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Potential Antidiabetic And Antioxidant Activitys Of Selected Medicinal Plants

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POTENTIAL ANTIDIABETIC AND ANTIOXIDANT ACTIVITYS OF SELECTED MEDICINAL PLANTS

by

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DEDICATION

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CHAPTER 1: INTRODUCTION

Diabetes mellitus is a global health threat

Diabetes mellitus is a group of metabolic diseases with multiple etiology, which is initially characterized by impairment of glucose homeostasis [1]. Non-insulin depended diabetes mellitus (NIDDM) is the most common type of diabetes, which accounts for 95% of the diabetic patients presenting [2]. The condition mainly initiated with the insulin resistance developed by body tissues with resultant hyperglycemia following inadequate insulin production by pancreatic beta cells [3]. Insulin resistance predominantly involves the peripheral utilization of glucose and regulation of hepatic glucose production thus the pathogenesis includes the abnormalities of carbohydrate, fat or protein metabolism due to deficient insulin action on target tissue [1-3].

Diabetes mellitus is one of the major global health threats that have been reaching devastating proportions. According to the international diabetic federation (IDF) summery report seventh edition, it has been estimated that nearly 415 million people worldwide suffer from diabetes currently and this figure is likely to reach six hundred forty two million by two thousand forty. It is further supposed that the three quarters of diabetic patients live in low and middle-income countries [4].

Traditional knowledge of herbal medicine for Diabetic management

These projections emphasize the necessity of seeking alternative approach for controlling diabetes mellitus. Traditional knowledge of herbal medicine consists of large number of formulas, which has been used over two thousand years for hypoglycemic activity [5]. Medicinal plants, based for those formulas are time tested through trial and error and proven for their safety and efficacy over generations [5, 6]. Most of the developing countries are rich with the traditional knowledge of herbal medicine and natural resources [7]. Treatment option developed

from this valuable resources for diabetic management would be easily accessible in the areas where there is persistent shortage of essential conventional medicine.

It is obvious that scientific study on traditional herbal medicine could give convincing clues for management of diabetes mellitus [8-10]. World health organization (WHO) has also suggested evaluating such systems to develop integrative approach for controlling this rising global burden [11]. Ethnobotanical findings reported that more than 1200 plant species have been identified for their hypoglycemic potentials [12, 13]. Many such herbs have demonstrated antidiabetic activity through various experimental techniques [13, 14]. Divers therapeutic techniques are being used for the conventional NIDDM management currently. This includes stimulation of insulin secretion, reduction of insulin demand, enhancement of insulin action on target tissue, inhibition of enzymatic activity on metabolic pathways that contribute for plasma glucose production [1, 15]

A wide range of plant derived active compounds have been isolated and analyzed for their possible use in treatment of NIDDM. Among those, alkaloids, glycosides, saponins, flavonoids, polysaccharides, peptidoglycans, guanidine, steroids, terpenoids, amino acids and inorganic irons are commonly present phytochemicals [16]. Those phytoconstituents are reported to possess good antioxidant properties [9, 16-18].

Enzymatic inhibition action of plants

Decreasing postprandial hyperglycemia is one of the most accepted therapeutic approaches for treating NIDDM [16, 19]. This can be achieved by retarding the membrane bound enzymes in the intestinal epithelium, which catalyze 1,4-alpha bonds in starch and disaccharides to glucose thus delaying glucose digestion and absorption through the brush boarder [8, 16, 20-22]. Inhibition of α-amylase and α-glucosidase, that elicits carbohydrate hydrolysis, results

reduction of postprandial plasma glucose elevation. Hence enzymatic inhibition capability is considered as a good indicator for containing anti-diabetic activity. This hypothesis was profoundly experimented and confirmed through animal and clinical studies using various α-glucosidase inhibitors. It was shown that diverse parts like fruit, leaf, flower, bark in various plants have possessed strong inhibitory action against key enzymes (α-amylase and α-glucosidase) linked to NIDDM [20, 21, 23]

Anti-oxidative properties of plants

It is identified that oxidative stress plays a major role in the etiology of both types of DM [8, 17, 24]. Previous studies have shown that elevation of reactive oxygen species (ROS) associated with apoptosis of pancreatic β cell leading to reduction of insulin secretion [25, 26]. Hence introduction of agents that elevate antioxidant capacity is a good therapeutic strategy for prevention further cellular damage[27]. Most of the herbal plants mentioned in traditional medicine have been identified to exert protective effect against oxidative stress in biological system. The antioxidants properties of such plants have also been scientifically validated through various in vivo and in vitro experimental procedures [6, 8-10, 17, 28, 29]. A number of assays are utilized for measuring antioxidant capacity of plant extracts [30, 31]. Each method depends on the generation of different radicals that are inhibited over the time by the reaction with the antioxidant systems presence in the sample [32]. *Tinospora cordifolia, Gymnema sylvestre* and *Aegle marmelos* are several of therapeutically most accepted medicinal plants for DM management in indigenous medicine in Southeast Asia.

Gymnema sylvestre

Gymnema sylvestre is tropical herb native to India and Sri Lanka commonly known as gymnema or Gudmar (sugar killer -because of its hypoglycemic properties) [33]. The plant is a vine belongs to family Apocynaceae. The qualitative analysis of gymnema leaf extract was reported the presence of alkaloids, terpenoids, flavonoids, steroids, saponins cardiac glycosides [33, 34]. The bio-active compound of the plant is a group of isolated glycosides known as gymnemic acid [35]. Gymnemic acids were identified for having an antidiabetic effect through reducing maltose transportation in rat intestine when combined with acorbose [35]. The aqueous extract of gymnema has shown to increase insulin secretion in mouse and human β-cells when used at a 0.125 mg/ml concentration [36]. An ethanol extract of *Gymnema* has demonstrated strong antioxidant activity in the assays including ABTS (2,2°-azinobis-3-ethylbenzothiazoline-6-sulfonic acid - 54%). Blood glucose levels and lipid peroxidation levels were decreased in the diabetic rats fed *Gymnema* extract [37].

Aegle marmelos

Aegle marmelos (Family Rutaceae) generally known as bael is a mid sized tree present along the Southeast Asian region. While fruit is widely used as a food, all parts of the tree are used in traditional medical systems throughout the region [38]. In traditional medicine the therapeutic value of bael leaves has been recognized as a very effective application for lowering elevated plasma glucose levels [38]. Chemical analysis of leaf extract suggests the presence of several biological active phytochemicals such as alkaloids, flavonoids, terpenoids, phenolics, steroids and many other aromatic compounds such as cineole, citral, limonene, and eugenol [39]. These compounds are recognized for biological and pharmacological activities against various chronic diseases such as diabetes mellitus and cancer. Antioxidant potentials of bael leaf extract

studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and TBA - lipid peroxidation activity, has revealed the bael is a potential source of antioxidants [40]. The study of the antiglycating, antidiabetic and antioxidant properties of the bael leaf extract demonstrated that the prevention of secondary complication in a model of STZ-induced diabetes mellitus and inhibition of protein glycation [41]. Leaves of bael were also identified for excellent alpha amylase inhibitory action implying the reduction of postprandial hyperglycemia [42]. Further it has been reported that bael leaf extract regenerated damaged pancreatic cell in diabetic rats and presented insulin like action by restoring the plasma glucose to normal levels [43].

Tinospora cordifolia

Tinospora cordifolia commonly known as guduchi is a vine of the family menispermaceae indigenous to tropical regions of Sri Lanka and India. It is a woody climber with succulent and elongated twining branches [44]. Guduchi is known for its healing and rejuvenating properties in ancient medical texts [45]. Even in present-day traditional medicine, it is widely used for therapeutic and preventive aspects for most chronic degenerative diseases like diabetes and cancer. Fresh stem is the mostly consumed part of the plant for medicinal preparations of antidiabetic applications [46]. Phytochemical screening of stem extract has revealed the presence of various groups of phytoconstituents like, Saponins, anthraquinones, terpenoids, tannins and alkaloids in addition to phenolics [47, 48]. Antioxidant potential of guduchi has *been* evaluated through invitro and invivo studies. Guduchi was found to be effective as a 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide radical (SOR) and hydroxyl (OH) scavenging agent. The antioxidant capacities were evaluated in vivo by using enzymatic (SOD, CAT) and non enzymatic (Vitamin C&E) antioxidant levels in streptozotocin induced diabetic rats [48]. The mechanisms of antidiabetic activity of guduchi were considered to be present through antioxidant

capacity as well as inhibition of mediators of insulin pathway preventing cellular absorption of glucose [49].

It is obvious these three plants are well known for their health benefits. Additionally their hypoglycemic and antioxidant properties have been studied using several experimental procedures [19, 37, 40, 47, 49, 50]. Though these plants have been highly explored through various scientific methods, still not enough confirmation of the most effective species giving the highest degree of antidiabetic activity. The previous experiments predominantly focus on a single plant or particular assay to evaluated their bioactivity. A comparative study using multi methods screening of antioxidant and enzyme inhibition ability of these three plants in relation to their phenolic content would provide a better understanding of their relative effectiveness against the diabetes mellitus. Hence the present study was planned to assess the antioxidant properties and enzyme inhibition activity of two different extracts of selected medicinal plants. Several different methods and different solvent extracts have been used for evaluating poly phenolic and antioxidant capacity of medicinal plants [28, 51-54]. The extraction method and the assay method employed, is extremely important for the accurate quantification of the properties of phytochemicals presented as well as the authentic comparison with the literature reports available [55, 56]. In the present study the 50% acetone extract and ethyl acetate extract of selected medicinal plants were evaluated using ORAC (oxygen radical absorbance capacity) assay, DPPH assay, ABTS assay, TPC (total phenolic content) assay and alpha glucosidase inhibition assay. Those data will be useful for caparison of selected plants for their antidiabetic activity [57].

CHAPTER 2: OBJECTIVE

The main objectives of this study were to examine the total phenolic content, oxygen radical scavenging capacity and α-glucosidase inhibition activity of *Tinospora cordifolia* (Guduchi), *Gymnema sylvestre* (Gymnema) and *Aegle marmelos* (Bael) and to determine the proper solvent system for extraction phytochemicals from these three plants consequently identify the most effectual plant extract to express potential antidiabetic activity.

CHAPTER 3: MATERIALS AND METHODS

Sample collection

The dried, finely ground powders of *Tinospora cordifolia* stem, *Gymnema sylvestre* leaves and *Aegle marmelos* leaves were purchased from Terrasoul Superfoods Company through Amazon.com.

Extract preparation

For 80 mg of each dry powder of sample, 800 mL of 50% acetone was added in an Erlenmeyer flask and stirred on a magnetic stirrer at 450 rpm for eight hours in room temperature. Then the extract mixture was filtered with 0.8 µm filter paper under vacuum filtration and the filtrate was stored at 4°C until use. The residue was re-extracted using 600 mL of 50% acetone. The 400 mL of filtrate was evaporated using rota-evaporator and dried 50% acetone extract was obtained. Remaining 400 mL of filtrate was transferred to a separation funnel and partitioned with equal amount of ethyl acetate (EtAc). The ethyl acetate fraction was concentrated with rotary evaporator and dried under the hood for obtaining the dried ethyl acetate extract. All the samples were stored at 4^0C. Both the 50% acetone fraction and EtAc fraction of each plant were subjected to all the analyses conducted.

Total phenolic content (TPC)

Folin-Ciocalteau reagent 2N (F-C), sodium carbonate (≥99%) and Gallic acid were purchased from Sigma Aldrich, MO. The TPC assay was performed to determine the total phenolic content in the samples by comparing to the standard phenolic compound gallic acid. The color intensity variance of the F-C reagent from yellow/green to blue in a basic media (sodium carbonate was added for pH adjustment) was measured spectrophotometrically (EnSpire multimode plate reader 2300-001L) to determine the phenolic reaction of the samples.

The TPC procedure was performed according to the lab protocol, which was adapted from Spanos and Wrolstad [58]. Six serial dilutions (0.5 mg/mL, 0.4 mg/mL, 0.3 mg/mL, 0.2 mg/mL, 0.1 mg/mL, 0.05 mg/mL) of Gallic acid from the Gallic acid stock and samples (1mg/mL) from each extract were prepared for the assay. Folin-Ciocalteau reagent (0.2N) and 20% sodium carbonate were prepared from the available stocks. A volume of 25 µL of each sample or gallic acid was mixed with 0.25 mL of ddH_2O, 0.75 mL of 0.2 N F-C reagents in a test tube. Mixture was shaken and 0.5 mL of 20% sodium carbonate was added. The tube was shaken on a vortex mixer and incubated in a dark place for 2 hours at room temperature. After that, the absorbance readings were measured by the spectrophotometer at 765 nm. Gallic acid and samples were measured against the 50% acetone, which was used as the blank. All readings were taken in duplicates. Gallic acid readings were used for plotting the regression between the absorbance vs. concentrations of the standard solutions. Concentrations of the samples were determined based on the regression, using Beer lambert law.

Oxygen radical absorbance capacity (ORAC) assay.

The ORAC assay is based on the competitive kinetics to monitor the peroxyl radical (generated by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)) scavenging capacity of the sample against the molecular probe fluorescein. Reaction of peroxyl radical with fluorescein results in the decay of fluorescence, which is monitored using a spectrophotometer. Trolox is used as the standard and the antioxidant power (which prevent the degradation of the fluorescence of the probe) of the samples is expressed as micromoles of Trolox equivalents per gram of sample. Results of the ORAC are determined using area under the kinetic curve calculations for samples, standards, and the blank.

The ORAC assay was applied according the methodology adapted by Prior et al. with modification to reagent based on test sample composition [59]. Samples of 0.5 mg/mL Concentration were prepared. Six calibration solutions (20, 40, 80, 100, 200, 500 µM) of Trolox were tested to establish the standard curve. Phosphate buffer (PB) with pH 7.4 was prepared from sodium phosphate dibasic heptahydrate and sodium phosphate monobasic (sigma Aldrich, MO) to the concentration of 75 mM with pH adjustment using sodium hydroxide solution. Fluorescein reaction mixture (0.008 mM) and AAPH working solution (0.36 M) were prepared based on the PB buffer for the solvent. One hundred and seventy five microliters of the fluorescence was recorded for 2 hours at excitation and emission wavelength of 485 nm and 535 nm respectively. For blanking the plate reader readings 175 µL of PB buffer and 25 µL of 50% acetone solutions were used. All the readings were taken in duplicates. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve.

$$AUC = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots. f_{i-1}/f_0 + 0.5(f_i/f_0)$$

The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was calculated and ORAC values were determined as µmol Trolox equivalents/g of sample extract using the standard curve.

DPPH Radical scavenging assay

Determination of antioxidant capacity of the samples through colorimetric analysis was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical reagent (Sigma Aldrich, WI).

The methodology was adapted from Bran-Williams.et.al, with modification according to the experimental sample [60]. The maximum absorption of DPPH is 515 nm, which disappears with the reduction by radical scavenging ability of the samples. Spectrophotometric

measurements were taken using clear 96-well micro plate and HTS7000 bioassay reader (Perkin Elmer, CT). The DPPH solution with concentration of 0.8 mM in 100% ethanol was prepared. All the samples were dissolved in 70% acetone and 1mg/mL concentration of each sample was tested to determine the best antioxidant source. Sample blank was included 100 µL of 70% acetone solution and 150 µL of 100% ethanol solution. Sample wells contain 100 µL sample extract and 150 µL of DPPH solution. Each sample was run in duplicate. Another set of duplicate wells, containing only 100 µL of sample were prepared for minimizing the background color interference. Absorbance readings were taken under room temperature at 515nm every 5 minutes for two hours. Background readings of each sample were subtracted from reaction mixture readings and radical scavenging activity was calculated using following formula.

$$\%\ DPPH^{\bullet}\ scavenging = [Abs_{control} - (Abs_{sample} - Abs_{sample\ background})]/Abs_{control} \times 100$$

ABTS decolonization assay

This assay uses the spectrophotometric analytical technique to measure the total antioxidant activity of solutions or pure compounds. The blue green $ABTS^{\bullet+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation that has absorption maxima at wavelengths 415nm and 734 nm was generated through the reaction between ABTS and potassium persulfate. The reduction of preformed radical cation due to the antioxidant activity results decolorizing of the media. The percent inhibition of the $ABTS^{\bullet+}$ by the sample is considered as a function of concentration and duration then calculated relative to the reactivity of Trolox under the similar experimental conditions.

The protocol was adapted from Re *et. al*. and Zhou *et. al*. with modifications by Wenjun Zhou [32, 61]. Both ABTS (MP Biomedicals, OH) and potassium persulfate (Acros, PA) were dissolved separately in ddH_2O. Equal amount of each solution was combined in to total 1 mL

volume and incubated at room temperature in dark for 12-16 hours to make ABTS stock solution that has final concentration of 7 mM ABTS and 2.5 mM potassium persulfate. The absorbance of 200 µL of stock was measured using HTS7000 bioassay reader at wavelength of 405 nm. Based on previous absorbance reading, $ABTS^{\bullet+}$ working solution was prepared by diluting the stock solution to absorbance value of 0.7(±0.02). From 1.5 mM Trolox stock solution, six serial dilutions (500, 200, 100, 80, 40 and 20 µM) were prepared. Ten microliters of each sample or Trolox and 200 µL of ABTS working solution were pipetted to 96-well plate and absorbance was measured at room temperature at 405 nm every minute for 30 minutes. Blank was prepared by adding 10 µL of 50% acetone and 200 µL of ABTS working solution. Each sample was run in duplicate. The percent scavenging capacity of $ABTS^{\bullet+}$ was calculated based on following equation.

$$\text{Scavenging power \%} = (Abs_{INITIAL} - Abs_{END}) / Abs_{INITIAL} \times 100$$

Percent inhibition Vs. Trolox concentration was plotted and trend line equation was determined. Trolox equivalent (TE) was calculated as x using y = mx-b for samples and divided that by the corresponding concentration to get the Trolox equivalent per unit gram.

Alpha-glucosidase inhibition assay

This assay was conducted for in vitro testing the potential pant extracts that could serve to inhibit α-glucosidase activity. The inhibitory activity was compared with the known antidiabetic agent 10 µg/mL of acorbose in 0.1 M PB (pH 6.8).

The procedure was followed according to Wang's protocol adapted from Kikkoman® (http://www.cy-bio.com/administrator/order/20091222125123b.pdf). For intestinal α-glucosidase, p-nitrophenyl-α-d-glucopyranoside (pNPG) was used as a substrate with 4 mM concentration in 0.1 M PB (pH 6.8). Alpha glucosidase acetone powder from rat intestine

(Sigma, MO) was dissolved in 0.1 M PB (pH 6.8) to achieve 1 g/40 mL concentration for the glucosidase source. Samples with 1mg/mL concentration were subjected to spectrophotometric analysis at 405 nm at 37^0C using HTS7000 bioassay reader. Sample blank was prepared by adding 58 µL of 50% acetone 22.5 µL of pNPG and 45 µL of enzyme. Fifty-eight micro liters of sample extract was mixed with 22.5 µL of pNPG and 45 µL of enzyme to prepare sample wells. For the positive control, 58 µL of acorbose was mixed with 22.5 µL of pNPG and 45 µL of enzyme. Absorbance measurements were taken after 30 minutes and 90 minutes. All the samples were run in duplicates. Glucose concentration was determined based on the following formula.

Glucose Concentration (mg/dL) = $(Abs_{sample} / Abs_{standard}) \times$ Standard Concentration (mg/dL)

Percent glucose inhibition was calculated by subtracting readings blank using following equation

$$\% \text{ inhibition} = (1 - ([glucose]_{sample} / [glucose]_{negative\ control})) \times 100$$

Statistical analysis

The data analysis was performed using Microsoft excel. One-way ANOVA was used to identify the mean differences among all the samples. To analyze the significance between each samples independent t- test was used. Since fifteen tests had to be applied, according to the Bonferroni correction alpha value is considered to be 0.0033 (=0.05/15). A Pearson's correlation test was conducted to determine the association among the results of different assays.

CHAPTER 4: RESULTS AND DISCUSSION

Total phenolic content

Total phenolic content of the samples were determined using the equation (Y = 0.3472X ± 0.0793) obtained from the calibration curve of gallic acid as the standard. The amount of phenolic compounds present is given as gallic acid equivalent (GAE) in mg per mg of sample (Figure 1).

The amount of total phenolics extracted from different plants using two different solvents varied widely. Differential amount of phenolics (0.1627 – 0.5060 mg GAE/mg) was present in all the fractions. The highest amount of phenolic compounds (0.5060 mg GAE/mg) is present in the ethyl acetate fraction (EA) of guduchi whereas, 50% acetone fraction (Ac) contains more yields of phenolic compounds for gymnema (Ac-gymnema) and bael (Ac-bael). It was 0.2450 mg GAE/mg and 0.4190 mg GAE/mg respectively. Despite the apparent implications of the graph, the differences between two fractions in each three samples are not significantly different. However there is a significant difference of TPC among the fractions with the higher yield of phenolics in each plant.

Various solvents have been applied for extracting chemical constituents from dried plant materials such as ethanol, acetone and ethyl acetate. The recovery of phytochemicals is influenced by the solubility of phenolic compounds in the solvent used for extraction [62]. The polarity of the solvent is crucial for the solubility of compound present in the botanical sample. Considering all the results obtained from the TPC analysis, it is evident that the extractability of phenolic compounds differs according to the polarity of the solvents. Both gymnema and bael samples might be higher with phenolics that are comparatively more polar in nature, which explains the higher solubility in 50% acetone fraction. However the trend of TPC in guduchi

indicates that though aqueous organic mixtures are appropriate for solvent extraction of gymnema and bael, less polar solvents work better with guduchi extraction. A Previous study has shown that ethyl acetate fraction exhibited better extractability of phenolics than 80% acetone [47]. Our result is compatible with that observation.

Aqueous solvent systems especially the acetone water mixtures are accepted for the extraction of phenolic compounds from plant tissues [51]. This could be due to the hydrophilic nature of the phenolic compounds as well as the wide range of phenols that could be dissolved in aqueous acetone mixture [63]. Furthermore acetone water mixtures are proper solvent systems for extracting polar anti oxidants [51, 64]. But TPC result of Guduchi is not supportive for this notion. For this argument, part of the plant subjected for these experimental procedures should be considered. Because presence of phenolics in the plant tissues is differed by the plant species, part of the plant, maturity, and many other environmental factors. Concentration of low molecular weight simple phenolics such as phenolic acids and acid derivatives are generally higher in younger and tender tissues such as leaves. With the maturity different phenolic acids condense to form more complex phenolic compounds. Consequently bark tissues usually rich with high molecular weight complex phenolic compounds that are more soluble in non-polar solvents[65]. Additionally through the accumulation of phenolics with the maturity, bark tissues posses higher phenolic and flavonoid content [66]. Phytochemical analysis of guduchi revealed the presence of some phytochemicals such as alkaloids, terpenoids, tannins, and anthraquinones [46, 47]. Their presence in different solvent fractions is depend on the small structural differences in the compound that are critical to their activity and solubility. Though tannins and terpenoids can be found in aqueous solvents they are often obtained with less polar solvents [47].

These specifications explain the distinctions of the chemical nature of phenolics present in Leaf powder of Beal and Gymnema and stem powder of guduchi.

Antioxidant properties of the extracted samples were evaluated through the radical scavenging capacities using ORAC. ABTS, DPPH assays. The results are shown in Figure 2, Figure 3, Figure 4. There is a vast variance in the anti oxidants present and their activity in plant materials [30, 67]. Therefore evaluating anti oxidant capacity of each plant sample turns out to be very complex. According to the chemical nature of the antioxidants, some react with the specific free radicals by scavenging and forming stable resonance structure. Different methods have been developed to estimate this particular scavenging ability, lipid peroxidation inhibition ability or metal ions chelating ability [53].

Oxygen radical absorbing capacity (ORAC)

The oxygen radical absorbance capacities of each sample fractions are shown in Figure 2. The anti oxidant capacities of each sample expressed in Trolox equivalents per gram of extract powder (μmol TE/g). All the samples were significantly differ from each other except the EA-bael and Ac-guduchi. The highest ORAC value 1970.9 μmol TE/g was shown by the EA-guduchi followed by Ac-gymnema (1906.3 μmol TE/g) and EA-gymnema (1885.8 μmol TE/g) consecutively. Both these two fractions of gymnema showed substantially high ORAC values similar to EA-guduchi. Bael had the lowest ORAC values (1780.5 μmol TE/g) among three plants. However the tendency of the ORAC result was not equivalent with that of other two-antioxidant assay's results. The correlation of ORAC with other antioxidant assays or TPC assay was not significant (Table 1).

ORAC assay is based on the degree of inhibition of peroxyl radical produced by AAPH azo initiator by the compound of interest consequently delay the loss of fluorescence [67].

However the peroxyl radical formation and nature of damaging reaction have never been characterized in vivo neither the ORAC values have been proven for any biological significance [68]. Still ORAC values could provide valuable guidance for the anti oxidant properties of the samples.

Percent scavenging capacity of ABTS$^{\bullet+}$

The percent scavenging capacity of ABTS$^{\bullet+}$ at 735nm by 50% acetone and ethyl acetate fractions of each plant samples was shown in Figure 3Figure 3. The ABTS$^{\bullet+}$ scavenging capacity of each fraction ranged from 53.03% to 83.12%. All samples showed considerably high radical scavenging effect. The highest scavenging effect 83.1% was expressed by the EA-guduchi fraction followed by Ac-bael (72.5%). For gymnema and bael, the 50% acetone fraction shows the better inhibition but for the guduchi it was ethyl acetate. The scavenging effects of those fractions are significantly different from each other.

ABTS decolonization assay is reported to be applicable for both lipophilic and hydrophilic antioxidants. The preformed radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), which is generated by oxidation of ABTS with potassium persulfate can be reduced by the hydrogen donating antioxidants [32]. DPPH is a stable organic pre radical, which contains unpaired valence electron at one atom of the nitrogen bridge that can be scavenged by hydrogen donating antioxidant. Both these radicals shows strong absorption band in the visible spectrum while the absorption changed respectively upon taking an electron or hydrogen atom from the antioxidant [69].

DPPH radical scavenging capacity

DPPH is a stable free radical that has a characteristic blue color with an absorption band at 515nm. In the presence of anti oxidant it gets reduced to diphenylpicrylhydrazine that is light

yellow in color. The DPPH radical scavenging assay evaluates the ability of experimental sample to donate hydrogen or scavenge free radical by assessing the absorption change spectrophotometrically.

Percent scavenging effect of DPPH radicals by each experimental sample over two-hour period of interaction were illustrated in Figure 4. The initial scavenging effect of each sample is notably varies from each other. Higher initial values indicate the fast initial reaction rate of the sample. Same trend of DPPH radical scavenging activity was observed for two different fractions of gymnema while Ac-gymnema kept higher scavenging capacity. Scavenging activity of Ac-bael increased sharply to more than 50% in the initial 25 minutes then gradually became a plateau. EA-guduchi on the other hand continually extended towards the higher scavenging capacity though the initial effect is slower than that of the Ac-bael. Similarly EA-bael demonstrated constantly high reaction rate, which implied the potentially high DPPH scavenging capacity if reaction time extended. All the samples except EA-gymnema showed significant scavenging effect after two hour period based on it initial value.

Figure 5 depicts the percent DPPH radical scavenging effect by each experimental sample at the half of the reaction time. The percent DPPH radical scavenging effect was ranged from 24.49% to 79.5% by each sample fractions. For the EtAc fraction, guduchi showed the highest (79.5%) scavenging effect fallowed by bael and gymnema. For 50% acetone fraction bael had the highest (76.8%) radical scavenging effect followed by guduchi and gymnema. All these variances were statistically significant.

Observation of this experiment revealed that the DPPH radical scavenging capacity depends on the intrinsic characteristics of anti oxidants present, reaction rate and the duration of the reaction. The variation of the DPPH radical scavenging activity displayed by the samples

could be primarily due to the intrinsic characteristics of plant compounds and the extracting solvents.

Correlation

To see the association between the outcome of different antioxidant methods and their relationship to total phenolic content of the samples, a regression analysis was performed. The correlation coefficients (R) were given in Table 1.

Significantly strong correlation (R=0.8272) was found between ABTS and DPPH results (Figure 7). ORAC values were less correlated with the results of other antioxidant assays employed. Similarly ORAC did not show strong correlation with the total phenolic content determined by the Folin- Ciocalteau method. Nevertheless ABTS outcome was significantly correlated (R=0.868) with TPC assay results (Figure 8) while DPPH had moderately good correlation (R=0.6791) with TPC too (Table 1).

Previous comparative study of anti oxidant activity based on thirty medicinal plants extracts, have also reported the significant correlation between ABTS and DPPH assays while least correlation between ORAC and others [70]. Distinct to other antioxidant assays ORAC depends on the competitive kinetic action of the antioxidant. Besides, the assay measures the antioxidants action only against the particular peroxyl radical regardless the vast variation of anti oxidants present in plant extract. These particularities might explain the discrepancy between results of ORAC and those of other methods and weak correlation with TPC as well. Strong correlation found between TPC and ABTS assay indicates the antioxidant potential of plant-derived phenolics. Most of the secondary metabolites of plants are rich with phenolic compounds such as flavonoids, isoflavonoids, and phenolic acids [57]. Various scientific evidences have been presented for the positive correlation of phenolic compounds and the antioxidant properties

of the plants. These antioxidant properties could be due to their redox properties as well as the capability of metal chelating and quenching of singlet oxygen [55, 57, 71]. Indeed the poly aromatic ring structure of phenolics that bearing one or more hydroxyl groups potentially able to form stable resonance structure by quenching free radicals [70].

On the other hand radical scavenging activity is not only particular to the poly-phenolic properties. There are many other plant derivatives such as alkaloids, terpenoids that have antioxidant activity. Moreover phenolic compounds react differently with the diverse radicals, depending on the number of phenolic groups they consist of [59]. It has been evident that the interaction of a potential antioxidant with particular radical depends on its structural conformation [60]. Accordingly total phenolic compounds in the extract might not totally contribute for the anti oxidants activity in the particular assay hence demonstrated diverse trend of correlation between different antioxidant assays and TPC assay.

Because of the simple, rapid, sensitive and reproducible nature, DPPH and ABTS radical scavenging methods have been most frequently applied to assess the antioxidant properties of the compounds [69]. Considering all three antioxidant assays applied for our samples, ABTS and DPPH assays show the significantly similar trend of antioxidant activity. These results also considerably correlated with the total phenolics values of the samples.

Though there are a number of chemical techniques developed in an attempt to measure the antioxidant capacity in vitro in food, these assays are based on discrete mechanisms, different radicals or substrates. Even the same assay deviates through modified versions. Therefore the results among various studies could be less favorable for comparison. Additionally the results of antioxidant activity generated through various in vitro methods might not be extrapolated to invivo effects. As anti oxidant molecules in the plant kingdom demonstrate wide range of

functions, there could be abundant, which are unrelated to the free radicals scavenging activity. Also there could be

α-glucosidase inhibitory effect

Inhibitory activity of key enzymes (α-amylase, α-glucosidase) linked to NIDDM is another well-recommended method for studying antidiabetic activity in plant extracts and natural compound. Outcomes of the inhibition activity of α-glucosidase in our experiment samples were shown in Figure 6.

In contrast to results of antioxidant assays, both 50% acetone and ethyl acetate fractions of gymnema exhibited significantly higher α-glucosidase inhibitory effect than all the other samples while guduchi presented the lowest effect. α-glucosidase inhibitory effect of EA-gymnema (18.2%) is significantly higher than that of Ac-gymnema (8.55%). Acetone (50%) fraction gave significantly higher inhibition effect than ethyl acetate for both bael (6.3%) and guduchi (4.1%). According to the entire out come of the assay most significant α-glucosidase inhibitory effect was presented by EA- gymnema.

Recent animal and human studies have demonstrated that dried leaf powder of *Gymnema sylvestre* posses strong hypoglycemic activity [5, 19, 37]. Orally feeding of leaf Powder of *Gymnema sylvestre* (500mg/rat) for 10 days has significantly prevented intravenous beryllium nitrate induced hyperglycemia in rats [72]. Oral administration of aqueous extract of *Gymnema sylvestre* demonstrated significant reduction of blood glucose on OGTT without significant effect on immuno reactive insulin levels [73]. Further it has been revealed that the efficacy of gymnema was inversely proportionate to the severity of diabetes [74]. *Gymnema Sylvestre* ethanol extract on STZ (streptozotocin treated) rats demonstrated significant improvement of fasting blood glucose and pancreatic regeneration [75]. However *Gymnema* extract did not affect

insulin activity in healthy rats indicating it has a normalizing effect on blood glucose [74]. All these findings indicate *Gymnema* for a safer and comparable hypoglycemic agent. The mechanism behind the hypoglycemic effect of *Gymnema sylvestre* has been reviewed through various aspects based on its chemical constituents and biological influences. Triterpene glycosides and gymnemic acid isolated from the plant inhibited glucose utilization in muscles and glucose uptake in the intestines respectively [76]. Gymnemic acid mixture has inhibited glucose absorption in small intestines thus suppressed the plasma glucose surge. Many of the studies provided strong evidence to the activity that Gymnema significantly decrease the glucose uptake from small intestines [77]. Some authorities demonstrated improvement of insulin secretion from pancreatic β cells and regeneration of pancreatic islets. Gymnema has been also indicated for improvement of hepatic glycogen reduction, glycolysis, gluconeogenesis and hepatic and muscle glucose uptake [74]. Though it constantly identified the reduction of glucose uptake in small intestine in the influence of gymnema, there were not any studies conducted could be found on enzymatic inhibition activity of *Gymnema sylvestre*. This study provide optimistic results for the enzyme inhibition activity by gymnema thus it could be one of the possible reasons for reducing glucose uptake from intestinal epithelium. Most of the studies limited to aqueous extract or the ethanolic extract for their experimental procedures. But our study indicates that more non-polar nature of the solvent extract would give better outcomes in enzymatic inhibition analysis for *Gymnema sylvestre*.

Comparison of the outcome of all the assays applied for studying the antidiabetic potential of selected three plants is presented in Table 2. Each plant is titled in the scientific name and the common name (within brackets). Total phenolic content (TPC) and α-glucosidase inhibition tests were conducted on 1mg/ml concentrated samples. Samples with 0.5 µg/µl

concentration were subjected to all radical scavenging assays (ORAC, ABTS, DPPH). All the sample concentrations indicated here were taken before adding to the sample mixture. In general both the ethyl acetate fraction of guduchi and 50% acetone fraction of bael presented considerably high anti oxidant activity and total phenolic content while ethyl acetate fraction of gymnema showed the highest potential of α-glucosidase inhibitory effect thus all these three plants comprise possible antidiabetic activity.

CHAPTER 5: CONCLUSION

Evaluation of the anti diabetic potential of three medicinal plants (*Tinospora cordifolia, Gymnema sylvestre, Aegle marmelos*) through TPC, ORAC, ABTS, and DPPH assays demonstrated comparable results for their total phenolic content and antioxidant activity. All three-plant samples exhibited potent radical scavenging effect whereas anti oxidant properties among these differ significantly with the different solvent extractions. The maximum antioxidant property and total phenolic content were obtained by guduchi followed by bael 50% acetone extract. The solvent used for the sample extraction was crucial for the outcome of the assays employed. The optimum radical scavenging activity and total phenolic content were presented by the ethyl acetate extract of guduchi, while it was 50% acetone for bael and gymnema. It would be applicable to evaluate anti oxidant activity using total phenolic content since ABTS and DPPH showed considerable correlation with TPC results. High phenolic content might be a major contributor for the antioxidant activity present in guduchi and bael. Thus ethyl acetate extract of guduchi and 50 % acetone extract of bael would be promising antioxidants rich plant sources. Additional studies should be carried out using, multiple sample size to minimize the statistical errors and more anti oxidant methods for better confirmation.

Overall it is found that extracting solvent significantly affects the total phenolic content and antioxidant properties of the plant. However the exact phenolic compounds presented or active ingredients responsible for anti oxidant activities are yet to be found. Since the present study provides strong evidence for significant total phenolic content and significantly higher radical scavenging activity in ethyl acetate extract in guduchi it is recommended further studies to identify active compounds.

Contradictory to the outcome of anti oxidant assays gymnema showed the significantly

higher α-glucosidase inhibitory effect than other two plants. Within the gymnema ethyl acetate extract was significantly effective than the 50% acetone extract for the activity of α-glucosidase inhibition. Many previous studies have revealed that gymnema has an influence in reduction of glucose uptake in small intestine. Inhibition of α-glucosidase enzyme could be one of the promising hypotheses for investigating the biological mechanism behind this.

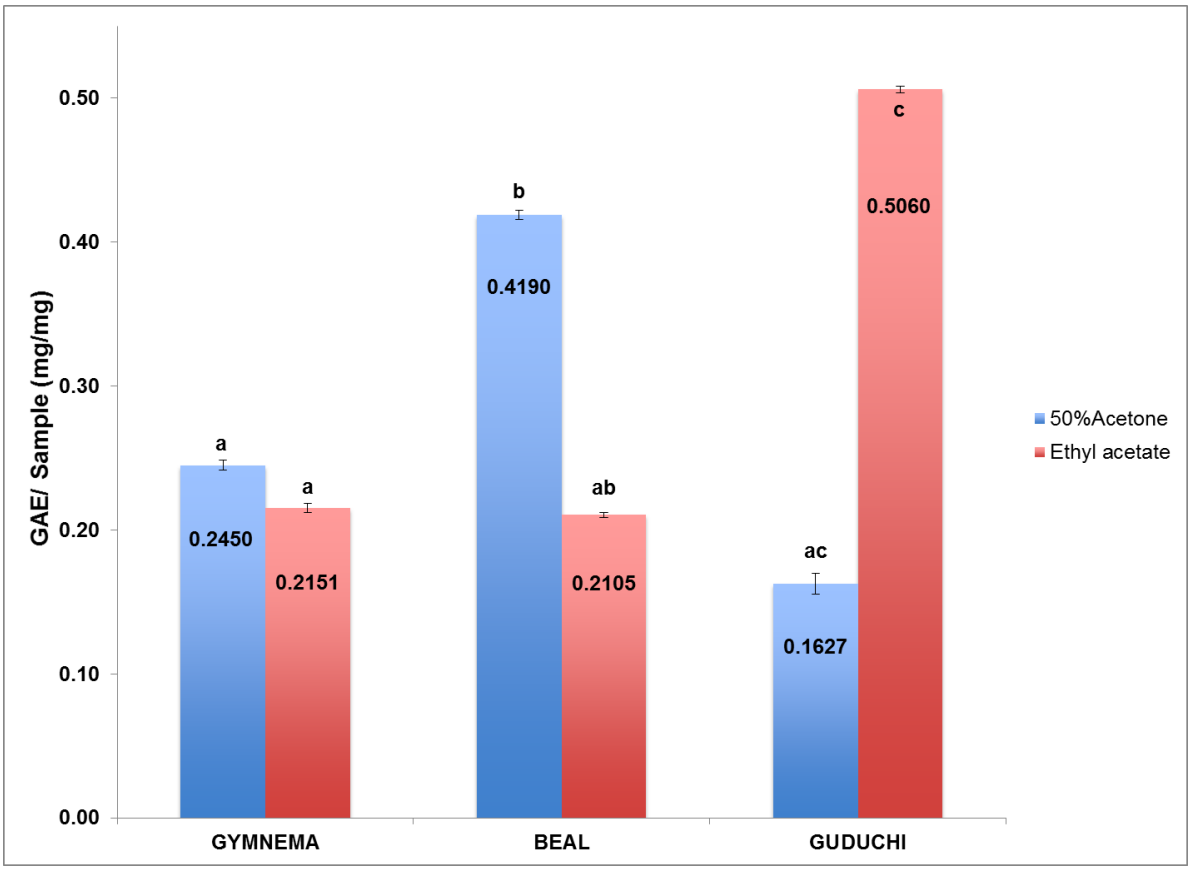

Figure 1. Total phenolic content present in 50% acetone and ethyl acetate fractions. All the samples have the same concentration. Total phenolic content values were calculated in GAE and expressed as mean ± standard deviation. Different letters indicate the significant difference at the level $p \leq 0.0033$.

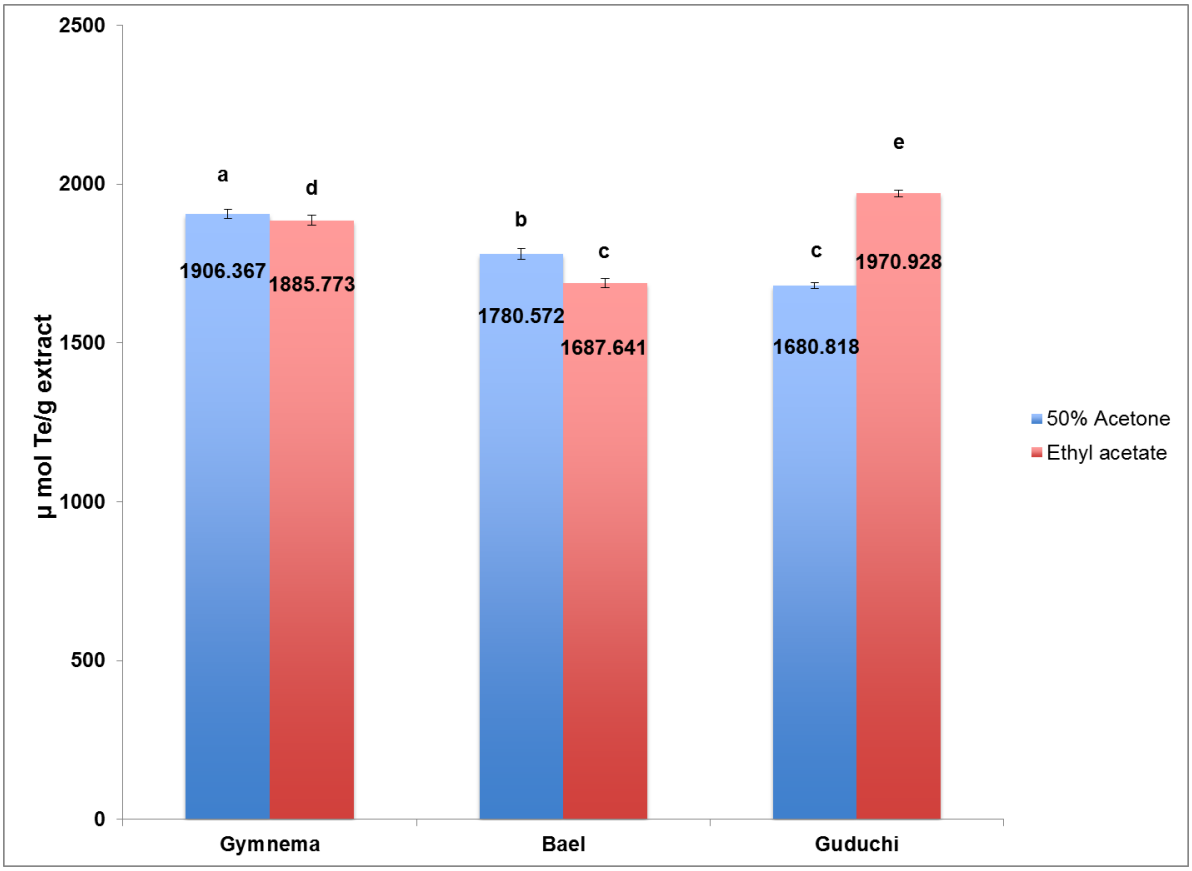

Figure 2. Oxygen radical absorbing capacity (ORAC) values in 50% acetone and ethyl acetate fractions of each plant samples.
All the samples have the same concentration. All the readings were calculated in micro molar of Trolox equivalent (TE) per gram of sample and expressed as mean ± standard deviation. Different letters indicate the significant difference at the level $p \leq 0.0033$.

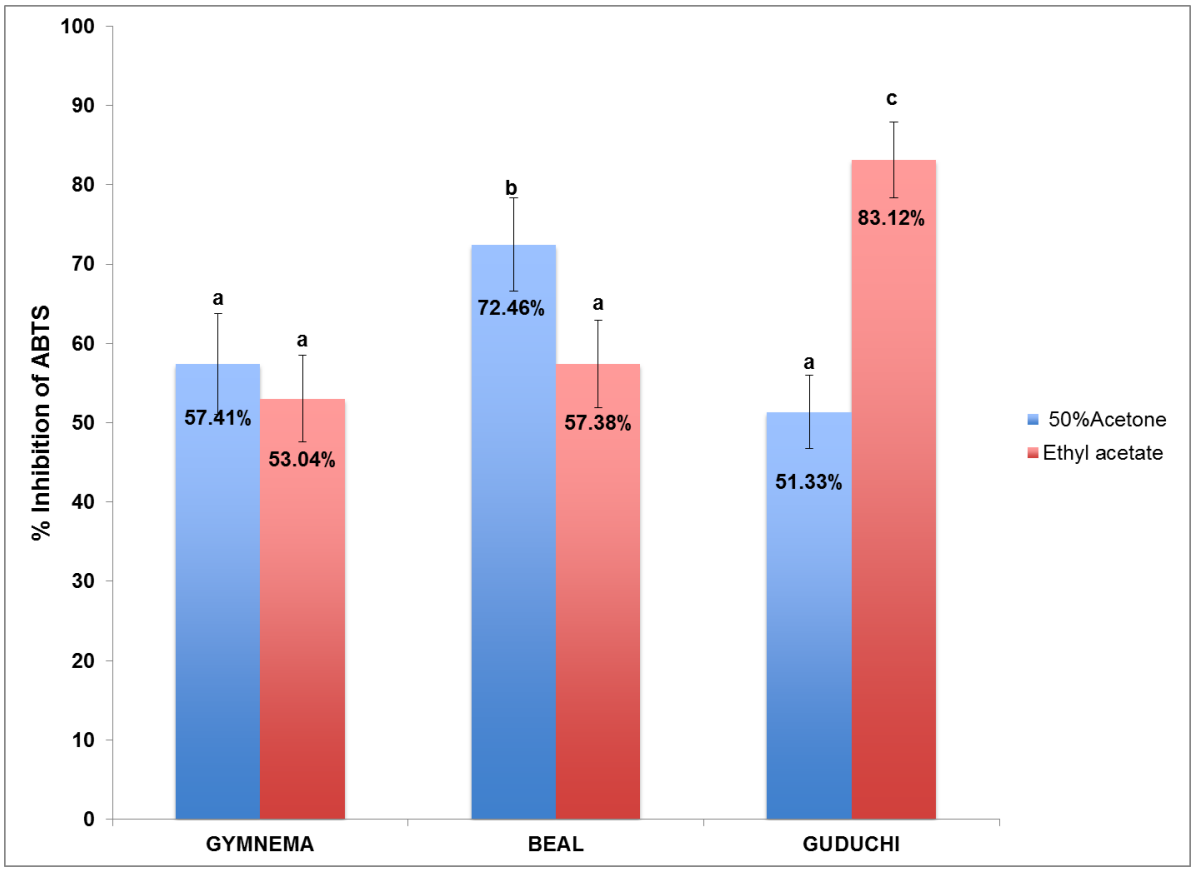

Figure 3. Percent scavenging effect/ inhibition of ABTS+• at 735 nm by 50% acetone and ethyl acetate fractions of each plant samples.
All the samples have the same concentration. Different letters indicate significant difference in the inhibition among each sample and between two fractions $p \leq 0.0033$.

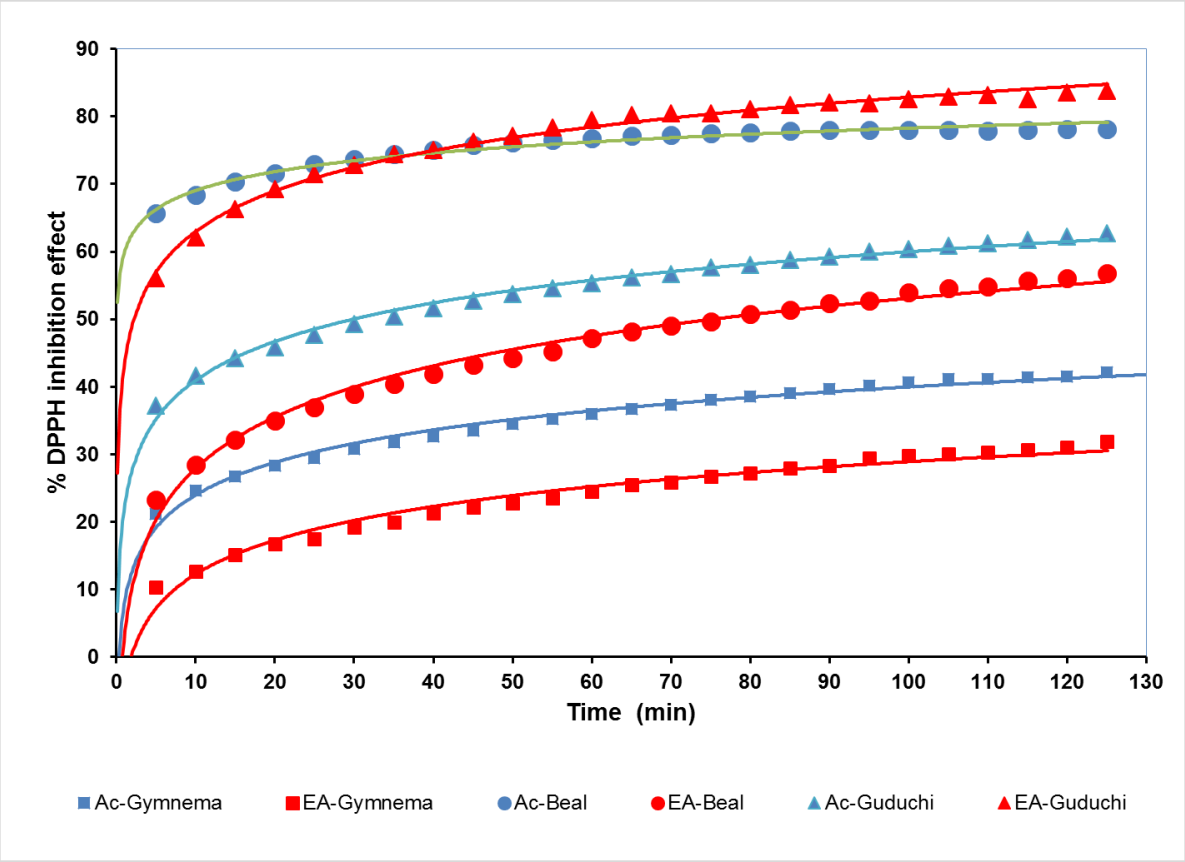

Figure 4. Percent DPPH radical scavenging effect of each experimental sample over two-hour interaction period.
Samples were taken in similar concentration. All the samples except EA-gymnema presented significant radical scavenging effect at the two hours time point when compare to initial point ($p < 0.05$).

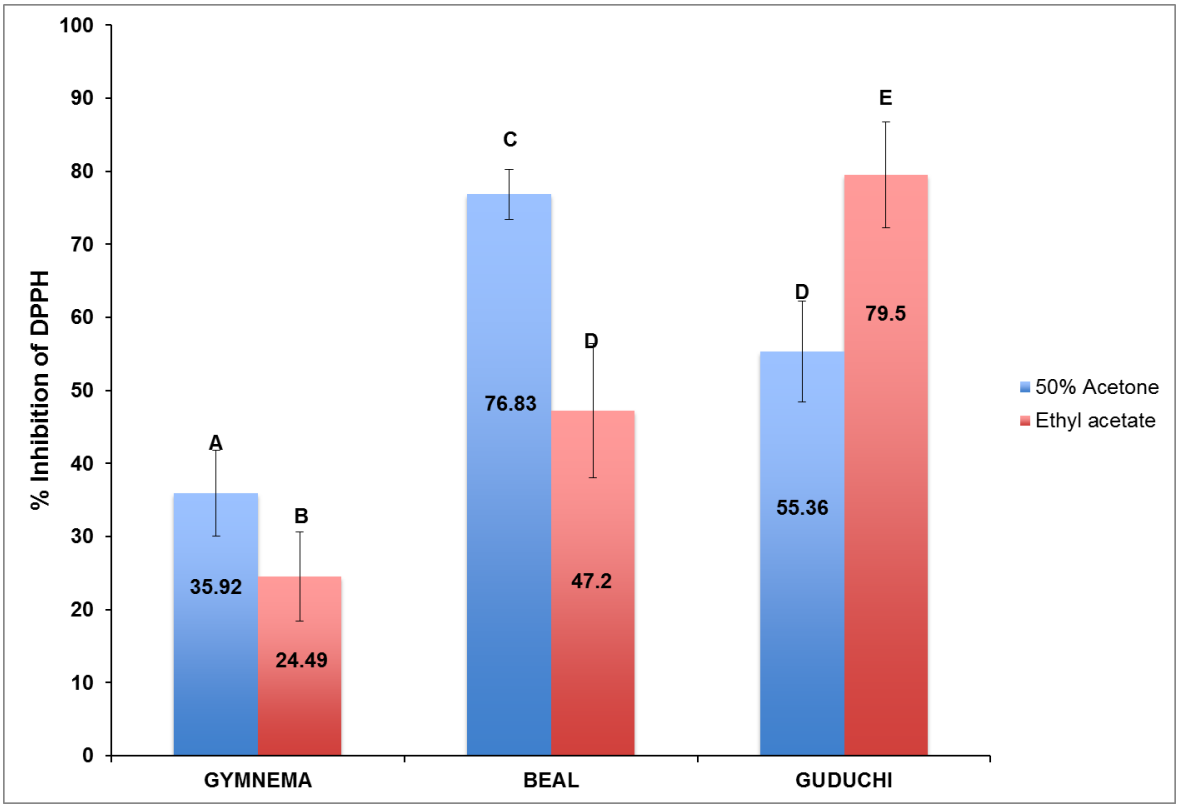

Figure 5. DPPH radical scavenging capacity by 50% acetone and ethyl acetate fractions of each plant samples at the sixty minutes of the reaction time.
All the samples have the same concentration. Different letters indicate the significant difference among each samples. ($p \leq 0.0033$).

Table 1. Correlation coefficient between assays used

	ORAC	DPPH	ABTS	TPC
ORAC				
DPPH	0.0113			
ABTS	0.512	0.8272*		
TPC	0.4023	0.6791	0.868*	

Significant correlation is indicated by * ($p \leq 0.05$)

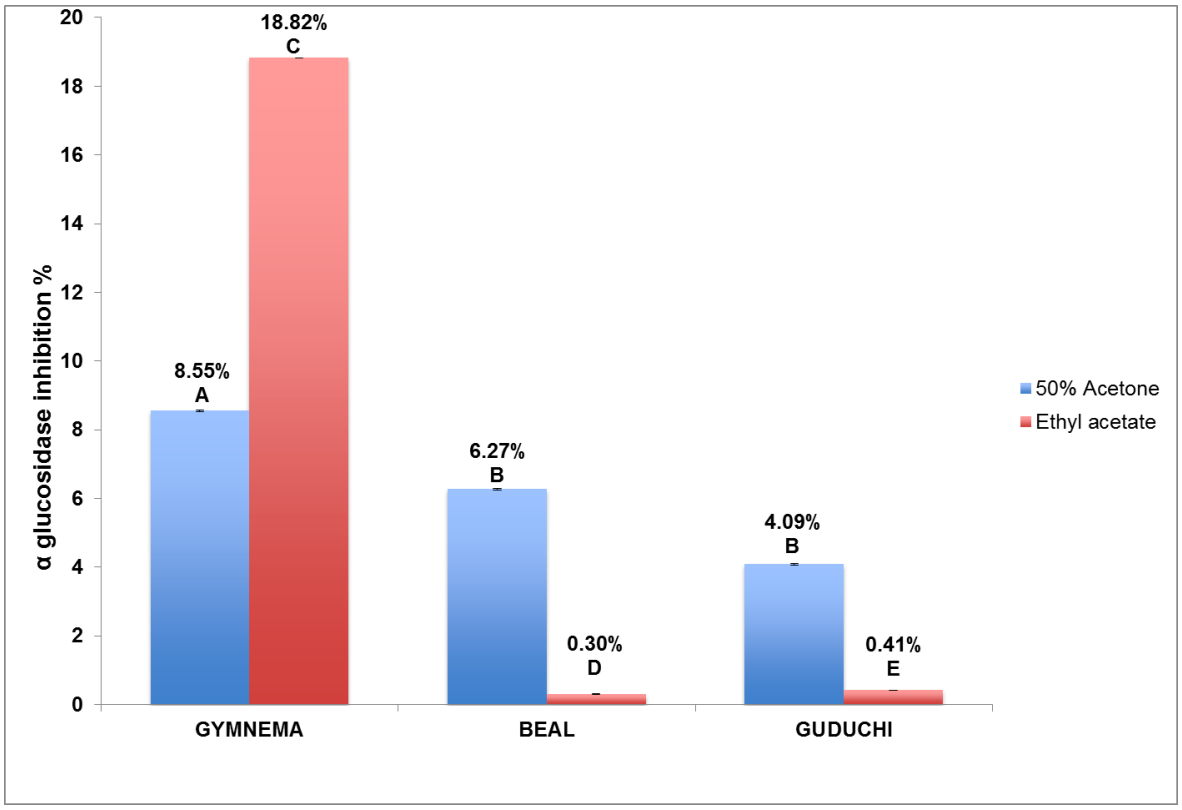

Figure 6. α-glucosidase inhibitory effect of experimental samples.
All the samples have the same concentration. Results were expressed as mean ± standard deviation. Different letters indicate the significant difference at the level $p \leq 0.0033$.between samples (n=2)

Table 2. Summery of assays

Plant	Part	Extract	TPC GAE(mg/mg)	ORAC µmol TE/g	ABTS %	DPPH%	α-glucosidase inhibition %
***Gymnema* sylvestre** **(Gymnema)**	Dry leaf powder	50% acetone	0.245±0.003	1906.4±14.4	57.4±6.4	35.9±5.8	8.549±0.033
		Ethyl acetate	0.215±0.003	1885.7±15.8	53±5.5	24.5±6.1	18.822±0.003
Aegle marmelos **(Bael)**	Dry leaf powder	50% acetone	0.419±0.003	1680.8±16.7	72.5±5.9	76.8±3.4	6.270±0.019
		Ethyl acetate	0.210±0.001	1970.9±15.2	57.4±5.5	47.2±9.1	0.302±0.014
Tinospora cordifolia **(Guduchi)**	Dry stem powder	50% acetone	0.163±0.007	1780.6±9.2	51.4±4.6	55.4±6.9	4.089±0.025
		Ethyl acetate	0.506±0.002	1687.6±10.4	83.1±4.8	79.5±7.2	0.413±0.009

Data are expressed as mean ± standard deviation.

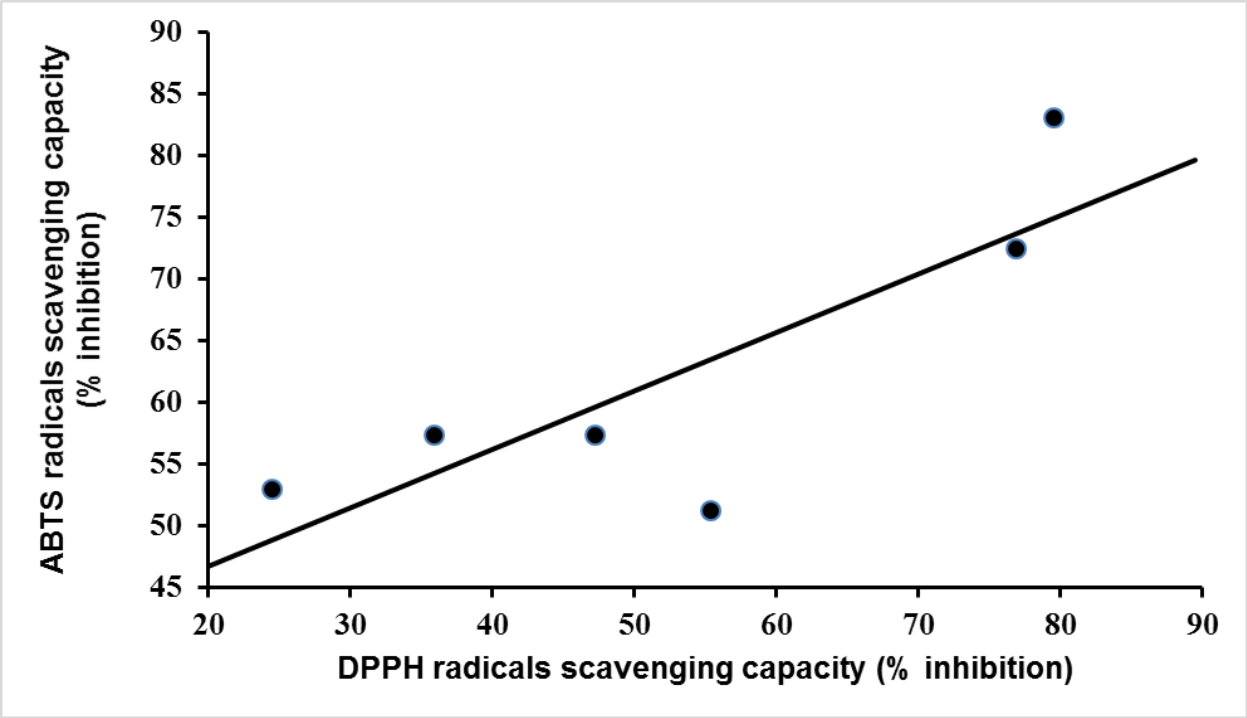

Figure 7. Correlation between ABTS and DPPH assays.
Correlation coefficient R = 0.8272

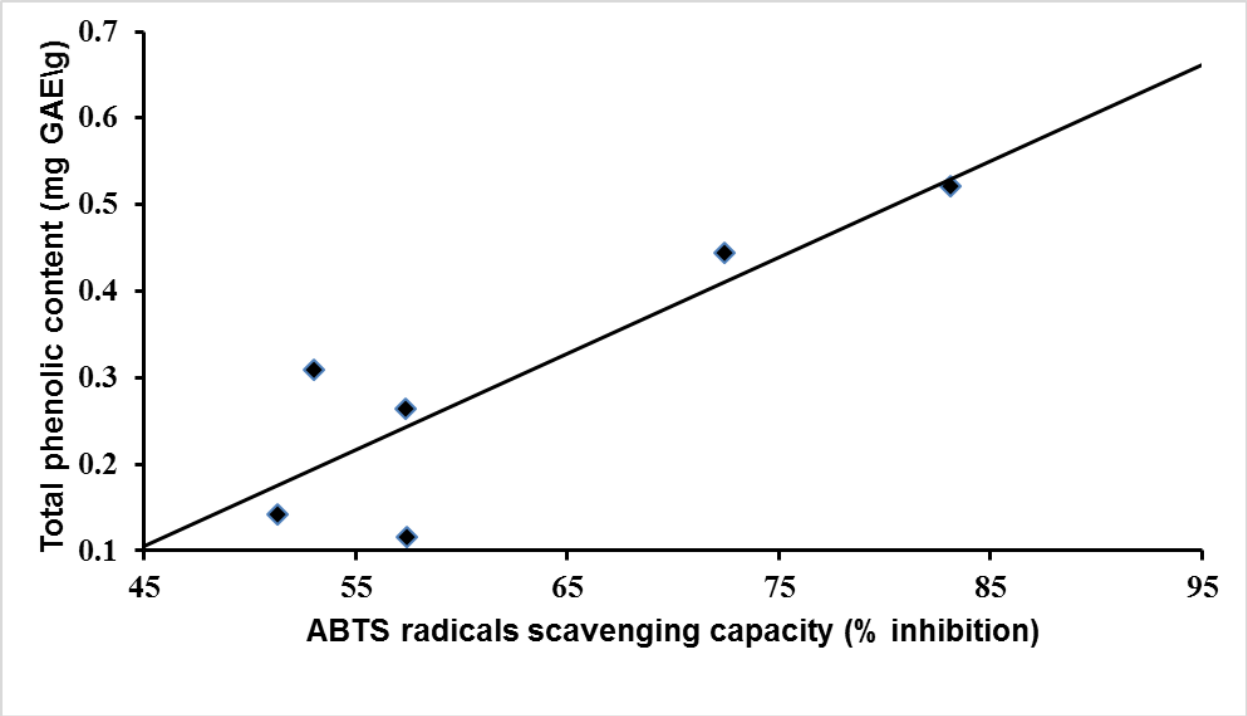

Figure 8. Correlation between ABTS and TPC assays.
Correlation coefficient R = 0.868

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ABSTRACT

POTENTIAL ANTIDIABETIC AND ANTIOXIDANT ACTIVITYS OF SELECTED MEDICINAL PLANTS

by

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Major: Nutrition and Food Science

Degree: Master of Science

Explore traditionally used medicinal plants for diabetes mellitus would be beneficial to find an alternative option for controlling global diabetic prevalence. Present study aims to compare and identify potential antidiabetic activity in *Tinospora cordifolia, Gymnema sylvestre* and *Aegle marmelos.* Fifty percent acetone and ethyl acetate extracts of each plant were studied for anti-diabetic activity using different assays (ORAC, DPPH, ABTS, TPC, α-glucosidase inhibition). Total phenolic content and anti oxidant properties were significantly different among three plants. *Tinospora cordifolia* ethyl acetate extract possessed significantly higher anti oxidant activity and total phenolic content. DPPH and ABTS results were significantly correlated with each other and with TPC assay thus phenolics could be the major contributor for the antioxidant activity. Ethyl acetate fraction of gymnema possessed significantly higher α-glucosidase inhibitory effect. Overall both the ethyl acetate fraction of guduchi and 50% acetone fraction of bael showed considerably high anti-oxidative activity and total phenolic content while gymnema showed the highest potential of α-glucosidase inhibitory effect thus all these three plants could be contain potential antidiabetic activity. Further investigation should be conducted using higher

sample size to confirm these observations. Anti diabetic activity of these samples could further be evaluated through animal studies

AUTOBIOGRAPHICAL STATEMENT

I was graduated in Bachelor of Ayurvedic Medicine and Surgery from University of Colombo, Sri Lanka in 2007. Following graduation I was appointed as an Ayurvedic community medical officer in Sri Lanka. There I got an opportunity to promote the status of public health and nutrition through awareness programs, educational workshops, and mobile clinics. While serving in that position I earned a Master's degree in Aromatic and Medicinal plants in 2011 from University of Sri Jayewardenepura. In 2013 I was accepted to pursue a Master of Science degree in Nutrition and Food science at Wayne state University, Detroit MI and would be completing the degree in December 2015.