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Antimicrobial Potential of Gemmo-modified Extracts of *Terminalia arjuna* and *Euphorbia tirucalli*

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ABSTRACT

The present study was conducted to investigate the *in vitro* antimicrobial potential of two medicinal plants *Terminalia arjuna* and *Euphorbia tirucalli.* Antimicrobial activity of methanolic, acetone, ethyl acetate, water and gemmomodified extracts of both plants was determined by using disc diffusion method against four bacterial (*Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli* & *Pasteurella multocida*) and two fungal strains (*Rhizopus solani*, A*spergillus niger).* Gemmomodified extract of *T. arjuna* showed broad spectrum antimicrobial potential. Strongest activity was detected with gemmo modified extract of *Terminalia arjuna* against *Bacillus subtilis* at the concentration of 1000 µg disc^{-1}. *T. arjuna* strongly inhibited the growth of tested fugal strains at all concentrations. Highest effect with methanolic extract of *E. tirucalli* was observed against *S. aureus* and *B. subtilis* at the concentration of 1000 µg. Gemmomodified extract of *E. tirucalli* was more effective against *E. coli* and strongly inhibited the growth of fungal strains. Gemmo modified extracts of both plants showed higher inhibitory effect on all tested microbial strains than other extracts.

Key Words: *Terminalia arjuna; Euphorbia tirucalli;* Gemmomodified; Disc diffusion; Methanolic extract

INTRODUCTION

Infectious diseases resulting from the presence of pathogenic microbial agents including bacteria, fungi and viruses are major healthcare problem in current century. These diseases are main reason of deaths in developing countries (Okusa *et al*., 2007; Mojab *et al*., 2008). Incidence of new and re-emerging infectious diseases and development of resistance to commonly used antibiotics is alarmingly increasing. In modern times treatment of infectious diseases has become a big problem due to severe side effects of some antibiotics, which includes hypersensitivity allergic reactions and immunosuppression. It is promising area of research to develop new antibiotics having less side effects and better activity against antibiotic resistant strains (Khan *et al*., 2009), as some of currently used antibiotics failed for the treatment of infectious diseases (Sieradzki *et al*., 1999; Janvoyska *et al*., 2003). Plants have great potential as antimicrobial agent, due to the presence of secondary metabolites.

Extraction solvent has great importance for extraction of bioactive components. Owing to complex nature and variable polarities of bioactive compounds, solvents of different polarities have been reported for extraction like water, methanol, ethyl acetate, acetone and n-hexane.

Gemmo-modified extracts are prepared with growing young shoots and buds of plants, which are mercerized in glycerin and methanol. Gemmo-modification is gaining interest for treatment of infectious diseases. In gemmotherapy embryonic parts of plants (Young shoots buds) are collected in growing season (spring) of plants, as at this stage metabolic activities of plants are very fast so their defense system is also very active, producing a large number of compounds. These compounds can be uses as antimicrobial agents. When gemmo-modified preparations are consumed by humans, it acts on organs to gently stimulate and promote elimination, detoxification, nourishment and RNA repair. Secondary metabolites are also synthesized in greater amount in growing parts due to fast metabolism, as high concentration of secondary metabolites help in defense system of plants. Secondary metabolites like alkaloids, polyphenols, could be better antimicrobial agents.

Terminalia arjuna (Arjun) is a versatile traditional medicinal plant. Ancient physicians used the powdered bark of *T. arjuna* for alleviating cardiovascular disease and wound healing. Many scientific studies proved its medicinal importance (Tiwari *et al*., 1990; Dwivedi & Agarwal, 1994; Ram *et al*., 1997; Chander *et al*., 2004; Ramya *et al*., 2008). *Euphorbia tirucalli* (milk bush) is also used in important tradition medicines. Roots of the plants are used as antimicrobial (Parekh *et al*., 2005), nephro-protective, anti-arthritic; purgative, carminative and anti-leprosy (Bani *et al*., 2007).

There is a great need for effective antimicrobial agents from natural sources in order to prevent the infectious diseases. Recent interest in gemmotherapy prompted this research work to explore the antimicrobial potential of

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gemmo-modified extracts of two indigenous medicinal plants. The objective of this study was to evaluate the comparative antimicrobial potential of gemmo-modified and other extracts of two indigenous medicinal plants *T. arjuna* and *E. tirucalli* against pathogenic bacterial and fungal strains.

MATERIALS AND METHODS

Extract preparation: The plant material (30 g) used to prepare extract containing active compounds, was subjected to 12 h sequential extraction with solvents of diverse polarities. The plant material was first extracted with n-hexane (300 mL), followed by acetone, ethyl acetate and methanol. The extract was then filtered and solvent was completely evaporated with rotary evaporator under reduced pressure approximately at 40°C. Yield of extracts was noted and stored in refrigerator at 4°C for further use.

Water extract: Dried plant material (30 g) was refluxed with water (300 mL) for 2 h. After completion of time extract was filtered and water was evaporated to get crude extract. Percentage yield of crude extract was calculated and stored in refrigerator at 4°C for further use.

Preparation of gemmo-modified extract: Paste of plant material (100 g), which was freshly harvested from plants during their growing stage, was macerated with one liter mixture of glycerin and methanol in a ratio of 1:2 and shaked vigorously. After one month, the macerate was filtered, solvent was removed with rotary evaporator and crude extract was stored in refrigerator.

Determination of antimicrobial activity: Antimicrobial screening was performed by disc diffusion method as described previously (Rios *et al*., 1988; Ripa *et al*., 2009). Different steps involve in this assay are detailed as follow:

Bacterial strains: Two Gram positive (*Staphylococcus aureus, Bacillus subtilus)* and two gram negative strains *(Escherichia coli, Pasteurella multocida)* of microorganisms were selected for determination of antibacterial activity. Microorganisms were collected from Department of Microbiology University of Agriculture Faisalabad.

Preparation of media: Nutrient agar was dissolved in distilled water and pH (7) of the media was adjusted by addition of acid or base. After properly plugged with cotton, this flask containing media was kept in autoclave for sterilization at 121°C for 15 min. After sterilization, this media was cooled at room temperature.

Dispersion of medium: Sterilized medium (15 mL) was spreaded into petri plates uniformly in the form of thin film of gel of 2-3 mm thickness. The poured plates were incubated overnight in oven at 37°C. The plates without any type of contaminations were selected for further procedure.

Antibacterial screening: Different concentrations of extracts were prepared by dissolving extracts (5, 10, 15, 20, 25, 50, 75, 100, mg) in 1 mL water to attain the concentrations in mg/mL. 10 uL of each solution was applied on sterile disc (6 mm) and allowed to dry solvent in antiseptic hood. Thus discs contained 50, 100, 150, 200, 250, 750, 500 and 1000 µg $disc^{-1}$ of extracts. Antibiotic chloramphenicol was used as standard to compare the activity.

Sterilized petri plates were injected with bacterial cultures. Discs permeated with different concentrations of extracts and standard were placed on this solid medium. Plates were incubated at 37°C for 24 h. At the end of incubation the zone of no growth (mm) were measured by zone reader.

Antifungal Activity

Fungal strains: Two fungal strains *Aspergillus niger* and *Rhizopus solani were* selected for antifungal screening.

Preparation of media: For the preparation of potato dextrose agar medium, glucose (20 g), potato starch (20 g) and agar agar (20 g) was dissolved in distilled water (1000 mL) in properly wool plugged flask and autoclaved at 121°C for 15 min to remove the contamination from medium. This sterilized medium was poured into petri plates and allowed to cool and set as gel of 2-3 mm thickness. These plates were incubated overnight at 37°C to check any type of contamination. Plates showing any growth of microorganism were discarded.

Antifungal screening: Different concentration of extracts was prepared similar to that prepared in antibacterial assay. Inoculated fungal culture was poured into medium. Filter paper discs (6 mm) with different concentrations of extracts and standard antifungal drug (fluconazole) were placed on medium. These plates were incubated at 37°C for 3-4 days. The diameters of inhibition zones were measured in (mm) with zone reader.

Statistical analysis: Each sample was analyzed in triplicate and data is expressed as mean±SD. Data was analyzed using analysis of variance ANOVA in SPSS 15 software. Tukey's Multiple Comparison test was used for comparison of means of different experiments ($p < 0.05$).

RESULTS

Antibacterial activity of *Terminalia arjuna*: Different extracts of *T. arjuna* showed varying level of antibacterial activity dose dependently (Table I). Gemmo-modified extract of *T. arjuna* showed superior antimicrobial activity against all tested microbes. Minimum inhibitory concentration was 250 µg $disc^{-1}$against all the tested microbial strains. Significantly ($p < 0.05$) strongest activity was observed with gemmo-modified extract of *T. arjuna* against *B. subtilis* at the concentration of 1000 µg $disc^{-1}$ and gave zone of no growth of 38±1.0 mm diameter, followed by *S. aureus* (31.00±1.00 mm zone diameter of inhibition), *P. multocida (*30±1.00 mm) and *E. coli* (28±1.00 mm). Methanolic extract of bark showed significantly ($p <0.05$) highest activity against *B. subtilis* followed by *P. multocida, S. aureus* and *E. coli.* Inhibitory effect of methanolic and gemmo-modified extracts were comparable with standard antibiotic against *S. aureus* (32.3±2.5mm) and *B. subtilis* (40±0.5) and less than standard antibiotic against *P. multocida* (38.33±1.52 mm) and *E. coli* (35.55±2.08 mm).

Table I: Antibacterial activity of different extracts of *T. arjuna*

Extracts	Zone of inhibition (mm)				
	Conc. (µg disc^{-1})	*E. coli*	*S. aureus*	*P. multocida*	*B. subtilis*
Methanolic extract of bark	250	15.000±1.000	8.000±2.000	0.000±0.000	18.000±2.000
	500	18.000±1.000	17.000±2.000	15.000±1.000	22.333±1.528
	750	20.000±1.000	19.000±2.000	18.000±1.000	23.000±1.000
	1000	23.000±1.000*#	23.000±1.0000*#	25.000±1.000*#	33.000±1.000*#
Acetone extract of bark	250	12.000±1.000	11.000±1.000	12.000±1.000	18.000±2.000
	500	14.000±2.000	14.000±1.000	14.000±1.000	20.000±1.000
	750	15.667±0.578*	19.000±1.000	14.333±2.082	21.667±2.082
	1000	19.000±1.000	21.000±1.000*	17.667±0.577*	26.333±1.528*
Ethyl acetate extract of bark	250	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
	500	9.333±1.155	9.667±1.528	11.000±0.5000	11.000±1.000
	750	12.000±1.000*	13.000±3.606	13.167±0.289	12.333±2.082
	1000	14.333±5.132	12.000±2.000*	15.100±0.173*	14.000±2.000*
Water extract of bark	250	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
	500	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
	750	11.000±1.000	17.000±1.000	13.000±4.359	14.000±2.646
	1000	15.000±1.000*	21.000±1.000*	16.000±1.000*	18.000±1.000*
Gemmo-modified extract	250	14.000 ±1.000	0.000±0.000	0.000± 0.000	18.000± 2.000
	500	19.833± 0.764	24.000± 0.000	17.000± 1.000	24.000± 2.000
	750	27.733± 0.643	27.000± 1.000	18.000± 1.000	32.667± 1.155
	1000	28.000± 1.0#*	31.000± 1.0#*	30.000± 1.0#*	38.000± 1.0*#
Standard	500	35.333±2.082	32.333±2.517	38.333±1.528	40.000±2.000

All values are mean of Inhibition zone (mm) ±S.D (n=3). * Significant difference between the mean zone of inhibition of extracts ($p < 0.05$). # Significant difference between bacterial strains ($p<0.05$)

There was significant difference ($p<0.05$) between the activity of methanolic and other extracts of bark. Acetone extract exhibited good activity against *B. subtilis* (26±1.58 mm), *S. aureus* (21.00±1.00 mm) *and E. coli.* Ethyl acetate extract showed less antibacterial activity. Water extract was most effective against *S. aureus* followed by *B. subtilis, P. multocida* and *E. coli.* Inhibitory effect of water extract against all microbes was observed only at 750 µg disc^{-1}.

Antifungal activity of *T. arjuna*: Extracts of *T. arjuna* strongly inhibited the growth of tested fugal strains at all concentrations (Table II). Gemmo-modified extract demonstrated excellent antifungal potential and inhibited the growth of *Rhizopus solani* (28.00±1.00 mm zone of inhibition) and *A. niger* (26±1.00 mm zone of inhibition) at the concentration of 1000 µg disc^{-1}. These results are comparable with standard antifungal drug against *R. solani* (32±1.00 mm) and *A. niger* (30.00±1.00 mm). There was no significant ($p<0.05$) difference in activity between gemmo-modified and methanolic extract of bark, however both extracts showed significantly ($p<0.05$) higher activity than other extracts. Methanolic extract of bark was highly active against *A. niger* and gave zone of inhibition of 25±1.00 mm and also inhibited the growth of *R. solani* and produced 23.66±1.00 mm zone of inhibition. Acetone extract also inhibited the growth of *R. solani* strain Ethyl acetate and water extracts showed low level of activity.

Antibacterial activity of *Euphorbia tirucalli*: Methanolic extract of dry plant and gemmo-modified extract showed strong antimicrobial activity (Table III). Gemmo-modified extract was sensitive against all tested strains and showed significantly ($p<0.05$) higher activity than other extracts. Highest effect with methanolic extract was observed against *S. aureus* and *B. subtilis* at the concentration of 1000 µg disc^{-1}. Gemmo-modified extract inhibited the growth of *S. aureus* and *B. subtilis.* Gemmo-modified extract was more active against *E. coli* than methanol. Inhibitory effect of methanolic and gemmo-modified extract were comparable with standard antibiotic against *S. aureus* and less than standard antibiotic against *B. subtilis*, P. *multocida* and *E. coli.* Acetone and ethyl acetate extracts exhibited good activity against *B. subtilis*. Both extracts showed low level of activity against other tested microbial strains. Water extract showed low activity against *B. subtilis* and not much effective against other strains.

Table II: Antifungal activity of various Extract of *T. arjuna*

Extracts	Zone of inhibition (mm)		
	Conc.(µg disc^{-1})	*Aspergillus niger*	*Rhizopus solani*
Methanolic extract of bark	250	12.000 ± 2.000	11.000± 2.000
	500	15.000 ± 2.000	15.000 ±1.000
	750	20.000 ± 2.000	18.000± 1 .000
	1000	25.000± 1.000*	23.667 ± 0.577*
Acetone extract	250	12.000± 2.000	11.000 ± 1.000
	500	12.000 ± 1.000	13.000 ± 1.000
	750	15.000 ± 1.000	16.000 ± 1.000
	1000	17.000 ± 1.000*	20.000± 1.000*
Ethyl acetate extract	250	9.333± 0 .577	10.000 ± 1.000
	500	11.000 ±1.000	12.000 ±1.000
	750	13.000 ± 1.000	14.000 ± 2.000
	1000	15.000 ± 1.000*	16.000 ±2.000*
Water extract	250	10.000 ± 2.000	7.000 ± 1.000
	500	11.333± 1.155	11.000 ± 1.000
	750	12.000 ± 1.000	11.000 ± 1.000
	1000	15.000 ±1.000*	14.000 ± 1.000*
Gemmo extract	250	14.000±1.000	15.000±1.000
	500	16.333 ± 0.577	20.000 ± 1.000
	750	19.000±1.000	23.000 ± 1.000
	1000	26.000 ± 1.000*	28.000± 1.000*
Standard	500	30.000 ± 2.000	35.000 ± 1.000

All values are mean of Inhibition zone (mm) ±S.D (n=3) * Significant difference between the mean zone of inhibition of extracts ($p<0.05$). # Significant difference between fungal strains ($p<0.05$)

Table III: Antibacterial activity of various extracts of *E. tirucalli*

Extracts	Zone of inhibition in mm				
	Concentration (µg disc^{-1})	*E. coli*	*S. aureus*	*P. multocida*	*B. subtilis*
Methanolic extract	250	0.000±0.000	0.000±0.000	0.000±0.000	15.000±1.000
	500	16.000±1.000	20.000±2.000	21.000±1.000	21.000±1.000
	750	19.000±1.000	22.000±2.000	23.000±1.000	24.000±1.000
	1000	24.000±1.000*	30.000±1.000*	27.333±1.528	30.000±1.0*#
Acetone extract	250	0.000±0.000	0.000±0.000	0.000±0.0*	0.000±0.0*
	500	0.000±0.000	14.000±1.000	15.000±1.000	19.000±1.000
	750	0.000±0.000*	15.000±1.000	17.000±1.000	21.000±1.000
	1000	0.000±0.000	21.000±1.000*	21.000±1.000	27.000±1.0*#
Ethyl acetate extract	250	0.000±0.000	0.000±0.000	0.000±0.0*0	0.000±0.000
	500	10.000±1.000	9.000±1.000	9.333±1.155	11.000±1.000
	750	14.000±1.000	10.000±2.000	10.000±2.000	12.000±1.000
	1000	19.000±1.000*	17.000±2.000*	18.000±1.0*	20.000±1.0*
Water extract	250	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
	500	11.667±2.082	13.000±1.000	11.000±1.000	13.000±1.000
	750	13.333±1.528	11.667±1.528	15.000±1.000	14.000±1.000
	1000	16.000±1.000*	16.000±1.000*	16.000±2.00*	17.000±1.0*
Gemmo extract	250	14.000 ±1.000	0.000±0.000	0.000± 0.000	18.000± 2.000
	500	19.833± 0.764	24.000± 0.000	17.000± 1.000	24.000± 2.000
	750	27.733± 0.643	27.000± 1.000	18.000± 1.000	28.667± 1.155
	1000	28.000± 1.000	31.000± 1.000*	29.000± 1.00*	32.000± 1.08
Standard	500	35.333±2.082*	32.333±2.517	38.333±1.528	40.000±2.000

All values are mean of Inhibition zone (mm) ±S.D (n=3). * Significant difference between the mean zone of inhibition of extracts ($p<0.05$). # Significant difference between bacterial strains ($p<0.05$)

Antifungal activity of *E. tirucalli*: Gemmo-modified extract strongly inhibited the growth of fungal strains and produced zone of inhibition 28±1.00 mm against *R. solani* and 23±1.00 mm against *R. niger* at high concentration (1000 µg disc^{-1}). Gemmo-modified extract showed significantly ($p<0.05$) better antibacterial activity than other extracts. Methanolic extract was second most effective extract and demonstrate zone of inhibition against *R. solani* and *A. niger*. Acetone extract demonstrated moderate activity, while ethyl acetate extract was found to be least effective. Water extract did not show any effect against fungal strains (Table IV).

Table IV: Antifungal activity of *E. tirucalli* extracts

Extracts	Zone of inhibition in mm		
	Concentration (µg disc^{-1})	A. *niger*	*R. solani*
Methanolic extract	250	0.000±0.000	0.000±0.000
	500	13.167±0.289	14.000±1.000
	750	15.500±0.500	17.000±1.000
	1000	19.667±0.764*	21.000±1.000*#
Acetone extract	250	0.000±0.000	0.000±0.000
	500	0.000±0.000	0.000±0.000
	750	15.000±1.000	13.000±1.000
	1000	17.000±1.000*	17.333±1.528*
Ethyl acetate extract	250	0.000±0.000	0.000±0.000
	500	0.000±0.000	0.000±0.000
	750	0.000±0.000	0.000±0.000
	1000	12.333±0.577*	11.000±1.000*
Water extract	250	0.000±0.000	0.000±0.000
	500	0.000±0.000	0.000±0.000
	750	0.000±0.000	0.000±0.000
	1000	0.000±0.000*	0.000±0.000*
Gemmo extract	250	14.000±1.000	15.000±1.000
	500	15.333±0.577	18.000±1.000
	750	17.000±1.000	20.000±1.000
	1000	23.000±1.000*	28.000±1.000*#
Standard	500	30.000±2.000	35.000±1.000

All values are mean of Inhibition zone (mm) ±S.D (n=3). * Significant difference between the mean zone of inhibition of extracts ($p<0.05$). # Significant difference between fungal strains ($p<0.05$)

DISCUSSION

In overall results *T. arjuna* and *Euphorbia tirucalli* showed strong broad spectrum antimicrobial activity and inhibited the growth of all tested strains. Gram positive strains (*B. subtilis* & *S. aureus*) seem to be more sensitive to the plant extracts than gram negative. Our findings are in harmony with previous reports (Papadopoulou *et al*., 2005; Brasileiro *et al*., 2006; Khan *et al*., 2009). This difference in sensitivity is associated with difference in cell wall structure of both strains, the less complex structure of gram positive strains make them more permeable for antimicrobial bioactive compounds (Walsh *et al*., 2003).

Gemmo-modified extracts of *T. arjuna* and *E. tirucalli* offered broad spectrum activity which is superior than their natively used parts. These plant extracts showed good potential against gram negative strains. These are significant finding, as gram negative strains are more resistant toward antibiotics. Among the other extracts, more polar extract (methanolic) was found to be more active than other extracts, so it may be concluded that bioactive antimicrobial compounds from these plants are polar in nature and high amount was extracted with methanol like polyphenols. These finding are in agreement with previous reports, that most of the antimicrobial compounds are extracted with methanol (Erdemgil *et al.,* 2004; Parekh *et al.,* 2005; Okusa *et al.,* 2007; Saravanakumar *et al.,* 2009). Acetone, water and ethyl acetate extracts of some plants also showed medium antimicrobial activity. Activity of these extracts may be due to some phytochemicals dissolved in these solvents like alkaloids, saponins, polyphenols and terpenoids. Antibacterial activity of several solvent extracts

has been reported in literature (Raghavendra *et al*., 2008). Polyphenols of selected plants may be responsible for their antimicrobial potential (Scalbert, 1991; Wen *et al*., 2003).

Finding of this study revealed that *E. tirucalli* showed good inhibitory effect against gram positive and gram negative strains like *E. coli.* Antibacterial activity of *E. tirucalli* against gram negative strain is not in agreement with the results of Brasileiro *et al*. (2006), who reported 32 plants species as antimicrobial agents and one of these was *E. tirucalli*, which inhibited growth of gram positive strain and showed no activity against *E. coli*. Our results are in agreement with Parekh *et al.* (2005), who reported the antimicrobial activity of methanolic extract of *E. tirucalli* against gram negative and gram positive strains. Lirio *et al*. (1998) also reported the activity of *E. tirucalli* against some microbial strains. Gemmo-modified extract of *E. tirucalli* showed better activity against the gram negative strains as compared to natively used parts. NO previous study is available about antimicrobial potential of gemmo-modified extract of *E. tirucalli.* Gemmo-modified extract of *E. tirucalli* showed better activity than natively used parts against the gram negative strains.

Terminalia species showed significant potential as antimicrobial agents, many species have been reported for antimicrobial potential like *T. bellerica* (Elizabeth, 2005), *T. chebula* (Kannan *et al*., 2009). Antibacterial activity of bark and leaves extracts of *T. arjuna* has earlier been reported by many scientists (Ramya *et al*., 2008), but no reference was available for gemmo-modified extract of *T. arjuna*, which could be a superior antibiotic source.

Results of antifungal activity showed almost similar trends as antibacterial activity. The results of this study revealed that diameter of the zone of inhibition for fungal strains was less than diameter measured for bacterial strains. Plant extracts showed greater inhibitory effect on bacterial strains as compared to fungal strains. This distinction is due to difference in cell wall structure and protein synthesis of fungal and bacterial strains. These findings are in agreement with the observations of Papadopoulou *et al*. (2005). Gemmo-modified extracts showed higher inhibitory effect on fungal strains. Among the other tested extracts; the methanolic extract demonstrated highest antifungal potential.

In conclusion, gemmo-modified extracts of understudy plants demonstrated broad spectrum antimicrobial activity than their natively used parts.

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