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Antioxidant and Antibacterial activity of *Digera muricata* (L.) Mart.

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Abstract: The present study was undertaken to determine the antioxidant and antibacterial activity of *D. muricata* against selected bacterial strains. The five solvents; hexane, chloroform, acetone, methanol and water were used to extract the plant material in ascending order of polarity. The antioxidant activity of these extracts was determined by using DPPH radical scavenging activity, Folin-Ciocalteu reagent method, total reducing power, superoxide radical scavenging activity, hydroxyl radical scavenging activity and nitric oxide radical scavenging activity. It was recorded that the methanolic extract exhibited maximum IC_{50} whereas water extract was found to exhibit least IC_{50} for all the antioxidant assays. The extracts were further tested for antibacterial activity against gram positive and gram negative bacteria by microbroth dilution assay. It was found that the hexane extract exhibited highest activity against all the tested pathogens. Chloroform and acetone extracts exhibited moderate activity. Hexane extract showed least MIC of 195 µg/ml towards *B. subtilis* and *S. aureus* as compared to others. Antioxidant and antimicrobial activity of *D. muricata* against tested organisms provides the platform for its utilization as herbal drug.

Keywords: D. muricata, DPPH, FRAP, Antioxidant activity, Antibacterial activity, IC_{50}

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Digera muricata (L.) Mart. is an indigenous herbaceous plant, belonging to Amarantaceae family, found as a weed throughout India. It is also known as False Amaranth, Latmahuria, Kunanjara and Aranya. It is commonly known as "Kondhra" in the local language of Haryana,"Aranya" in Sanskrit and "Latmahuria" in Hindi. It is an annual herb, growing 20-70 cm in height. Its cultivation occurs in northeast tropical Africa (Ethiopia) and in the Indian subcontinent (India). It is widely distributed in eastern tropical Africa (from Sudan and Ethiopia south to Tanzania) and subtropical Asia (from Yemen to Afghanistan, Pakistan, India, Malaysia and Indonesia) and is usually collected from the wild parts of Ethiopia (Konso region) (Townsend et al. 2010).

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The medicinal properties of this plant are being commercially exploited throughout the world due to the presence of a large number of secondary metabolites including phenols, flavonoids, alkaloids, terpenoids, saponins, tannins, cardiac glycosides and anthraquinones (Khan et al. 2011). The traditional medicinal system of India has reported the plant for its various pharmacological activities like antidepressant (Sundar et al. 2014), antioxidant (Mety et al. 2011), hepatoprotective (Paulsson et al. 2001; Friedman et al. 2003; Taubert et al. 2004; Svensson et al. 2003; Klaunig, 2008; Tong et al. 2004) antimicrobial (Mathad et al. 2010), anti-diabetic (Jagatha et al. 2011; Patel et al. 2013), anthelmintic (Hussain, 2008), allelopathic (Bindu et al. 2011), nephrotoxicity (Anjaria et al. 2002), protective and anti-testicular toxicity (Khan et al. 2009). Its boiled root infusion is recommended to the mother for lactation purpose, after childbirth. The leaves and young shoots of this plant are used locally as a vegetable in Africa and in India, and are given to relieve constipation (Gupta et al. 2005). The decoction of leaves given once in a day seems to be very effect-

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tive for treatment of kidney stone (Aggarwal et al. 2012; Sharma et al. 2011a). In folk medicine, it is used to treat renal disorders, causing reduction in generation of reactive radicals, which are involved in lipid peroxidation and the accumulation of dysfunctional proteins thereby, preventing reduction in kidney injuries. Its leaves, flowers and seeds are also reported to treat hepatitis (Durairaj et al. 2014). The plant is also used as an alternative for secondary infertility (Hocking, 1962).

D. muricata is described as anti-oxidant and antimicrobial in the folk literature. Therefore, the present study was performed to explore its anti-oxidant and antimicrobial activity.

MATERIALS AND METHODS

Chemicals and reagents

Folin-Ciocalteu reagent, Na_2CO_3, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, $FeCl_3$, DPPH, Nitro blue tetrazolium, NADH, phenazine methosulphate, 2-deoxyribose, $FeSO_4$-EDTA, thiobarbituric acid (TBA), NaOH, 2-deoxyribose, $FeSO_4$-EDTA, thiobarbituric acid (TBA) and NaOH were purchased from Hi-Media.

Collection of Plant Material

The whole plant of *D. muricata* was collected from local area of Bhiwani, Haryana, India during September, 2013. The collected plant material was identified by Department of Botany, Maharshi Dayanand University, Rohtak, India (Voucher specimen no. 12/14) and further cross authentication of selected plant was done with the help of Flora of Haryana (Jain et al. 2009).

Extraction

The leaves of *D. muricata* were washed and dried under shade for 3 weeks. The properly dried leaves were crushed and ground to fine powder. The powder was extracted three times for 72 h with five different solvents (100 ml each) in ascending order of polarity i.e. hexane, chloroform, acetone, methanol and water. The extracts were filtered and solvents were evaporated to dryness under temperature below 50°C to yield a crude extracts. The extracts were stored at -80°C till further use.

Determination of percentage yield of plant extract

The percentage yield of the plant extract was obtained by using the formula,

Extraction yield (%) = $w_1 \times 100 / w_2$

Where, w_1 = weight of dried crude plant extract (after extraction); w_2 = weight of the dried plant material (before extraction)

The % yield of the plant extract obtained was 0.072%, 1.67%, 1.68%, 7.42% and 18.01% in hexane, chloroform, acetone, methanol and water respectively.

Antioxidant Assays

Determination of Phenolic content

Folin-Ciocalteu reagent based assay was used to determine total phenol content. To one mL extract (100 µg/mL) in methanol, 5 mL of tenfold diluted Folin-Ciocalteu reagent and 4 mL (75 g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20°C for 30 min and the absorbance was recorded at 765 nm using UV-VIS spectrophotometer (McDonald et al. 2001). 1mL aliquots of 10, 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used to prepare standard calibration curve. Total phenol value was obtained from the regression equation and expressed as mg gallic acid equivalent (GAE)/g dry weight using the formula,

Phenolic Value (C) = cV/M

Where C = total content of phenolic compounds in mg GAE, c = the concentration of gallic acid (mg/L) established from the calibration curve, V = volume of extract (0.5mL) and M = the weight of pure plant methanolic extract.

Scavenging activity against 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH)

DPPH radical scavenging activity of crude extracts was measured as described earlier (Braca et al. 2001; Rajeswara et al. 2001). Extract solutions were prepared by dissolving 0.05 g of dry extract in 50 mL of methanol. An aliquot of 2 mL of 0.004% DPPH solution in methanol was mixed with 1 mL of plant extracts in methanol at various concentrations (10, 20, 40, 60, 80, 100 µg/mL) and incubated at 25°C for 30 min. Absorbance of the test mixture was read at 517 nm using a spectrophotometer against a DPPH control containing only 1 mL of methanol in place of the extract. All experiments were repeated thrice along with gallic acid (standard). Percent inhibition was calculated using the following expression,

% Inhibition = $(A_{blank} - A_{sample} / A_{blank}) \times 100$

Where "A_{blank}" and "A_{sample}" stand for absorption of the blank sample and absorption of tested extract solution, respectively.

Ferric reducing antioxidant power assay (FRAP)

The reducing antioxidant power of the extracts was determined by the method of Oyaizz (Oyaizz, 1996). Various concentrations of extracts (10, 20, 40, 60, 80, 100 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (3.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated in an incubator at 50°C for 20 min. After incubation, 2.5 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The samples absorbance was read at 700 nm

against a blank using UV-VIS spectrophotometer. Gallic acid was used as standard. Higher absorbance indicated a higher reducing power. EC_{50} values were calculated and indicate the effective concentration at which the absorbance was 0.5 for reducing power. The % inhibition of was calculated using the formula,

% inhibition= $(A_C - A_S / A_C) \times 100$

Where "A_C" is absorbance of control, "A_S" is absorbance of sample.

Determination of superoxide radical scavenging activity

Measurement of superoxide radical scavenging activity of the extracts was done by using standard methods followed by slight modification (Nishikimi et al. 1972). Nitro blue tetrazolium (NBT) reduction method was used to determine superoxide scavenging activity. The reaction mixture was prepared by mixing 1.0 mL of NBT solution (312 µM NBT in 100 mM phosphate buffer, pH 7.4), 1.0 mL NADH solution (936 µM NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 mL different extracts of *D. muricata* at different concentrations (10, 20, 40, 60, 80, 100 µg/mL). Gallic acid was used as standard. Further 100 µL of phenazine methosulphate solution (120 µM PMS in 100 mM phosphate buffer, pH 7.4) was added to the mixture. The tubes were incubated for 15 minutes and the optical density was measured at 560 nm. The percent inhibition of superoxide generation was calculated by comparing the absorbance values of the control and experimental tubes using the following formula.

% scavenging = $(1- A_e / A_o) \times 100$

Where "A_o" is the absorbance of mixture without sample and "A_e" is the observance of mixture with the sample.

Hydroxyl radical scavenging activity

Deoxyribose method was used to study the effect of extracts on hydroxyl radical (Klein et al. 1981). The reaction mixture was prepared by adding 450 µL of 0.2 M sodium phosphate buffer, pH 7.0), 150 µL of 10 mM 2-deoxyribose, 150 µL of 10 mM $FeSO_4$-EDTA, 150 µL of 10 mM H_2O_2, 525 µL of H_2O and 75 µL of sample solutions of different concentrations (10, 20, 40, 60, 80, 100 µg/ml) in respective solvents and gallic acid solution was prepared in methanol. The reaction was started by the addition H_2O_2. After incubation at 37°C for 4 hours, the reaction was stopped by adding 750 µL of 1 % thiobarbituric acid (TBA) in 50 mM NaOH, the solution was boiled for 10 min and then cooled in water. The absorbance of the solutions was measured at 520 nm. The ability to scavenge the hydroxyl radical was calculated using the following equation:

% radical scavenging activity = $(1- A_s / A_c) \times 100$

Where, "A_s" is the absorbance of the sample and "A_c" is the observance of control, respectively.

Nitric oxide radical scavenging activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosvoy reaction (Garratt, 1964). The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (10, 20, 40, 60, 80, 100 µg/ml) of the test solution in a final volume of 3 mL. After incubation for 150 minutes at 25°C, 1 mL sulphanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 mL to the incubated solution and allowed to stand for 5 min. Then 1mL of Napthyl ethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured by using gallic acid as standard and % scavenging was calculated by using the following formula.

% scavenging activity = $(A_o - A_c) \times 100$

Where "A_C" is observance of control, "A_o" is observance of the sample.

Calculation of inhibition concentration (IC_{50}) values for scavenging assays

IC_{50} value is defined as the amount of antioxidant necessary to decrease the initial concentration of the free radical activity by 50%. IC_{50} values were calculated from the graph plotted between % scavenging activity against the concentrations of the samples.

Antibacterial screening test organisms

Lyophilized cultures of Gram positive (*Bacillus cereus* NCDC66, *Bacillus subtilis* NCDC70, *Staphylococcus aureus* NCDC110) and gram negative bacteria (*Escherichia coli* NCDC135, *Salmonella typhi* NCDC113) were obtained from National Dairy Research Institute (NDRI), Karnal in September 2013. *Streptococcus pyogens* MTCC1927 (Gram negative bacteria) was purchased from Indian Institute of Microbial Technology (IMTECH), Chandigarh.

In-vitro antibacterial screening of *Digera muricata*

Antibacterial activities of the extracts were determined by the microbroth dilution assay in 96-well culture plates (Khond et al. 2009; Arif et al. 2009). Stock solution of 10 mg/ml concentration was prepared in 10% (v/v) DMSO (for hexane, chloroform extract), 5% (v/v) DMSO (for acetone, methanol extract) and sterile double distilled water (for water extract). To each wells of the culture plates, 100 µl of autoclaved nutrient broth was added and 100 µl of test material was added to the first row of microtiter plate. Two fold serial dilutions of test extracts were made. 2 X resazurin (20 µl) was added as indicator in each well. Finally, from bacterial suspension

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10 µl volume was taken and added to each well to achieve a final concentration of 5×10^6 CFU/ml. Each plate was wrapped loosely with cling film to avoid the dehydration of bacterial culture. The experiment was run in duplicate. Proper controls were kept for each experiment. The culture plates were incubated at 37°C and change in colour was examined after 18 hrs for the growth of test bacteria. Appearance of purple colour indicates the death of bacteria whereas; pink colour indicates the presence of live bacteria in each well containing bacterial inoculum. The results were expressed as minimum inhibitory concentration (MIC).

RESULTS

Antioxidant Activities

D. muricata leaf extract prepared in different solvents (chloroform, acetone, methanol and water) was evaluated for its antioxidant activity by analysis of phenolic content, FRAP, DPPH, superoxide radical, hydroxyl radical and nitric oxide radical scavenging activity assays.

Determination of Phenolic content

Results obtained in the present study revealed maximum total phenolic content for methanol extract followed by acetone, chloroform and water extract. (Table 1, Fig. 1)

DPPH radical scavenging assay

The DPPH radical scavenging activity of *D. muricata* leaf extract is shown in figure 2. The IC_{50} values of scavenging DPPH radicals for methanol and acetone extracts were found to be 55.17 and 48.99 µg/ml. IC_{50} value of gallic acid was found to be 39.71 µg/ml, which was quite low as compared to all the tested extracts. The maximum % scavenging activity was recorded in the metanol extract while the minimum % scavenging activity was found in water extract (Table 1).

FRAP radical scavenging assay

The reducing power of methanol and water extract is shown in figure 3. The result indicates that reducing ability of the extracts increased with the concentration. The methanol extract showed better reducing power as compared to water extract. IC_{50} value of methanol and water extract were found to be 39.06 and 33.99 µg/ml (Table 1).

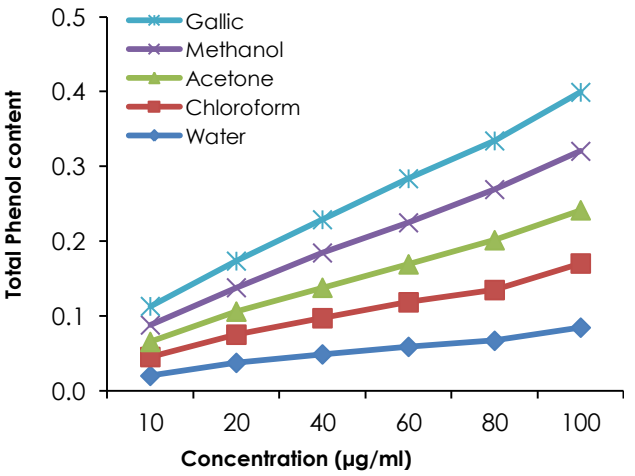

Figure 1 Total phenolic content of different extracts of *D. muricata*

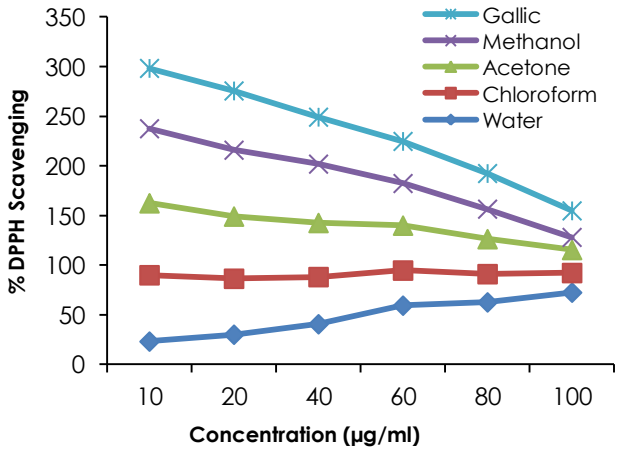

Figure 2 DPPH Scavenging activity of different extracts of D. muricata

Superoxide radical scavenging assay

The % superoxide radical scavenging was found to be greater in the methanol extract as compared to water extract of *D. muricata* (Fig. 4). Methanol extract was found to exhibit IC_{50} value of 73.27 µg/ml while water extract was found to possess IC_{50} of 61.53 µg/ml. When compared to Gallic acid, which had an IC_{50} value of 52.34 µg/ml, the IC_{50} value of water was found to be high (Table 1).

Table 1 Scavenging activities of prepared plant extract in different solvents

D. muricata extracts	DPPH radical scavenging [IC_{50} (µg/ml)]	Total phenolic content [mg GAEs/g dry wt]	Ferric reduction activity potential [IC_{50} (µg/ml)]	Superoxide radical scavenging [IC_{50} (µg/ml)]	Hydroxyl radical scavenging activity [IC_{50} (µg/ml)]	Nitric oxide radical scavenging [IC_{50} (µg/ml)]
Gallic acid	39.71	50.70	46.81	52.34	33.86	49.33
Chloroform	48.41	48.97	-	-	54.78	42.82
Acetone	48.99	49.21	-	-	55.16	29.94
Methanol	55.17	49.98	39.06	73.27	64.97	40.47
Water	36.58	46.89	33.99	61.53	52.27	29.09

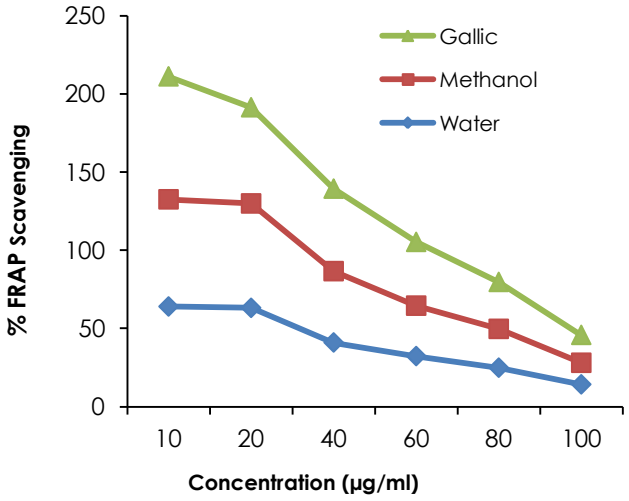

Figure 3 Reducing antioxidant power of methanol and water extracts of *D. muricata*

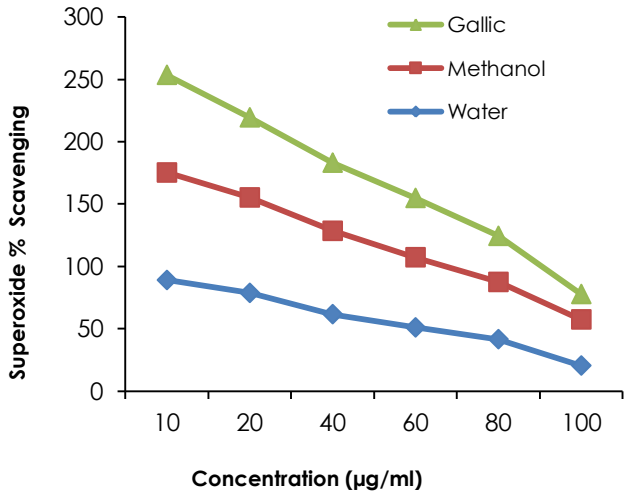

Figure 4 Superoxide scavenging activity of methanol and water extracts of *D. muricata*

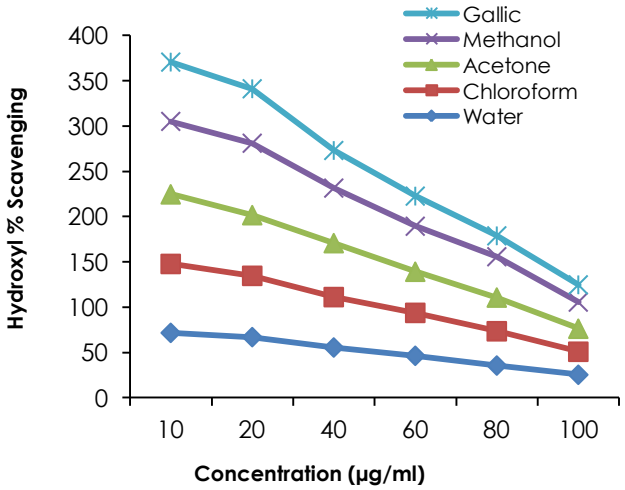

Figure 5 Hydroxyl radical scavenging activity of different extracts of *D. muricata*

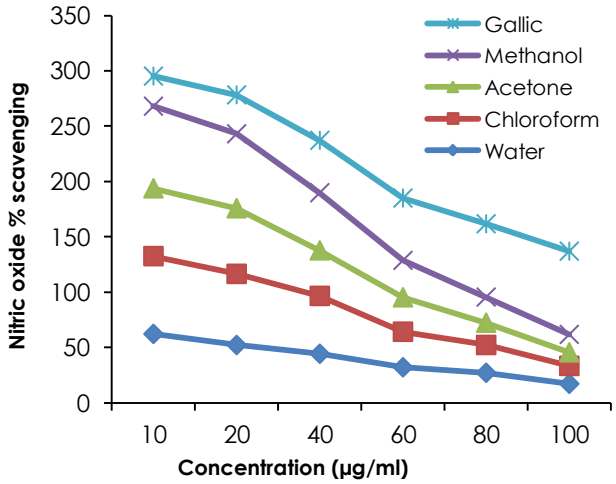

Figure 6 Nitric oxide scavenging activity of different extracts of *D. muricata*

Hydroxyl radical scavenging assay

Methanol extract of *D. muricata* displayed strong H_2O_2 scavenging activity of 64.97 µg/ml, whereas that of standard, gallic acid exhibited 33.86 µg/ml. The scavenging activities of water, chloroform and acetone extracts were recorded to be 52.27, 54.78, 55.16 µg/ml respectively (Table 1). The % hydroxyl radical scavenging activity was found to be maximum in the methanol extract of *D. muricata* followed by acetone, chloroform and water (Fig. 5).

Nitric oxide radical scavenging assay

The maximum nitric oxide scavenging activity was found in methanol extract and minimum was reported in water extract (Fig. 6). The nitric oxide activity values were found to be lower than that of gallic acid, having IC_{50} value of 49.33 µg/ml. Among the extracts tested, chloroform extract had IC_{50} value of 42.82 followed by methanol extract having IC_{50} value of 40.47 µg/ml. Aqueous extract had the least reducing ability of 29.09 µg/ml (Table 1). Figure 7 summarizes the comparative antioxidant activities of *D. muricata* extract in different solvents.

Antibacterial activity

Leaves of *D. muricata* were evaluated for their antimicrobial potential against six bacteria by microbroth dilution assay. Plant extracts showing MIC >5 mg/ml are considered to be significant. Table 2 summarizes the MICs of all the plant extract dissolved in different solvents presenting significant activity against micro-organisms. Ampicillin and Streptomycin (1 mg/ml) were used as positive control against different bacterial strains. Figure 8 represents the comparative evaluation of antibacterial activity of different extracts

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Table 2 Antibacterial activity of D. muricata (L.) Mart. extract in different solvents

D. muicata extracts	*B. cereus* (mg/ml)	*B. subtilis* (mg/ml)	*S. aureus* (mg/ml)	*S. pyogens* (mg/ml)	*S. typhi* (mg/ml)	*E. coli* (mg/ml)
Hexane	0.156	0.0195	0.0195	0.156	1.25	2.5
Chloroform	0.156	0.156	0.0195	0.039	2.5	5.0
Acetone	0.156	0.0195	0.0195	0.156	2.5	5.0
Methanol	2.5	1.56	5.0	1.25	2.5	-
Aqeuous	5.0	-	-	1.25	-	-

of *D. muricata* against tested bacterial pathogens. The hexane extract exhibited least MIC while chloroform and acetone extracts exhibited considerable MIC against all the tested pathogens. In gram positive bacteria, hexane extract exhibited MIC of .0195 mg/ml in *B. subtilis* and *S. aureus* followed by 0.156 mg/ml in *B. cereus* and *S. Pyogens*. In gram negative bacteria, *E. coli* exhibited considerable MIC of 2.5 mg/ml. In chloroform extracts, gram positive bacteria exhibited MIC ranging from 0.0195 mg/ml to 0.156 mg/ml. Among these *S. aureus* exhibited lowest MIC 0.0195 mg/ml. In acetone extracts the MIC 0.0195 mg/ml was observed against *B. subtilis* and *S. aureus*. Acetone extracts was found to have least MIC in case of gram negative bacteria. However, methanol extract exhibited highest MIC toward gram positive bacteria and not found to be effective against gram negative bacteria. It exhibited 1.56 and 1.25 mg/ml MIC against *B. subtilis* and *S. pyogens*. Aqueous extracts of the plant showed activity only against *S. pyogens* where MIC was found to be 1.25 mg/ml.

DISCUSSION

D. muricata (L.) Mart is an important medicinal plant possessing hepatoprotective, antimicrobial, antioxidant, anti-diabetic, anthelmintic and anti-testicular toxicity. Plant is rich source of phenols, tannins, terpenoids, flavonoids and glycosides. In the present study, various extracts of *D. muricata* has been

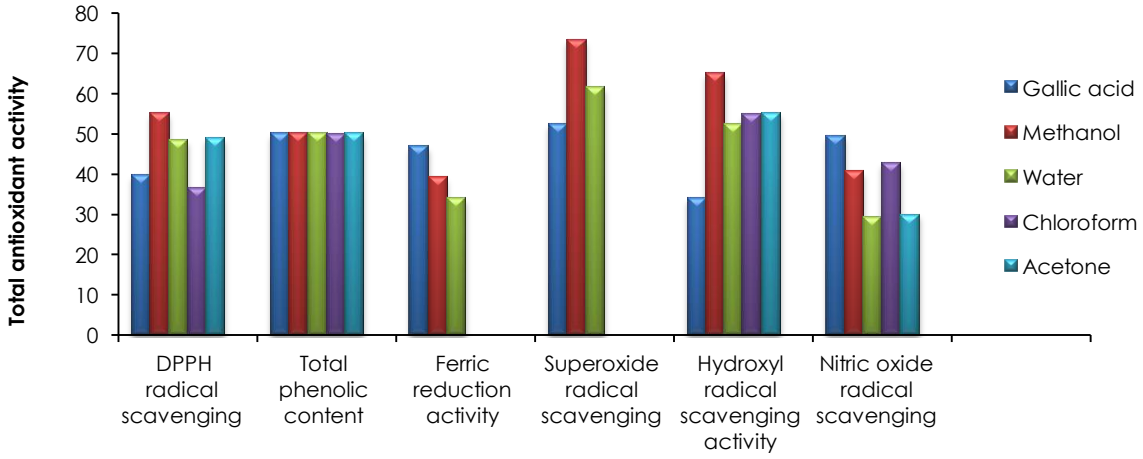

Figure 7 Antioxidant activities of different extracts of *D. muricata*

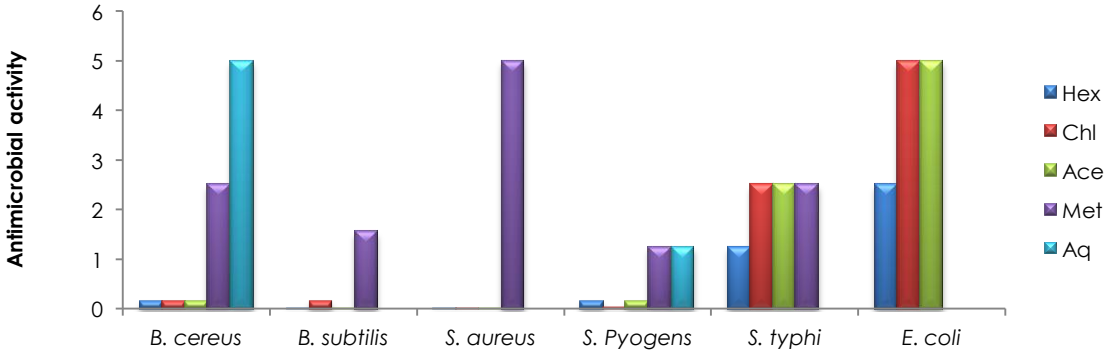

Figure 8 Evaluation of antibacterial activity of different extracts of *D. muricata* against tested bacterial pathogens

evaluated for its free radical scavenging and antibacterial activity. Although, similar studies on free radical scavenging and antimicrobial activity on *D. muricata* has been performed previously but the present study also supports the antioxidant nature of the plant. Mety et al. carried systematic evaluation of the antioxidant activity of this plant and revealed that methanol extract was found to be most active followed by ethanol and water extract and least activity was recorded in hexane extract (Mety et al. 2011), while in the present study maximum anti-oxidant activity was reported in methanol extract and least in water extract. IC_{50} for total phenolic content was found to be maximum for methanol extract followed by acetone, chloroform and water. It was found that the methanolic extract had the maximum IC_{50} for DPPH radical assay followed by acetone and chloroform and minimum IC_{50} was recorded in water extract. The maximum IC_{50} for reducing power was recorded in methanol extract and minimum IC_{50} was recorded in water extract. For superoxide radical scavenging assay IC_{50} was found to be maximum in the methanol extract at a concentration of 10 µg/ml. Hydroxyl radical scavenging activity was recorded maximum in the methanolic extract followed by acetone, chloroform and water extract. In nitric oxide radical scavenging assay, methanolic extract was found to have maximum IC_{50} followed by chloroform, acetone and water extract. Previous study reported that stem and root extract of this plant showed maximum activity against *P. aeruginosa* and *E. coli*. Leaf extract of this plant has also been reported for minimum zone of inhibition against *P. aeruginosa* and maximum zone of inhibition against *E. coli* (Mathad et al. 2010). Methanol extract of this plant has also been found to show maximum activity against tested test bacteria and fungi (Sharma et al. 2011b). The results of the present study clearly indicate that the hexane extract exhibited least MIC while chloroform and acetone extracts exhibited consid-erable MIC against all the tested pathogens. Among gram positive bacteria, *S. aureus* exhibited lowest MIC 0.0195 mg/ml in chloroform extract. Highest MIC toward gram positive bacteria is recorded in methanol extract. However, methanol extract is not found to be effective against gram negative bacteria. Aqueous extract showed activity only against *S. pyogens*.

Hence, the present investigation clearly indicated the antioxidant and antibacterial effect of *D. muricata* thereby enhancing the possibility of finding and exploring potent bioactive molecule possessing antibacterial property.

Conclusion

D. muricata exhibits antioxidant and antimicrobial activity against tested organisms which provides the platform for its utilization as herbal drug. It is therefore essential that efforts should be made to develop novel herbal drugs to control pathogenic bacteria which pose threat to human health and act as cheaper, safer and more effective medicine. Furthermore, *in vivo* studies can also explore the mechanism of antioxidant action.

Conflict of interest statement

We declare that we have no conflict of interest.

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