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RESEARCH ARTICLE

Quantitative measurement of bioactive compounds from leaves of *Syzygium samarangense* with antioxidant efficacy

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Abstract: Botanicals have been explored for their multitude of pharmaceutical and therapeutic potential. In this study, crude methanolic extract (MSSL) and organic soluble fractions of *Syzygium samarangense* leaves were evaluated for antioxidant activity using *in vitro* and *in vivo* models. To evaluate the antioxidant activity, total antioxidant capacity, free radical scavenging ability (DPPH) and reducing power, assays were conducted *in vitro.* Lipid peroxidation and activities of different antioxidant enzymes were measured *in vivo*. Quantitative phytochemical analyses such as the measurement of total phenolic, flavonoid, flavonol, and proanthocyanide contents were also evaluated. The ethyl acetate fraction (ESSL) showed significant effects in DPPH assay (IC_{50} = 38.84 ± 0.08 µg/mL) and reducing power assay. The effect of ESSL was found to be concentration-dependent with remarkable antioxidant activity as compared to other tested samples. In addition, administration of the extract/fractions (200 and 400 mg/kg body weight) to male Wistar rats increased the percentage of inhibition of reduced glutathione, superoxide dismutase and catalase significantly ($p < 0.05$). The level of lipid peroxidation decreased at 400 mg/kg body weight after seven days in hepatotoxic rats. The pharmacological findings of this study clearly indicate that extracts and/or fractions of *Syzygium samarangense* leave could be a source of natural antioxidants.

Keywords: Antioxidant, flavonoids, phenol, proanthocyanidin, *Syzygium samarangense.*

INTRODUCTION

Syzygium samarangense (Blume) Merr. & L.M. Perry is a plant species in the family Myrtaceae, commonly known as 'Wax Apple' native to the Malay Peninsula and the Andaman and Nicobar Islands (Peter *et al*., 2011). This plant is cultivated extensively for its fruits in tropical countries. In Bangladesh, it is locally called as '*Jamrul*'. Different parts of *S. samarangense* such as leaves, fruits, root, and bark have been used for the treatment of various diseases including diabetes mellitus, bronchitis, asthma and inflammation syndromes (Gurib-Fakim, 1991). The leaves and seeds of *S. samarangense* have been studied extensively for antimicrobial activities against some specific pathogenic microorganisms such as *Pseudomonas aeruginosa, Klebsiella pneumonia* and *Cryptococcus neoformans* (Chandrasekaran & Vankatesalu, 2004). *S. samarangense* has shown potent free radical scavenging potential, antihyperglycaemic ability, antioxidant, antimutant, and anticancer activities (Mario *et al*., 2008). The leaves of *S. samarangense* contain a large amount of phytochemicals, including ellagitannins, flavanones, flavonol glycosides, proanthocyanidins, anthocyanidins, triterpenoids, chalcones, and volatile terpenoids (Shen *et al*., 2012).

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S. samarangense is cultivated in India for its edible fruits that contains flavonol glycosides as well as epigallocatechin gallate (EGCG), epicatechin 3-*O*-gallate, and samarangenins A and B, which has been reported to have potential antioxidant and anti-inflammatory properties (Harborne & Baxter, 1999; Asif, 2014). The pear-shaped fruits of *S. samarangense* with a subtly sweet taste and aromatic flavour are rich sources of phenolics, flavonoids and several antioxidant compounds (Moneruzzaman *et al*., 2012).

Antioxidants, as food additives, are used worldwide to improve the protection level against oxidative degradation of foods by free radicals (Gulcin *et al*., 2002). From the ancient time, spices have been added to different types of foods not only to improve flavours but also for their antioxidant capacities (Madsen & Bertelsen, 1995). In recent times, studies have been performed to find plant extracts with natural antioxidant properties with less toxic effects. Although various synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been used in food preservation, these substances are harmful for chronic human consumption (Ito *et al*., 1986; Lobo *et al.,* 2010). Some researches have proved their possible toxic characteristics as a major threat for human health and the environment (Ito *et al*., 1986). The consumption of many fruits and vegetables can be beneficial as they are rich in flavonoids and phenolic compounds, which show potent antioxidant effects. Flavonoids include a large variety of compounds with a common diphenyl propane structure ($C_6C_3C_6$) with different phases of hydroxylation, oxidation, and substitution. These compounds are also called polyphenols, which commonly work as glycosides in plants (Pietta, 2000). The high antioxidant activity of anthocyanin is attributed to its complicated structure with easy donation capacity of a H-atom from the aromatic hydroxyl group that improves the capacity to support the impaired electron *via* delocalisation around the pi-electron system. Furthermore, in case of inhibition of lipoprotein oxidation and platelet aggregation, anthocyanin works as an effective scavenger of reactive oxygen radicals, and in protection against cardiovascular diseases (Ghiselli *et al*., 1998).

The present study was designed to investigate the antioxidant properties of the methanolic extract (MSSL) and organic soluble fractions of *S. samarangense* leaves in various *in vitro* and *in vivo* models. The outcome of this research provides some specific insights on the significant use of *S. samarangense* as a natural source of antioxidant agents.

METHODOLOGY

Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose and α-tocopherol were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid, ethylene-diamine-tetra-acetic acid (EDTA), Folin-Ciocalteu's phenol reagent and hydrogen peroxide were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Thiobarbituric acid (TBA) was purchased from Sigma Company (St. Louis, MO, USA). All the other solvents and chemicals were of analytical grade. All the experiments were performed at room temperature of 28 °C.

Preparation of *S. samarangense* extracts and fractions

Leaves of *S. samarangense* were dried in an oven at 37 ± 2 °C and powdered with a household grinder, passed through a no. 40 sieve and stored in an air tight container. The dried powdered material (1.5 kg) was refluxed with MeOH and placed in a shaking incubator at room temperature for 3 hrs. The filtrate was concentrated to dryness *in vacuo* at 40 °C to render the MeOH extract (MSSL; 490 g). This extract was suspended in H_2O and then successively in CH_2Cl_2 (CSSL, 200 g) and EtOAc (ESSL, 60 g) fractions along with the residue (120 g) present in the aqueous (H_2O) phase (WSSL). The resulting fractions including the aqueous fractions were evaporated to dryness by using a rotary evaporator followed by freeze drying. For further analysis, rest of the dried fractions were stored in brown coloured glass vials (in order to prevent oxidation) at -20 °C.

Experimental animals

For the *in vivo* experiment, male Wistar rats with a mean weight of 170 ± 6.4 g were used. The rats were purchased from Samtaco Korea (Osan, Korea). The animals were fed with a commercial diet (Purina Korea Inc., Seoul, Korea) and water *ad libitum* throughout the experiment. For the *in vivo* animal experiment, all protocols were approved by the institutional animal ethical committee and conducted in accordance with the Principles of Laboratory Animal Care (CCAC, 1993), and the internal guidelines of the Kyunpook National University Animal Ethical Committee were strictly followed. All the animals were acclimated to the laboratory environment for at least 1 wk prior to commencement of the experiment.

Quantitative measurement of major bioactive components of crude extract and solvent fractions

Determination of total phenolic content

Total phenolics were determined by using the Folin-Ciocalteu method as described previously (Palanisamy *et al*., 2008). An aliquot of the sample extract was mixed with 2 mL of Folin-Ciocalteu reagent (1:10 v/v) and 2 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15 s for colour development and the absorbance was measured at 760 nm with a UV-spectrophotometer (Shimadzu, USA). Samples of the extract were evaluated at a final concentration of 0.1 mg/mL. Total phenolic contents were expressed in terms of gallic acid equivalent, GAE ($Y = 0.0086x + 0.0105$, $R^2 = 0.9997$), mg of GAE/g of dry extract.

Determination of total flavonoids

Flavonoids content was determined by the aluminum chloride colourimetric method (Chang *et al*., 2002). To 0.5 mL of the sample, 1.5 mL of methanol, 100 µL of 10 % aluminium chloride, 100 µL of 1 M potassium acetate solution and 2.8 mL of distilled water were added. After 1 h, the samples were incubated for 30 min at room temperature, and the absorbance was measured at 420 nm. The extract samples were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids content was expressed in terms of quercetin equivalent, QAE ($Y = 0.0135x + 0.0085$, $R^2 = 0.9984$), mg of QAE/g of dry extract.

Determination of total flavonols

Total flavonols in the extract samples were estimated using the method by Kumaran and Karunakaran (2007). To 2.0 mL of sample (standard), 2.0 mL of 2 % $AlCl_3$ in ethanol and 3.0 mL of sodium acetate (50 g/L) were added. The absorption at 440 nm was read after 2.5 h at 20 °C. Extract samples were evaluated at a final concentration of 0.1 mg/mL. Total content of flavonols was expressed in terms of quercetin equivalent, QAE ($Y = 0.0255x + 0.0069$, $R^2 = 0.9995$), mg of QAE/g of dry extract.

Determination of total proanthocyanidins

Determination of the proanthocyanidin content was based on the procedure reported by Aiyegoro and Okoh (2010). A volume of 0.5 mL of 0.1 mg/mL extract solution was mixed with 3 mL of 4 % vanillin-methanol solution and 1.5 mL of hydrochloric acid, and the mixture was allowed to stand for 15 min at room temperature. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/mL. Total content of proanthocyanidin was expressed in terms of catechin equivalent, CAE ($Y = 0.567x - 0.024$, $R^2 = 0.9801$), mg of CAE/g of dry extract.

***In vitro* antioxidant activity**

Determination of total antioxidant capacity

Total antioxidant activity of the MeOH extract and several fractions was evaluated by the phosphomolybdenum method according to a procedure reported previously (Prieto *et al*., 1999). The experiment method was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The antioxidant activity was expressed as the number of equivalents of ascorbic acid using the following formula:

Antioxidant activity: $C = (c \times V)/m$

where C is represented as the total antioxidant activity in mg/g of plant leaf extracts in ascorbic acid. c is the concentration of the ascorbic acid established from the calibration curve in mg/mL; V is the volume of the extract in mL and m is the weight of pure plant extract in grams.

Determination of DPPH free radical scavenging activity

Free radical scavenging activity of the extracts/fractions, based on the scavenging activity of the stable DPPH free radical, was determined by a previously described method (Braca *et al*., 2001). Plant extract/fractions (0.1 mL) were added to 3 mL of 0.004 % MeOH solution of DPPH. Absorbance at 517 nm was measured after 30 min and the percentage inhibition activity was calculated from the following formula:

Inhibition (%) $= [(A_0 - A_1) / A_0] \times 100$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard. IC_{50} value was calculated from the equation of the line obtained by plotting a graph of concentration (µg/mL) *versus* percentage inhibition.

Reducing power assay

The reducing power of the extract/ fractions was determined according to the method previously described by Oyaizu (1986). Extracts at different concentrations in 1 mL of 10 % DMSO were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [K Fe (CN)] (1 %), and then the

mixture was incubated at 50 °C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

In vivo antioxidant activity

Animal grouping and extract administration

Twenty male Wistar rats were randomised into 7 groups consisting of 5 in each group. Group 1 served as the control and was given 0.5 mL of saline per day for 7 days with the aid of oropharyngeal cannulae. Group 2 animals served as the hepatotoxic control, treated with CCl_4 in a single dose of 0.5 mL administered orally for 7 days. Group 3 to 6 and 7 were treated like the control except that they received 0.5 mL of the extract corresponding to 200 and 400 mg/kg body weight, and 100 mg/kg of ascorbic acid, respectively. Group 3 to 6 and 7 were given 0.5 mL of CCl_4 on the 7^{th} day after 6 h of extract administration. All the animals from each group were sacrificed by ether anesthesia, 24 hrs after their respective 21 daily doses of the extract and saline. The liver from each animal was excised and rinsed in ice cold 0.25 M sucrose solution. A 10 % (w/v) homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 5,000 rpm for 60 min at 4 °C. The supernatant obtained was used for the estimation of catalase, superoxide dismutase, lipid peroxidation (TBARS) and reduced glutathione.

Lipid peroxidation assay

Lipid peroxidation in the liver was estimated calorimetrically by thiobarbituric acid reactive substances (TBARS) using a modified method (Niehius & Samuelson, 1968). In this method, 0.1 mL of liver homogenate (10 % w/v) was treated with 2 mL of 1:1:1 TBA: TCA: HCl reagent (0.37 % thiobarbituric acid, 15 % trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min and cooled. The amount of malondialdehyde formed in each of the samples was assessed by measuring the absorbance of the clear supernatant at 535 nm against reference blank. Percentage inhibition was calculated using the following equation:

$$\% \text{ lipids inhibition} = \{A_o - A_1\}/A_o \times 100$$

where A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Estimation of reduced glutathione (GSH) level

The GSH level of the liver tissue was determined as described by Ellman *et al*. (1951). The tissue homogenate (in 0.1 M phosphate buffer, pH 7.4) was taken and an equal volume of 20 % trichloroacetic acid (TCA) containing 1 mM EDTA was added to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 2,000 rpm. The supernatant (200 µL) was then transferred to a new set of test tubes and added with 1.8 mL of the Ellman's reagent [5,50 -dithiobis-2- nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1 % of sodium citrate solution]. All the test tubes were made up to the volume of 2 mL. After completion of the total reaction, absorbance was measured at 412 nm against blank and the GSH value was expressed as µg/g of liver tissues.

Estimation of superoxide dismutase (SOD) levels

The method described by McCord and Fridovich (1969) was used. To 50 µL of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by the spectrophotometer. One unit of enzyme activity was 50 % inhibition of the rate of auto-oxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD was expressed as units/mg protein.

Estimation of catalase levels

Catalase activity in erythrocyte lysate can be determined using a previously reported method (Aebi, 1984). 50 µL of the lysate was added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity was measured at 240 nm for 1 min using the spectrophotometer. The molar extinction coefficient of 43.6/M/cm for H_2O_2 was used to determine the catalase activity. One unit of activity was equal to 1 mM of H_2O_2 degraded per min and was expressed as units / mg protein.

Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test of significance. All statistical analyses were performed with Prism 4.0 (GraphPad software Inc., San Diego, CA). $p < 0.05$ was considered to be significant.

RESULTS

Polyphenolic, flavonoid, proanthocyanidin and flavonol compounds

The results of total phenol, total flavonoid, total flavonol and total proanthocyanidin contents in the methanolic extract and organic soluble fractions of *S. samarangense* leaves have been presented in Table 1. The contents of total phenolic, flavonoid, proanthocyanidin and flavonols in MSSL were found to be 100.48 ± 0.21, 111.40 ± 0.23, 9.26 ± 0.45, 46.40 ± 0.70 mg/g, respectively. Soluble solvent fractions isolated from MSSL also showed varied total phenolic and flavonoid contents. Among the fractions, ESSL showed the highest total phenolic content (148.57 ± 0.22 mg/g) followed by WSSL (48.43 ± 0.41 mg/g), and CSSL (38.86 ± 0.15 mg/g). In the case of flavonoid content ESSL again showed the highest flavonoid content (207.40 ± 0.12 mg/g) followed by CSSL (64.40 ± 0.32 mg/g) and WSSL (30.20 ± 0.24 mg/g). The results showed that ESSL (16.87 ± 0.58 mg/g) had the highest content of total proanthocyanidin compared to CSSL (6.81 ± 0.02 mg/g) and WSSL (2.84 ± 0.33mg/g), while the content of total flavonol in the organic fractions decreased in the order of CSSL (71.47 ± 0.47) > ESSL (57.80 ± 0.05) > WSSL (16.00 ± 0.55). Therefore, it can be suggested that among all the solvent soluble fractions, ESSL was potent for the tested compounds.

Table 1: Total amount of phenolic compounds, flavonoids, flavonols, proanthocyanidin and total antioxidant capacity of methanolic extracts and its various organic soluble fractions of *Syzygium samarangense* leaves

Sample	Total phenol mg/g plant extracts (in GAE)[a]	Total flavonoid mg/g plant extracts (in QAE)[b]	Total proanthocyanidin mg/g plant extracts (in CAE)[c]	Total flavonols mg/g plant extracts (in QAE)[b]
MSSL	100.48 ± 0.21[c]	111.40 ± 0.23[d]	9.26 ± 0.45[a]	46.40 ± 0.7[b]
CSSL	38.86 ± 0.15[b]	64.40 ± 0.32[c]	6.81 ± 0.02[a]	71.47 ± 0.47[d]
ESSL	148.57 ± 0.22[c]	207.40 ± 0.12[d]	16.87 ± 0.58[a]	57.80 ± 0.05[b]
WSSL	48.43 ± 0.41[d]	30.20 ± 0.24[c]	2.84 ± 0.33[a]	16.00 ± 0.55[b]

Superscripts in different columns of same row not sharing a common superscript are significantly different at $p < 0.05$ by Duncan's multiple range test. [a] Galic acid equivalents (GAE, mg/g of each extract) for the total phenolic content; [b] quercetin equivalent (QAE, mg/g of each extract) for the total flavonoid and flavonol content; [c] cathecin equivalent (CAE, mg/g of each extract) for the total catechin; MSSL = methanolic extract; CSSL = dichloromethane fraction; ESSL = ethyl acetate fraction; WSSL = water fractions of *Syzygium samarangense* leaves. Values are means ± SD of three experiments.

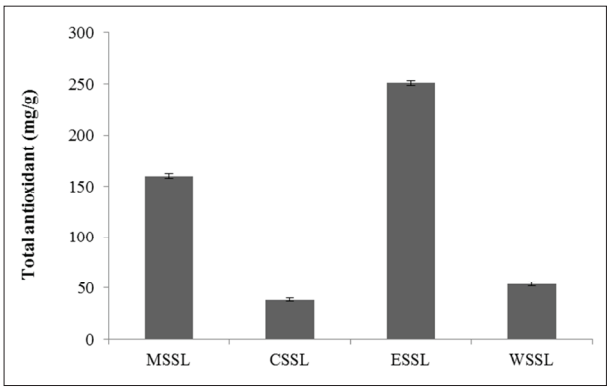

Figure 1: Total antioxidant capacity of extract/fractions of *Syzygium samarangense* leaves is expressed as the number of equivalents of ascorbic acid (ASC, mg/g of each dry extract). Values are means ± SD of three experiments

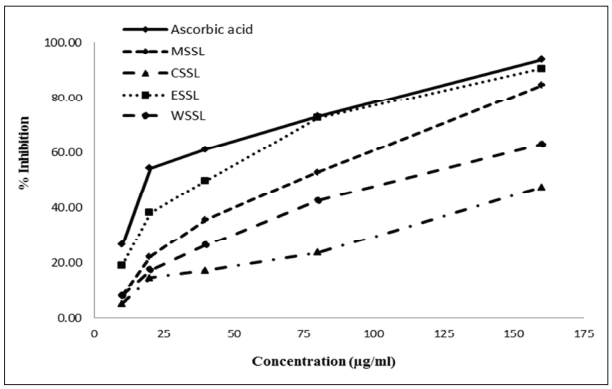

Figure 2: DPPH scavenging activity of extract/fractions of *Syzygium samarangense* leaves. Results are mean ± SEM of three parallel measurements.

Total antioxidant capacity

Total antioxidant capacity of the methanolic extract and organic soluble fractions of *S. samarangense* is given in Figure 1. MSSL showed the total antioxidant activity as 159.99 ± 0.33 mg/g. The total antioxidant capacity of various fractions of *S. samarangense* was also expressed as the number of equivalents of ascorbic acid (ASC, mg/g of each dry extract) and was found to be the highest for ESSL (251.01 ± 0.23 mg/g) followed by WSSL (54.12 ± 0.02 mg/g) and CSSL (38.7 ± 0.45 mg/g) equivalents of ascorbic acid. It was observed that MSSL has higher total antioxidant activity than the other solvent soluble fractions (ESSL, WSSL and CSSL).

DPPH radical scavenging activity

Figure 2 shows the dose-response curve of DPPH radical scavenging activity of various fractions of *S. samarangense* leaves in comparison with ascorbic acid. All the fractions of *S. samarangense* leaves demonstrated proton-donating activity. In this assay, ESSL showed the highest DPPH scavenging activity with an IC_{50} value of 38.84 ± 0.28 µg/mL, followed by WSSL with an IC_{50} value of 137.63 ± 0.08 µg/mL. CSSL had no activity within the experimental concentration range whereas standard ascorbic acid showed an IC_{50} value of 18.84 µg/mL. MSSL also showed potent activity with an IC_{50} value of 75.24 ± 0.16 µg/mL, which is lesser than ESSL and ascorbic acid but higher than WSSL and CSSL.

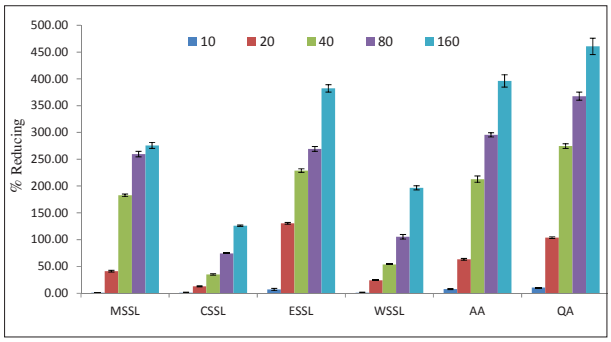

Figure 3: Reducing power of MeOH extract, fractions of *Syzygium samarangense*, ascorbic acid (AA) and gallic acid (GA) by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation. Results are mean ± SEM of three parallel measurements.

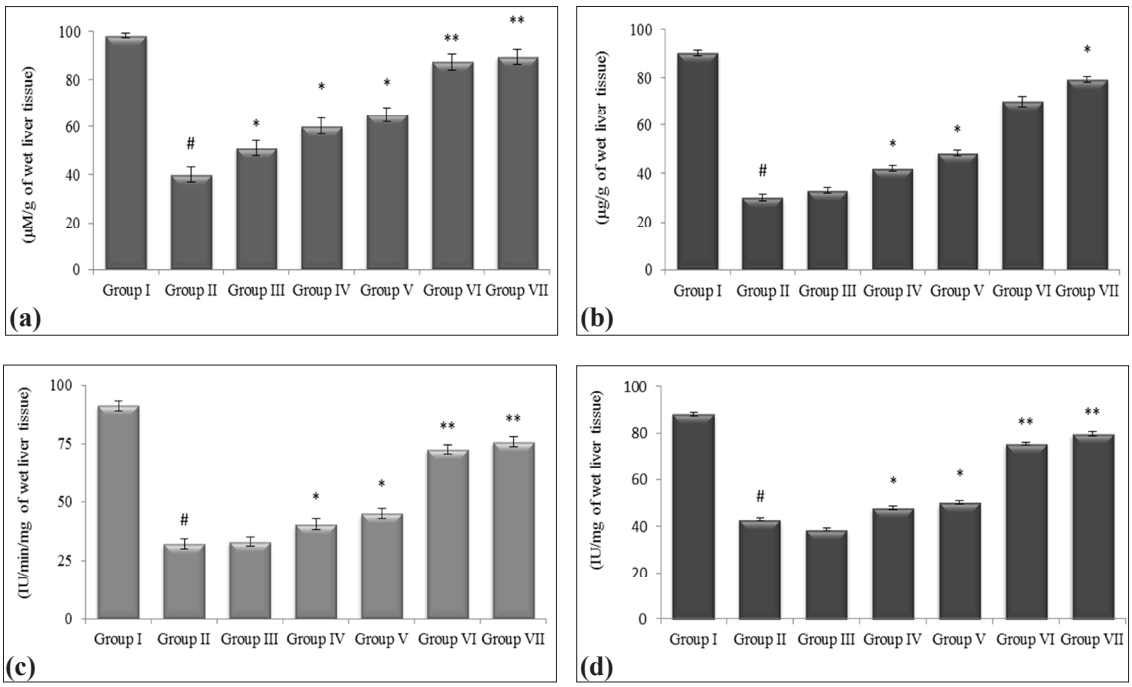

Figure 4: Effect of *Syzygium samarangense* on lipid peroxidase (MDA) (a); reduced glutathione (GSH) (b); superoxide dismutase (SOD) (c); and catalase (CAT) (d) in CCl_4 treated rat. Each point represent the mean ± SEM. (n = 6 mice per group); # $p < 0.05$ statistically significant when compared with normal saline group; * $p < 0.05$ statistically significant when compared with CCl_4 treated control group; ** $p < 0.005$ statistically significant when compared with CCl_4 treated control group. Group I animals received normal saline (5 mL/kg); group II received CCl_4 treated control; group VII received ascorbic acid 100 mg/kg body weight; groups III and IV were treated with 200 and 400 mg/kg body weight (p.o.) of the MSSL, respectively; group V and VI were treated with 200 and 400 mg/kg body weight (p.o.) of the ESSL, respectively.

Reducing power ability

For the measurement of reductive ability, Fe^{3+} to Fe^{2+} transformation in the presence of extract and organic fractions was investigated. Similar to the antioxidant activity, the reducing power of *S. samarangense* leaves increased with increasing concentration of the sample. Figure 3 shows the reductive capabilities of the *S. samarangense* leaf extract/fractions compared with quercetin and ascorbic acid. The extract and all the fractions derived from *S. samarangense* showed higher activities at the tested concentrations and these differences were statistically significant ($p < 0.05$).

Lipid peroxidation (LPO), enzymatic (CAT, SOD) and non-enzymatic (GSH) antioxidant system

Reduced activities of enzymatic (CAT, SOD) and non-enzymatic (GSH) antioxidant systems along with the lipid peroxidation (LPO) level of liver homogenate are summarised in Figure 4. In this assay, MSSL at the concentration of 400 mg/kg showed reduced lipid peroxidation at the level of 60.25 ± 1.10. After administration of the solvent soluble fraction ESSL at 400 mg/kg and ascorbic acid (100 mg/kg) to CCl_4 treated rats, the level of lipid peroxidation decreased ($p < 0.05$) by 87.32 ± 2.10 and 89.59 ± 1.04, respectively relative to the CCl_4 control group (Figure 4a). Reduced glutathione levels were found to be significantly ($p < 0.05$) elevated towards the normal level upon administration of crude methanol extract (MSSL) at 400 mg/kg when compared to the CCl_4 control group (Figure 4b).

Administration of methanol crude extract (MSSL) at 200 and 400 mg/kg drastically ($p < 0.05$) increased the levels of SOD and CAT in a dose-dependent manner (Figures 4c and 4d, each $3^{rd} \sim 4^{th}$ lane) relative to that of the CCl_4 control group. The solvent soluble fraction (ESSL) showed almost similar activity to the standard compound ascorbic acid for both parameters (Figure 4, each 7^{th} lane).

DISCUSSION

It is well known that phenolic compounds contribute to the quality and nutritional value of food in terms of modifying the colour, taste, aroma, and flavour as well as exerting health beneficial effects. Examining the polyphenolic content of *Syzygium samarangense* may be of great benefit to understand the health aspects of both traditional and modern uses of *S. samarangense*. Flavonoids are hydroxylated phenolics with potent antioxidant ability, which helps in scavenging free radicals and preventing oxidative cell damage (Gupta *et al*., 2005). It is estimated that human beings consume between a few hundred milligrams to one gram of flavonoids every day. The range of biological activity of flavonoids is large; in addition to scavenging free radicals and reactive oxygen species (ROS), flavonoid actions include anti-inflammatory, antiallergenic, antiviral, antibacterial, antifungal, antitumor, and antihaemorrhagic (Ozgen *et al*., 2010). Flavonoids also inhibit a number of enzymes, including aldose reductase, α-glucosidase, xanthine oxidase, monooxygenase, lipoxegenase and cyclooxygenase (Aiyegoro & Okoh, 2010). In this study, the ESSL fraction of *S. samarangense* showed the presence of higher amount of flavonoids than the other solvent fractions investigated. In addition, proanthocyanidins are a group of polyphenolic bioflavonoids, which have a protective effect in eliminating hydroxyl radicals (Pataki *et al*., 2002). The proanthocyanidin content was relatively high in the ESSL extract. This is in agreement with the findings of Loots *et al*. (2007), who reported a higher concentration of proanthocyanidins (polyphenols) in the leaf gel extract of *A. ferox*, which may serve as a potential source of bioactive agents in the treatment of oxidative stress or free radical associated diseases.

The upshots of oxidative stress are serious and sometimes manifested by increased activities of enzymes involved in oxygen detoxification (Gupta *et al*., 2005). Therefore, the identification of new antioxidants may reduce the risk of various chronic diseases involving free radicals. To determine the efficacy of natural antioxidants either as a pure compound or as a plant extract, a great number of *in vitro* methods have been developed in which antioxidant compounds act *via* several mechanisms. The knowledge of total antioxidant activity can be useful in the analysis of changes in plasma antioxidant activity related to oxidative stress, or in the understanding of structure-activity relationships of pure antioxidant species. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the compounds having antioxidant properties and is successfully used to quantify vitamin E in various samples (Prieto *et al*., 1999).

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Lompo *et al*., 2007), and is usually used as a substrate to evaluate the antioxidant activity of a variety of compounds (Braca *et al*., 2001). Based on the data obtained in this study, DPPH radical scavenging activity of ESSL was found to be IC_{50} 38.84 ± 0.28 µg/mL. Moreover, it was revealed that ESSL showed proton

donating ability, confirming its role as a free radical inhibitor or scavenger. A direct correlation between the antioxidant capacity and the reducing power of certain plant extracts has been reported (Nakayama *et al*., 1993). The reducing properties are generally associated with the presence of reducing groups, which have been shown to exert antioxidant action by breaking the free radical chain upon donating a hydrogen atom (Tanaka *et al*., 1988). Since a substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atoms (Jayprakash *et al*., 2001), the ferric reducing property of plant extracts (Figure 3) implies that they are capable of donating hydrogen atoms in a dose-dependent manner.

CCl_4 is one of the most commonly used hepatotoxins in experimental studies of liver damage (Lee *et al*., 2001). The hepatotoxic effect of this chemical is mostly based on membrane lipid peroxidation. Consequently, the trichloromethyl radical leads to severe cell damage (Curtis & Mortiz, 1972). In the present study, a single dose of CCl_4 developed significant hepatic damage and oxidative stress, leading to increased lipid peroxidation. The treatment with different fractions of *S. samarangense* leaves was able to reduce the level of lipid peroxides in a dose-dependent manner as compared with the hepatotoxic group.

Superoxide dismutase (SOD) has been reported as one of the most important enzymes in the enzymatic antioxidant defense system (Curtis & Mortiz, 1972). It removes superoxide anions by converting it to hydrogen peroxide and prevents the toxic effect caused by this radical. In this assay, CCl_4 induced hepatic damage led to the decrease in the percentage inhibition of SOD. However, the administration of plant extract/fractions increased the percentage inhibition of SOD, revealing the efficient protective mechanism of this plant.

Catalase is another antioxidant enzyme, which is widely distributed in animal tissues. Catalase decomposes H_2O_2 and protects the cells from highly reactive hydroxyl radicals (Chance & Greenstein, 1992). Yeh and Yen (2006) reported that four different phenolic acids were able to induce antioxidant enzymes including SOD, catalase and glutathione peroxidase. In the present study, it was found that when administered, *S. samarangense* increased the percentage inhibition of SOD, probably due to the presence of phenolic compounds in its extract/fractions.

Reduced glutathione (GSH) is a tripeptide, non-enzymatic biological antioxidant present in the liver. It protects cellular proteins against ROS generated from the exposure to CCl_4 (Arivazhagan *et al*., 2000). In this assay, the ability of the extract/fractions derived from *S. samarangense* to reactivate the hepatic glutathione reductase was reflected by decreasing the level of lipid peroxidation, and the results were in strong agreement with earlier reports (Bhandarkar & Khan, 2004).

In addition, Manaharan *et al*. (2013) also observed insulin-like and/or insulin-sensitising effects of *Syzygium aqueum* leaf extract and its six bioactive compounds (4-hydroxybenzaldehyde, myricetin-3-O-rhamnoside, europetin-3-O-rhamnoside, phloretin, myrigalone-G and myrigalone-B) in 3T3-L1 adipocytes. *Syzygium aqueum* leaf extract (0.04 – 5 µg/mL) and its six bioactive compounds (0.08 – 10 µM) at non-cytotoxic concentrations effectively enhanced adipogenesis, as well as stimulated the glucose uptake and increased adiponectin secretion in 3T3-L1 adipocytes. In a further study, Manaharan *et al*. (2012) isolated and identified six flavonoid compounds, 4-hydroxybenzaldehyde, myricetin-3-O-rham-noside, europetin-3-O-rhamnoside, phloretin, myrigalone-G and myrigalone-B, from the ethanolic leaf extracts of *S. aqueum*. Compounds myricetin-3-O-rham-noside, and europetin-3-O-rhamnoside showed high inhibitory activities with EC_{50} values of 1.1 lM and 1.9 lM against α-glucosidase, and EC_{50} values of 1.9 lM and 2.3 lM against α-amylase, respectively. These findings provide a strong rationale to establish *S. aqueum*'s capability as an antihyperglycaemic agent. Similarly, Amor *et al*. (2005) isolated a dihydrochalcone flavonoid compound from the leaves of *S. samarangense* with anticholinesterase activity. Raga *et al*. (2011) also isolated a few terpene compounds including cycloartenyl stearate, lupenyl stearate, sitosteryl stearate, and 24-methylenecycloartanyl stearate from the leaves of *S. samarangense*, which exhibited potent analgesic and anti-inflammatory activities at effective doses of 6.25 mg/kg body weight and 12.5 mg/kg body weight, respectively. Further, Reddy and Jose (2011) analysed the chemical composition and antibacterial activity of the volatile oil from the leaves of *S. samarangense*, supporting its biological significance.

CONCLUSION

The results of the present study indicate that the methanolic extract (MSSL) and various solvent soluble fractions of *Syzygium samarangense* leaves exhibit interesting antioxidant properties in various *in vitro* and *in vivo* models. The MSSL showed higher antioxidant activity than the other solvent soluble fractions. Among all the solvent fractions, ESSL showed better results than others in each tested antioxidant assay, suggesting it to be a potential candidate of significant antioxidant

efficacy. These findings reinforce the suggestions that *S. samarangense* could be a suitable candidate for the treatment of various free radical mediated diseases. However, the results of this investigation does not reveal which chemical compound is responsible for the aforementioned activity.

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