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In vitro Antioxidant and Antibacterial Efficacy of Condensed Tannins Containing Tree Leaves Extract of Jammu Province

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ABSTRACT

The present study was conducted to assess the antioxidant potential and antibacterial efficacy of lyophilized condensed tannins (CT) extract from locally available tree leaves (*Acacia nilotica*, *Eugenia jambolana*, *Ficus religiosa*, *Leucaenea leucocephala* and *Psidium guajava*) against bacterial species (viz. *Enterococcus faecalis, Escherichia coli*, *Salmonella enteritidis* and *Staphylococcus aureus*). Antioxidant activity was determined by 1, 1-Diphenyl-2- picryl-hydrazyl (DPPH) radical scavenging and total reducing power assays. Lyophilized CT extracts of *P. guajava* and *E. jambolana* showed significantly ($P<0.05$) higher antioxidant potential compared to standard ascorbic acid and other CT sources. Antibacterial efficacy was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using micro dilution method. The MIC and MBC values of CT extracts were significantly ($P<0.05$) higher for *F. religiosa* and *L. leucocephalla* then that of *A. nilotica, E. jambolana* and *P. guajava*. The MBC value for *S. enteritidis* was lower than *E. coli and. S. aureus*. Lower MIC and MBC values against *E. coli*, *S. aureus* and *S. enteritidis* in CT extracts of different sources showed better results compared to *E. faecalis* which showed statistically non-significant difference among all CT sources. It may be concluded that all CT sources possess antioxidant and antibacterial efficacy and were found to be effective against *E. coli, S. aureus* and *S. enteritidis* except *E. faecalis* and the comparison among the CT sources, *E. jambolana* and *P. guajava* were found to be most potent CT source as well as potent alternative antioxidant and antibacterial agents.

Keywords: Antibacterial, Antioxidant, Condensed tannins, Lyophilized extract, Tree leaves

Synthetic antibiotics and antioxidants as feed additives in poultry nutrition have long been used as growth promoters. However, the development of bacterial resistance against presently available antibiotics and toxicity due to continuous use of synthetic antioxidants has necessitated the need to search for alternatives having antibacterial and antioxidant properties. This situation has forced scientists to search for alternatives from plants as novel antibacterial chemotherapeutic agents (Abiramasundari *et al*., 2011).

In recent years, there has been a considerable interest in finding natural antioxidants and antimicrobials from plants; especially from tanniferous tree leaves. The plant secondary metabolites (PSMs), particularly condensed tannins (CTs), have been reported to inhibit the propagation of free radicals, protect the animals from parasitic, bacterial and other stress related diseases (Anderson and Teuber, 2001; Jayasri *et al*., 2009; Pathak, 2013). Moreover, the use of synthetic antioxidants and antimicrobials has been questioned because of their health hazards (Duong *et al*., 2006). Therefore, there have been numerous researches on these bio-resources to search for natural, possibly economic and effective alternative antioxidant and antibacterial agents to replace the synthetic ones.

It is believed that CT extracts from different tree leaves could be acting directly on pathogenic bacteria thereby destroying them. There is also evidence for direct inactivation of pathogenic bacteria: low CT concentrations modify the morphology of microorganism (Brownlee *et al.*, 1990). Furthermore, the use of CTs at low to moderate levels are advantageous for their easy availability, less

cytotoxicity, and economical for therapeutic purpose as well as for maintaining normal health, production and well being (Shan *et al*., 2007, Pachanawan *et al*., 2008).

Therefore, the screening and evaluation of locally available tanniferous tree leaves as natural antioxidant and antibacterial may be a step forward to overcome the problem of synthetic antioxidants and antibiotics, their toxicity, resistance and residual effects in food products. Though, the information regarding the antioxidants and antibacterial properties of CT containing tree leaves extracts in Jammu province is scanty. Thus, the study aimed to screen locally available tanniferous tree leaves for their chemical composition, presence of CTs, antioxidant and antibacterial potential of lyophilized CT extracts warrant investigation.

MATERIALS AND METHODS

Tree leaves' collection, processing and screening

Five promising tree leaves viz. *Acacia nilotica*, *Eugenia jambolana*, *Ficus religiosa*, *Leucaenia leucocephala* and *Psidium guajava* were selected for *in-vitro* study based on their availability, accessibility and possibility of containing condensed tannins (CT) from Faculty premises, R.S. Pura, Jammu.

The matured tree leaves were lopped and transported to the laboratory in fresh state. The samples were dried at about 45–50°C using a forced air oven. Once the materials were dried, they were kept in a cool, dark and dry place only after it has dry matter content of > 90 %. A representative sample was taken for their chemical composition and CT analysis.

Nutritional and statistical analysis

Selected promising tree leaves were screened for their proximate composition as per the standard method of AOAC (1995). The extraction and estimation of CTs were done as per Makkar (2000). Experimental data under this study were subjected to analysis of variance and treatment means were ranked using Duncan's multiple range tests (Snedecor and Cochran 1994) and analyzed by SPSS (SPSS version 10.0 for windows).

Preparation of lyophilized CT extract

The extraction was made as per the method described by Barrau *et al*. (2005) with slight modifications as per Pathak *et al.* (2013a, b). Briefly, 200 g dry, ground material from each tree leaves were extracted with 2 x 1200 ml of 70:30, acetone: water; (v/v) containing ascorbic acid (1 g l^{-1}) in the large size stoppard conical flask on electric shaker for 2 hrs followed by homogenization. The extracts were lyophilized in freeze drier. The lyophilized extracts were kept in the deep freezer at -20°C temperature till further used for *in vitro* biological assay.

Antioxidant activity

The *in-vitro* antioxidants activities of lyophilized CT extracts of tree leaves were performed by using 1, 1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and total reducing power assays. The free radical scavenging activity of various tree leaves extracts was measured in terms of hydrogen donating or radical scavenging ability of the stable DPPH free radical as per the method described by Braca *et al*. (2001), whereas, the total reducing capacity of the prepared and lyophilized tree leaves extracts was determined according to the method of Oyaizu (1986). In the present experimental study ascorbic acid was used as standard.

Antibacterial property

Antibacterial efficacy of CT from *A. nilotica*, *E. jambolana*, *F. religiosa*, *L. leucocephala* and *P. guajava* was assessed *in vitro* against some pathogenic bacteria and clinical isolates. The antibacterial activity of various CT extract were ascertained by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of some tested bacterial species which are most common pathogenic microorganism of poultry.

Bacterial species

Pathogenic strains of *Enterococcus faecalis* MTCC 9845, *Escherichia coli* MTCC 1610, *Salmonella enteritidis* and *Staphylococcus aureus* were selected for *in-vitro* experimental study. The pathogenic strain of *E. faecalis* and *E. coli* were purchased from Institute of Microbial Technology, Chandigarh, India, whereas, *S. enteritidis*

and *S. aureus* were obtained from Division of Veterinary Pathology and Division of Veterinary Microbiology, respectively.

Bacterial suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard (Andews, 2005). The 1:100 dilutions of initial suspension were additionally prepared into sterile 0.85 % normal saline.

Table 1: Chemical composition of locally available tanniferous tree leaves

Attributes	*Acacia nilotica*	*Eugenia jambolana*	*Ficus religiosa*	*Leucaena leucocephala*	*Psidium guajava*
DM	89.44	90.17	89.64	89.05	91.83
CP	8.99	9.93	11.64	17.93	8.26
EE	4.73	4.61	3.72	5.23	3.2
CF	8.11	12.93	11.68	7.92	13.78
TA	6.97	9.22	10.11	10.08	7.83
CT	1.97	8.18	1.95	3.02	8.34

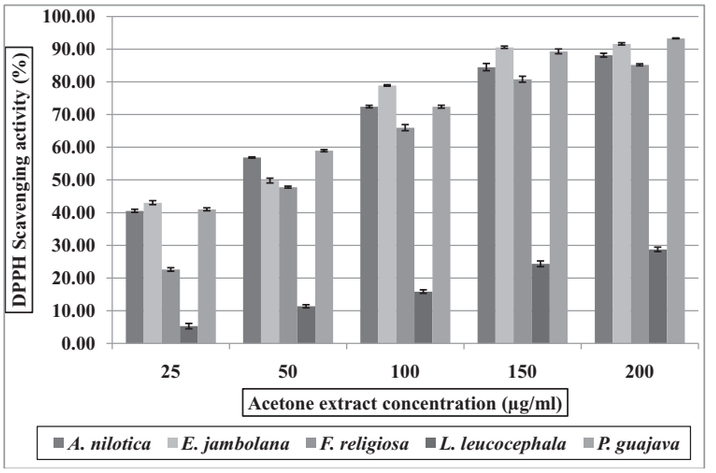

Fig. 1: DPPH scavenging activity (%) of condensed tannins from various tree leaves extract at different concentrations

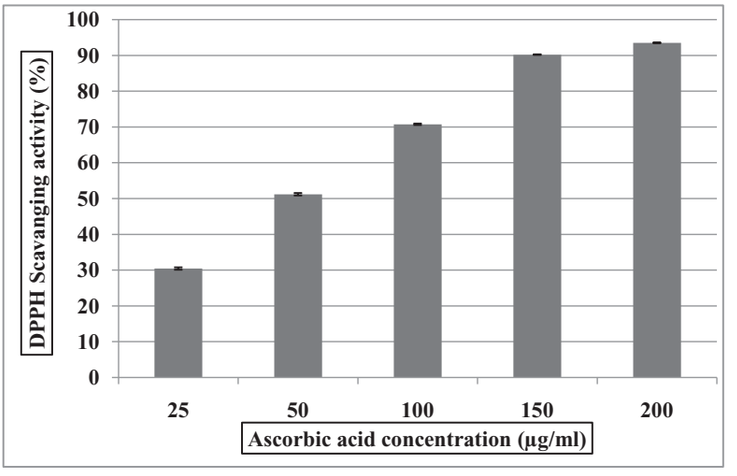

Fig. 2: DPPH scavenging activity (%) of ascorbic acid as standard at different concentrations

Micro dilution method

Antimicrobial activity was tested by determining the MIC and MBC by using micro dilution method with resazurin (Sarker *et al*., 2007). The 96-well plates were prepared by dispensing 100 µl of nutrient broth and/or Mueller–Hinton broth for bacteria into each well. Lyophilized extracts were obtained by dissolving in DMSO and then diluted into Mueller-Hinton broth to achieve a concentration of 10% DMSO. Solvent control test was performed to study the effects of 10 % DMSO on the growth of microorganism. It was observed that 10 % DMSO did not inhibit the growth of bacterial species. Also, in the experiment, the concentration of DMSO was additionally decreased because of the two-fold serial dilution assay (the working concentration was 5 % and lower).

Each test included growth control and sterility control. A 100 µl from the stock solution of tested extracts (concentration of 640, 320, 160, 80 and 40 mg/ml) was added into the first row of the plate. Then, two-fold, serial dilutions were performed by using a multichannel pipette. A 10 µl of diluted bacterial suspension was added to each well to give a final concentration of 5 $\times 10^5$ CFU/ml for bacteria. Finally, 10 µl of resazurin solution was added to each well inoculated with bacteria. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. The inoculated plates were incubated at 37 °C for 24 hrs for bacteria.

All tests were performed in triplicate and MICs were constant. The MBC was determined by plating 10 µl of samples from wells, where no indicator colour change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as MBC.

RESULTS AND DISCUSSION

The chemical composition of locally available tanniferous tree leaves is presented in table 1. A wide variation in the chemical composition of locally available tanniferous tree leaves was evident. The chemical composition of target tree leaves in the present study was comparable with the values reported by previous workers (Dey *et al*., 2006; Pathak *et al*., 2015; Singh *et al*., 2015; Zargar *et al*., 2016), except some nutrient specific differences usually observed in such tree leaves. The wide range of CT in tree leaves (1.95- 8.34 %) are in conformity with the findings of earlier workers (Pathak *et al*., 2015; Singh *et al*., 2015; Zargar *et al*., 2016). The levels of CT in tree leaves vary greatly between species, within species, stage of development, from location and from year to year (Makkar *et al*. 2007; Pathak *et al*., 2015).

Antioxidant activity of CT extract

The DPPH scavenging assay was performed in lyophilized extract of different CT sources. The results of the present study varied with each CT source. The results of DPPH scavenging activity of CT extract of various leaves sources and standard ascorbic acid are represented in the graphical form in figures 1-2. The DPPH radical scavenging assay is an easy, rapid and sensitive method for screening of various tree leaves extracts for their antioxidant activity. Figures 1-2 shows the dose-response curve of DPPH radical scavenging activity of various CT sources and ascorbic acid as standard, respectively. The results showed that CT extracts of *E. jambolana* and *P. guajava* have the highest radical scavenging activity which was followed by *A. nilotica* and *F. religiosa*. The lowest radical scavenging activity was shown by *L. leucocephala* leaves extract. At a concentration of 200 µg/ml the DPPH radical scavenging activity of *P. guajava* CT extract reached 93.28±0.12, which at the same concentration, that of *L. leucocephala* CT extract was 28.74 ±0.68.

As the CT extract concentration of each tree leaves increased, the DPPH radical scavenging activities increased significantly ($P<0.05$). In DPPH assay, the decrease in absorbance of reaction mixture at 517 nm in spectrophotometer at different concentration of CT extracts from different leaves sources and the reaction mixture color is de-colorized which clearly indicated that it increases the radical scavenging activity of tree leaves extracts due to the presence of CT as natural antioxidant. The CTs which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Ebrahimzadeh, *et al.*, 2009 and Nabavi *et al.*, 2008). Present findings (CT extract concentration and radical scavenging activity) are in accordance with the results reported by Zargar *et al*. (2016) using aqueous extract of same CT sources, however, DPPH radical scavenging activity of *P. guajava* and *E. jambolana* of acetone extract of present study at higher concentration showed better

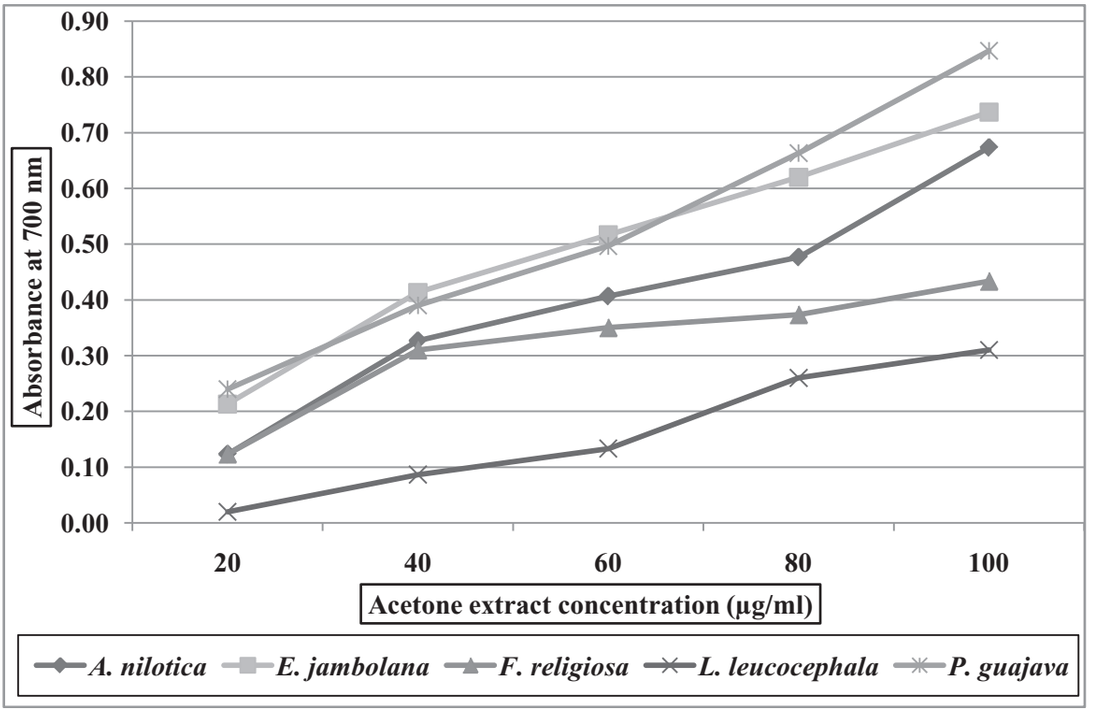

Fig. 3: Total reducing power of condensed tannins from various tree leaves extract at different concentrations

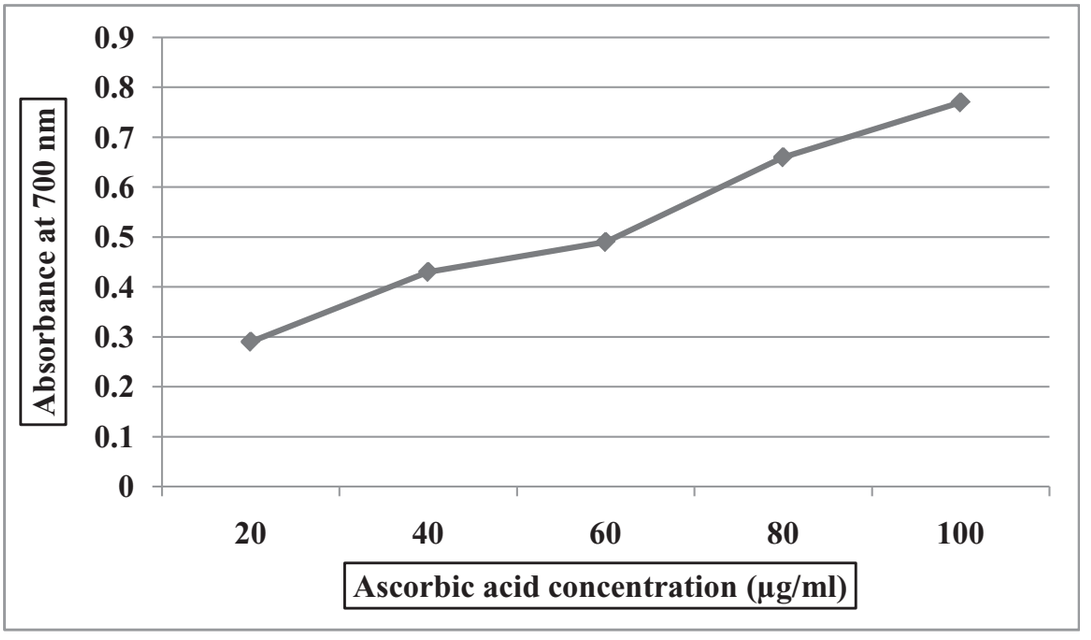

Fig. 4: Total reducing power of ascorbic acid as standard at different concentrations

Table 2: Minimum inhibitory concentration (MIC) of tanniferous tree leaves extract against tested bacteria

Leaf source	Bacterial species				Mean ± SE
	Escherichia coli	*Staphylococcus aureus*	*Salmonella enteritidis*	*Enterococcus faecalis*	
Acacia nilotica	3.33	2.50	2.17	40.00	12.00^{a} ±4.88
Eugenia jambolana	4.17	4.17	2.17	40.00	12.63^{a} ±4.78
Ficus religiosa	10.00	8.33	8.33	80.00	26.67^{b} ±9.30
Leucaena leucocephala	8.33	10.00	8.33	80.00	26.67^{b} ±9.30
Psidium guajava	3.33	3.33	1.83	40.00	12.13^{a} ±4.86
Mean ± SE	5.83^{a} ±0.83	5.67^{a} ±0.86	4.57^{a} ±0.92	56.00^{b} ±5.24	

^{ab}Means with different superscripts within a row and column differ significantly (P<0.05)

Table 3: Minimum bactericidal concentration (MBC) of tanniferous tree leaves extract against tested bacteria

Leaf source	Bacterial species				Mean ± SE
	Escherichia coli	*Staphylococcus aureus*	*Salmonella enteritidis*	*Enterococcus faecalis*	
Acacia nilotica	6.67	6.67	5.00	80.00	24.58^{a} ±9.66
Eugenia jambolana	8.33	10.00	5.00	80.00	25.83^{a} ±9.45
Ficus religiosa	20.00	20.00	20.00	160.00	55.00^{b} ±18.28
Leucaena leucocephala	16.67	20.00	16.67	160.00	53.33^{b} ±18.60
Psidium guajava	6.67	8.33	3.33	80.00	24.58^{a} ±9.68
Mean ± SE	11.67^{a} ±1.67	13.00^{a} ±1.60	10.00^{a} ±1.94	112.00^{b} ±10.47	

^{ab} Means with different superscripts within a row and column differ significantly (P<0.05)

antioxidant activity compared to aqueous extract of same CT source reported by Zargar *et al*. (2016), which might be due to the type of solvent, extraction processes and activity of CT.

In another antioxidant assay (total reducing power), the absorbance at 700 nm correspond high reducing power potential of *P. guajava* (0.85±0.05) at the concentration of 100 µg / ml. Results of the present study show that all tree leaves extract exhibited potent with varying reducing power ability interestingly *P. guajava* was higher (0.85±0.05), *A. nilotica* and *E. jambolana* were as near as (0.67±0.04 and 0.74±0.04, respectively), while *F. religiosa* and *L. leucocephala* was lower (0.43±0.03 and 0.31±0.03, respectively) to the reductive ability of ascorbic acid (0.773±0.004) at same concentration. In the present study we observed a concentration-dependent increase in the absorbance of reaction mixture for CT extract of all leaves sources as well as for ascorbic acid as standard antioxidants (Fig. 3 and 4), respectively.

The increase in absorbance of reaction mixture at 700 nm in spectrophotometer which clearly indicated the greater the reducing power of leaves extracts (at different concentration) from various sources due to the presence of CT which showed potent antioxidant property. Total reducing power of CT is related to its electron transfer ability and may therefore serve as an indicator of its antioxidant potential and higher absorption of reaction mixture indicates a stronger reducing power. The observed reducing power activity of all tree leaves extract could be attributed to the presence of CT. The present findings are in line with the previous studies which reported that the reducing power ability of tree leaves extract correlate with the CT content (Rosalind *et al*., 2013; Zargar *et al*., 2016). Reducing power assay is the measurement of the reductive ability of antioxidant (Gulcin *et al*., 2003).

Antibacterial activity of CT extract

The MIC values of CT extracts from different sources are presented in table 2. MIC value was significantly higher for *F. religiosa* and *L. leucocephalla* then *A. Nilotica, E. Jambolana* and *P. guajava*. But MIC values between *A. nilotica, E. jambolana* and *P. guajava* were statistically non-significant. MIC values *against E. coli*, *S. enteritidis*, *S. aereus* and *E. faecalis* ranged from 4.57 to 56 (mg/ml) above leaf sources which was statistically significant ($P<0.05$). *E. coli, S. enteritidis* and *S. aereus* were significantly ($p<0.05$) more sensitive than that of *E. faecalis*, however, MIC values against *E. coli*, *S. enteritidis* and *S. aureus* were statistically non-significant among each other.

The MBC values of CT extracts from different sources have been presented in table 3. MBC values were significantly higher for *F. riligiosa* and *L. leucocephalla* then that of *A. nilotica, E. jambolana* and *P. guajava*, while MBC values between *A. nilotica, E. jambolana* and *P. guajava* did not differ significantly. MBC values against bacterial species viz. *E. coli, S. enteritidis, S. aereus* and *E. faecalis* ranges from 10 to 112 (mg/ml). *E. coli, S. enteritidis, S. aereus* were significantly ($P<0.05$) more sensitive than that of *E. fecalis*. In the present study *E. faecalis* was found to be more resistant against CT extracts of different leaves as compared to other tested bacterial species and it needs higher concentration of CT extracts which clearly indicated by showing higher MIC and MBC values compared to other bacterial species.

There was significant variation in the antibacterial activities (MIC and MBC values) of CT extracts from different sources. The MIC was recorded as the least concentration of extracts that completely inhibit the growth of the test bacteria. This is done to determine whether the CT extracts of tested tree leaves can inhibit the bacterial strains of Gram-positive and Gram-negative, since there is the possibility of CT which is a natural compound that most of the spread in the tropical tree leaves is capable of inhibiting bacterial cell wall synthesis and damage the germ cell plasma membrane of Gram-positive and Gram-negative, so it is necessary to study the anti-bacterial activity of the CT.

The MIC and MBC values were significantly lower for *A. nilotica, E. jambolana* and *P. guajava* compared to *F. religiosa* and *L. leucocephalla*. Similarly, Buvaneswari *et al.* (2011) stated that methanolic extract of *P. guajava* L. was more effective in inhibiting various Gram positive and Gram negative bacteria. Aqueous extract of *P. guajava* L. could inhibit all tested bacterial species. Similar to our results, Abubakar (2009) reported that aqueous extract of *P. guajava* L. was effective in inhibiting *Proteus mirabilis, Streptococcus pyogenes, E. coli, S. aureus and Pseudomonas aeroginosa*.

In the present study *E. faecalis* was found to be more resistant against CT extracts of various sources as compared to other tested bacteria and it needs higher concentration extracts which clearly indicated by showing higher MIC and MBC values compared to other bacterial species. Differences in antibacterial activities of various tree leave extracts against tested bacteria are expected as the activities are based not only on different structures of bacteria but also on their susceptibilities. Tested bacteria that had low MIC values also showed low concentrations of MBC. In the present study, results showed that the extracts exhibited bacteriostatic activities at lower concentrations and bactericidal activities at higher concentrations. Therefore, the MIC and MBC values are useful as guideline to the choice of appropriate and effective concentrations for therapeutic substances.

Kumar and Vaithuyanathan (1990) suggested that tannins directly inhibit microbial function by complexing with bacterial cells or indirectly by reducing the availability of nitrogen and sulphur for microbial protein synthesis. De Bruyne *et al*. (1999) evaluated the antimicrobial activity of CTs and verified that MIC was >100 µg/ml for *E. coli*, *Piromonas aerugenosa*, *Salmonella paratyphe*, *Enterobacteria cloacae*, *Mycobacterium spp.*, *S. aureus* and *Candida albicans*. Dijpa *et al*. (2000) correlated the antibacterial activity demonstrated by *Syzygium jambos* to its high tannin contents.

The antibacterial potential of *F. religiosa* was reported by Hemaiswarya *et al*. (2009). According to their study the chloroform extract of *F. religiosa* leaves inhibited the growth of various *Salmonella spp.*, *P. vulgaris*, *E. coli*, *B. Subtilis* and *K. Pneumonia*, which revealed the antibacterial potential of the plant (Hemaiswarya *et al*., 2009). In another study, different extracts (methanol, aqueous, chloroform) of *F. religiosa* barks has inhibitory effect on the growth of three enteroxigenic *E. coli,* isolated from the patients suffering from diarrhoea (Uma *et al*.,

2009). It has been reported that the antimicrobial activity of *P. guajava* leaf extracts were attributable to the presence of tannins, triterpenoids and flavonoid glycosides in the leaves themselves (Arima and Danno, 2002; Begum *et al*., 2002; Fukuda *et al*., 2003). However, the extracts used in the present study consist mainly of CTs, because triterpenoids, low molecular tannins, flavonoids glycosides and polysaccharides were washed out by extraction process, it required several steps in various solvents (Dauer *et al*., 2003). These CTs are known to have antibacterial effects (Guyot *et al.*, 1999). Therefore, CTs are known for their antibacterial activity against Gram-positive and Gram-negative bacteria (Afolayan and Meyer, 1997). The properties of the CTs could have attributed to the results of the antibacterial activities observed in the present study.

Another mechanism by which, CTs works as antibacterial that it can form hydrogen bonds with the protein contained in bacterial cells, if the hydrogen bonds formed between CTs with proteins it will be denatured proteins possibility that bacterial metabolism becomes impaired. Allegedly based on the reaction of CTs can inhibit the growth of *E. coli*, *S. aureus* and *S. enteritidis*. The CTs can also inhibit growth and kill bacteria by reacting with the cell membrane. The CT will damage the cell membrane, causing leakage of essential metabolites that inactivate the bacterial enzyme system. It is believed that CT extracts from tree leaves could be acting directly on pathogenic bacteria thereby destroying them.

CONCLUSION

It may be concluded that the lyophilized extract of all CT sources in the present study possess antioxidant and antibacterial activities against gram-positive and gram-negative bacteria and were found to be effective against *E. coli, S. aureus* and *S. enteritidis* except *E. faecalis*. Comparison among the CT sources *E. jambolana* and *P. guajava* were found to be most potent CT source as well as potent natural antioxidant and broad spectrum antibacterial efficacy against pathogenic *E. coli, S. aureus* and *S. enteritidis*. Further research is needed to determine the antibacterial efficacy against other bacterial species so that they can be used as alternative natural feed additives for poultry to produce socioeconomic organic food products.

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