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Antifungal and haemolytic activities of organic extracts of *Tecoma stans* (Bignoniaceae)

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Abstract

The present study was undertaken to examine the antifungal and haemolytic activities with selected herbal preparations. The antiyeast and antifungal activities were tested by the drop diffusion method. There were nine different plant species investigated in the present study. Amongst, *Tecoma stans* found to give the best zone of inhibition against the fungal activity. The extract of *Tecoma stans* with chloroform produced the 6 different colours of bands in TLC plate. The Rf values of different colour bands were also calculated. Results showed that *Tecoma stans* did not lyse the RBC of A,B,O blood groups.

1. Introduction

Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro organisms, animals and plants. One of such resources is folk medicine. Systematic screening of them may result in the discovery of novel effective compounds. *Arnica* and *Propolis* have been used for thousands of years in folk medicine for several purposes. They possess several biological activities such as anti inflammatory, antifungal, antiviral and tissue regenerative, among others. Although the antibacterial activity of *Propolis* has already been demonstrated very few studies have been done on bacteria of clinical relevance in dentistry. Also, the antimicrobial activity of Arnica has not been extensively investigated by Koo *et al.* (2000).

The aqueous and methanolic extract of *Thonningia sanguinea* root, as well as seven fractions obtained by TLC from the methanol extract, has been shown to posses varying degrees of antimiocrobial activity (Ohiri and Uzodinma, 2000). Phongpaichit *et al.* (2005) studied 36 extracts derived from 10 plant species were selected to screen for their antifungal activity against clinical isolates of *Candida albicans, Cryptococcus neoformans* and *Microsporum gypseum*. Selected was based on their use by traditional Thai healers or their reported antimicrobial activities in an attempt to find bioactive medicines for use in the treatment of opportunistic fungal infections in AIDS patients The disc diffusion and hyphal extension inhibition assays were primairily used to test for inhibition of growth. Minimum inhibitory concentration was determined by dilution methods. The chloroform extracts of *Alpinia galangal* and *Boesenbergia pandurata* had pronounced antifungal activity against *C.neoformans* and *M. gypseum*, but exhibited weak activity against *C. albicans*. Alpinia galangal and *B. pandurata* are excellent candidates for the development of a remedy for opportunistic fungal infections in AIDS patients.

In recent years, antifungal properties of Indian medicinal plants had been increasingly reported. However, a majority of traditionally used Indian medicinal plants have not yet to be systematically screened against various microbial pathogens (Alma *et al.*, 2003). The present study was undertaken to examine the antifungal and haemolytic activities with selected herbal preparations.

2. Materials and Methods

2.1. Preparation of test samples

5g of each powered sample was successively extracted with 15ml of petroleum ether, chloroform and 70% ethanol for overnight extraction. The extracts were dried and dissolved in corresponding solvents and tested for antifungal activities. DMSO (di methyl sulfhonyl oxidant) fractions of the herbal preparations were extracted by using solvents (chloroform, 70%ehanol, dichloro methane).

2.2. Source of microorganisms

To study the antimicrobial activities of selected herbal preparations, the strains of yeast and moulds were collected from microbiology laboratory at PRIST University, Thanjavur. The selected microorganisms such as *Candida albicans* (yeast), *Cryptococcus neoformans* and *Microsporum gypseum* (moulds). The clinical isolates were collected from different diseases infected patients.

2.3. In vitro experiments for antifungal activities

2.3.1. Drop diffusion method

The antiyeast and antifungal activities were tested by the drop diffusion method. The medium used for evaluating antiyeast and antifungal activities was Sabourauds dextrose agar (SDA). Control studies with ketoconazole 0.01gm/ml and the solvent were done concurrently. The plates were incubated at 28° C for 24 hours.

2.3.2. Minimum Inhibitory Concentration method

The antiyeast and antifungal activities were tested using serial dilution method as mentioned for evaluating antifungal activity. The Sabourauds dextrose broth was used for evaluating antiyeast and antifungal activities and the plates were incubated at 25° C for overnight. A positive control containing each organism with sterile medium was also maintained.

2.3.3. Thin Layer Chromatography

Thin layer chromatography (TLC) method was used for identified substances and purifies the compounds. TLC method was relatively quick and requires small quantities of material. Separation in TLC involves distribution of mixure of two or more substances between a stationary phase and a mobile phase. The stationary phase was a thin layer of adsorbent (usually silica gel or alumina) carried the samples with it. Components of the samples would separate based on the mobile phase of the samples from the stationary phase. Each components of the mobility would different from others.

2.3.4. Preparation method for Chamber

The jar with tightly fitted lid adds enough amount of dichloro methane. So that it was 0.5 to 1cm deep in the bottom of the jar. Next, place a piece of filter paper into the jar so that it lines the walls and was immersed in the liquid. Close the jar tightly, and led it stand for about 30mints so that the atmosphere in the jar becomes saturated with solvent.

2.3.5. Plate preparation

With a pencil, etch two small notches into the adsorbent about 2cm from the bottom of the plate. The notches should be on the etches of the plate, and each notch should be the same distance from the bottom of the plate. The notches must be farther from the bottom of the plate than the depth of the solvent in the jar used a drawn out capillary tube, spot the samples on the plate so that they line up with the notches etched. After prepared the development chamber and spotted the samples, the plates were ready for development. Handle the plates only by the edges, and try to leave the development chamber uncovered for as little time as possible carefully when the plates were removed from the chamber. The solvent was quickly traced with a pencil.

2.3.6. Spot identification

If the spots were present, they were outlined with a pencil. If no spots were obvious, the most common visualization technique is to hold the plate under a UV lamp. Many organic compounds could be seen using this technique, and many often contain a substance which aids in the visualization of compounds.

2.3.7. Data Interpretation

The Rf value for each spot should be calculated. Rf stand for "ratio of fronts". Rf values can be compared to those of unknown substances to aid in their identifications

$$Rf = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$

2.3.8. Haemolytic Test

Human blood of A, B, O group was collected from volounteers in tubes containing heparin anticoagulant. Then, it was centrifuged at 3,000 rpm for 3 minutes and the hRBCs were collcted. The cells were washed with PBS solution repeated until the supernatant was colourless. Again the erythrocytes were resuspended with PBS (pH-7.0) solution to make 4% cells. The haemolytic assay was carried out in a micro well plate. The wells were filled with 100µl of PBS in each. Then in the appropriate rows 100µl of 4% hBCs were added in all the walls. The peptide solutions (10%TCA,PBS filtrate,30% ammonium sulphate extractions) were serially diluted from 100µg to 3µg in the appropriate wells. The hRBCs alone and 4% hRBCs in 1% Triton 100X were used as 0%(-ve) and (+ve) haemolytic controls respectively. After 1 hour incubation, the wells were observed visually for button formation.

3. Result

In the present study, the anti fungal activity of *Tecoma stans* against the fungal species (*Candida albicans, Cryptococcus meoformans, Microsporum gypseum*) were carried out.

3.1. Plants screened by drop diffusion method

There were nine different plant species investigated in the present study. *Osimum santum* screened against the selected three fungal species (*Candida albicans, Cryptococcus meoformans, Microsproum gypseum*) followed by *Solanum trilobactum*, *Tabebuia impetiginosa*, *Parthenum argentatum*, *Lippia gracilis*, *Xylopia sericea*, *Arthemus sativa*, *Mikania triangularis* and *Tecoma stans*. Among the nine plants screened in the present investigation, *Tecoma stans* (Table 1) found to give the best zone of inhibition against the fungal activity.

Table 1. Screening of *Tecoma stans* against different micro organisms by Drop Diffusion method

Micro organisms	*Candida albicans*		*Cryptococcus neoformans*		*Microsporum gypseum*	
PE(S)	G	G	G	G	G	G
CF(S)	2.5	2.8	2.5	2.6	G	G
70%E(S)	G	G	3	2.5	G	G
PE	G	G	G	G	G	G
CF	G	G	G	G	G	G
70%E	G	G	G	G	G	G
K(PE)	1.3	1.1	1.5	1.2	1.4	1.2
K(CF)	2.6	2.5	1.8	2	2	2.5
K(70%E)	2.6	2.6	3.2	3.2	2	1.8

PE-Petroleum ether; CF- Chloroform; E- Ethanol; K- Ketoconazole; G-Growth (cm)

3.2. Thin Layer Chromatography

The *Tecoma stans* extract with chloroform produced the 6 different colours of bands in TLC plate (Fig. 1). The different colour bands of Rf values present in Table 2.

3.3. Bands screened by drop diffusion method

The growth and non growth of three different organisms were tested by using the different bands of *Tecoma stans* through the drop diffusion method (Table 3). The zone of inhibition of *M. gypseum* treated with bands 3&4 of *Tecoma stans* in drop diffusion method (Fig. 2). The zone of inhibition of *M. gypseum* treated with bands 5&6 of *Tecoma stans* in drop diffusion method (Fig. 3).

3.4. Bands screened by MIC method

The different bands of *Tecoma stans* with *C. neoformans* and *M.gypseum* are presented in Table 4 & Table 5 respectively in MIC method. The zone of inhibition of *M. gypseum* treated with different bands of *Tecoma stans* in MIC method (Fig. 4).

3.5. Bands Tested by Haemolytic Assay

Tecoma stans did not lyse RBC of A,B,O blood groups (Table 6).

Table 2. Different bands produced by chloroform extract of *Tecoma stans* in Thin Layer Chromatography

Bands	Colour	Rf value (cm)
Band 1	Green	0.3
Band 2	Yellow	1.1
Band 3	Ash	2
Band 4	Light Ash	2.9
Band 5	Pink	7
Band 6	Orange	11.5

Table 3. Different micro organisms tested by using the bands of *Tecoma stans* in Drop Diffusion method

Bands	*Candida albicans*		*Cryptococcus neoformans*		*Microsporum gypseum*	
Band 1	G	G	G	G	G	G
Band 2	G	G	2.7	2.2	2.1	2.4
Band 3	G	G	2.5	2.3	2.5	2.5
Band 4	G	G	2.6	2.2	2.7	2.8
Band 5	G	G	1.9	2.1	2.3	2.5
Band 6	G	G	2.2	2.5	2.7	2.5

G – Growth (cm)

Table 4. Different Bands of *Tecoma stans* treated with *Cryptococcus neoformans* in Minimum Inhibitory Concentration

Bands	10mg		1mg		0.1mg		0.01mg	
Band 1	G	G	G	G	G	G	G	G
Band 2	G	G	G	G	G	G	G	G
Band 3	G	G	G	G	G	G	G	G
Band 4	1.0	1.2	G	G	G	G	G	G
Band 5	G	G	G	G	G	G	G	G
Band 6	2.9	2.7	G	G	G	G	G	G

G – Growth (cm)

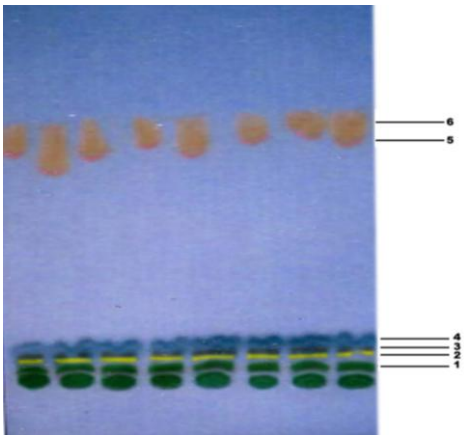
Table 5. Different Bands of *Tecoma stans* treated with *Microsporum gypseum* in Minimum Inhibitory Concentration

Bands	10mg		1mg		0.1mg		0.01mg	
Band 1	G	G	G	G	G	G	G	G
Band 2	G	G	G	G	G	G	G	G
Band 3	G	G	G	G	G	G	G	G
Band 4	G	G	G	G	G	G	G	G
Band 5	G	G	G	G	G	G	G	G
Band 6	2.4	2.6	G	G	G	G	G	G

G – Growth (cm)

Table 6. Different concentrations of *Tecoma stans* extract tested against different blood groups by heamolytic test

hRBCs of Different Blood Groups	Concentrations							
	100µg	50µg	25µg	12µg	6µg	3µg	(-) ve control	(+)ve control
A	+	+	+	+	+	+	+	-
B	+	+	+	+	+	+	+	-
O	+	+	+	+	+	+	+	-



Band 1 – Green; Band 2 – Yellow; Band 3 – Ash; Band 4 - Light Ash; Band 5 – Pink; Band 6 - Orange

Fig. 1. Bands separation of *Tecoma stans* in TLC

Fig. 2. The zone of inhibition of *Microsporum gypseum* treated with and 3&4 of *Tecoma stans* in drop diffusion method

Fig. 3. The zone of inhibition of *Microsporum gypseum* treated with band 5&6 of *Tecoma stans* in drop diffusion method

Fig. 4. The zone of inhibition of *Microsporum gypseum* treated with band 6 of *Tecoma stans* in MIC method.

4. Discussion

The plant *Tecoma stans* differ significantly in their activities against the microorganisms tested and most of the extracts showed antifungal activity against *Candida albicans, Cryptococcus neoformans* and *Microsporum gypseum.* Oh *et al.* (2001) reported that the antifungal activity of *Belamcanda chinensis* was evaluated by a single cell bioassay method. An active fraction was separated by silica gel column chromatography and reverse phase HPLC. The isolated compound was found to be identical to tectorigenin (5, 7-dihydroxy-3-(4-hydroxy phenyl1)-6-methoxy-4H-1-benzopyran-4-one) which has formerly appeared in the literature without any remarks on its antimicrobial activity. Antimicrobial activity was investigated against 17strains of fungi and 6 strains of bacteria. This compound showed marked antifungal activity against dermatophytes of the genera Trichophyton, the minimum inhibitory concentration (MIC) being in the range of 3.12-6.25 mg/ml.

Ethanol and aqueous extracts of 20 Palestinian plant species used in folk medicine were investigated by Ali Shtayeh *et al.* (1998). Their antimicrobial activities against five bacterial species (*Staphylococcus aures, Escherichia coli, Klebsiella pnuemoniae, Proteus vulgaris, Pseudomonas aeruginosa*) and one yeast (*Candida albicans*). The plants showed 90% of antimicrobial activity with significant difference in activity between the different plants. The most antimicrobial active plants were *Phagnalon rupestre* and *Micromeria nervosa*, whereas, the least active plant was *Ziziphus spinachristi*. Only ten of the tested plant extracts were active against *Candida albicans* with the most active from *Migosporum nervosa* and *Inula viscosa* and the least active from *Ruscus aculeatus*. In the present study, petroleum ether, 70% ethanol and chloroform extract from the leaves of *Tecoma stans* showed antifungal activities. However, little activity was observed from the 70% ethanol. Generally, petroleum ether and chloroform showed broad spectra of activity against the tested organisms. The activities of chloroform extract were found to be higher on test fungi. It is of interest to note that, the growth of *Candida albicans, Cryptococcus neoformans* and *Microsporum gypseum* were inhibited by the extracts at the tested concentration.

The methanolic crude and methanol-aqueous extract of *Alstonia macrophylla* leaves and n-butanol part of the crude extract showed antimicrobial activity against various strains of *Staphylococcus aureus,*

Staphylococcus saprophyticus, Streptococcus faecalis, Escherichia coli,Proteus mirabilis, Trichophyton rubrum, Trichophyton mentagrophytes var. *mentagrophytes* and *Microsporum gypseum*. The Minimum Inhibitory Concentrations (MIC) values ranges from 64-1000 microg/ml for bacteria and 32-128 mg/ml for dermatophytes. However the strains of *Pseudomonas aeruginosa, Klebsiella* sp. and *Vibrio cholarae* showed resistance against in vitro treatment of the extracts up to 2000 mg/ml concentration, while the two yeast species were resistant even at 128 mg/ml concentration. The stem bark extract prepared similarly was found to be less active compared to the leaves. Phytochemical study indicates that the crude extract contains tannins, flavonoids, saponins, sterols, triterpene and reducing sugars. Further fractionation and purifications of n-butanol part of the extract showed the presence of beta-sitosterol, ursolic acid, beta sitosterol glucoside and a mixture of minor compounds only detected in TLC (Chattopadhyay *et al* ., 2001).

The methanolic extract of the roots of *C. majus* revealed a high resistance to *Fusarium* (Matos *et al*., 1999). Several flavonoids and phenolic acids were isolated from the aerial parts that exhibit interesting antiviral and antimicrobial properties both *in vitro* and *in vivo*. A glycoprotein was isolated from *C. majus* exhibits good antibacterial activity against methicillin resistant *staphylococci* and multiresistant *enterococci* (Fik *et al*., 1997). According to the liquid dilution screened method for antifungal activity of higher plants reported by Van Den Berghe and Vlietinck (1991), a prominent antibacterial effect, worthy of further investigation, was obtained if not only the ½, but also the 1/8 and 1/32 dilutions show inhibitory activities. An inhibition shown for the ½ dilution only was less promising for further investigation.

Plants containing pyrrolizidine alkaloids (*T. farfara*) could be toxic for man or livestock. The hepatotoxic potential of conventional drugs was well known while herbal medicines are often assumed to be harmless. *C. majus* was frequently prescribed to treated gastric and biliary disorders but it might be the cause of cholestatic hepatitis (Benninger *et al.,* 1999).

Feresin *et al.* (2001) reported that the eighteen extracts from *Acaena magellanica, Baccharis grisebachii, Ephedra breana, Oxalis erythrorhiza, Pachylaena atriplicifolia,* and *Satureja parvifolia* are assessed for antimicrobial activity against bacteria and fungi with the agar dilution method. In the present study, the leaves of *Tecoma stans* had highest antifungal activity. All the extracts showed various degrees of antifungal activity on the microorganisms tested in the present study. The chance to find antifungal activity was more apparent in ethanol than water extracts of the same plants. *Tecoma stans* did not lyse the RBC of A,B,O blood groups. Hence these plant extracts could be used as new antibiotic compounds against the anti fungal activity.

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