

Original Article

IN VITRO ANTIOXIDANT POTENTIAL, FREE RADICAL SCAVENGING AND CYTOTOXIC ACTIVITY OF SIMAROUBA GLAUCA LEAVES.

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Received: 22 Nov 2014 Revised and Accepted: 15 Dec 2014

ABSTRACT

Objective: To investigate the total phenolic, flavonoids, tannin contents of *Simarouba glauca* leaves and evaluate the antioxidant potential and cytotoxic activity in different human cell lines.

Methods: The methanol, ethanol and water extracts of *S. glauca* leaves were examined for total phenolics, flavonoid and tannin content. Its antioxidant properties using FRAP, Phosphomolybdenum, Ferric ferrozine assays and free radical scavenging using DPPH were determined. Further, the reducing power and iron chelating effect of the extract using spectrophotometric assays were estimated. Finally, the cytotoxic activity against few human cancer cell lines were also examined using MTT assay.

Results: The phytochemical investigations revealed that *S. glauca* leaves has only 0.14 to 0.18% of flavonoids, 250-400µg/mg phenolics and 67-200µg/mg tannin content in various solvent extracts. The extracts exhibited good reducing power with similar EC₅₀ values approximately in the range of 57-61µg/ml. Further, the leaf extracts showed iron chelation effect which was more pronounced in aqueous extract with IC₅₀ value of 332µg/ml and exhibited very strong DPPH radical scavenging activity with IC₅₀ values ranging from 9-13µg/ml in various extracts. The methanolic extracts showed good antioxidant potential using FRAP and phosphomolybdenum methods, whereas the aqueous extract exhibited more pronounced antioxidant activity using sensitive ferric ferrozine assay with 580µg AAE/mg extract. The methanolic extract showed strong cytotoxic effect on SCC9 cancer cell line and less potent on HCT116 cancer cells.

Conclusion: The findings suggests that *Simarouba glauca* leaf extract contain bioactive molecules which exhibit antioxidant activity that could be synergistically influencing the cytotoxic activity in selected cancer cell lines.

Keywords: *Simarouba glauca*, Phenolics, Tannins, DPPH, FRAP, Reducing power, Iron chelation, Cytotoxicity.

INTRODUCTION

Stress in the human body results in the production and accumulation of reactive oxygen species (ROS) in terms of free radicals, which leads to oxidative stress that damage cellular components such as DNA, lipids, sugars and proteins [1]. This results in an imbalance of ROS homeostasis. The imbalance between oxidants and antioxidants has led to searching for novel additional antioxidant molecules that could be helpful in maintaining cellular homeostasis even though the body is equipped with the antioxidant defense system. Plants provide naturally occurring phytochemicals as a rich source of antioxidants having free radical scavenging property. Many of these dietary components, including flavonoids and phenolic acids contribute to the protective properties against cancer and other ill effects in humans. These phytochemicals act independently or in combination as contributing factors to the antioxidant activity in circumventing cancer by various mechanisms [2-5].

Exploration of the plant kingdom for the presence of naturally occurring biologically active phytochemicals against cancer has led to the identification of several plants with beneficial properties on human health. *Simarouba* are monospecific belonging to the family simaroubaceae and widely documented in several pharmacopoeias for their high medicinal value [6]. *Simarouba amara* (synonym *Simarouba glauca*) is commonly known as a paradise tree or laxmitaru in India and known for its high medicinal value. It is native of El Salvador that has been introduced in India during 1960 [7]. Several phytochemicals have been isolated from *Simarouba*. The main active phytochemicals are quassinoids, known as bitter principles in this plant. These quassinoids are known for important pharmacological activities including anticancer and antiplasmodial activity. The plant is used against several dermatological disorders [6]. The plant exhibit strong antiplasmodial [8], antibacterial [9], antifungal activity [10]. Extracts of *Simarouba glauca* have been used for treatment of gastrointestinal disorders [11].

Glaucarubinone, a natural product from *Simarouba* is an antimalarial drug [12] and has shown to possess anticancer activity via down regulation of P21-activated kinases (PAK) in pancreatic cancer [13]. Further, glaucarubinone extends lifespan in *C. elegans* by promoting mitochondrial metabolism and reducing body fat [14]. Several other bioactive compounds including six canthin 6-one type alkaloid derivatives and a triterpenoid, 14-deacetylerylene exhibiting cytotoxic activity against several human cancer cell lines have also been characterized [15].

Extraction solvent mixtures are known to have significant impact on antioxidant activity [16,17], free radical scavenging [18] and antioxidant effects are believed to differ among cancerous cell lines in terms of cytotoxic properties. Therefore, the present study was carried out aiming to evaluate the total phenolic compounds, flavonoids, total condensed tannin content and their antioxidant potential from methanolic, ethanolic and aqueous leaf extracts of *S. glauca* utilizing more than one type of antioxidant activity measurement. Further, the extracts were tested to evaluate the *IN VITRO* anticancer activity using the MTT assay on various human cancer cell lines.

MATERIALS AND METHODS**Chemicals**

1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), Gallic acid, Quercetin, Catechin, 2, 4, 6-tripyridyl-s-triazine (TPTZ), FeCl₃·6H₂O, ferrozine, FeCl₂·4H₂O, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade purity and procured from Himedia, Mumbai.

Preparation of plant extracts

Leaves of *Simarouba glauca* were collected from Jnanabharathi campus, Bangalore University, Bangalore. The leaves were shade

dried at room temperature for two weeks and powdered mechanically of mesh size 1 mm. About 10 gms of powder were extracted twice in 200 ml of methanol with random shaking, after two days, the extract was filtered through whatmann filter paper No.1, the filtrate was mixed and evaporated through the rotary vacuum evaporator at 40°C to get approximately 900 mg methanol crude extract [SGME]. Similar extraction was done with ethanol, which yielded about 650mg crude extract [SGEE]. Aqueous extract was obtained by boiling the leaves for 30 minutes, filtered to yield approximately 950mg of extract [SGAE]. All the extracts were stored at 4° C for further phytochemical and *IN VITRO* investigations.

Estimation of Total phenolic content (TPC)

Determination of the total phenolic content was performed as previously described [19]. Extracts were used in concentration from 0.025 to 0.250 mg/ml. Gallic acid, prepared in varied concentrations ranging from 0.005 to 0.050 mg/ml was used as a standard. The total phenolic compound concentration in the extracts was expressed as micrograms of gallic acid equivalent per mg (μ g GAE/mg) of extract.

Estimation of total flavonoids

Aluminium chloride colorimetric method was used to determine the total content of flavonoids [19]. Test samples were prepared in concentrations of 25.0 to 250 μ g /ml, while quercetin solutions were prepared ranging from 10 to 100 μ g/ml and used as a standard. The total flavonoid content was expressed in μ g of quercetin equivalent (QE) per mg of extract.

Estimation of total condensed tannins

Determination of total condensed tannins (content of proanthocyanidins) was based on the method as previously described [20]. In brief, to 50 μ L of diluted sample, 3 ml of 4% vanillin solution in methanol and 1.5 ml of concentrated HCl was added. The mixture was incubated at room temperature for 15 min, and absorption was measured at 500 nm against methanol as a blank. The total content of proanthocyanidins was expressed in terms of catechin equivalent (standard curve equation: $y = 0.0101x + 0.1209$, $R^2=0.9984$), μ g of CAE/mg of extract. All samples were analyzed in triplicate.

Ferric-ferrozine total antioxidant capacity assay

The assay was carried out according to Berker et al [21]. In brief, Ferric- Ferrozine complex solution was prepared to give a final iron (III) concentration 2.0×10^{-3} M and ferrozine concentration 1.0×10^{-2} M. This ferric-ferrozine complex solution was always freshly prepared. To 500 μ L antioxidant solution diluted in ethanol, 1.5 ml of ferric-ferrozine solution, 2.0 ml 0.2 M acetate buffer (pH 5.5) and 0.5 ml water were added so as to make the final volume 4.5 ml. The absorbance against a reagent blank was measured at 562 nm after 30 min standing at room temperature.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability

Radical scavenging activities of *S. glauca* leaf extracts was determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as previously described [22]. Ascorbic acid was used as the positive control. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Percentage inhibition} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

The IC_{50} value is the concentration of the plant extract required to scavenge 50% of the total DPPH radicals available.

Ferric reducing antioxidant power (FRAP) Assay

The antioxidant activity based on the ferric reducing ability of *S. glauca* leaf extracts was estimated based on the assay [23] with some modifications. A working reagent was prepared fresh by mixing 10 ml of 300 mM acetate buffer with 1 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM of hydrochloric acid (HCl) and

1 ml of 20 mM $FeCl_3 \cdot 6H_2O$. The freshly prepared FRAP reagent was pre-warmed at 37°C after which a blank reading was taken at 595 nm. Subsequently, 30 μ l of sample / standard, 90 μ l of water was added to 900 μ l of the FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent and again at 30 min after the start of the reaction. The change in absorbance in the 30 min reaction was calculated by comparison to the absorbance changes of ascorbic acid against a standard curve tested in parallel. Results were expressed as micromoles of ascorbic acid equivalents (AAEs) per milligram of extract. All experiments were carried out in triplicate.

Phosphomolybdenum assay (Total antioxidant capacity)

Total antioxidant activity of the extract was assessed by the phosphomolybdate method [24] using ascorbic acid as a standard. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against the blank. Methanol (0.1 ml) in the place of extract is used as the blank. Ascorbic acid equivalents were calculated using standard graph of AA. The experiment was conducted in triplicates and values were expressed as the equivalent of ascorbic acid per mg of extract.

Chelating activity on Fe^{2+}

The extracts were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. The chelating ability of ferrous ions by various extracts was estimated by the method [25]. Extracts (0.1-1.0 mg/ml), were added to a solution of 0.6 mM $FeCl_2 \cdot 4H_2O$ (0.1 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.1 ml); the mixture was shaken vigorously and allowed to stand at room temperature for 5 min. Absorbance of the solution was then measured at 562 nm against the blank performed in the same way using $FeCl_2$ and water. EDTA (0.5-50 μ g/ml) served as the positive control, and a sample without extract or EDTA served as the negative control. All tests were carried out in triplicate and averaged. The percentage of inhibition of ferrozine- Fe^{2+} complex formation was calculated using the formula:

$$\text{Chelating activity \%} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100$$

Cell Culture

In this study, we have used four different cancer cell lines derived from human tongue squamous cell carcinoma (SCC9), colorectal carcinoma (HCT116), human lung adenocarcinoma epithelial (A-549) and human breast adenocarcinoma (MCF-7) which were obtained from ATCC. HCT116 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) and other cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Cytotoxic activity (MTT assay)

The cytotoxic activity detects the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) by mitochondrial dehydrogenase to blue insoluble formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. Briefly 5.0×10^4 cells of SCC9, MCF-7, A549 and HCT116 were plated in triplicate in 96 well plates with RPMI-1640 without FBS or DMEM and incubated for 24 hrs at 37°C. Plant extracts were tested at 0-320 μ g/ml in serum free RPMI media and incubated for 24 hr in CO_2 incubator at 37°C. After incubation with plant extracts, the media was removed from the wells and added a 100 μ l/well of the MTT reagent (0.5mg /ml in PBS) and incubated for 3-4 hrs. After incubation, the supernatant was then aspirated, and 100 μ L of dimethyl sulfoxide was added to the wells, to dissolve the colored formazan crystals produced from the reaction of cells

with MTT. Plant extracts treated cells were compared to untreated cell control wells (DMSO as vehicle control). The optical density values were measured using a Tecan microplate reader at a wavelength of 590 nm. The percentage inhibition was determined using the formula.

$$\% \text{ Growth Inhibition} = \frac{100 - \text{OD of individual test group}}{\text{OD of control group}} \times 100$$

Statistical analysis

All the experiments were carried out in triplicates (n=3) and the results were expressed as mean \pm standard deviation (SD). Statistical tests as well as mean and SD calculations were performed using Graph Pad Prism v 5.

RESULTS

Total phenolic and flavonoids content

In the present study, the content of the total phenolics in different extracts of the plant were determined using Folin-Ciocalteu method and expressed as gallic acid equivalents as shown in fig. 1. Total phenolics content of different *S. glauca* extracts were solvent dependent. The aqueous extract contained higher phenolic content of $402.4 \pm 56.01 \mu\text{g}/\text{mg}$ GAE compared to alcoholic and methanolic extracts. The total phenolic content in the extracts decreased in the order of SGAE > SGME > SGEE extract. Flavonoids, the secondary plant phenolics considered to be contributing factors for antioxidant and chelating properties were found to be less in all the extracts tested. The total flavonoid content (fig. 1) in methanolic, ethanolic and aqueous extract were found to be 17.87 ± 5.052 , 14.98 ± 3.498 and 16.96 ± 4.801 respectively.

Condensed tannins

The content of proanthocyanidin compounds ($\mu\text{g}/\text{mg}$) in different extracts of *S. glauca* was determined (fig. 1) and expressed as catechin equivalent (CE). The condensed tannins in methanolic and ethanolic leaf extract were determined spectrophotometrically as $134.93 \pm 18.08 \mu\text{g}$ (CE) / mg and $207.7 \pm 18.09 \mu\text{g}$ (CE) / mg of extract respectively. The aqueous extract had only $67.27.00 \pm 14.55 \mu\text{g}$ (CE) / mg of extract, indicating differential extraction in various solvents.

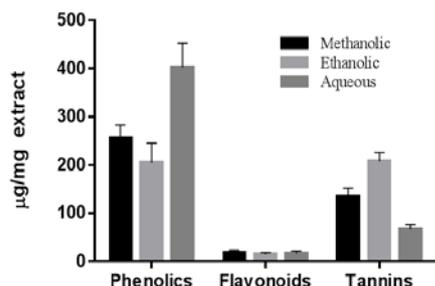


Fig. 1: Total phenolics, flavonoids and tannin content of *S. glauca* leaf extract

Reducing power

The reducing power of the crude extracts was examined as a function of their concentration. The reducing capacity for investigated extracts of *S. glauca* is illustrated in fig. 2.

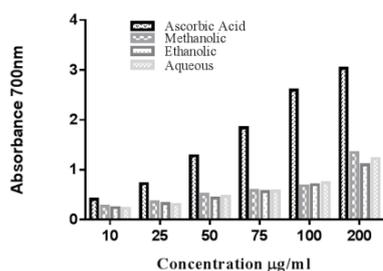


Fig. 2: Reducing power of various extracts of *Simarouba glauca*

Out of three extracts, the methanolic extract exerted the highest reductive capacity with an absorbance of 1.34 at $200 \mu\text{g}/\text{ml}$ followed by aqueous and ethanolic extracts. Also, the reducing ability of each extract was concentration dependent and comparable to that of the control ascorbic acid with an absorbance of 1.2743 at $50 \mu\text{g}/\text{ml}$ and the EC_{50} value of 18.884.

Ferrous ion chelating ability

Metal chelating activity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. Fig. [3] shows concentration dependent chelating effect of the extracts from *S. glauca* on the Fe^{2+} - ferrozine complex. The aqueous extract displayed the highest chelating activity [IC_{50} $332.690 \mu\text{g}/\text{ml}$], whereas methanolic and ethanolic extracts showed a relatively moderate chelating activity, with higher concentration resulting in precipitation indicating interference of other components inhibiting pronounced chelating activity. Chelating agents may serve as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. Accordingly, it is suggested that the low to moderate ferrous ion chelating effect of these extracts would be somewhat beneficial to protect against oxidation damage.

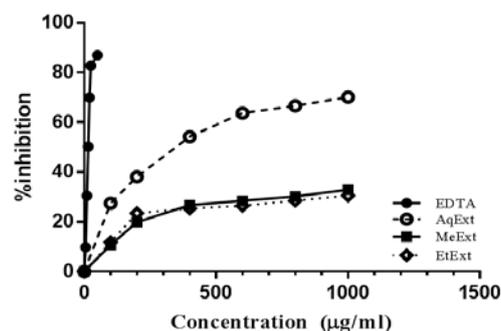


Fig. 3: Iron chelation activity *S. glauca* extracts. (MeExt- Methanolic extract, EtExt-Ethanolic extract, AqExt-Aqueous extract) (n=3)

Radical scavenging activity

The crude methanol extracts of *S. glauca* showed a highly effective free radical scavenging in the DPPH assay. These extracts exhibited a noticeable antioxidant effect at low concentrations (fig. 4). All the extracts of *S. glauca* exhibited a greater antioxidant effect at $50 \mu\text{g}/\text{ml}$ (83%, 84%, and 85%, respectively) compared with the effect of ascorbic acid at the same concentration (95%). The values of IC_{50} of aqueous, methanolic and ethanolic extract were in the range of 9.327, 12.45 and $13.12 \pm 1.54 \mu\text{g}/\text{ml}$ respectively, whereas ascorbic acid had IC_{50} values of $6.297 \mu\text{g}/\text{ml}$ implying relatively high antioxidant activity in methanolic extract.

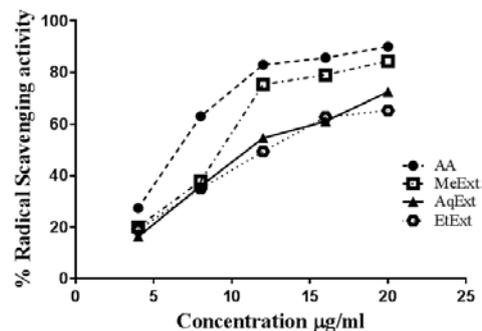


Fig. 4: DPPH scavenging effect of *S. glauca* extracts. (AA-Ascorbic acid, MeExt-Methanolic extract, EtExt-Ethanolic extract, AqExt-Aqueous extract) (n=3)

Ferric reducing antioxidant power (FRAP) Assay

The antioxidant effect of a substance can be measured in the reaction medium in terms of its reducing ability using the FRAP assay. Antioxidant potential of the leaf of *S. glauca* was estimated for their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. The antioxidant capacities of leaf extract in methanol, ethanol and water varied significantly (Table 1). The methanol and ethanol extracts showed FRAP values of 175.96±2.25 µmol AAEs / mg and 150.42±10.18 µmol AAEs / mg respectively. Whereas, the aqueous extract showed slightly lower reducing ability with FRAP value of 133.54±14.098 µmol AAEs / mg indicating more pronounced antioxidant power in methanolic extract.

Phosphomolybdenum assay

The quantitative phosphomolybdenum method was utilized to evaluate the total antioxidant capacity of the *S. glauca* extract. The leaf extract in various solvents exhibited different degrees of activity as shown in table 1. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity. The results indicate that the methanolic extract had comparatively higher antioxidant activity with differences in the degree of Mo reduction between three types of extracts used. Experiments indicated methanolic extracts showing a higher degree (427±28.70 µg AAE / mg extract) of antioxidant capacity than the ethanolic extract (337.9±23.32µg AAE / mg extract). However, the antioxidant activity noticed in aqueous extract was comparatively less with 311.1±30.11µg AAE / mg extract.

Ferric-Ferrozine TAC assay

For measuring the total antioxidant capacity, more sensitive Ferric – ferrozine assay was used which proceeds via the reduction of Fe(III) to Fe(II) with ferrozine at near neutral pH and its subsequent determination at 562 nm using spectrophotometer. The results of this assay on *S. glauca* leaves exhibited 501.1±28.77 µg AAE / mg, 486.1± 66.55 and 580.4±76.71 µg AAE / mg respectively for methanolic, ethanolic and aqueous extracts (Table 1) indicating the total antioxidant activity (TAC) being more in aqueous extract unlike FRAP and phosphomolybdenum assays.

Table 1: Quantitative estimation of antioxidant activity of *S. glauca* extracts

Extract	Phosphomolybdenum assay µg AAE / mg extract	Ferric ferrozine assay µg AAE / mg extract	FRAP assay µM AAE / mg extract
Methanolic extract	427±28.70	501.1±28.77	175.96±2.25
Ethanolic extract	337.9±23.32	486.1± 66.55	150.42±10.18
Aqueous extract	311.1±30.11	580.4±76.71	133.54±14.098

Table 2: Quantitative estimation of radical scavenging, Iron chelation and reducing power of *S. glauca* extracts

Extract	Reducing power (EC ₅₀ ^a)	DPPH radical scavenging assay (IC ₅₀ ^b)	Iron chelation (IC ₅₀ ^b)
Methanolic extract	58.85	9.38	NA
Ethanolic extract	60.078	13.12	NA
Aqueous extract	57.95	12.45	332.69

^aEC₅₀ (µg/ml): effective concentration at which the absorbance is 0.5

^bIC₅₀ (µg/ml) : effective concentration at which 50% of radicals are scavenged/chelated

Cytotoxic activity

Methanol extract of *S. glauca* were investigated in tumor cell lines in range concentrations of 10 to 320 µg/ml in six concentration points, and the results are shown in fig. (5). The most effective cell growth inhibition activity were observed in squamous oral carcinoma cell line (SCC9) for methanol extract of *S. glauca* (IC₅₀ = 312.2 µg/ml). Similar concentration exhibited nearly 30% cell growth inhibition in colorectal cancer cells (HCT116) compared to SCC9 cells. However, the water extracts showed negligible amount of inhibitory effect on above cell line due to its varied qualitative compositions, while both methanolic and water extract didnot exhibit any potent cytotoxic activity in breast cancer cells (MCF-7) and lung adenocarcinoma epithelial (A-549) cell lines indicating differential activity of bioactive compounds in the extract on different cancer cell lines.

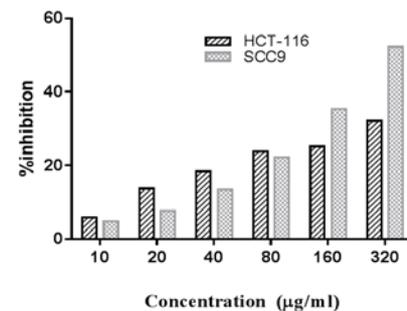


Fig. 5: Cytotoxic activity of *S. glauca* extracts on human cancer cell lines

DISCUSSION

Recent years have seen an increase in epidemiological evidence of close relationship between several active biomolecules of plant origin and human health, particularly in treating cancer with minimal side effects. This has led to exploration of plants for their active biomolecules for development of new plant derived pharmaceuticals and herbal formulations. The present investigation aims at the evaluation of antioxidant, free radical scavenging and cytotoxic potential of *Simarouba glauca* using multiple complementary tests, where the previous studies are sparse.

The health benefit results from the secondary metabolites synthesized in the plant that includes the bioactive constituents like phenolics, flavonoids and tannins. The amount of phenolics in leaf extract was highest in water extract with 402.4±10.4µg, whereas the lowest in ethanolic (204.7±40.33µg/mg). Earlier studies have shown that *S. glauca* leaf extract scavenge H₂O₂ with IC₅₀ being 4.46±0.2 µg/ml for methanol extract [26] and this could be because of the polyphenolic reducing properties as hydrogen or electron donating agents for free radical scavenging [2]. The flavonoid content in leaf extracts was in the range of 0.14 to 0.18% of the extract. Flavonoids are considered to be the best candidates for a protective effect in the diet being highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [27] and known to inhibit metastasis of cancer [28]. Since the amount of flavonoids in leaf extracts is too less compared to other bioactive components, it could only have ameliorative effects along with phenolics and tannins in addition to known chelating activity [29].

Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention [30] and as biological antioxidants [31]. The amount of tannins in *S. glauca* leaf extract varied from approximately 10-20%. The highest amount was present in the ethanolic extract (207.7±18.04µg/mg extract) and low in water (67.27±29.55 µg/mg). These tannins are generally known for its antioxidant activity [32]. Further, anticarcinogenic potentials of tannins may be related to their antioxidative property, which is important in protecting cellular oxidative damage, including lipid peroxidation [33, 34]. Maranhao et al [35] have reported the hepatoprotective effect of *Simarouba amara* bark extract by inhibiting free radical formation

by catechins as evident by the recovery of the antioxidant enzymes and by the decreased lipid peroxidation.

Free-radicals play an important role in the oxidative damage of biological systems [36]. Several complementary methods have been adopted to trap free radicals through antioxidant activity, among which DPPH* assay is the most common. DPPH* being a stable and deep purple color free radical with a strong absorption in the range of 515–520 nm, turns to yellow in the presence of antioxidant compounds by accepting an electron resulting in more stable DPPH in reduced form. Leaf extracts of *S. glauca* exhibited a dose dependent [fig 4] scavenging activity with IC₅₀ values of 9.38, 13.12, 12.45 [table 2] respectively for methanolic, ethanolic and aqueous extracts. This low IC₅₀ values implies the much stronger scavenging of free radicals than other simaroubaceae members like *Ailanthus altissima* [37,38] and *Brucea amarissima* [39].

Antioxidants are considered as reductants and inactivators of oxidants [40]. The presence of reductants (antioxidants) in any extract causes the reduction of the Fe³⁺/ ferricyanide complex to the ferrous form. The ferrous ion can be measured at 700 nm which give the semiquantitative determination of polyphenolics involved in redox reactions [41]. The *S. glauca* leaf extracts showed quantitatively similar reducing ability with methanolic extract being far superior to other extracts at higher concentrations. However, the reducing ability was approximately 3.4 times lower than that of ascorbic acid tested. Further, this was complemented with FRAP assay, which showed the superiority of methanolic extract with FRAP value of 175.96±2.25 μmol AAE/mg compared to other extracts indicating strong reducing ability and antioxidant power. Similar but varied FRAP values have been reported for *Ailanthus altissima* [38] collected from different habitats.

Redox metals like Iron have the ability to produce reactive radicals and considered to be toxic in large amounts. Excessive accumulation of metal ions leads to oxidative stress resulting in increased ROS, which is responsible for several ill effects including cancer. Chelating activity of antioxidants is a preventive mechanism of oxidative damage [42] and polyphenols can act as metal chelators [43]. The chelating activity for ferrous ion of the extracts was assayed by the inhibition of formation of red coloured ferrozine and ferrous complex. The metal chelation of *S. glauca* aqueous extract was superior and showed highest chelation activity with IC₅₀ value of 332 μg/ml, whereas methanolic and ethanolic extract showed up to 40% chelation at the highest concentration used [fig 3]. This moderate chelating activity may be due to interference from other components in the extract. Similar interference was observed when extracts were tested for nitric oxide scavenging with aqueous extract being superior (data not shown).

The total antioxidant activity was evaluated by phosphomolybdenum assay, which is based on the reduction of MoVI to MoV by antioxidant analytes and the formation of a green phosphate Mo (V) complex at acid pH which is estimated quantitatively. All the three extracts exhibited strong antioxidant activity with methanol having 427±28.70 μg/ml AAE/mg extract followed by ethanol and aqueous extracts [table 1]. Further, the antioxidant power was also measured using ferrozine as reagent [21] as this method is a very sensitive method that works at near physiological pH for evaluating antioxidant activity compounds having even a weak reducing potential. The *S. glauca* aqueous extract having antioxidant capacity of 580.45 μgAAE/mg, ethanolic 452.69 μg AAE/mg and methanolic having 433.42 μg AAE/mg extract, indicating aqueous extract having high antioxidant capacity.

Herbal infusions have the potential to inhibit cell growth in cancer cells [44] and cytotoxicity of chemotherapeutic agents could be enhanced by using antioxidants [45] as adjuncts. Several studies have shown that quassinoids, the active principles of the family simaroubaceae are potential anticancer compounds [46]. The results of cytotoxicity of *S. glauca* leaf extract exhibited potent inhibitory effect in human cell lines. The methanolic extract showed strong cytotoxic activity with IC₅₀ values of 312 μg/ml on squamous oral carcinoma cell [SCC9] and less potent on HCT116 colorectal cancer cells, whereas MCF-7 and A549 cell line didn't show any notable activity, indicating differential bioactivity of the extract in different

cell lines. Further, the solvent used for extraction would also have a major influence on cytotoxic activity as the water extract did not exhibit potential anticancer activity in these cell lines. *Simarouba versicolor* extracts of various solvent have exhibited pronounced cytotoxic activity with IC₅₀ less than 5 μg/ml [47]. Though the criteria of the American National Cancer Institute, 30 μg/ml is the upper IC₅₀ limit considered promising for purification of a crude extract [48], the present results give ample scope for further activity guided purification of bioactive molecules which exerts selective cytotoxicity as has been done on several other human cell lines using *S. glauca* leaves [15].

CONCLUSION

In summary, antioxidant activities of *Simarouba glauca* leaves were evaluated in this study with different *IN VITRO* testing systems. Extracts showed meagre amount of flavonoids along with substantial amount of phenolics and tannins. Extracts were highly effective in scavenging free radicals such as DPPH and chelating ferrous iron. The extracts also exhibited potential antioxidant activity with different complementary assays. Further, extracts showed selective cytotoxic activity in various human cell lines with differential effect indicating the significance of solvent used for extraction. It is suggested that the active biomolecules of *S. glauca* with antioxidant activity could be synergistically influencing the cytotoxic activity *IN VITRO*. However, it would be interesting to know whether a similar activity exists under *in vivo* conditions, which would largely depend on the bioavailability and bioaccessibility of these phytochemicals under the test model.

CONFLICT OF INTERESTS

Declared None

REFERENCES

- Halliwell B, Gutteridge J. Free Radicals in Biology and Medicine, 4 Ed. Oxford University Press: Oxford; 2007.
- Rice-Evans CA, Miller N, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152-9.
- Neergheen VS, Bahorun T, Taylor EW, Jen L, Aruoma O. Targeting specific cell signaling transduction pathways by dietary and medicinal phytochemicals in cancer chemoprevention. Toxicol 2010;278(2):229-41.
- Fresco P, Borges F, Marques MPM, Diniz C. The anticancer properties of dietary polyphenols and its relation with apoptosis. Cur Pharm Design 2010;16(1):114-34.
- Huang WY, Cai YZ, Zhang YB. Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. Nut Cancer An Int J 2010;62(1):1-20.
- Moron J, Merrien MA, Polonsky J. Sur la biosynthèse des quassinoides de *Simarouba glauca* (Simarubaceae). Phytochem 1971;10:585.
- Patel DP, Verma VD, Loknathan TR, Bhatt KC, Mishra JB. *Simarouba glauca*—a non traditional oil source. Ind Farm 1997;47:7-9.
- Franssen FFJ, Smeijsters LJJW, Berger I, Aldana BEM. *In vivo* and *in vitro* antiplasmodial activities of some plants traditionally used in Guatemala against malaria. Antimicrob Agents Chem 1997;41(7):1500-3.
- Valdes AF, Martínez JM, Lizama RS, Paul Cos MV, Maes L. *In vitro* antimicrobial activity of the Cuban medicinal plants *Simarouba glauca*, *Melaleuca leucadendron* and *Artemisia absinthium*. Mem Inst Oswaldo Cruz Rio de Janeiro 2008;103(6):615-8.
- Mikawlawng K, Sandeep Kaushik, Pushker AK, Suresh Kumar, Kameshwar Singh M, Sharma GS. Comparative *in vitro* antifungal activities of *Simarouba glauca* against *Fusarium oxysporum* and *Aspergillus parasiticus*. J Med Plant Stud 2014;2(3):1-7.
- Lidia MG, Fereire V, Alonzo A, Caceres A. Ethnobotanical survey of the medicinal flora used by the caribs of Guatemala. J Ethnopharmacol 1991;34:173-87.
- Monjour L, Rouquier F, Alfred C, Polonsky J. Therapeutic trials of experimental murine malaria with the quassinoid, glaucarubione Comptes Rendus de l'Academie des. Sci-Serie III 1987;304(6):129-32.

13. Yeo D, Nhi Huynh, Beutler JA, Christophi C, Shulkes A, Baldwin GS, et al. Glucarubinone and gemcitabine synergistically reduce pancreatic cancer growth via down-regulation of P21-activated kinases. *Cancer Let* 2014;346:264-72.
14. Zarse K, Bossecker A, Müller-Kuhrt L, Siems K, Hernandez MA, Berendsohn WG, et al. The phytochemical glucarubinone promotes mitochondrial metabolism, reduces body fat, and extends lifespan of *Caenorhabditis elegans*. *Horm Metab Res* 2011;43:241-3.
15. Rivero-Cruz JF, Lezutekong R, Lobo-Echeverri T, Ito A, Mi Q, Chai H, et al. Cytotoxic constituents of the twigs of *Simarouba glauca* collected from a plot in Southern Florida. *Phyt Res* 2005;19(2):136-40.
16. Zhao H, Dong J, Lu J, Chen J, Li Y, Shan L, et al. Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). *J Agric Food Chem* 2006;54(19):7277-86.
17. Boeing JS, Barizão EO, Costa e Silva B, Montanher PF, Almeida VC, Visentainer JV. Evaluation of solvent effect on the extraction of phenolic compounds and antioxidant capacities from the berries: application of principal component analysis. *Chem Centr J* 2014;8:48.
18. Akowuah GA, Ismail Z, Norhayati I, Sadikun A. The effects of different extraction solvents of varying polarities on polyphenols of *Orthosiphon stamineus* and evaluation of the free radical-scavenging activity. *Food Chem* 2005;93(2):311-7.
19. Umesh TG, Abhirami D. Evaluation of antioxidant potential and reducing power of callus induced from leaves of *Asystasia gangetica* (L.) T Anderson *Int J Pharm Pharm Sci* 2014;6(8):532-8.
20. Sun B, Richardo-Da-Silvia JM, Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agri Food Chem* 1998;46:4267-74.
21. Berker KI, Guclu K, Demirata B, Apak R. A novel antioxidant assay of ferric reducing capacity measurement using ferrozine as the colour forming complexation reagent. *Anal Methods* 2010;2:1770-8.
22. Umesh TG. *In vitro* callus induction and antioxidant potential of *Decalepis hamiltonii* (wight and arn). *Int J Pharm Pharm Sci* 2014;6(6):452-6.
23. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Anal Biochem* 1996;239:70-6.
24. Prieto P, Pineda M, Aguilar M. Spectrophotometric Quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem* 1999;269:337-41.
25. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Arch Biochem Biophys* 1994;315:161-9.
26. Lakshmi KS, Sangeetha D, Sivamani S, Tamilarasan M, Rajesh TP, Anandraj B. *In vitro* antibacterial, antioxidant, haemolytic, thrombolytic activities and phytochemical analysis of *Simarouba glauca* leaves extracts. *Int J Pharm Sci Res* 2014;5(2):432-7.
27. Bravo L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev* 1998;56:317-33.
28. Fresco P, Borges F, Diniz C, Marques MPM. New insights on the anticancer properties of dietary polyphenols. *Med Res Rev* 2006;26(6):747-66.
29. Van Acker SABE, Berg DVD, Tromp MNJL, Griffioen DH, Van Bennekom WP, Van Der Vijgh WJF, et al. Structural aspects of antioxidant activity of flavonoids. *Free Rad Bio Med* 1996;20(3):331-42.
30. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of Intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogens* 1989;10:1003-8.
31. Hagerman AE, Riedl KM, Alexander Jones G, Sovik KN, Ritchard NT, Hartzfeld PW, et al. High molecular weight plant polyphenolics (Tannins) as biological antioxidants. *J Agric Food Chem* 1998;46:1887-92.
32. Koleckar V, Kubikova K, Rehakova Z, Kuca K, Jun D, Jahodar L, et al. Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Rev Med Chem* 2008;8:436-47.
33. Gulcin I, Huyut Z, Elmastas M, HassanY, Aboul-Enein. Radical scavenging and antioxidant activity of tannic acid. *Arab J Chem* 2010;3(1):43-53.
34. Chung K, Yee Wong T, Wei C, Huang Y, Lin Y. Tannins and Human Health: a review. *Cri Rev Food Sci Nut* 1998;38(6):421-64.
35. Maranhao HML, Vasconcelos CFB, Rolim LA, Rolim Neto PJ, Silva Neto J, da Silva Filho R, et al. Hepatoprotective effect of the aqueous extract of *Simarouba amara* Aublet (Simaroubaceae) stem bark against carbon tetrachloride (CCl4)-induced hepatic damage in rats. *Mol* 2014;19:17735-46.
36. Scalbert A, Manach C, Morand C, Remsy C. Dietary of polyphenols and the prevention of diseases. *Cri Rev Food Sci Nut* 2005;45:287-306.
37. Rahman A, Kim E, Kang S. Antibacterial and antioxidant properties of *Ailanthus altissima* swingle leaf extract to reduce foodborne pathogens and spoiling bacteria. *J Food Saf* 2009;29:499-510.
38. Albouchi F, Hassen I, Casabianca H, Hosni K. Phytochemicals, antioxidant, antimicrobial and phytotoxic activities of *Ailanthus altissima* (Mill.) Swingle leaves. *S Afr J Bot* 2013;87:164-74.
39. Nonita PP, Mylene MU. Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants. *J Med Plants Res* 2010;4(5):407-14.
40. Siddhuraju P, Becker K. The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. *Food Chem* 2007;101:10-9.
41. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem* 2004;84:551-62.
42. Sugihara N, Ohnishi M, Imamura M, Furuno K. Differences in antioxidative efficiency of catechins in various metal-induced lipid peroxidations in cultured hepatocytes. *J Health Sci* 2001;47:99-106.
43. Brown JE, Khodr H, Hider RC, Rice-Evans CA. Structural dependence of flavonoid interactions with Cu²⁺ ions: implications for their antioxidant properties. *Biochem J* 1998;330:1173-8.
44. Kaliora AC, Kogiannou DAA, Kefalas P, Papassideri IS, Kalogeropoulos N. Phenolic profiles and antioxidant and anticarcinogenic activities of Greek herbal infusions; balancing delight and chemoprevention? *Food Chem* 2014;142:233-41.
45. Chinery R, Brockman JA, Peeler MO, Yu Shyr, Beauchamp RD, Robert JC. Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21^{WAF1/CIP1} via C/EBP β . *Nat Med* 1997;3:1233-41.
46. Houel E, Stien D, Bourdy G, Deharo E. Quassinoids: anticancer and antimalarial activities. In: KG Ramawat, JM Me'rrillon. Editors. *Natural Products*. Berlin Heidelberg Springer-Verlag; 2013. p. 3775-802.
47. Mesquita ML, Paula J, Pessoa C, de Moraes M, Costa-Lotuf LV, Grougnat R, et al. Cytotoxic activity of brazilian cerrado plants used in traditional medicine against cancer cell lines. *J Ethnopharmacol* 2009;123:439-45.
48. Suffness M, Pezzuto JM. Assays related to cancer drug discovery. In: Hostettmann K, Edited. *Methods in Plant Biochemistry: Assays for Bioactivity*. London: 6. Academic Press; 1990. p. 71-133.