

Study of total phenolic and flavonoid content, antioxidant activity and antimicrobial properties of medicinal plants

Abstract

The anti-oxidant activity and total phenolic content of alcoholic extracts from seven medicinal plants (*Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Piper betel*, *Citrus aurantifolia*, *Catharanthus roseus*, and *Polyalthia longifolia*) were evaluated by using a model system consisting of β-carotene, DPPH free radical and Folin-Ciocalteu method. The total Phenolic content of the extracts was determined spectrophotometrically according to the Folin-Ciocalteu procedure and ranged from 366mg/100g to 212mg/100g on fresh weight basis. The total flavanoid content of extracts determined by Aluminium chloride colorimetric assay and ranged from 39.84mg/100g to 15.94mg/100g of fresh weight. The highest antioxidant activity was demonstrated by *Citrus aurantifolia* (87.05%) followed by *Ocimum sanctum* (81.80%) and *Catharanthus roseus* (71.4%). The highest tannin content was found to be in *Catharanthus roseus* (7.14%) while in case of anthocyanin content the highest value was found to be in *Polyalthia longifolia* (0.65mg/l). As far as antimicrobial activity is concerned, *Ocimum sanctum* and *Citrus aurantifolia* were found to be most potent against *Escherichia coli* and *Staphylococcus aureus* whereas *Piper betel* showed no effect. Except *Piper betel* all the extracts were able to inhibit the two bacterial strains and the zone of inhibitions ranged from 19.6mm to 13.5mm. The minimum inhibitory concentration against *E. coli* in case of *Ocimum sanctum* is 10% and against *S. aureus* it is 20% while in case of *Citrus aurantifolia* extracts were active even at 10% concentration for *E. coli* and 15% for *S. aureus*.

Keywords: anti-oxidant activity, phenolic content, flavanoid content, antimicrobial activity, minimum inhibitory concentration

Introduction

Medicinal plants, as source of remedies, are widely used as alternative therapeutic tools for the prevention or treatment of many diseases. The recent studies have investigated that the antioxidant effect of plant products is mainly attributed to phenolic compounds such as flavonoids, phenolic acids, tannins etc.^{1,2} Accumulation of free radicals can cause pathological conditions such as ischemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementia. Natural antioxidants have become the target of a great number of research studies in finding the sources of potentially safe, effective and cheap antioxidants.³ Herbal drugs containing free radical scavengers are known for their therapeutic activity.⁴

Seven medicinal plants were selected for this study (*Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Piper betel*, *Citrus aurantifolia*, *Catharanthus roseus*, and *Polyalthia longifolia*) considering their medicinal properties. Medicinal plants are considered as clinically effective and safer alternatives to the synthetic antibiotics.⁵ Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, ascorbic acid, etc to prevent the oxidation of the susceptible substrate. Antioxidants are usually applied to prevent lipid peroxidation in the food industries.^{6,7} In this investigation, along with Phenolic and antioxidant properties of selected plants, antimicrobial activity of plant extracts against *Escherichia coli* and *Staphylococcus aureus* are also tested.

Materials and methods

Plant material used

The leaves of medicinal plants (*Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Piper betel*, *Citrus aurantifolia*, *Catharanthus roseus*, and *Polyalthia longifolia*) were obtained from Lovely Professional University campus and Defense Colony, Jalandhar of Punjab (India).

Microorganisms procured and their maintenance

For determination of antimicrobial activity the test organisms *Staphylococcus aureus* (MTCC96) and *Escherichia coli* (MTCC723) were procured from IMTECH, Chandigarh. The selective media used for growth of *E. coli* was Eosin Methylene Blue (EMB) media and for *S. aureus* it was Manitol Salt Agar (MSA) media. For the antimicrobial test Mueller Hinton Agar Medium was used. Inoculated bacterial cultures were incubated at 37°C for 24hr.⁸

Chemicals

Sodium acetate, potassium chloride, 2-diphenyl-1-picrylhydrazyl-hydrate (DPPH), Folin-Ciocalteu reagent, catechol, beta carotene, Tween-20, sodium carbonate, sodium hydroxide, acetone, butanol, chloroform, ethanol and aluminium chloride were obtained from purchased from E-merck, Mumbai, India. Manitol salt, Mueller Hinton Agar and Eosin Methylene Blue were obtained from Hi-media Ltd. All the chemicals used were of analytical grade.

Preparation of plant samples

The plant leaves were cleaned and cut into small pieces. Samples (2g) were homogenized in 80% aqueous ethanol at room temperature and centrifuged at 10,000 rpm for 15min and the supernatant was preserved for estimation of various parameters. The residue was re-extracted twice with 80% ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature. Residue was dissolved in 5ml of distilled water and stored at 4-8°C in a refrigerator for further analysis.⁹

Preparation of plant samples for antimicrobial activity

Twenty five grams of the plant leaves were weighed and then grinded with methanol. The leaves were then soaked into 50ml methanol (98%) for 72hours. Then the methanol was allowed to evaporate in water bath. The concentrated methanolic extracts were weighed and preserved for further use.¹⁰

Determination of total phenolic, total flavonoid, total anthocyanin and tannin content

Total Phenolic Content was determined by using Folin-Ciocalteu method with catechol as standard. One-hundred microlitres of each sample extract was diluted to 3ml with distilled water and 0.5ml of Folin-Ciocalteu reagent was added. After 3min, 2ml of 20% sodium carbonate was added and the contents were mixed thoroughly. The colour was developed and absorbance measured at 650nm in spectrophotometer (Shimadzu UV-1800) after 60min.¹¹ Different concentrations of catechol solution (5mg/100ml) were used to plot the calibration curve. Results were expressed as mg catechol/100g of fresh weight material using the

Formulae equivalent:

Where A₆₅₀ is absorbance at 650nm, V is volume made up, D.F is

$$\frac{O.D(Sample) \times A_{650} \times V \times D.F \times 100}{w \times 1000}$$

dilution factor and w is weight of sample in grams

Total Flavanoid Content was determined by aluminum chloride colorimetric assay.¹² An aliquot (1ml) of extract or a standard solution of catechol (10mg/100ml) was added to 10ml flask containing 4ml of distilled water. To this 0.3ml 5% NaNO₂ was added. After 5minutes, 0.3ml of 10% AlCl₃ was added. After 6min, 2ml of 1M NaOH was added and the total volume was made up to 10ml with distilled water. Absorbance was read at 510nm with a spectrophotometer.

Total monomeric Anthocyanin (TA) was estimated by pH-differential method.¹³ Two dilutions of the sample were prepared, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5. These dilutions were left to equilibrate for 15min. The absorbance of each dilution at 520nm and at 700nm, against a blank cell filled with distilled water was measured. Calculation of TA concentration is based on the molecular weight (MW) and the molar extinction coefficient (ε) (26900) of cyanidin-3-glucoside (449.2g), the most common anthocyanin in nature.¹ The absorbance of sample (A) was calculated as follows:

$$A = (A_{520} - A_{700}) \text{ pH } 1.0 - (A_{520} - A_{700}) \text{ pH } 4.5$$

$$TA(\text{mg/L}) = \frac{(A \times MW \times DF \times 1000)}{(\epsilon \times 1)}$$

Determination of condensed tannin content (proanthocyanidins) (TC) was determined by the method of Porter et al.¹⁴ In a glass test tube, 0.50ml of the tannin extract was diluted with 70% acetone. The quantity of acetone was large enough to prevent the absorbance (550nm) in the assay from exceeding 0.6. It will depend on the quantity of condensed tannin expected in the sample, and occasionally will need to be determined by trial and error. To the tubes 3.0ml of the butanol-HCl reagent and 0.1ml of the ferric reagent was added. The contents in the tubes were evenly mixed by vortex mixer. The mouth of each tube was covered and the tubes were put in a boiling water bath for 60min. The tubes were allowed to cool and absorbance was recorded at 550nm. For each sample mixture comprising 0.5ml of the extract, 3ml of butanol and 0.1ml of the ferric reagent were used.

Condensed tannins is expressed as leucocyanidin equivalent (460) and calculated by the formula:

$$\frac{A_{550} \times 78.26 \times DF}{\% \text{Tannin content}}$$

Beta carotene bleaching method

β-Carotene (2mg) was dissolved in 20ml of chloroform. A 4ml aliquot of the solution was added to a conical flask with 40mg linoleic acid and 400mg Tween-40. Chloroform was removed with a rotary evaporator at 50°C. Oxygenated distilled water (100 ml) was added to the β-carotene emulsion mixed well and aliquots (3ml) of the oxygenated β-carotene emulsion and 0.2ml of alcoholic extracts were placed in capped culture tubes and mixed well. The tubes were immediately placed in a water bath (Optics technology) and kept at 50°C for 10minutes. Oxidation of the β-carotene emulsion was monitored spectrophotometrically, taking absorbance at 470nm for 100min. A control consisted of 0.2ml distilled water instead of plant leaf extracts.¹⁵

Antioxidant activity was expressed as per cent inhibition relative to control using the equation below:

$$1 - \frac{(A_{omin} - A_{100min}) \times 100}{A_{omin} - A_{100min}}$$

DPPH scavenging method

DPPH scavenging activity (%) was determined following the method of Thirunavakkarasu et al.¹⁶ To 50μl of samples, 950μl of 90μM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was added and made up to a final volume of 4ml with 95% ethanol. After the mixtures were vigorously shaken, they were incubated at room temperature for 2 hours in dark. The reduction of solution colour caused by free radicals (DPPH) was measured at 515nm using a spectrophotometer. The capability of samples to reduce DPPH was determined by sample colour reduction effect with control (mixture without the sample) using following equation and expressed in % values:

$$= 1 - \frac{A_{Sample}}{A_{DPPH \text{ Solution}}}$$

Agar well diffusion assay

The antimicrobial activity was measured by Agar well diffusion assay.¹⁷ The plant extract were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Petri plates containing 20ml Mueller Hinton medium were seeded with the bacterial strains. Each labeled medium plate was uniformly

inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. Wells were punched and 100 μ l of the methanolic plant extracts were added. The plates were then incubated at 37°C for 24 hours. Erythromycin (0.05%) was used as positive control and analysis was done in triplicates. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. The diameter of zone of inhibition can be measured in millimeters.¹⁸

Determination of minimum inhibitory concentration (MIC) of crude methanoic extracts

The minimum inhibitory concentration was determined of those plant methanolic extracts which showed significant zones of inhibition against tested microorganisms (*Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Citrus aurantifolia*, *Catharanthus roseus*, and *Polyalthia longifolia*). Different dilutions (20, 40, 60, and 80)% of the plant extracts were assayed against the test organisms. Distilled water was used as negative control. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth.¹⁹ *Piper betel* was unable to show any significant effect on the test organisms, so its MIC was not determined. The extracts which exhibited zone of inhibition at even 20% concentration, they were being tested at lower concentrations viz. 5, 10 and 15%.

Results and discussion

The amount of total phenolics content (TPC) is expressed as mg/100g of fresh weight (Table 1). *Citrus aurantifolia*, *Asparagus racemosus*, *Ocimum sanctum* showed maximum and *Piper betel* showed the minimum phenolic content among the selected extracts. Content varied widely among the samples and ranged from 366mg/100g to 212mg/100g of fresh weight. *Ocimum spp.* showed high phenolic content according to the results by Veeru et al.,²⁰ while *Asparagus racemosus* is also having comparable phenolic content contributing to its medicinal uses²¹ *Citrus aurantifolia* is reported to have comparatively higher phenolic content as per the study conducted by Ahmed et al.,²² *Citrus aurantifolia*, *Cassia fistula* and *C. roseus* showed maximum and *Asparagus racemosus* showed the minimum flavonoid content among the extracts (Table 1). Ghafar et al.,²³ also found the flavonoid content of *Citrus aurantifolia* to be the highest. The total Flavonoid Content varied widely among the samples and ranged from 39.84mg/100g to 15.94mg/100g of fresh weight. Figures 1 shows the comparative β -carotene bleaching rates of the control and plant extracts. It shows a decrease in absorbance of β -carotene in the presence of different extracts due to the oxidation of β -carotene and linoleic acid. This indicates that all tested extracts possessed antioxidant capacity. All plant extracts showed moderate to high antioxidant capacity. The highest antioxidant activity was demonstrated by *Citrus aurantifolia* (87.05%) followed by *Ocimum sanctum* (81.80%). *Catharanthus roseus* (71.4%), *Cassia fistula* (77.8%) & *Polyalthia longifolia* (73.01%) showed moderate antioxidant activity, whereas *Piper betel* (52.4%) and *Asparagus racemosus* (59.94%) exhibited comparatively low activity. Yao et al.,²⁴ also reported the very high antioxidant activity of *Citrus aurantifolia* and suggest major contribution of non-vitamin factors to total in vitro antioxidant potency of *C. aurantifolia*. Chu et al.,²⁵ also reported to have similar results regarding antioxidant activity of *Asparagus racemosus*. Rosmarinic acid present in the chemical composition of *Ocimum sanctum* acts as the powerful antioxidant.²⁶

DPPH scavenging ability of seven medicinal plants were screened in ethanol solvent system as shown in Figure 2. *Polyalthia longifolia* leaf (76.84%) showed strong inhibition of DPPH radical followed by the leaf extract of *Cassia fistula* with 75.78% inhibition and *Ocimum sanctum* leaf (74.73%), while others exhibited the DPPH radical scavenging capacity ranging from 35-65% in ethanolic solvent. The high scavenging activity of *Polyalthia longifolia* is also being supported by the work of Mukherjee et al.,²⁷ When total anthocyanin content was estimated, the highest was found to be in *Polyalthia longifolia* (0.65mg/l) and minimum in *Citrus aurantifolia* (0.04mg/l) (Table 2). This may be due to the deep green color of *Polyalthia longifolia* leaves. Tannin was estimated as percentage dry matter, the highest was found to be in *Catharanthus roseus* (7.14%) and lowest in *Citrus aurantifolia* (2.0%) (Table 2). Although tannins in general exhibit antioxidant activity specific research is needed to test the relationship between tannin content and antioxidant activity.

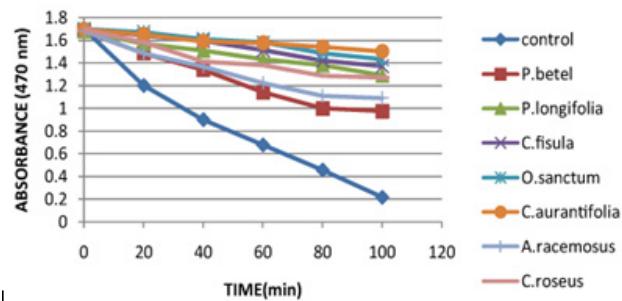


Figure 1 Antioxidant activity (beta carotene assay) of leaf extracts.

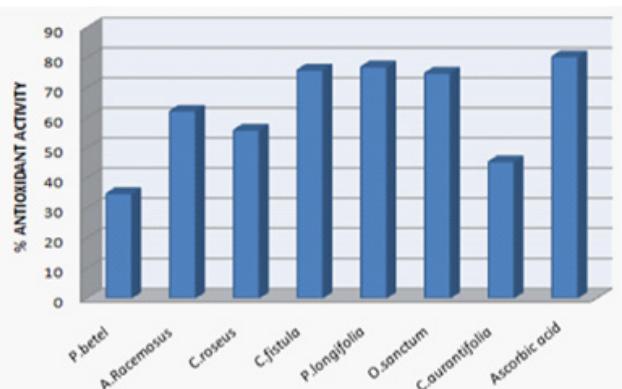


Figure 2 Antioxidant activity (DPPH scavenging method) of leaf extracts.

Table 1 Total Phenolic and flavanoid content of medicinal plant samples

Times	Total phenolic content (mg/100 g)	Total flavanoid content (mg/100 g)
P.betel	212±0.50	39.84±0.15
A.racemosus	365±0.45	15.94±0.10
C.roseus	285±0.30	41.68±0.25
C.aurantifolia	366±0.13	39.03±0.20
C.fistula	264±0.52	38.15±0.19
O.sanctum	365±0.25	20.50±0.21
P.longifolia	244±0.60	27.11±0.30

Data expressed as mean±SE.

Relationship between total phenolic content and antioxidant activity of medicinal plants

The anti-oxidant activity of extracts could not be explained just on the basis of their phenolic content but also required their proper characterization. This lack of relationship is in agreement with other literature.²⁸ It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity.^{29,30} Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities. Our study is in agreement with Sengul et al.,³¹ which reported no correlation between total phenolic content and antioxidant capacities of a number of medicinal plant extracts no correlation between total phenolic content and antioxidant capacity in our plant samples is possible owing to the presence of the following antioxidant capacity observed was not solely from the Phenolic contents, but could possibly be due to the presence of some other phytochemicals such as ascorbic acid, tocopherol and pigments etc which also contribute to the total antioxidant capacity. Also, phenolic content determined according to the Folin-Ciocalteu method is not an absolute measurement of the amount of phenolic materials. Different types of phenolic compounds have different antioxidant activities, which is dependent on their structure. The extracts possibly contain different type of phenolic compounds, which have different antioxidant capacities.

Table 2 Anthocyanin content and percentage tannin content of medicinal plant samples

Plant sample	Anthocyanin content (mg/l)	Percentage Tannin content
P.betel	0.134	14.6
A.racemosus	0.13	3.76
C.roseus	0.34	7.14
C.aurantifolia	0.04	2.0
C.fistula	0.62	13.6
O.sanctum	0.37	14.37
P.longifolia	0.65	9.74

Antimicrobial activity

In the present study, methanolic leaf extracts of seven medicinal plants were investigated against two bacterial species (*Staphylococcus aureus* and *Escherichia coli*). Some extracts had conspicuous zone of inhibition while some had moderate or little or no zone of inhibition. Methanolic extracts of *Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Citrus aurantifolia*, *Catharanthus roseus* and *Polyalthia longifolia* were active against *Staphylococcus aureus* (gram positive) and *Escherichia coli* (gram negative). *Piper betel* was inactive against both *E. coli* and *S. aureus*. Table 3 summarizes the microbial growth inhibition of methanolic extracts of the plant species and their MIC. Out of the seven plants, *Ocimum sanctum* was found to be most potent against all the microbes showing 19.6mm and 17.6mm zone of inhibition against *S. aureus* and *E. coli* respectively whereas *Cassia fistula* showed comparatively lower effect of 13.5mm and 14.6mm respectively. The study done by Joshi et al.,³² also exhibits comparable inhibition against *S. aureus* by *Ocimum sanctum*. *Citrus aurantifolia* has shown 16.3mm of inhibition zone against *S. aureus*, which is similar to the study conducted by Penecilla et al.,³³ Onyeagba

et al.,³⁴ also supports the strong inhibition demonstrated *C. aurantifolia* (18mm zone of inhibition). Study conducted by Lawrence et al.,³⁵ (19mm inhibition observed) and Goyal et al.,³⁶ (zone of inhibition 18.5mm) is also in agreement with this study about antimicrobial activity of *Ocimum sanctum*. The antibacterial activities of medicinal plants are attributed due to the presence of flavonoids, tannins and steroidal alkaloids.³⁷ In *Ocimum sanctum* carvacrol and terpene are the antibacterial agents present. Sesquiterpene and caryophyllene also serve the same purpose. This constituent is FDA approved food additive which is naturally present in *Ocimum spp.*²⁶ The zone of inhibitions observed in case of *Asparagus racemosus* is also observed by Sinha et al.,³⁸ against *E. coli* and *S. aureus*.

Table 3 Antimicrobial activity of the Extracts Data expressed as mean±SE, ---- showed no significant effect of extract on the microorganisms, -NA- not applicable

Plant species	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>	
	Zone of Inhibition	MIC	Zone of Inhibition	MIC
<i>Ocimum sanctum</i>	19.6±0.33mm	10%	17.6±0.41 mm	20%
<i>Citrus aurantifolia</i>	16.33±0.40 mm	10%	17.3±0.40mm	15%
<i>Asparagus racemosus</i>	18.5±0.40mm	20%	16.3±0.41mm	20%
<i>Cassia fistula</i>	13.5±0.7mm	60%	14.6±0.41mm	60%
<i>Catharanthus roseus</i>	14.33±0.41mm	40%	16.33±0.40mm	40%
<i>Polyalthia longifolia</i>	16.33±0.41mm	20%	16.5±0.70mm	20%
<i>Piper betel</i>	----	-----	----	-----
Control	23.5±0.42mm	-NA-	19.8±0.40mm	-NA-

Table 3 gives details of minimum inhibitory concentration against *S. aureus* and *E. coli* by the methanolic extracts. *Ocimum sanctum* is found to inhibit *S. aureus* at a concentration of 20% and *E. coli* at a concentration of 10% and *C. aurantifolia* inhibited *S. aureus* and *E. coli* at 15% and 10% concentration respectively, while the methanolic extracts of *Cassia fistula* was only able to inhibit *E. coli* and *S. aureus* at 60% concentration. Agarwal et al.,³⁹ found that *Ocimum sanctum* extracts can inhibit Streptococcus species at even 4% concentration. Methanolic extract of *Polyalthia longifolia* against *E. coli* and *S. aureus* was active at concentration of 40% and 60% respectively. Chanda et al.,⁴⁰ also found similar results for *Polyalthia longifolia*. The phytochemical study of *Polyalthia longifolia* leaves shows plant mainly contains tannins, phenolic acids, glycosides and steroids. Presence of flavonoids and tannins in *Polyalthia longifolia* are responsible for antibacterial activity.⁴¹

Phytochemical studies revealed the presence of phenolics, flavonoids, tannins, anthocyanins which contributes to the antimicrobial activity of these plants. In recent years multiple drug resistance in human pathogenic microorganisms has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases, making it a global growing problem. In addition to this problem antibiotics are sometimes

associated with adverse effects on host including hypersensitivity, immune suppression and allergic reactions.⁴² Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infections obtained from various sources such as medicinal plants. The antibacterial activity found in this present study may be attributed to the presence of secondary metabolites of various chemical types present in the plant material either individually. The discovery of a potent remedy from plant origin will be great advancement in microbial infection therapies.

Conclusion

The present study reported the antioxidant activity, total phenolic and flavonoid contents of seven medicinal plants (*Asparagus racemosus*, *Ocimum sanctum*, *Cassia fistula*, *Piper betel*, *Citrus aurantifolia*, *Catharanthus roseus*, and *Polyalthia longifolia*). In order to realize the health benefits from potential plant sources, it is important to measure the anti-oxidant activity using various radicals and oxidation systems. *Ocimum sanctum* and *Asparagus racemosus* were found to possess the highest phenolic content while *Citrus aurantifolia* showed the highest antioxidant activity, followed by *Ocimum sanctum*, thus attributing to their use as medical plants. The two bacterial strains (*Staphylococcus aureus* and *Escherichia coli*) used in this experiment are responsible for an array of human diseases such as cholecystitis, urinary tract infection, skin infections etc. However, these human pathogenic strains were significantly inhibited by the methanolic leaf extracts of the medicinal plants. *Ocimum sanctum* and *Citrus aurantifolia* was most potent against the microorganisms, their extracts were effective even at 10% concentration against *Escherichia coli* whereas against *Staphylococcus aureus* it was 20% and 15% concentration respectively. Therefore, the study provides support to the plant's traditional and alternative use against various diseases and infections. Further, the biomolecules present in the extract which are active against these microbes needs to be characterized. Use of natural products has been encouraged due to less or no side effects, cost effectiveness and development of resistance to conventional synthetic antibiotics. Hence, this study holds importance in using medicinal plants as an alternative source for treating various diseases. Additional information on the dietary intake of medicinal plants selected in this study, and enhancing their bioavailability after various processing operations need to be elucidated in future.

Future work

Extensive research in the area of isolation and characterization of the active principles of these plants are required so that better, safer and cost effective drugs for treating bacterial infections can be developed.

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None.

Conflict of interest

The author declares no conflict of interest.

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