dry matter, 4661.17 mg GAE/100 g dry weight and 4.7 mg GAE/g dry basis, respectively. At the optimum conditions, the TP extracted from the fruits was similar to the TP of henna stems (5554.15 ± 73.04 mg GAE/100 g dry weight) (Tan et al., 2013). The TP at optimum conditions was lower than the findings of Bouterfas et al. (2014) in white horehound (293.34 ± 14.60 mg GAE/g dry weight). The reasons behind the variability of TP under optimum conditions might be: (1) different solvents, temperatures and times used for extraction, (2) different samples having dissimilar genetic constituents, geographic locations, seasonal variations, post-harvest handlings etc (Solar et al., 2006). At the optimum extraction conditions, the

experimental values of TF, DRS and TA were 9.2 ± 0.1 mg CE/g powder, 89.6 ± 0.8 mg AAE/g powder and 36.2 ± 0.4 mg AAE/g powder, respectively. After optimizing extraction conditions, the weight of the dried extracts extracted at normal and optimized conditions for comparison were 29.31% (1.47 ± 0.08 g/5 g) and 30.34% (1.52 ± 0.09 g/5 g) of the dry weight of powder, respectively. Our results were higher than those found by Hossain et al. (2013) (11.2% with 99% methanol in *S. apetala*); whereas, lower than the finding of Do et al. (2013) (33.66% with 75% aqueous acetone in *Limnophila aromatic*). The reason might be that under the same extraction time and temperature, solvent polarity and composition of sample are known as the most important parameters affecting extraction yield (Do et al., 2013). In case of our findings, increased yield at optimized condition might be due to high temperature heating, which can decrease the cell barrier by weakening integrity of the cell wall and membrane (Mohamad et al., 2013). Thus, the solvent can easily get in contact with phytochemical compounds and affect the extraction yield. However, compounds other than phenolics may have been extracted and contribute to higher yield.

According to Rao (1996), optimization is the act of obtaining the best result possible or the effort for achieving the optimal solution under a given set of circumstances, while extraction means separation of soluble phenolic compounds by diffusion from a solid matrix (plant tissue) using a liquid matrix (solvent). From the above findings it can be clearly visualized that it had been possible to optimize the extraction of polyphenols and antioxidants from the fruits of *S. apetala* in this experiments and under the optimum conditions, highest amount of TP, TF, DRS and TA had been found. It is a very big challenge for the food, pharmaceuticals and other industries to produce a new generation of products with enriched content of natural antioxidants. Thus, *S. apetala* fruits could be a great and cheap source of polyphenols and antioxidants in near future, if maximum amounts of polyphenols and antioxidants could be extracted by an optimum, cost efficient and safer extraction method.

Chapter Seven

CONCLUSION

CONCLUSION

From these results it could be concluded that TP, TF, DRS and TA extracted from the fruits of *S. apetala* were significantly affected by the type of solvents, extraction temperatures, pH, and extraction times. Preextraction heat treatment (heating temperatures and times) also significantly affected the extraction of TP, TF, DRS and TA. Optimal extraction conditions were found to be preextraction heating at 90 °C for 1 h, methanol : ethanol (1:1) mixture as the best solvent, pH 3, extraction temperature 30 °C and extraction time 20 h, at which the highest amounts of TP, TF, DRS and TA were extracted. Other experimental conditions such as the solid-to-solvent ratio, the number of extractions, the nature of the extraction method etc. would also be considered in future. Further detailed chemical studies for the identification and purification, followed by pharmacological and toxicological assessment are still required to examine the specific compounds responsible for the high polyphenol content and antioxidant capacity of the extracts, under the optimum conditions. This is the first report demonstrating the optimization of extraction of polyphenols and antioxidants from the *S. apetala* fruits and these results are the first steps for further large scale implementation process. The fruits of *S. apetala* contain high amounts of polyphenols and antioxidants, which could be optimally extracted by these conditions. Thus, the results of this report could contribute to enhancing the utilization of the fruits of *S. apetala* in food, pharmaceutical, nutraceutical, cosmetic industries, and so on.

Chapter Eight

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APPENDIX

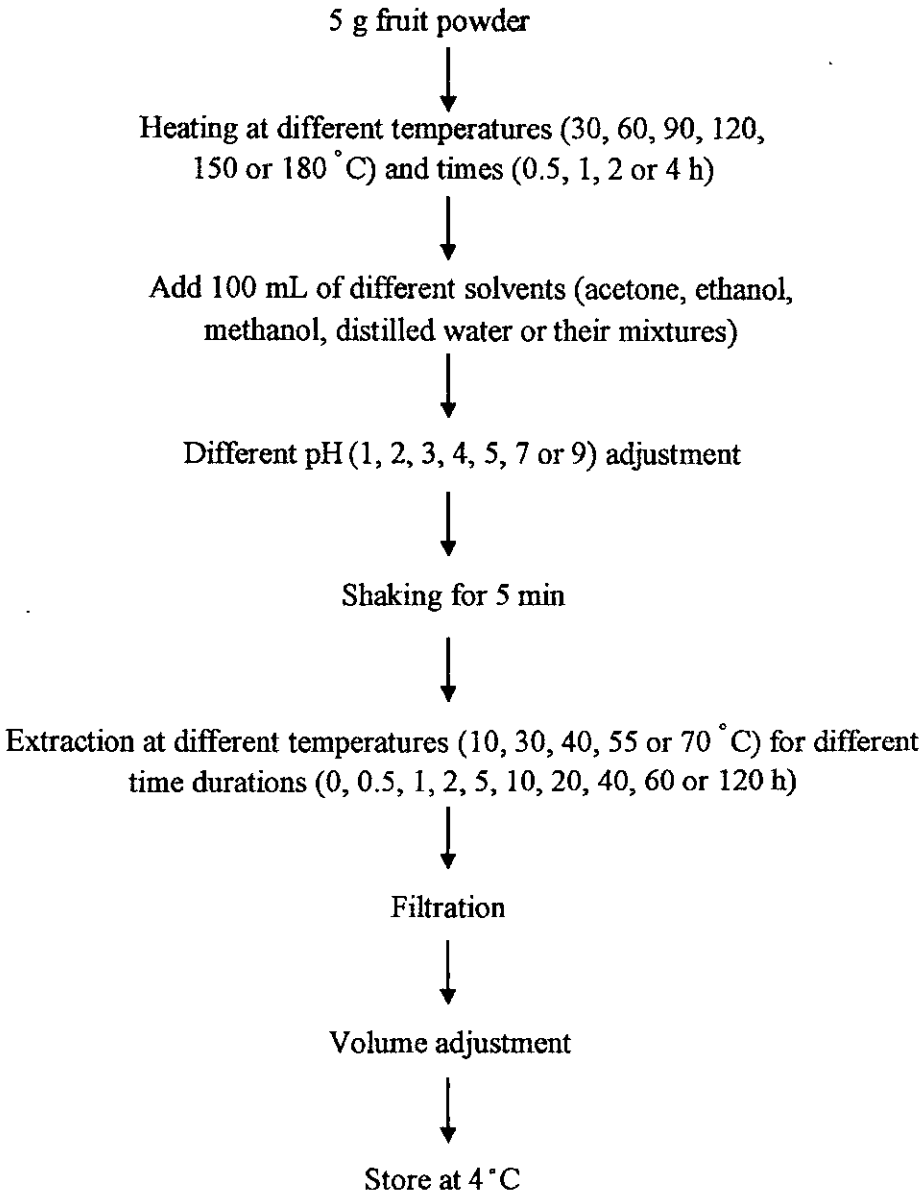

Figure 9.1: Flow chart of overall extraction process.