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Antimicrobial properties of tecoma stans stem bark extracts against antibiotic-resistant pathogenic bacteria and toxin producing molds

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ABSTRACT

Tecoma stans stem bark possesses a substantial amount of polyphenolic antimicrobial compounds that can be used for controlling the growth of pathogenic microorganisms. The purpose of this study was to assess antibacterial and antifungal activity of *Tecoma stans* stem bark extracts against antibiotic-resistant pathogenic bacteria and toxin producing molds, respectively. *Tecoma stans* stem bark was subjected to polyphenolic extraction using different solvents viz., water, ethanol, acetone, and methanol. Antibiotic-resistant strains of *Staphylococcus aureus, Enterococcus faecalis*, *Enterobacter aerogenes, Salmonella typhimurium,* and *Escherichia coli* were screened for the antibacterial activity of different grape extracts. Antibacterial activity was analyzed using agar well diffusion method. *Penicilliumchrysogenum*, *Penicilliumexpansum*, *Aspergillusniger* and *Aspergillusversicolor* were screened for the antifungal activity. Antifungal activity was determined by counting nongerminated spores in the presence of peel extracts. As compared to other solvent extracts, methanol extracts possessed high antibacterial and antifungal activity. *S.typhimurium* and *E. coli* showed complete resistance against antibacterial action at screened concentrations of grape peel extracts. Maximum zone of inhibition was found in case of *S.aureus,* i.e., 22 mm followed by *E.faecalis* and *E.aerogenes,* i.e., 18 and 21 mm, respectively, at 1080 mg tannic acid equivalent (TAE)/ml. The maximum and minimum percent of growth inhibition was shown by *P.expansum* and *A.niger* as 73% and 15% at 1080 TAE/ml concentration of grape peel extract, respectively. Except *S.typhimurium* and *E.coli*, growth of all bacterial and mold species were found to be significantly ($P < 0.05$) inhibited by all the solvent extracts.

Keywords: Antibacterial activity, antifungal activity, polyphenolic compounds, *Tecoma stans*, zone of inhibition

INTRODUCTION

Today scientist community is running a race of making drugs and antimicrobial systems for limiting the growth of antibiotic-resistant pathogenic bacterial species and toxin producing molds. In this race, extracts containing polyphenols of plant origin gained more attention of researchers for their use against drug-resistant food borne pathogens.[1] Moreover, antimicrobials or antibiotics from these sources have been found to work more efficiently with fewer side effects and less cost of production.[2],[3]

Tecoma stans (common name yellow bell) also known as yellow trumpet bush belongs o the family bignoniaceae. It is an ornamental plant. It is an erect, branched, sparingly hairy or nearly smooth shrub two to four meters in height. The leaves are opposite, odd-pinnate, Up to 20 centimeters in length with 5 to 7 leaflets. The leaflets are lanceolate to oblong-lanceolate, 6 to 13 centimeters long, pointed at both ends and toothed on the margins. Trumpet shaped flowers are yellow faintly scented and borne in short, dense, terminal clusters. The calys is green. 5 to 7 millimeters long and 5 toothed. Flowering can begin as early as April and continue in to fall. The flowers are followed by 6 inch long, tan pods that are filled with small, papery winged seeds.4

As an antimicrobial agent, these polyphenols can penetrate the semi permeable cell membrane where they react with the cytoplasm or cellular proteins. This potential is higher in grape peel extract as phenolic acids are present in un-dissociated form.[5] Therefore, these highly negative charged antimicrobial polyphenolic compounds can be used for combating the growth of antibiotic resistant pathogenic bacteria and toxin producing molds. From the extraction point of view, different kind of solvents can be used as the solubility of polyphenols depends on the aqueous and nonaqueous medium.[6-8]

The objective of this in vitro study was to screen antimicrobial activity of different *Tecoma stans* stem bark extracts against antibiotic resistant bacterial species and toxin producing molds. Antibacterial activity was assessed against Staphylococcus aureus, Enterococcus faecalis, Enterobacter aerogenes, Salmonella typhimurium and Escherichia coli. These bacteria are well known for food borne pathogenesis. Their growth may cause the death of the patient. Antifungal activity was analyzed against Penicillium chrysogenum, Penicillium expansum, Aspergillus niger and Aspergillus versicolor. The molds used in this investigation are known for their mycotoxins production and toxic nature. A. niger, A. versicolor, P. expansum (blue mold) and P. chrysogenum produces ochratoxin, sterigmatocystin, patulin and citrinin, respectively. Ochratoxin and citrinin are nephrotoxic; sterigmatocystin is mutagenic and tumigenic while patulin is genotoxic in nature.[9]

MATERIALS AND METHODS

Plant extraction

The stem bark of *Tecoma stans* were collected in the month of May 2015 from Rasipuram (Namakkal District) Tamil Nadu. A herbarium specimen of the plant was deposited in the Department of Pharmacognosy. The plant was identified by Dr.G.V.S.Murthy, Joint Director of the Botanical Survey of India, Southern circle, All the chemicals used were procured from Himedia and Merck (Mumbai, India).

Microbial Cultures

Amoxycillin, cloxacillin, and vancomycin-resistant S. aureus; amoxycillin and ampicillin resistant E. aerogenes; vancomycin and gentamycin resistant E. faecalis; ciprofloxacin resistant S. typhimurium; cloxacillin and gentamycin resistant E. coli (collected from human infections) were provided by Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India). Before use, the antibiotic resistant nature was further verified by us. P. chrysogenum, P. expansum, A. niger and A. versicolor were provided by Department of Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (India).

Preparation of Extracts

Stem barks were washed thoroughly with water to remove dirt particles and mixed with different solvents for the solvent extraction in the ratio 1:10 (grapes: Volume of solvent). Thereafter, the mixture was placed in a shaker incubator at 50°C temperature and 150 rpm for 12 h. Each extract was then filtered with whatman paper number 1 to separate polyphenols of palnt in solvent. This process was repeated up to 3 times to ensure complete migration of polyphenolic compounds to solvent, which was indicated by the color of marc. Every extract was vacuum oven dried at 50°C for the assessment of total phenolic content, antibacterial and antifungal activity.

Total Phenolic Content Determination

The total phenolic content of the extracts was determined by the Folin–Ciocalteau method with some modifications.[10] 5 g/50 ml of sample was filtered with whatman number 1 paper. To 0.5 ml of the sample, 2.5 ml of 0.2 *N* Folin–Ciocalteau reagent was added and kept at room temperature for 5 min. After that, 2 ml of sodium carbonate (75 g/l) was added to reaction mixture and the total volume was made up to 25 ml using distilled water. The solution was then kept for incubation at room temperature for 2 h. Absorbance was measured at 760 nm using 1 cm cuvette in ultraviolet - 1800 spectrophotometer (Shimadzu, Kyoto, Japan). All procedures were performed with three replicates. Tannic acid (0.1–0.5 mg/ml) was used to produce a standard calibration curve. The correlation equation constructed with tannic acid was y = 1.633X (R^2 = 0.985). Total phenolic content was expressed as milligrams of tannic acid equivalents per gram of dry extract (mg TAE/g).

Antibacterial Activity Determination

S. *aureus, E. aerogenes, E. faecalis S. typhimurium* and *E. coli* were cultured in Mueller hinton (MH) broth at 37°C. The agar well diffusion method as described by Uhlman *et al*.[11] was used for screening the antagonistic activity of the extracts against different pathogenic microbes. MH agar (38 g/l) plates were inoculated with 10^6 colony forming units per ml of overnight cultures of the corresponding indicator bacterial strain. Wells were done on agar with the back of a sterile pasteur pipette and 10 µl of each extract containing 260 mg TAE/ml (A_1), 540 mg TAE/ml (A_2) and 1080 mg TAE/ml (A_3) of extract were inoculated in each well. After diffusion, plates were incubated at 37°C for 24 h. Bacterial growth was inhibited by different extracts leads to the formation of inhibition zones. Antimicrobial activity was evaluated by measuring the diameter of inhibition zones with no bacterial growth in mm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration where no viability was observed after 24 h on the basis of zones of growth. All the determinations were conducted in triplicates.

Antifungal Activity

Antifungal activity determined as a percent of inhibition of conidia germination. The effect of extracts on the conidia germination was carried out according to the method described by Droby et al.[12]*P. chrysogenum* NCIM 709, P. expansum MTCC 2006, A. niger NCIM 596 and A. versicolor NCIM 698 were stored on potato dextrose agar (PDA) slants at 4°C and grown on PDA plates for 1 week at 25°C. From 2 weeks old cultures grown on PDA, was used to prepare spore suspensions. Conidia were removed from the surface of the cultures with a sterile bacteriological loop in 5 ml of sterile distilled water. Thereafter, suspensions were filtered through four layered muslin cloth to remove fungal mycelia. Spore concentration was estimated with a hemacytometer and the concentration was adjusted to 6×10^6 spores/ml. Aliquots of 100 µl of spore suspension of indicator fungal species were added to the wells of tissue culture plates containing 900 µl of PDA containing 260 mg TAE/ml, 540 mg TAE/ml and 1080 mg TAE/ml of extract. Thirty µl aliquots were placed on sterile microscope slides and incubated at 30°C for 24 h under dark conditions in sterile petri dishes lined with moist filter paper. After that, germinated and nongerminated spores were visualized under a microscope and represented as an antifungal activity. All treatments consisted of three replicates and experiments were repeated 3 times.

Antifungal activity (percent of inhibition of growth) = Spores not germinated/number of spores present in control × 100.

STATISTICAL ANALYSIS

Statistical significance was tested by employing one-way and two-way analysis of variance and comparison between means was made by least significant difference pair-wise comparison with the help of Microsoft excel and Systat software.

RESULTS

Total Phenolic Content

It can be clearly seen from [Figure 1] that acetone extract exhibited high phenolic content, i.e., 62.44 mg/g of dry sample. All the finding were statistically justified at $P < 0.05$ and found significantly different. The ability to extract polyphenols was as follows:

Acetone extracts > Methanol extracts > Ethanol extracts > Water extracts

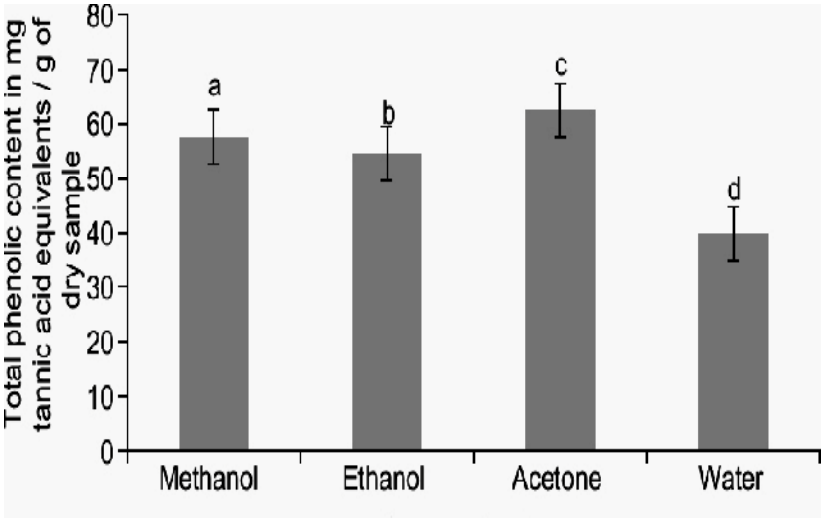

Figure 1: Total phenolic content of extracts. Each value is mean ± standard error of mean of triplicate samples and bars with different letters are significantly different ($P < 0.05$)

Antibacterial Activity

Antibacterial activity of different extracts is represented in [Table 1]. Methanol and acetone extracts showed MIC of 260 mg TAE/ml polyphenols against S. aureus, E. aerogenes and E. faecalis. Ethanol extracts followed the same pattern of MIC at 260 mg TAE/ml except against E. faecalis, which showed MIC of 540 mg TAE/ml. Whereas, water extracts showed the MIC 540 mg TAE/ml against S. aureus, E. aerogenes, and E. faecalis. Data from [Table 1] clearly indicates that *S. typhimurium* and *E. coli* was found completely resistant against the antibacterial action of extracts screened at all concentrations.

Table 1: Antibacterial activities of different *Tecoma stans* extracts

Groups	S. aureus			E. aerogenes			E. faecalis			*S. typhimurium*			*E. coli*		
Methanol	0.9±0.71^{Aa}	19±1.21^{Ab}	22±1.31^{Ac}	09±1.2^{Ad}	16±0.6^{Ae}	21±0.7^{Af}	07±1.8^{Ag}	11±1.6^{Ah}	18±1.5^{Ai}	ND	ND	ND	ND	ND	ND
Ethanol	0.7±0.51^{Ba}	10±0.81^{Bb}	15±0.90^{Ba}	06±0.8^{Bd}	9±0.8^{Be}	12±1.2^{Bf}	ND	8±1.2^{Be}	10±1.3^{Bh}	ND	ND	ND	ND	ND	ND
Aceone	0.8±0.51^{Cc}	19±1.01^{Ab}	21±1.01^{Ac}	08±0.5^{Cg}	13±0.5^{Cd}	19±1.4^{Cb}	07±0.9^{Ag}	10±1.4^{Ce}	15±0.3^{Cf}	ND	ND	ND	ND	ND	ND
Water	ND	09±0.40^{Da}	12±0.91^{Db}	ND	7±0.6^{Dc}	10±1.1^{Da}	ND	7±0.3^{Dc}	9±0.8^{Da}	ND	ND	ND	ND	ND	ND

[a]All the digits in the table shows inhibition zones in mm of different extracts as A1 is 260mg TAE/ml, A2 is 540mg TAE/ml and A3 is1080mg TAE/ml of extract. [b] Each value is the mean±SD of experiments performed in triplicate. [c]mean±SD in the same column with different capital letters superscript indicating significant difference at P<0.05,. [d] mean±SD in the same row with different small letter superscripts indicating significant difference at p<0.05. [e]ND= Not detected, SD= Standard deviation. TAE= Tannic acid equivalents.

Antifungal Activity

The extracts at a concentration of is 260, 540 and 1080 mg TAE/ml were screened against the growth of different molds; *P. chrysogenum, P. expansum, A. niger* and *A. versicolor*. The trend of mold growth inhibition observed was as follows [Figure 2]:

Methanol extracts > Acetone extracts > Ethanol extracts > Water extracts

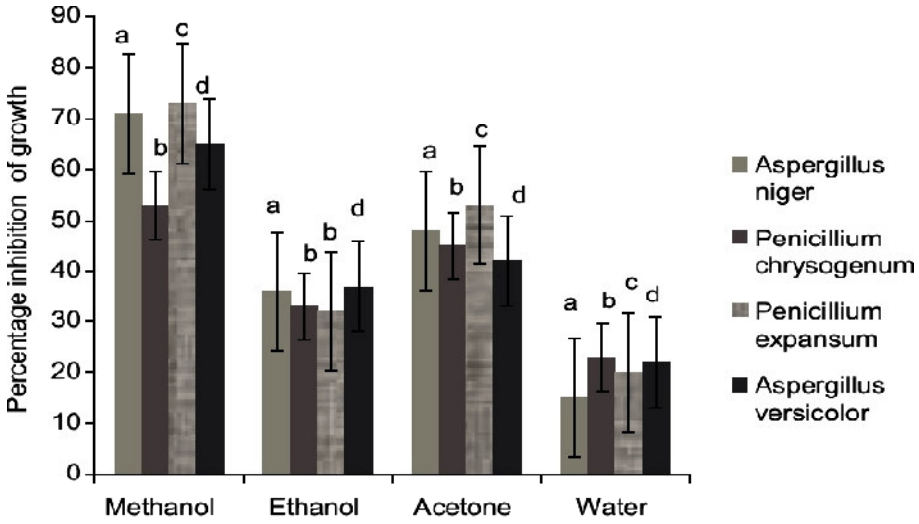

Figure 2: Growth inhibition of different molds by solvent extracts (1080 mg tannic acid equivalent/ml). Each value is mean ± standard error of mean of triplicate samples and within the group, bars with different letters are significantly different ($P < 0.05$).

The results were found significantly ($P < 0.05$) different among the fungistatic effects of *Tecoma stans* in all four solvents. As the concentrations of extracts were increasing, the percentage of inhibition was increasing. The MIC of all extracts (except ethanol and water extracts against A. niger) was found at 260 TAE/ml. On the other hand, MIC of ethanol and water extracts was noticed at 540 TAE/ml. P. expansum showed maximum (73%) and A. niger exhibited least percent of growth inhibition (15%) on antibacterial action of methanol and water extract (at 1080 TAE/ml), respectively.

DISCUSSION

Total Phenolic Content

Tecoma stans contains anthocyanins, which is present in a huge amount as compared to other polyphenolic compounds, due to the presence of the anthocyanins.[13] Thus, the solvent extract which possess higher polyphenolic contents will have maximum extractability of anthocyanins. In the present study, acetone was proved to be a better solvent for the extraction polyphenols from different plant exract fractions, showing resemblance with Cheng *et al*.[8] who found that acetone was more efficient than methanol and ethanol for the extraction of polyphenols from *Tecoma stans*. Although, Oki *et al*.[7] reported methanol and 70% acetone as better solvents for catechin and procyanidins extraction, respectively. Thus, found 3 times higher value of total soluble solids and polyphenols on the extraction of red-hulled rice using methanol rather than water. However, Arts and Hollman [6] obtained maximum catechin yields in case of both acetone and methanol (On the other hand, methanol is more acceptable to work with). They also found that the extraction process is greatly influenced by type and concentration of the solvent. Variations were justified by the well-known tendency of phenols to combine themselves through polymerization reactions during oxidation.[12]

Antibacterial Activity

Tecoma stans contains a higher amount of dimers and trimers of (-) epicatechin which possess a higher antimicrobial activity than monomer ones. Scalbert [12] proposed that the antibacterial activity of tannins could be due to the inhibition of extracellular microbial enzymes, can be a reason for

antibacterial action of plant. Moreover, the complexation of metal ions from the bacterial growth environment could also be a possible mechanism for their antimicrobial properties. Almost similar results were obtained by Nirmala and Narendhirakannan [14] found that the inhibition zone of ethanolic extract against S. *aureus* and *E. faecalis* was 7 mm and 5.9 mm at 250 mg/ml. The maximum inhibition zone was observed in methanolic extracts than acetone, ethanolic and water extracts, infers that antimicrobial compounds from plant are more soluble in methanol, showed resemblance with Cheng *et al*.[8] The present study was found complementary to those reported by Baydar *et al*.[15] and Ozkan *et al*.[16] in which they showed that different mixture of solvents containing polyphenols from *Tecoma stans*, significantly inhibited the growth of *E. aerogenes, E. faecalis* and *S. aureus*. Our study was also found in agreement with Papadopoulou *et al*.[17] demonstrated that *S. aureus* was most sensitive against antibacterial action of wine extracts and showed 25 mm inhibition zone at 21,200 mg gallic acid equivalent/liter. Our findings revealed that *S. typhimurium* and *E. coli* were resistant at all concentrations of polyphenols. *S. typhimurium* and *E. coli* are Gram-negative bacteria. It may be possible that the lipo-polysacharidic wall of Gram-negative bacteria represented a great barrier for extracted polyphenols to get into the cytoplasm, hence no inhibition was achieved. Similarly, Nirmala and Narendhirakannan [14] Ozkan *etal*.[16] and Papadopoulou *et al*.[17] found that *S. typhimurium* and *E. coli* were quite resistant against antimicrobial action of *Tecoma stans* stem bark extracts.

Antifungal Activity

Tecoma stans contains significant amount of catechins and epicatechins.[3] Catechins and other polyphenols are highly negative charged phytochemicals, which can be related to antifungal activity.[11],[18] Even though, acetone extract was having great amount of polyphenols, but it showed less antifungal activity as compared to methanol extract. Solvent differential solubility of antifungal compounds leads to variation in the percentage of growth inhibition. Some growth retarding effect has been attributed to isolated phenolic acids such as coumaric acid, caffeic acid, ferulic acid, and sinapic acid as well as to isolated flavonoids such as (+)-catechin, kaempferol and quercetin.[11],[9] Hence, the presence and solubility of some of these compounds in extracts is likely to be responsible for the growth inhibitory effect of different extracts.

CONCLUSIONS

So far, acetone was very active in extracting polyphenolic compounds but it was not effective as methanol in extracting antibacterial and antifungal compounds from *Tecoma stans*. Except *S. typhimurium* and *E.coli*, growth of all bacterial and mold species were found to be greatly inhibited by all the solvent extracts.

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