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Assessment of total phenolic content and antimicrobial activity of herbal extracts and their combinations

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Abstract

Plants have been known to be a reservoir of secondary metabolites which are being exploited as source of bioactive substance for various pharmacological purposes. In the present study, dry leaf extracts of eight plants were prepared using ethanol, methanol and distilled water. Total phenolic content of each extract was carried out using FCR method. Based on the TPC, three best extracts were selected and were combined in ratio of 1:1, 1:2 and 2:1. Further, antimicrobial activity of crude extracts as well as herbal combinations was assessed. Results revealed that yield of all the extracts was higher in ethanol and methanol solvent compared to distilled water. Meanwhile, irrespective of extraction solvents, castor yielded higher total phenols in all the leaf extracts followed by coral vine, touch me not, banyan and clerodendron. Among the extraction solvents, ethanol gave good results for extraction of phytochemicals compared to methanol and distilled water. Further, it was observed that among the single herbal extract, the crude extract of castor showed higher zone of inhibition against bacterial (*S. aureus* & *E.coli*) and fungal (*A. niger*) species compared to clerodendron and banyan extracts. However, among the herbal combinations, castor : banyan (1:1), castor : clerodendron (1:2) and castor : banyan (2:1) combinations showed greater zone of inhibition against all the test organisms implying very good antibacterial and antifungal activity. Thus, the information regarding antimicrobial activity of herbal extracts and their combinations can be utilized by pharmacists, chemists, pathologists, food processing units for development of innovative drugs.

Keywords: *Aspergillus niger*, *Escherichia coli*, Extraction, *Staphylococcus aureus*, Total phenolic content

Introduction

India has about 45,000 plant species, among them, several thousands have been claimed to possess medicinal properties. Although a significant number of studies have used known purified plant chemicals, very few screening programmes have been initiated on crude plant materials (Gupta and Laha, 2007). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals and plants; one such resource is folk medicine. Systematic screening of herbs used in folk medicines

may result in the discovery of novel effective compounds.

The metabolites of plant are commercially important and find its use as raw material for various scientific investigations. In recent times, the blind dependence on synthetic compounds is surpassed over to the fact that the herbal agents are cost effective, easily available and most importantly, with negligible side effects (Sharma *et al*., 2010). Phytochemical investigations of crude plant extracts depict the presence of active constituents in the plant parts like bark, leaves, flowers, roots, fruits and seeds. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties (Veerachari and Bopaiah, 2012). The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plant (Pranoothi *et al*., 2014). Secondary metabolites are synthesized in a specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products (steroids, quinines, alkaloids, terpenoids and flavonoids) which are used for varied applications.

With the increase in resistance of microorganisms to the currently used antibiotics and the high cost of production of synthetic compounds, researchers are now looking for alternatives. Medicinal plants could be those alternatives because most of them are safe with little side effects (if any), cost effective and affect a wide range of antibiotic resistant microorganisms. The demand for new effective antimicrobials is urgent and of great importance in the clinical health. Allied with this demand is the need for assays to detect new and previously undiscovered antimicrobials from plant sources (Oskay *et al*., 2009). Many studies have been carried out to extract various herbal products for screening antimicrobial activity but attention has not been focused intensively on studying the combinatorial effect of the medicinal plant sources.

Hence, the present study was designed with an aim to extract the phytochemicals from traditionally used medicinal plants using different solvents and to assess the total phenolic content & antimicrobial activity of the herbal extracts and their combinations.

Materials and methods

Selection of plant sources

Plants possessing medicinal properties such as banyan (*Ficus benghalensis*), castor (*Ricinus communis*), cassia (*Cassia sericea*), clerodendron (*Clerodendron inerme*), coral vine (*Antigonon leptopus*), peepal (*Ficus religiosa*), prickly chaff (*Achyranthes aspera*) and touch me not (Mimosa pudica) were selected for the present study.

Herbal extraction

The matured leaves of the selected plants were collected and cleaned with distilled water and shade dried at room temperature to remove the traces of moisture. Leaves were crushed to fine powder using mechanical grinder. Two grams of dry leaf powder of each sample were weighed and mixed with 25 ml (w/v) of each solvent (ethanol, methanol and distilled water) separately. The extracts were incubated for 24 hours at room temperature, later centrifuged at 5000 rpm at room temperature (REMI C-24 Plus refrigerated centrifuge) and the supernatants were separated. Residue was re-extracted with 25 ml of the respective solvent and the process was repeated. The supernatants obtained were pooled and the extracts obtained were measured and filtered using Whatman filter paper No. 40 (125 mm) (Babel *et al*., 2013).

Total phenolic content (TPC)

Total phenolic content in the extracts was determined by Folin-Ciocalteu assay method (Singleton and Rossi, 1965) with little modification using gallic acid as the reference standard. All the solvent extracts were diluted to appropriate volumes and were mixed with 2 ml of 10 per cent sodium bicarbonate solution, incubated at room temperature for 3 minutes, later 100 µl of Folin-Ciocalteu reagent was added to the mixture. The resulting solution was incubated for 90 minutes at room temperature under dark, the absorbance was measured at 765 nm using the UV-Vis Spectrophotometer (BioMate 3S UV-Visible Spectrophotometer) with interface VisionLite software. The TPC was expressed as gallic acid equivalent (GAE) in milligrams per gram of dry leaf.

Formulation of herbal combinations

The beneficial antimicrobial effects of plant materials typically result from the combinations of secondary metabolites present in the plant (Pranoothi *et al*., 2014). Therefore, in the present study, a novel idea of mixing the plant extracts in different ratios to determine the combinatorial antimicrobial activity was carried out. Three plant sources and one solvent with maximum total phenolic content were selected and mixed in different ratios such as 1:1, 1:2 and 2:1 (v/v) and screened for their combinatorial antimicrobial activity against bacterial and fungal strains.

Procurement of bacterial cultures

The bacterial cultures *viz.* Gram positive *Staphylococcus aureus* (ATCC 6538) and Gram negative *Escherichia coli* (ATCC 8739) were procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh.

Isolation and identification of fungal strain

In the present study, the fungal strain was isolated from peanuts by direct transfer technique. The infected peanuts were stored in a moist chamber prepared by placing moist filter papers in sterilized *petri* plate for 7 days to allow the moulds to grow and sporulate. Later on, the spores were transferred onto sterile potato dextrose agar using sterile inoculating loop and incubated for seven days at 30 ^{0}C. Further, the identification of fungal strain was done based on the morphological characters observed under research compound microscope (Carl Zeiss, Germany). The fungus produced septate mycelium and aerial conidiophores bearing hyline single celled conidia on phialides inturn on the vesicle, a characteristic feature of *Aspergillus niger* as described by Barnett (1960).

Antimicrobial activity of herbal extracts and their combinations

The antimicrobial activity of the herbal extracts and their combinations was assessed as per Agar well diffusion method (Oskay *et al*., 2009). Nutrient media and nutrient broth was prepared separately in distilled water and autoclaved at 120 ºC for 15 minutes at a pressure of 15 lb. A loopful of bacterial (*S. aureus* and *E. coli*) cultures was mixed separately in the nutrient broth and kept under shaking condition for 24 hours. The nutrient media was poured in the sterilized *petri* plates and allowed to solidify (lower layer) under aseptic conditions. Later, 150 ml nutrient media was inoculated with 1ml of bacterial working culture and poured on the solidified media (upper layer) and allowed to solidify. Four wells equidistant to each other were created using a cork borer. About 5 µl of plant extract was loaded to each well and the plates were incubated for 24 hours at 37 °C. Seventy per cent ethanol

was used as control. Zone of inhibition around the well was recorded in millimeters. Similar procedure was followed for assessing antifungal activity against *A. niger* using potato dextrose agar and potato dextrose broth. The plates were incubated at 28 °C for 48 hours and the observations were recorded.

Results and discussion

Yield of the herbal extracts

Yield of the extracts depends on the type of solvents with varying polarities, extraction time, temperature, sample to solvent ratio as well as the chemical composition and physical characteristics of plant source. The yield of prickly chaff, cassia and touch me not leaves was found to be high in ethanol and methanol (40 ml, 40ml & 43ml/ 50 ml) as compared to distilled water (30 ml, 36ml & 37ml/50 ml). However, coral vine, clerodendron and castor leaves produced higher yield in ethanol (40 ml, 42ml & 40ml/ 50 ml) against methanol (38 ml, 38ml & 39ml/ 50 ml) and distilled water (37 ml, 35ml & 34ml/ 50 ml). On the other hand, peepal and banyan leaves produced higher yield in methanol (39 ml & 36 ml/ 50 ml) followed by ethanol (37 ml & 35 ml/ 50 ml) and distilled water (32 ml & 33 ml/ 50 ml), respectively (Fig. 1).

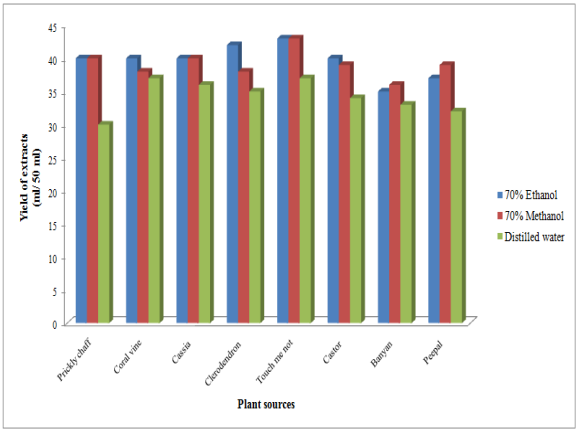


Figure 1: Yield of plant extracts (ml/ 50 ml of solvent).

In general, irrespective of the plant sources, yield of all the extracts was higher in ethanol and methanol solvent compared to distilled water because alcohols have the ability to penetrate the cellular membrane and extract the intracellular ingredients (polyphenols) from the plant material thereby resulting into lighter extracts. Almey *et al*. (2010) suggested that both ethanol and methanol gave best results to extract phenolic compounds because of the presence of polar groups. The authors found that as polarity of the solvent increased, higher extraction yields of total soluble solids and total extractable phytochemicals were obtained. Moreover, it was observed that extracting solvent significantly affected the yield of extracts indicating that different extracting solvents influenced different yields of extracts.

The results are in line with the study conducted by Vastrad *et al.* (2015) who revealed that the yield of plant extracts was higher in ethanol and methanol solvents. Contrary results were found by Vastrad *et al*. (2016) who concluded that distilled water produced higher yield of aromatic plant extracts compared to ethanol and methanol solvents.

Total Phenolic Content (TPC)

Table 1 highlights on the total phenolic content of the herbal extract (mg/ g of dry leaf). It is observed that, banyan leaf extracts gave higher TPC in methanol (29.302 mg/ g) followed by ethanol (27.214 mg/ g) and distilled water (18.472 mg/ g) extracts. However, cassia, touch me not and coral vine leaves showed higher TPC in ethanol (19.087 mg/ g, 27.496 mg/ g & 28.751 mg/ g) extract in comparison to methanol (16.932 mg/ g, 24.20 mg/ g & 22.397 mg/ g) and distilled water (7.479 mg/ g, 9.324 mg/ g & 16.682 mg/ g) extracts, respectively.

Table 1. Total phenolic content of herbal extracts.

Sl. No.	Plant sources	Total phenolic content (mg/g dried leaf)			Mean
		70% Ethanol	70% Methanol	Distilled water	
1.	Banyan	27.214 ± 2.312**	29.302 ± 2.10**	18.472 ± 1.527**	**24.996 ± 1.980**
2.	Cassia	19.087 ± 1.911**	16.932 ± 1.877**	7.479 ± 1.777**	**14.499 ± 1.855**
3.	Castor	33.522 ± 2.519**	33.305 ± 2.521**	26.78 ± 2.306**	**31.202 ± 2.449**
4.	Clerodendron	26.708 ± 3.362**	26.248 ± 2.048**	19.194 ± 2.023**	**24.050 ± 2.478**
5.	Coral creeper	28.751 ± 3.212**	22.397 ± 2.113**	16.682 ± 1.983**	**22.610 ± 2.436**
6.	Peepal	17.004 ± 1.853**	19.426 ± 2.034**	17.582 ± 2.285**	**18.004 ± 2.057**
7.	Prickly chaff	7.258 ± 1.785**	13.985 ± 2.202**	12.044 ± 1.603**	**11.096 ± 1.863**
8.	Touch me not	27.496 ± 3.422**	24.20 ± 1.465**	9.324 ± 1.473**	**20.340 ± 2.120**
Mean		**23.380 ± 2.547**	**23.224 ± 2.045**	**15.945 ± 1.872**	
C.D. (0.01)	Sources	1.866			
	Solvents	1.142			
	Sources × Solvents	3.231			
C.V. (%)		**9.316**			

Mean ± Standard deviation, **Highly significant @ 1 per cent level of significance

On the other hand, castor and clerodendron leaves recorded maximum TPC in ethanol (33.522 mg/ g & 26.708 mg/ g) and methanol (33.305 mg/ g & 26.248 mg/ g) extracts followed by distilled water (26.78 mg/ g & 19.194 mg/ g) extract, respectively. Further, peepal and prickly chaff leaves recorded maximum total phenols in methanol (19.426 mg/ g & 13.985 mg/ g) in comparison to distilled water (17.582 mg/ g & 12.044 mg/ g) and ethanol (17.004 mg/g & 7.258 mg/ g) extracts. In general, irrespective of extraction solvents, castor yielded higher total phenols in all the leaf extracts followed by coral vine, touch me not, banyan and clerodendron. However, least TPC was obtained in prickly chaff extracts. Meanwhile, among the extraction solvents, ethanol gave good results for extraction of phytochemicals compared to methanol and distilled water in terms of quality and quantity. Statistically, the results were found to be highly significant with respect to plant sources and solvents. Hence, based on the availability of the plant sources, castor, banyan & clerodendron with ethanol solvent were selected for formulation of herbal combinations.

Huda-Faujan *et al.* (2007) mentioned that the different levels of TPC may be attributed to different plants, procedures and standards used to express the TPCs; the colour measurement of Folin-Ciocalteu reagent and perhaps presence of other components that can react with Folin-Ciocalteu reagent such as ascorbic acid.

Apart from that, the results also suggested that extraction by ethanol could give higher phenolic content as compared to methanol. The findings were likely in agreement with Perez *et al*. (2007) who found that ethanol was the most efficient solvent as compared to methanol and water for extracting phenolic compounds. Ethanol is said to be the most suitable solvent in the extraction of phenolic compounds due to its ability to inhibit the reaction of polyphenol oxidase that causes the oxidation of phenolics and its ease of evaporation compared to water (Almey *et al*., 2010).

The results are supported by the study conducted by Vastrad *et al.* (2014) who

concluded that methanolic and ethanolic extracts of forest species exhibited maximum amount of TPC. Vastrad *et al*. (2015) and Vastrad *et al*. (2016) also mentioned that leaf extracts with ethanol exhibited higher total phenols than methanol and aqueous extracts.

Antimicrobial activity of herbal combinations

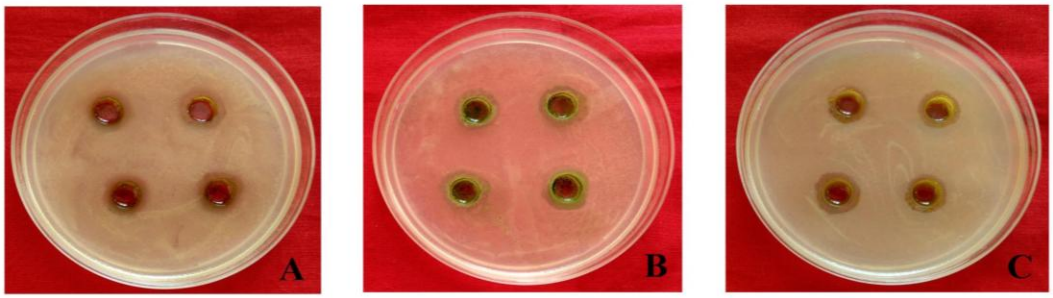
The antimicrobial activity of herbal extracts and their combinations against bacterial and fungal species is recorded in Table 2.

It is observed that among the single herbal extract, castor extract exhibited maximum zone of inhibition against both *S. aureus* (14 mm) and *E. coli* (12 mm) compared to clerodendron (12 mm – *S. aureus* & 10 mm – *E. coli*) and banyan (11 mm – *S. aureus* & 12 mm – *E. coli*) extracts. Similarly, castor extract showed greater zone of inhibition against *A. niger* (13 mm) than clerodendron (12 mm) and banyan (10 mm). However, 70 per cent ethanol (control) exhibited least antibacterial (09 mm – *S. aureus* and 08 mm – *E. coli*) and antifungal (09 mm) activity (Plate 1a).

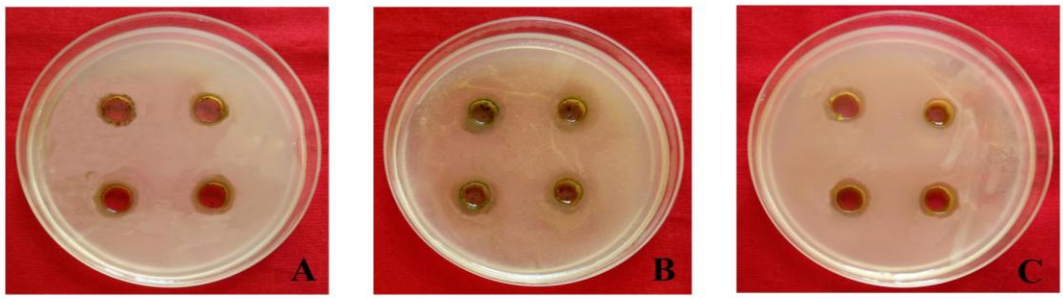
Table 2. Antimicrobial activity of herbal extracts and herbal combinations.

Sl. No.	Extracts	Zone of inhibition (mm)		
		Antibacterial activity		Antifungal activity
		S. aureus	***E. coli***	***A. niger***
1.	Control (70 % ethanol)	09 ± 0.82	08 ± 0.82	09 ± 0.82
2.	Castor	14 ± 1.83	12 ± 1.63	13 ± 1.71
3.	Banyan	11 ± 0.96	12 ± 2.22	10 ± 1.26
4.	Clerodendron	12 ± 0.96	10 ± 0.82	12 ± 1.26
	Herbal combinations			
	1 : 1 (v/v)			
5.	Castor : Banyan	22 ± 1.50	18 ± 2.16	11 ± 1.50
6.	Castor : Clerodendron	14 ± 1.29	12 ± 1.71	09 ± 1.50
7.	Banyan :Clerodendron	11 ± 0.82	12 ± 1.29	NI
	1: 2 (v/v)			
8.	Castor : Banyan	10 ± 0.96	14 ± 0.96	NI
9.	Castor : Clerodendron	12 ± 1.29	10 ± 1.63	10 ± 1.29
10.	Banyan :Clerodendron	09 ± 1.89	16 ± 1.29	NI
	2: 1 (v/v)			
11.	Castor : Banyan	17 ± 1.50	15 ± 1.71	13 ± 1.29
12.	Castor : Clerodendron	11 ± 1.26	09 ± 0.96	10 ± 1.71
13.	Banyan :Clerodendron	12 ± 1.41	11 ± 0.96	NI

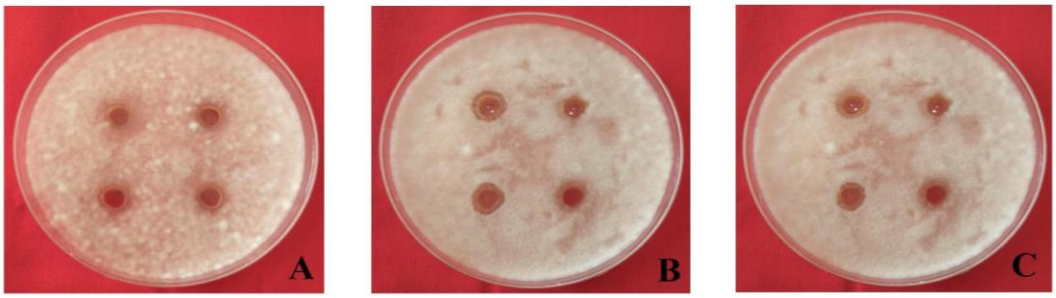
v/v: volume/volume
Mean ± S.D.
NI: No zone of inhibition
S. aureus – Staphylococcus aureus
E. coli – Escherichia coli
A. niger – Aspergillus niger



Antibacterial activity against *Staphylococcus aureus:* Castor (A), Banyan (B) & Clerodendron (C)

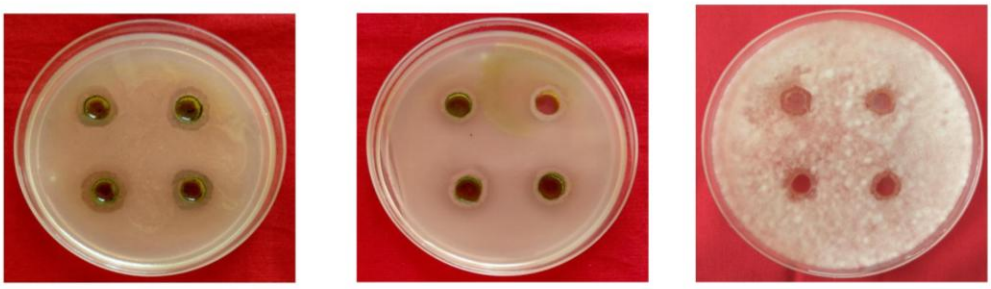


Antibacterial activity against *Escherichia coli*: Castor (A), Banyan (B) & Clerodendron (C)

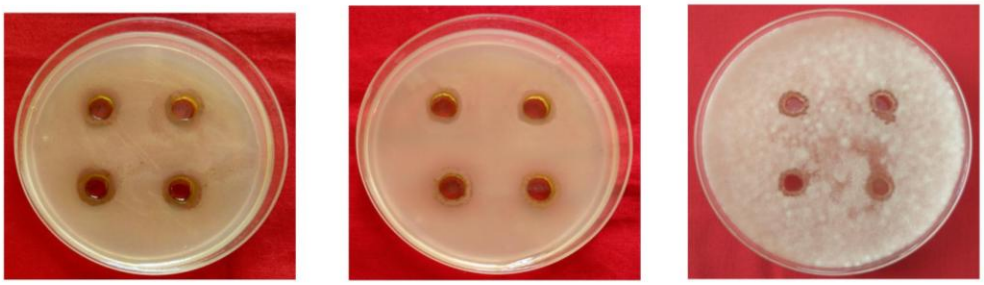


Antifungal activity against *Aspergillus niger:* Castor (A), Banyan (B) & Clerodendron (C)

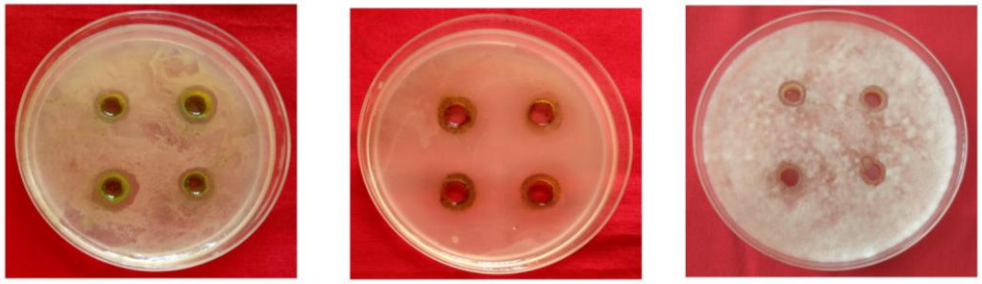
Plate 1a. Antimicrobial activity of herbal extracts



1:1 C:B combination: *S. aureus* (A), *E. coli* (B) & *A. niger* (C)



1:2 C:Cl combination: *S. aureus* (A), *E. coli* (B) & *A. niger* (C)



2:1 C:B combination: *S. aureus* (A), *E. coli* (B) & *A. niger* (C)

Plate 1b. Antimicrobial activity of herbal combinations

Further, among 1:1 (v/v) combination, C:B extract showed greater zone of inhibition against *S. aureus* (22 mm) and *E. coli* (18 mm) in comparison to C:Cl and B:Cl extracts. Likewise, C:B extract showed greater zone of inhibition against *A. niger* (11 mm) than C:Cl (09 mm) extract. However no antifungal activity was observed in case of B:Cl extract. Meanwhile, in case of 1:2 (v/v) combination, C:Cl extract exhibited good antibacterial activity against both *S. aureus* (12 mm) and *E. coli* (10 mm) as compared to C:B and B:Cl extracts. On the other hand, antifungal activity was noticed only in C:Cl extract with zone of inhibition of 10 mm (Plate 1b). C:B extract recorded highest zone of inhibition against both bacterial species (17 mm – *S. aureus* & 15 mm – *E. coli*) than C:Cl and B:Cl extracts in case of 2:1 (v/v) herbal combination. Correspondingly, good antifungal activity was observed in C:B (13 mm) and C:Cl (10 mm) extracts.

The variation in the antibacterial activity of the plant extracts can be attributed to inoculum size, type of media used, type of solvent used for extraction, extraction procedure, incubation time and temperature, part of the plant used and its time of collection, method of extraction procedure, incubation time and temperature, method of antibacterial assay and strain activity (Jahan *et al*., 2011). Meanwhile, among the bacterial species, higher inhibition zone was observed against Gram positive (*Staphylococcus aureus*) bacteria than Gram negative (*Escherichia coli*) bacteria. Similar observations have been made by many researchers who reported that Gram positive bacteria are more susceptible to plant's extracts as compared to Gram negative bacteria.

Moreover, the herbal combinations exhibited better antimicrobial activity than the crude extracts of respective plant sources may be because of the higher concentration of bio-active compounds possessing antimicrobial activities in the combined extracts thereby increasing the resistivity of herbal combinations.

Conclusion

The growing concern for development of eco-friendly and user-friendly products has created a way for the utilization of biodegradable plant based products. Also, the non edible plants and their parts are thrown as waste due to the lack of knowledge about the active constituents present in them. Therefore, the information regarding phytochemical analysis of plant extracts can be utilized by pharmacists, chemists, pathologists, food processing units for development of innovative drugs as well as for textile processing units for development of eco-friendly antimicrobial agents. Further, the novel idea of combining the herbal extracts in different ratios can reduce the quantity of plant source thereby conserving the natural environment yet exhibiting good antimicrobial activity. Besides, the herbal extracts and their combinations can be used in formulation of eco-friendly disinfectants and household/ consumer chemicals for better health and sanitation.

References

Almey, A.A. A., Khan, A.J.C., Zahir, S.I., Suleiman, M.K., Aisyah, M.R. and Rahim, K.K., 2010. Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leaves. Int. Food Res. J. 17, 1077-1084

Babel, S., Mogra, D., Rajvanshi, R., Agrawal, N. and Sharma, S., 2013. Ecofriendly finishing of fabric with *Jatropha curcas* leaves. Res. J. Family, Community & Consumer Sci. 1, 7-9

Barnett, H.L. 1960 Illustrated Genera of Imperfect Fungi, Burgess Publishing Company, Minneapolis, USA

Gupta, D. and Laha, A. 2007. Antimicrobial activity of cotton fabric treated with *Quercus infectoria* extract. Indian J. Fibre Text. Res. 32, 88-92

Huda-Faujan, N., Noriham, A., Norrakiah, A.S. and Babji, A.S., 2007. Antioxidative activities of water extracts of some Malaysian herbs. Asean Food J. 14, 61-68

Jahan, F., Lawrence, R., Kumar, V. and Junaid, M. 2011. Evaluation of antimicrobial activity of plant extracts on antibiotic susceptible and resistant *Staphylococcus aureus* strains. J. Chem. Pharm. Res. 3, 777-789

Oskay, M., Oskay, D. and Kalyoncu, F. 2009. Activity of some plant extracts against multi-drug resistant human pathogens. Iranian J. Pharmaceutical Res. 8, 293-300

Perez, M.B., Calderon, N.L. and Croci, C.A. 2007. Radiation-induced enhancement of antioxidant activity in extracts of rosemary (*Rosmarinus officinalis* L.). Food Chemistry, 104, 585-592

Pranoothi, E.K., Narendra, K., Joshi, D.S., Swathi, J., Sowjanya, K.M., Rathnakarreddi, K.V.N., Emmanuel, S., Padmavathi, S.J. and Satya, A.K. 2014. Studies on qualitative, quantitative, phytochemical analysis and screening of *in vitro* biological activities of *Leucas indica* (L). Int. J. Herbal Medicine, 2, 30-36

Sharma, A., Yadav, A., Barman, N. and Malwal, M. 2010. Quantification of primary metabolites of *Moringa oleifera* Lam. The Bioscan. 5, 403-405

Singleton, V.L. and Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144-158

Vastrad, J.V., Byadgi, S.A., Goudar, G. and Kotur, R. 2014. Characterization of phytoconstituents in leaf extracts of forest species for textile applications. Forest Products J. 64, 259-264

Vastrad, J.V., Goudar, G., Byadgi, S.A., Devi, R.D. and Kotur, R. 2015. Identification of bio-active components in leaf extracts of *Aloe vera*, *Ocimum tenuiflorum* (*Tulasi*) and *Tinospora cordifolia* (*Amrutballi*). J. Medicinal Plants Res. 9, 764- 770

Vastrad, J.V., Goudar, G., Walmiki, L., Mariyappanavar, S. and Mahale, G. 2016. Empowerment of women through dyeing technology – Inspirations from nature. Tech. Bull., Univ. Agric. Sci., Dharwad (India), pp. 27

Veerachari, U. and Bopaiah, A. K. 2012. Phytochemical investigation of the ethanol, methanol and ethyl acetate leaf extracts of six cassia species. Int. J. Pharma and Bio Sci. 3, 260-270