International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 3, 2016

Original Article

EVALUATION OF *IN VITRO* ANTIOXIDANT POTENTIAL OF THE TOTAL CRUDE ALKALOID EXTRACT OF *GLYCOSMIS PENTAPHYLLA* (RETZ.) CORREA LEAVES

PRESSY P. PRAKASIA[1], ASHALATHA S. NAIR[2]

[1]Research Scholar, Department of Botany, University of Kerala, Thiruvananthapuram, [2]Professor, Department of Botany, University of Kerala, Karyavattom, Thiruvananthapuram 695581
Email: dearpressy@gmail.com

Received: 08 Oct 2015 Revised and Accepted: 13 Jan 2016

ABSTRACT

Objective: The present investigation was undertaken to appraise the antioxidant properties and the total crude alkaloid extract of *Glycosmis pentaphylla* leaves.

Methods: Radical attenuating abilities of total crude alkaloid extract were ascertained by six *in vitro* assays such as 2, 2-diphenyl 1-picryl hydrazyl ($DPPH^{\cdot}$) radical scavenging assay, ferric reducing antioxidant power assay (FRAP), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) radical scavenging assay, nitric oxide (NO) scavenging assay, hydroxyl radical ($OH^{\cdot}$) scavenging assay, and reducing power capacity assessment.

Results: The total crude alkaloid extract showed a considerable antioxidant potentiality in comparison with the standards as evidenced by the IC_{50} values obtained in the $DPPH^{\cdot}$ (966.93 µg/ml), FRAP (510.81 µg/ml), ABTS•+(400.47 µg/ml), $OH^{\cdot}$ (1805.28 µg/ml), NO (1426.50 µg/ml). In the reducing power assay, the total crude alkaloid extract showed a concentration dependent reducing power potential.

Conclusion: These results suggest the potential of *Glycosmis pentaphylla* as a curative against free-radical-associated oxidative damage diseases.

Keywords: *Glycosmis pentaphylla,* Antioxidant assay, Total crude alkaloid, IC_{50}

INTRODUCTION

Life evolved from an intense free radical-rich primordial 'soup', and it would have never got past the first cell division without the simultaneous evolution of powerful antioxidants. The appearance of large quantities of molecular oxygen, which in itself is a free radical some 2.2 billion years ago, provoked the need to adapt further to the anaerobic environment with a requirement for specialist protection against reactive forms of oxygen [1]. Antioxidant adaptation likely involved subtle changes in emulating structural integrity and compartmentalization of reactants within cells [2].

Free radicals and related species have attracted a great deal of attention in recent years, which is mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in the human body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states. ROS are highly reactive molecules which include free radicals such as, superoxide ions ($-O2^{-}$), hydroxyl radicals ($OH^{\cdot}$), nitric oxide radical (NO), singlet molecular oxygen ($O_2^{\cdot}$), peroxynitrite radicals and hydrogen peroxide (H_2O_2).

The free radicals react with the cell membranes and other structures such as DNA, lipids and proteins by stealing electrons from these molecules, thereby causing damage, and while attacking these essential molecules, they, in turn, change into free radicals themselves causing a chain reaction that can lead to the destruction of a cell. Lipids are highly prone to free-radical damage, in turn, resulting in lipid peroxidation that can lead to adverse alterations. Free radical damage to the protein can result in loss of enzyme activity and such damage caused to DNA can result in mutagenesis and carcinogenesis [3].

Antioxidants are compounds able to neutralize free radicals by using their own electrons to the free radicals thereby preventing cellular damage. After neutralizing a free radical, the antioxidants become inactive, which means they need to be constantly re-supplied to the bodies through the right nutrients. The natural antioxidants come mainly from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid or carotenoids. Thus, knowledge of plant antioxidants and tapping their potential is of increasing interest [4].

The group commonly known as alkaloids constitutes tremendous varieties of nitrogenous compounds which are usually, but not always of plant origin and heterocyclic. Alkaloids are reported to have a beneficial effect in various neurodegenerative and neuropsychiatric disorders whilst also exhibiting their capability in modulating neurotransmitters and their receptor systems in the brain [5]. Its protective effect in neuro disorders such as Alzheimer's, cerebral ischemia, mental depression, schizophrenia, and anxiety is highlighted in many studies. There have been few studies of alkaloids as antioxidant compounds, but under some conditions, some have been reported to be highly active. An un protonated, basic nitrogen atom might be expected to be a potent electron donor and, therefore, a potentially good antioxidant. A possible antioxidant role of alkaloids could be as quenchers of singlet oxygen [6].

Synthetic antioxidants such as butyl hydroxy anisole (BHA) or butyl hydroxytoluene (BHT) have been used widely in food industries, but there have been concerns regarding its side effects on humans. Recent researches have shown that the antioxidants of plant origin with free-radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress [7]. Plant extracts and phytoconstituents found effective as radical scavengers and inhibitors of lipid peroxidation [8, 9]. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant [10].

Glycosmis pentaphylla, belonging to the Rutaceae family, is a shrub or small (1.5–5 m) tree, widely distributed, spanning from India, Malaysia and Southern China to the Philippine Islands where it occurs in tropical forests at low altitudes. It has been used as folk medicine for the treatment of fever, liver complaints, jaundice, cough, eczema, anemia, diarrhea, and rheumatism [11, 12]. Chemical studies of *Glycosmis pentaphylla* revealed the presence of acridone, quinolones, carbazoles, quinozoline, quinolinone, furoquinoline alkaloids. Studies have shown the extracts of *Glycosmis pentaphylla*

having potent anthelmentic, antipyretic, hepatoprotective, antibacterial, antioxidant, antidiabetic and antinociceptive properties [13, 14]. Based on the traditional claims surrounding *Glycosmis pentaphylla* of its potential pharmacological properties, the objective of this study was to evaluate the antioxidant potential of the total crude alkaloid through free radical scavenging methods.

MATERIALS AND METHODS

Materials

Procurement and authentication of the plant

Fresh leaves of *Glycosmis pentaphylla* were collected from its natural habitat from the district of Thiruvananthapuram (Latitude-8.54 °N and Longitude-76.91ºE), Kerala, India. The titled plant was botanically identified by Curator, department of Botany, the University of Kerala and a voucher specimen of the titled plant has been deposited under the code number KUBH 5858 at the herbarium of Botany Department, University of Kerala, India. Healthy, uninfected and undamaged leaves were used for the study. The leaves were washed under running tap water and dried under shade at room temperature for two weeks. The dried leaves were powdered using an electric blender and stored in airtight bottles to free from moisture and humidity until further experimental usage.

Chemicals

All chemicals and solvents were of the analytical grade obtained from Sigma Chemical Company, U. S. A, Merck India Pvt. Ltd, and Himedia, Bombay.

Methods

Preparation of total crude alkaloid extract

The total alkaloids were extracted by the method of Gonzales *et al.* with modifications [15]. 20 g of ground leaf powder moistened with sufficient amount of 95 % ethanol. This was made alkaline with liquor ammonia and kept undisturbed for overnight. The sample is placed in a soxhlet apparatus on the next day using 95 % ethanol as the solvent. The sample was extracted for 5 h. The ethanol extract filtered and concentrated using a rotary evaporator at 60 °C. The crude extract was treated with 1.0 N hydrochloric acid. This was filtered and the filtrate was alkalified with liquor ammonia and placed in a separatory funnel. Equal quantities of chloroform were added into the separatory funnel, mixed and shaken 100 times slowly up and down manner and allowed to separate into two layers. The lower layer of chloroform contained the alkaloids and the upper layer was extracted until the last chloroform extract was found negative to Dragendorff's reagent. The combined chloroform extract was concentrated in a rotary evaporator. The residue weighed, and the percentage yield was calculated using the formula.

$$\% \text{ yield} = \frac{\text{weight of the alkaloid residue}}{\text{weight of ground sample}} \text{x}100$$

Investigation of antioxidant potential

The antioxidant potential of the extracted total crude alkaloid of *Glycosmis pentaphylla* leaves was determined by following methods.

1-2, 2-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging method

The radical scavenging activity of different extracts was determined by using $DPPH^{\cdot}$ assay according to Chang *et al.* [16]. The principle of this assay is that 1, 1-diphenyl-2-picryl hydrazyl is a stable free radical with red color which turns yellow when scavenged. The $DPPH^{\cdot}$ assay uses this character to show free radical scavenging activity. The scavenging reaction between ($DPPH^{\cdot}$) and an antioxidant (H-A) can be written as,

$$DPPH^{\cdot} + [H\text{-}A] \rightarrow DPPH\text{-}H + (A)$$

Antioxidants react with $DPPH^{\cdot}$ and reduce it to DPPH-H and as a consequence, the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The decrease in the absorption of the $DPPH^{\cdot}$ solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10 mg/ml DMSO) was used as a reference. 0.1 mM $DPPH^{\cdot}$ solution was prepared by dissolving 4.0 mg of $DPPH^{\cdot}$ in 100 ml of methanol. Different volumes (1.25-20 µl) of plant extracts were made up to 40 µl with DMSO and 2.96 ml $DPPH^{\cdot}$ (0.1 mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 517 nm. Methanol was taken as the blank and 3.0 ml of $DPPH^{\cdot}$ as a control. The percentage scavenging activity at different concentrations was determined and the IC_{50} value of the fractions was compared with that of standard ascorbic acid.

$$\% \text{ activity} = \frac{A0-A1}{A0} \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the sample

Ferric reducing power assay (FRAP)

The experiment was processed according to Benzie and Strain method [17]. FRAP assay is based on the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ (2, 4, 6-tripyridyl-s-triazine), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. The absorbance decrease is proportional to the antioxidant content. Various concentrations of the extract were added to 270 µl of FRAP reagent (10 mM TPTZ in 40 mM HCL, 20 mM $FeCl_3.6H_20$ in water solution). The mixture was incubated in the dark for 30 min at room temperature. The absorbance was determined at 593 nm against the blank, which was water. The reducing ability of the crude alkaloid extract was evaluated in terms of percentage by relating the absorbance value of the sample extract and the standard. Ascorbic acid was used as the standard. The percentage ferric reducing antioxidant activity was calculated using an equation below

$$\% \text{ reduction ability} = \frac{A0-A1}{A0} \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the sample

2, 2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) scavenging assay (ABTS•+)

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] ABTS•+radical cation decolourization assay according to the method of Re *et al.* [18]. The principle of ABTS•+assay is the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2, 2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, ABTS•+, a soluble chromogen that is green in colour and can be determined spectrophotometrically at 734 nm. Antioxidants suppress the production of the radical cation in a concentration-dependent manner, and the color intensity decreases proportionally. 2.0 mM ABTS•+(0.0548 g in 50 ml) was prepared in distilled water. Potassium persulphate 70 mM (0.0189 g in 1.0 ml) was prepared in distilled water. 200 µl of potassium per sulphate and 50 ml of ABTS•+were mixed and used after 2 h. This solution was used for the assay. To the 0.5 ml of various concentrations of the extract and standard ascorbic acid, 0.3 ml of ABTS•+solution and 1.7 ml of phosphate buffer pH 7.4 was added and the absorbance was measured at 734 nm. The inhibition was calculated in the following way:

$$\% \text{ inhibition} = \frac{A0-A1}{A0} \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the sample.

Nitric oxide scavenging assay (NO)

Nitric oxide radical scavenging activity was measured spectrophotometrically according to the method of Garrat described by the use of Griess Illosvoy reaction [19]. When sodium nitroprusside was mixed with an aqueous solution at physiological pH, suddenly it generates nitric oxide, which reacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. NO scavengers compete with oxygen leading to reduced production of

nitrite ions. Sodium nitroprusside (5 mmol/l) in phosphate buffered saline, pH 7.4, was mixed with a different concentration of the extract (125-2000 µg/ml) prepared in methanol and incubated at 25 °C for 30 min. A control without the test compound but an equivalent amount of methanol was taken. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1 % sulphanilamide, 2 % phosphoric acid and 0.1 % N-1-naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphthyl ethylenediamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard gallic acid. The percentage scavenging of nitric oxide of the sample and standard compound was calculated using the following formula:

$$\text{NO scavenged (\%)} = \frac{A0-A1}{A0} \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the sample

Hydroxyl radical scavenging assay (Fenton reaction based radical (FRBR) assay)

Hydroxyl radicals ($OH^{\cdot}$) were generated by a Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2 system), and the scavenging capacity towards the hydroxyl radicals was measured by using deoxyribose method [20]. The reaction mixture in a final volume of 1.0 ml contained 100 µl of 2-deoxy 2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 100 µM $FeCl_3$ (1:1 v/v), 1.04 mM EDTA (100 µl), 100 µl H_2O_2 (1.0 mM), 1.0 mM ascorbic acid (100 µl) and various concentrations (125-2000 µg/ml) of the test sample. Test samples were kept at 37 °C for 1 hour. The free radical damage imposed on the substrate deoxyribose was measured using the thiobarbituric acid test. About 1.0 ml of 1 % thiobarbituric acid (TBA) and 1.0 ml 2.8 % trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100 °C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Ascorbic acid was used as positive control. The hydroxyl radical scavenging activity of the extracts is reported as % inhibition of deoxyribose degradation and is calculated as,

$$\text{OH scavenged (\%)} = \frac{A0-A1}{A0} \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the sample

Ferric (Fe^{3+}) reducing power capacity assessment

The reducing power was determined according to the method of Oyaizu [21]. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferrous ferric complex that has an absorption maximum at 700 nm. An aliquot of extracts (1.0 ml) at various concentrations was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 ml) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 25 ml of 10 % trichloroacetic acid, the mixture was centrifuged at 6500 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% iron (III) chloride (0.5 ml) and the absorbance was measured at 700 nm using phosphate buffer as blank. Ascorbic acid at various concentrations was used as a standard. The increased absorbance of the reaction mixture indicates an increase in reducing power.

Calculations of 50% inhibitory concentrations (IC_{50})

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process by half. In other words, it is the half maximal (50 %) inhibitory concentration (IC) of a substance (50 % IC, or IC_{50}). Sometimes, it is also converted to the pIC_{50} scale (-log IC_{50}), in which higher values indicate exponentially greater potency. According to the Food and Drug Administration (FDA), IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in vitro*. The concentration of the fractions that was required to scavenge 50 % of the radicals was calculated by using the percentage scavenging activities versus the concentration of extract using nonlinear regression analysis (curve fit) at five different concentrations of the extract and standard, as well.

Statistical analysis

All the aforementioned experiments were evaluated statistically with the use of SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Three independent measurements of each experiment were pooled and subjected to statistical analysis. The results were represented in mean±SEM (standard error of mean). One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) to find out any significant differences in antioxidant activities resulting from the analytical experiments were carried out. P value less than 0.05 were adopted as statistically different.

RESULTS AND DISCUSSION

In pharmacology studies, it has been reported that the alkaloids were found to have antifungal, analgesic, cardiotonic, thermoregulation, vascular regulation and anti-tumor activities. As many naturally occurring molecules, such as flavonoids, phenols, and quinones, alkaloids also have antioxidant properties, including the inhibitory effect of free radicals and other damaging oxygen activated products such as hydroxyl radical, superoxide, hydrogen peroxide and lipid peroxides. However, there is a little study of the antioxidant property of total crude alkaloid from *Glycosmis pentaphylla* leaves, so far known.

Extraction efficiency

Extraction is a very decisive step for the isolation of bioactive constituents from plants in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products. The solvents, nature, the age of the plant materials and the conditions used for extraction greatly influence the yield and bioactivities. The extractability of alkaloids tends to pose a serious challenge due to their high polarity. Hence, a significant affinity to the acid-base extraction method is the mainstay of alkaloid separation and in almost all cases is the simplest way to achieve purity and good yield [22, 23]. However, according to Phillipson, quaternary alkaloids cannot be extracted easily by these methods because; in general they remain in the alkaline aqueous phase when partitioned with immiscible organic solvent [24]. If the alkaline aqueous phase is acidified with mineral acids, then quaternary alkaloids can be precipitated. In the present study extraction of total crude alkaloid extract involved soxhlet extraction of leaf powder using 95 % ethanol followed by acidification and basification and finally extraction of the crude alkaloid from the basified extract with the chloroform. The extraction was based on previously developed method modified accordingly in the present study [25].

It can be gleaned from table 1 that 10 g ground leaf powder yielded with an average weight of a crude extract of 2.56±0.12 w/w gram and after acid-base extraction of the same yielded 1.27±0.03 w/w percentage of total crude alkaloid. In a similar study done by Shahrear Ahmad *et al.* dried leaf powder yielded 1.43 % (w/w) total crude alkaloid [26]. By the conventional acid-base protocol the percentage yield of crude alkaloid was 4 % w/w in *Litsea polyantha* bark [27]. But by altering the pH and the temperature the percentage yield was increased from 4 % to 17.3 % w/w, inferring that pH and temperature affect the yield value of the crude extract.

***In vitro* evaluation of antioxidant efficiency**

The principle of antioxidant activity is based on the availability of electrons to neutralize free radicals. Performing a single assay to evaluate the antioxidant properties would not give the correct result because the antioxidant activity of plant extract is influenced by many factors, for example, the test system and composition of the extract. Therefore, it is important to carry out more than one type of antioxidant capacity measurement to cover the various mechanisms of antioxidant action [28]. In this study, the antioxidant activity of the total crude alkaloid extract of *Glycosmis pentaphylla* leaves was evaluated by various *in vitro* methods.

Table 1: The percentage yield of total alkaloid extract using acid-base extraction technique

Weight of leaf powder	Solvent	Volume of solvent	Weight of extract (g)	% yield of total alkaloid after acid-base processing
10 g	95 % ethanol	200 ml	2.56±0.12	1.27±0.03

Data are expressed mean±standard error done in triplicate.

DPPH· radical scavenging method

The DPPH are often used to evaluate the ability of antioxidants to scavenge free radicals, which are known to be a major factor in biological damages caused by oxidative stress [29]. In this assay, radicals are reduced to their stable or less reactive derivatives by the antioxidant compounds. In the present investigation, the total crude alkaloid extract exhibited scavenging activity against DPPH· radical in a significant dose-dependent fashion (P<0.05). The present findings are in harmony with reports describing the dose-dependent antioxidant activity of alkaloids [30]. Maximum scavenging activities, expressed as percentage inhibition, was found at the concentrations of 2000 µg/ml (table 2) with an IC_{50} value 966.93 µg/ml, where standard ascorbic acid showed free radical scavenging with an IC_{50} value of 152.33 µg/ml. Crude methanolic extract of *Glycosmis pentaphylla* leaves showed higher antioxidant activity with an IC_{50} value of 37.62 µg/ml than the result of the present study [31]. The effect of higher antioxidant activity in the methanolic extract than the total crude alkaloid extract could be due to the high percentage of major secondary metabolites as well as other minor components in small quantities or the synergistically actions between the both. From the results, it seems that alkaloids are capable of scavenging the free radical DPPH·. Several reports confirm these data [32-34]. The DPPH· assay involves a reduction mechanism; therefore, it is possible that the total crude alkaloid extract probably had lesser DPPH· reductions compared to that of the standard ascorbic acid. This might explain the marked differences in DPPH· IC_{50} values between the sample and control. Apart from this, the variation in the antioxidant mechanism of active compounds in the extracts could also lead to these differences [35].

FRAP

The FRAP assay evaluates total antioxidant power and is chosen to assess the presumable effects of medicinal plants [36]. FRAP assay depends upon the ferric tripyridyl triazine (Fe (III)–TPTZ) complex to form ferrous tripyridyl triazine (Fe (II)–TPTZ) by a reductant at low pH. Fe (II)–TPTZ has an intensive blue color and can be monitored at 593 nm [37]. In the present investigation of FRAP assay, the reducing power was observed over the concentration range 100–500 µg/ml. The results (table 3) for FRAP were calculated from calibration graph which was linear over the calibration range with R^2 value of 0.993 for total alkaloid extract and 0.975 for standard ascorbic acid (fig. 2). In the present study, the trend for ferric ion reducing activities of total crude alkaloid extracts of *G. pentaphylla* leaf showed a decrease in absorbance (absorbance values not shown), due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration. Decrease in absorbance value is inversely proportional to increase in reducing the capacity of the extract/sample. The total crude alkaloid extract showed a significant dose-dependent activity, and it reached up to 48.95±2.10 % free radical inhibition 500 µg/ml. However, an ascorbic acid which was used as a positive control showed a better radical scavenging effect, 85±2.35 % at the concentration of 500 µg/ml. The effect of the extract was significant (p<0.05) comparable with that of standard ascorbic acid. A similar result has been reported in *Glycosmis mauritiana* species [38]. A connected study conducted on the ripened fruits of *Glycosmis pentaphylla* has shown antioxidant characteristics. Present study carried hereunder shows similar characteristics for the leaf on the same plant. Similar antioxidant characteristics exhibited by different entities of the same plant suggest that the antioxidant characteristics for the plant are inherent. According to Paulido *et al.*, FRAP assay measures ferric to-ferrous reduction capacity of water-soluble antioxidants in acidic pH such as pH 3.6 [39]. The disadvantage found in this method is that the FRAP assay does not react fast with some antioxidants such glutathione [40]. But according to Schafer and Buettner FRAP assay still can be used for assessment of antioxidant activity in plant materials as humans only absorb limited amount of glutathione [41].

ABTS•+scavenging assay

The principle behind the ABTS•+assay technique involves the reaction between ABTS•+and potassium persulphate to produce the ABTS radical cation (ABTS•+) a blue, green chromogen. In the presence of an antioxidant reductant, the colored radical is converted back to colorless ABTS, the absorbance of which is measured at 734 nm. It is often used in evaluating the total antioxidant power of single compounds and complex mixtures of various plants [42]. The scavenging ability of *Glycosmis pentaphylla* leaf on ABTS•+free radical is shown in table 3. In this, ascorbic acid acted as the comparison standard. It is found that radical scavenging activity increased as the concentration of total crude alkaloid increased. The results show that at 500 µg/ml concentration standard ascorbic acid shows maximum inhibition of ABTS•+with 81.57 %, while at the same concentration, total crude alkaloid extract showed very low inhibition of ABTS•+with only 56.33 % (table 3). From the plot of concentration against percentage inhibition, a linear regression analysis was performed in Microsoft Excel 2007 to obtain the IC_{50} value. The total crude alkaloid extract of *Glycosmis pentaphylla* leaf showed maximum IC_{50} value being 400.47 µg/ml, suggesting a weak free radical scavenging activity compared to a standard ascorbic acid having IC_{50} value of 120.38 µg/ml. The presence of specific alkaloid compounds in the extracts of *Glycosmis pentaphylla* may inhibit the potassium persulfate activity and hence reduced the production of ABTS•+. Statistical analysis of ABTS•+scavenging activity was significantly different from each other (p<0.05). A similar kind of trend could be observed in a study made in the same plant by Gupta N *et al.* where an ethanol extract of *Glycosmis pentaphylla* plant showed ABTS•+free radical scavenging activity with IC_{50} value of 26.2 µg/ml compared to an ascorbic acid having an IC_{50} value of 22.8 µg/ml [14].

Table 2: DPPH·, OH·, NO scavenging activity of total alkaloid extract of *Glycosmis pentaphylla* leaves

Concentration (µg/ml)	Percentage Inhibition					
	DPPH·		OH·		NO	
	Ascorbic acid (standard)	Alkaloid extract	Ascorbic acid (standard)	Alkaloid extract	Gallic acid (standard)	Alkaloid extract
125	45.54±0.11^{e}	16.14±1.01^{e}	43.14±1.16^{e}	10.76±0.04^{e}	47.82±1.20^{e}	09.36±0.01^{e}
250	65.94±0.25^{d}	24.81±0.01^{d}	55.33±0.52^{d}	16.47±0.21^{d}	56.53±0.52^{d}	15.54±0.05^{d}
500	81.63±1.01^{c}	32.46±2.10^{c}	64.84±1.52^{c}	30.13±1.10^{c}	60.86±1.92^{c}	36.27±1.20^{c}
1000	89.36±3.01^{b}	51.25±0.01^{b}	70.36±0.93^{b}	41.15±0.10^{b}	65.22±2.50^{b}	48.23±2.15^{b}
2000	92.46±2.01^{a}	62.73±2.32^{a}	77.72±0.67^{a}	52.14±1.90^{a}	78.26±1.55^{a}	52.38±1.30^{a}
IC_{50}	152.33	966.93	195.96	1805.28	156.29	1426.50

Each value is expressed as mean±standard error done in triplicates. Data were analyzed by ANOVA SPSS version 12.0 for windows followed by Duncan Multiple Range Test (DMRT) for comparison at P= 0.05 level of significance. Mean values followed by different superscript in a column are significantly different.

The scavenging of the ABTS•+radical by the extracts was found to be much higher than that of DPPH· radical. Factors like stereo selectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals [43]. Wang *et al.* found that some compounds which have ABTS•+scavenging activity did not show DPPH· scavenging activity [44]. The ABTS•+radical is stable and soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compound/ samples. However, as the results obtained for samples are related to an antioxidant compound that shows different kinetic behavior, the results provided by this assay are dependent on the time of analysis [45].

Nitric oxide (NO) scavenging assay

Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control of vasodilation and control of blood pressure [46, 47]. However, the elevation of the NO results in several pathological conditions, including cancer. The plant/plant products may have the property to counteract the effect of NO formation and in turn, may be of considerable interest in preventing the ill effects of excessive NO generation *in vivo*. Incubation of solutions of sodium nitroprusside in PBS at 25 °C for 30 min resulted in linear time dependent nitrite production, which is reduced by the tested total crude alkaloid extract of *Glycosmis pentaphylla* leaves. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. NO scavenging capacity is determined by the decrease in the absorbance at 550 nm, induced by antioxidants. In order to evaluate the antioxidant potency through NO scavenging by the test samples, the change of optical density of NO was monitored. The percentage inhibition at various concentration (200-500 µg/ml) of total crude alkaloid extract as well as standard ascorbic acid (12.5-100 µg/ml) were calculated using Microsoft Office Excel 2007. The IC_{50} values are calculated from the graph using linear regression. The total crude alkaloid extracts of *Glycosmis pentaphylla* leaves significantly ($p<0.05$) inhibited the generation of NO in a concentration dependent manner with IC_{50} being 1426.50 µg/ml as compared with the concurrent standard ascorbic acid having IC_{50} value of 156.29 µg/ml (table 2). The total crude alkaloid extract leaves showed the maximum activity of 52.38 % (moderate activity) at 2000 µg/ml, whereas ascorbic acid at the same concentration exhibited 78.26 % (high activity) inhibition. This may be due to the presence of the antioxidant principle present in the extract which inhibited the binding of oxygen to nitric oxide. The reports regarding the inhibition of NO by the total alkaloid extract of *Glycosmis pentaphylla* are unavailable. However, crude extracts of *Glycosmis pentaphylla* have been reported to inhibit NO radicals *in vitro* [48]. In that study aqueous extract reported to scavenge NO by 52.07 % at 50 µg/ml concentration. In a similar study made in the same plant, the ethanolic extract of *Glycosmis pentaphylla* leaves exhibited NO radical scavenging activity of 61.06 % at 50 µg/ml concentration with IC_{50} value being 31 µg/ml [14].

FRBR assay

Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals [49]. Hydroxyl radical can cause oxidative damage to DNA, Proteins and lipids [50]. The hydroxyl radical scavenging ability of the extracts was found in its ability to contend with deoxyribose for hydroxyl radical generated by the Fenton reaction. In this study *Glycosmis pentaphylla* crude alkaloid extract was found to compete with deoxyribose and diminish the formation of chromogen significantly ($P<0.05$) and in a dose-dependent manner. A similar dose-dependent hydroxyl radical scavenging activity was observed for all the extracts of *Glycosmis pentaphylla* and in which of all the extracts put into the account, ethanolic extract showed better hydroxyl radical scavenging activity compared to standard ascorbic acid [48]. The hydroxyl radical-scavenging effect of the total crude alkaloid extract in a concentration of 2000 µg/ml was found to be 52.14±1.90and ascorbic acid was used as a standard since it is reported to be significantly effective in inhibition of hydroxyl radicals, showed 77.22±0.67 scavenging effect at the same concentration (table 2). In the present study, the radical scavenging activity of the alkaloid extract is moderate when compared to standard (ascorbic acid). Obviously, the components possessing the scavenging ability of hydroxyl radicals might not be present in the total crude alkaloid extract. The IC_{50} value of alkaloid extract and ascorbic acid was found in the concentration of 1805.28 µg/ml and 195.96 µg/ml respectively (table 2). Contrary to the present study ethanolic extract of *Bauhinia variegata* showed lower IC_{50} value than the standard ascorbic acid [48].

Reducing power capacity assessment

The reducing power of a compound is related to its electron transfer ability and may therefore serve as a significant indicator of its antioxidant activity [51]. A direct correlation between antioxidant activities and reducing the power of plant extracts has been reported in many studies [14]. Reducing power is the measure of the reductive ability of antioxidant and it is evaluated by the transformation of $Fe^{3+}$to $Fe^{2+}$by the reductones (antioxidant) present in the extracts [52]. In the reducing power assay, the total crude alkaloid extract of *Glycosmis pentaphylla* leaves showed a concentration dependent reducing power potential. Fig. 1 shows the reducing power of the total crude alkaloid extract and standard ascorbic acid as a function of their concentration. Increased absorbance at 700 nm indicates an increase in reducing power. The present study depicts that the reductive effect of total crude alkaloid extract increased (in terms of absorbance) with a concentration of extracts (fig.1). Similar results were obtained in many studies [53, 54]. At a maximum concentration of 500 µg/ml the extract showed the absorbance of 0.46±0.01. However, its reducing power was weaker than that of ascorbic acid, which exhibited the strongest reducing power at the same concentration (0.93±0.01). Gupta N *et al.* have reported a good reducing power activity of the ethanolic extracts of *G. mauritiana* [14]. Anandhan *et al.* reported that essential oil from leaves of *G. pentaphylla* showed excellent reducing capacity potential compared to the standard ascorbic acid at the concentration 100 µg/ml [55]. In a study conducted by Panduranga Murthy G *et al.* on the tribal medicine found in BR hills in Karnataka, one of the formulated components in the tribal medicine is *Glycosmis pentaphylla,* which reported a moderate to high reducing power activity [56]. In the present study, the crude alkaloid extract of the leaves showed a moderate level of reducing power activity. Whilst two observations are in conjunction, clearly highlights the reducing power characteristics of the plant. Jayanthi *et al.* found in her study that the reducing ability of the plant extract is dependent on time, probable reason being the decrease in reducers which would have converted the Fe^{3+}/ferricyanide complex to the ferrous form within a short time [57].

Table 3: FRAP, ABTS•+scavenging activity of total alkaloid extract of *Glycosmis pentaphylla* leaves

Concentration (µg/ml)	Percentage Inhibition			
	FRAP		ABTS•+	
	Ascorbic acid (standard)	Alkaloid extract	Ascorbic acid (standard)	Alkaloid extract
100	45.15±0.72^{e}	11.35±0.01^{e}	48.63±1.52^{e}	12.04±1.20^{e}
200	52.19±1.20^{d}	23.54±0.58^{d}	55.35±0.55^{d}	29.55±1.54^{d}
300	62.14±1.52^{c}	31.17±0.32^{c}	59.25±1.66^{c}	42.76±0.94^{c}
400	79.27±0.55^{b}	39.24±1.50^{b}	72.86±1.24^{b}	49.97±2.73^{b}
500	85.33±2.35^{a}	48.95±2.10^{a}	81.57±2.55a	56.33±0.08^{a}
IC_{50}	168.89	510.81	120.38	400.47

Each value is expressed as mean±standard error done in triplicates. Data were analysed by ANOVA SPSS version 12.0 for windows followed by DMRT for comparison at P= 0.05 level of significance. Mean values followed by different superscript in a column are significantly different.

Calculations of 50% inhibitory concentrations (IC_{50})

The input data consists of values for the percentage concentration for each sample and the corresponding percentage inhibition observed for the same for 5 test samples with varying incremental concentration being observed to determine the percentage inhibition characteristics. A plain graph is plotted with the obtained values to form a smooth curve scatter graph in Microsoft Excel 2007 with the concentration read along the X-axis and the percentage inhibition read along the Y-axis. The smooth line curve, thus formed will give the characteristic of the sample which in some cases will for a linear graph whereas some samples will exhibit a logarithmic curve. In each case, the corresponding trendline is plotted onto the graph, thereby obtaining the trendline equation (Linear equation or Logarithmic equation) for the curve which will yield the unknown constants for the graph. Thus, solving a new similar equation for its 'X' value with the obtained constants from the graph and 50% inhibition for 'Y' value will give the corresponding concentration for the sample.

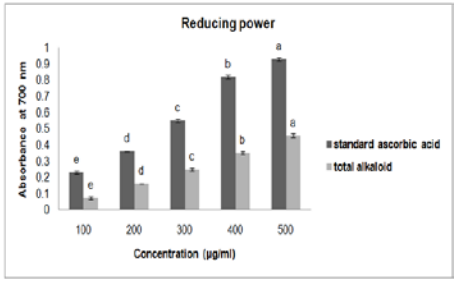

Fig. 1: Reducing the power of total crude alkaloid extract of *Glycosmis pentaphylla* leaves. Each value is expressed as mean±standard error done in triplicates. Data were analysed by ANOVA SPSS version 12.0 for windows followed by DMRT for comparison (p<0.05). Mean values followed by different superscript in a column are significantly different

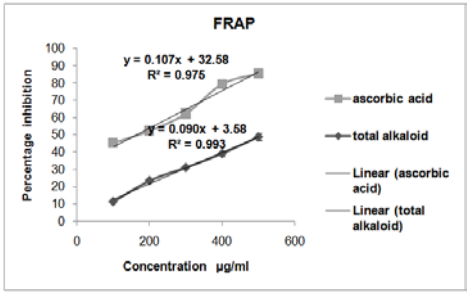

Fig. 2: Smooth curve scatter graph (Microsoft Excel 2007) plotted for FRAP assay of total crude alkaloid extract of *Glycosmis pentaphylla* leaves and standard ascorbic acid. Regression line equation and the R^2 of the sample and standard are represented

CONCLUSION

Alkaloids are reported to possess a variety of activities, including neuroprotective and antioxidant effects. Alkaloids have strong potential in inhibition and prevention of Alzheimer's disease mainly through both choline esterases and beta-amyloids pathways, and additionally through antioxidant capacities. The results documented in this study indicate that the total crude alkaloid extract of *Glycosmis pentaphylla* leaf attenuated moderating the oxidative stress through its antioxidant properties. The combined effect of various other antioxidant metabolites or a pro-oxidative activity may reduce the total antioxidant strength of the plant extract. As the plant *Glycosmis pentaphylla* is rich in phytochemicals and many alkaloids contained therein would clearly have beneficial uses in the development of therapeutic and preventive agents. Overall, present data on the potentiality of crude alkaloids as antioxidant agents and, along with other published data on *Glycosmis pentaphylla* alkaloids highlight a possible therapeutic potential of neurological diseases such as Alzheimer's, Parkinson's and other oxidative stress-related diseases. With a number of alkaloids currently under investigation, more effective therapeutic candidate for the treatment of is much anticipated in the near future.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Department of Botany, University of Kerala for the facilities.

CONFLICT OF INTERESTS

Authors declare that they have no conflict of interest concerning this research paper

REFERENCES

1. Halliwell B, Gutteridge JMC. The chemistry of free radicals and related reactive species'. In: Free radicals in biology and medicine. 4th edn. Oxford University Press: Oxford; 2007. p. 30-79.
2. Halliwell B. "Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life". Plant Physiol 2006;141:312-22.
3. Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: current status and future prospects. J Assoc Physicians India 2004;52:794-804.
4. Nawal Kishore Dubey. Plants as a source of natural antioxidants. 1st ed. United Kingdom: CABI publishers; 2014. p. 242.
5. Kulkarni SK, Dhir A. Berberine: a plant alkaloid with therapeutic potential for central nervous system disorders. Phytother Res 2010;24:317-24.
6. Richard Larson A. Naturally occurring antioxidants. United States: CRC Press LLC Lewis Publishers; 1997. p. 145.
7. Ramchoun M, Harnafi H, Alem C, Benlys M, Elrhaffari L, Amrani S. Study on antioxidant and hypolipidemic effects of polyphenol rich extract from *Thymus vulgaris* and *Lavendula multifida*. Pharmacognosy Res 2009;1:106-12.
8. Dash DK, Yeligar VC, Nayak SS, Ghosh T, Rajalingam D, Sengupta P, *et al.* Evaluation of hepatoprotective and antioxidant activity of *Ichnocarpus frutescens* (Linn.) R. Br. on paracetamol-induced hepatotoxicity in rats. Trop J Pharm Res 2007;6:755-65.
9. Yildirim A, Oktay M, Bulaloulu V. The antioxidant activity of the leaves of *Cydonia vulgaris*. Turk J Med Sci 2001;31:23-7.
10. Nagulendran K, Velavan S, Mahesh R, Begum VH. *In vitro* antioxidant activity and total polyphenolic content of *Cyperus rotundus* rhizomes. E J Chem 2007;4:440-9.
11. Kirtikar KR, Basu BD. Indian medicinal plants. Dehradun: International Book Publisher; 1993. p. 1621-2.
12. Ghani A. Medicinal plants of bangladesh. 2nd ed. Bangladesh: Asiatic society of Bangladesh; 2003. p. 1-17.
13. Jaya Raju N, Rao BG. Evaluation of hepatoprotective activity of *Glycosmis pentaphylla* roots against CCl_4 induced acute liver injury in rats. Int J Pharm Pharm Sci 2010;4:81-6.
14. Gupta N, Agarwal M, Bhatia V, Jha SK, Dinesh J. *In vitro* antioxidant activity of crude extracts of the plant *Glycosmis pentaphylla* Correa. Int J Pharm Sci Rev 2011;6:159-62.
15. Gonzales, Maria Victoria M, Tolentino, Angelina G. Extraction and isolation of the alkaloids from the *Samanea Saman* (acacia) bark: its antiseptic potential. Int J Life Sci Biotechnol Pharma Res 2014;3:119-24.
16. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant activity of extracts from *Acacia confusa* bark and heartwood. J Agric Food Chem 2001;49:3420-4.
17. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': the FRAP assay. Anal Biochem 1996;239:70–6.
18. Roberta Re, Nicoletta Pellegrini, Anna Proteggente, Ananth Pannala, Min Yang, Catherine Rice-Evans. Antioxidant activity

is applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med 1999;26:1231-7.

19. Garrat DC. The quantitative analysis of drugs. 3rd ed. Japan: Springer; 1964. p. 456-8.
20. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple 'test tube' assay for determination of rates constants for reactions of the hydroxyl radical. Anal Biochem 1987;165:215-24.
21. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr 1986;44:307-15.
22. Zanolari B, Guilet D, Marston A, Queiroz EF, Paulo MQ, Hostettmann K. Tropane alkaloids from the bark of *Erythroxylum vacciniifolium*. J Nat Prod 2003;66:497-502.
23. Zhang X, Yea W, Zhaoa S, Che CT. Isoquinoline and isoindole alkaloids from *Menispermum dauricum*. Phytochem 2004;65:929-32.
24. Phillipson JD. Alkaloids. Pharm J 1983;102-3.
25. Gonzales, Maria Victoria M, Tolentino, Angelina G. Extraction and isolation of the alkaloids from the *Samanea Saman* (Acacia) bark: its antiseptic potential. Int J Sci Tech Res 2014;3:119-24.
26. Shahrear Ahmad, Amin MN, Ashik Mosaddik. Studies on moisture content, biomass yield (Crude Plant Extract) and alkaloid estimation of *in vitro* and field grown plants of *Rauvolfia serpentine*. Pak J Biol Sci 2002;5:416-8.
27. Manik Ghosh, Barij Sinha N, Julio A Seijas, M Pilar Vázquez-Tato, Xesús Feás, Optimization process for increasing the yield of crude alkaloid from *Litsea polyantha* JUSS. Bark. Sciforum.net/conference/ecsoc-18/2555. p. 1-5.[Last accessed on 05 sep. 2015].
28. Gan RY, Xu XR, Song FL, Kuang L, Li HB. Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. J Med Plants Res 2010;4:2438-44.
29. Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne HD. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J Food Compos Anal 2006;19:669-75.
30. Houcine Benmehdi, Nadia Bounoua, Abdelillah Amrouche, Driss Lahcene, Abdelhak Maazouzi. The phytochemical study, antioxidant and antimicrobial activities of *Euphorbia Resinifera* L. Int Res J Pharm 2013;4:44-50.
31. Amran Howlader, Farhana Rizwan, Shapna Sultana, Mohammad Rakibur Rahman, Shams-Ud-Doha KM, Rumana Mowla, *et al.* Antimicrobial, antioxidant and cytotoxic effects of methanolic extracts of leaves and stems of *Glycosmis pentaphylla* (Retz.). J Appl Pharm Sci 2011;1:137-40.
32. Rackova L, Majekova M, Kostalova D, Stefek M. Antiradical and Antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects. Bioorg Med Chem 2004;12:4709-15.
33. Yanxuan W, Fuhou L. Studies on the antioxidant activities of total alkaloids from the fruits of *Evodia rutaecarpa* (Juss) Benth. Adv Mat Res 2012;396-398:52-5.
34. Maiza Benabdesselam F, Khentache S, Bougoffa K, Chibane M, Adach S. Antioxidant activities of alkaloid extracts of two Algerian species of *Fumaria*: *Fumaria capreolata* and *Fumaria bastardii*. Rec Nat Prod 2007;1:28-35.
35. Diplock AT. Will the good fairies please prove to us that vitamin E lessens human degenerative disease? Free Radical Res 1997;27:511–32.
36. Szollosi R, Varga IS. Total antioxidant power in some species of Labiatae (Adaption of FRAP method). Acta Biol Szeg 2002;46:125-7.
37. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem 1996;239:70-6.
38. Ebenezer T, Kadamban D, Shekhawat MS, Justin Packia Jacob S. Phytochemical evaluation and antioxidant activity of *Glycosmis mauritiana* (Lam.) tanaka leaf extract. Int J Pharm Res Rev 2013;2:16-23.
39. Pulido R, Bravo L, Saura-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem 2000;48:3396-402.
40. Guo C, Yang J, Wei J, Li Y, Xu J, Jiang Y. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutr Res 2003;23:1719-26.
41. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/gluthathione couple. Free Radical Biol Med 2001;30:1191-212.
42. Aoxue Luo, Yijun Fan, Aoshuang Luo. *In vitro* free radical scavenging activities of polysaccharides from *Polygonum multiflorum Thunb*. J Med Plants Res 2011;5:966-72.
43. Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M. Free radical scavenging properties of wheat extracts. J Agric Food Chem 2002;50:1619-24.
44. Wang M, Li J, Rangarajan M, Shao Y, La Voie EJ, Huang T, *et al*. Antioxidative phenolic compounds from Sage (*Salvia officinalis*). J Agric Food Chem 1998;46:4869-73.
45. Aline Augusti Boligon, Michel Mansur Machado, Margareth Linde Athayde. Technical evaluation of antioxidant activity. J Med Chem 2014;4:517-22.
46. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524-6.
47. Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 1989;86:3375-8.
48. Bhatia L, Bishnoi H, Chauhan P, Kinja K, Shailesh S. *In-vitro* comparative antioxidant activity of ethanolic extracts of *Glycosmis pentaphylla* and *Bauhinia variegata.* Recent Res Sci Technol 2011;3:1-3.
49. Umamaheswari M, Chatterjee TK. *In vitro* antioxidant activities of the fractions of *Coccinia Grandis* L. leaf extract. Afr J Tradit Complementary Altern Med 2008;5:61-73.
50. Spencer JPE, Jenner A, Aruoma OI. Intense oxidative DNA damage promoted by L-DOPA and its metabolities, implications for neurodegenerative disease. FEBS Lett 1994;353:246-50.
51. Ajila CM, Naidu KA, Bhat SG, Prasad Rao UJS. Bioactive compounds of mango peel extract. Food Chem 2007;105:982-8.
52. Gulcin I, Oktay M, Kirecci E, Kufrevioglu I. Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L) seed extract. Food Chem 2003;83:371-82.
53. Duh PD. Antioxidant activity of burdock (*Arctium lappa* Linne.): Its scavenging effect on free-radical and active oxygen. J Am Oil Chem Soc 1998;75:455-61.
54. Duh PD, Du PC, Yen GC. Action of methanolic extract of Mung beans hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. Food Chem Toxicol 1999;37:1055-61.
55. Anandhan Vignesh, Purushothaman Rama, Govindhan Lakshmanan, Kandasamy Murugesan. Chemical composition and antioxidant activity of essential oil from *Glycosmis pentaphylla.* Int J Bot Res 2014;4:19-28.
56. Panduranga Murthy G, Chandrasekhar KB, Leelaja BC, Lokesh S. Antimicrobial and antioxidant activities of tribal medicine formulation (TMF) accomplished for wound related remedies in biligirirangana hill area of chamarajanagara district, Karnataka (India). Int J Pharm Chem 2015;5:261-76.
57. Jayanthi P, Lalitha P. Reducing power of the solvent extracts of *Eichhornia crassipes* (MART.) SOLMS. Int J Pharm Pharm Sci 2011;3:126-8.