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In vitro evaluation of phytochemical components and antimicrobial activity of the methanolic extract of *Tridax procumbens* L. against pathogenic microorganisms

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Abstract
Tridax procumbens Linn. is renowned for its traditional use as ayurveda in folk medicine all over the world. In this study, the plant was screened to ascertain its bioactive compounds and to determine the antimicrobial activity against different pathogenic microorganisms. Methanolic extract of the leaves of *T. procumbens* L. was examined at different concentrations on both bacteria and fungi by disc diffusion method and agar well diffusion method. Both gram positive and negative bacteria including *E. coli, V. cholera, B. subtilis, B. cereus* and *S. aureus* showed sensitivity to 250 µg/ml and 500 µg/ml plant extract except *Salmonella typhi* was resistant to both concentrations. Their zone of inhibition was within 8-14mm range in diameter for both concentrations. Several fungal species including *C. albicans*, *A. niger* and *A. fumigatus* were also tested for its susceptibility to methanol extract and showed significant value within the range of 9-15mm zone of inhibition with great potency against *C. albicans*. MIC and MFC was also determined at different concentrations to evaluate the efficacy of methanol extract. The findings of current study suggested that *T. procumbens* L. can be used as a potential source of alternative drugs in present days to combat the drug resistance phenomenon of different microorganisms.

Keywords: Antimicrobial activity, methanol extract, bacteria, fungi, MIC, MFC

1. Introduction
Nature is enriched with uncountable number of medicinal resources. Long before the existence of medical facilities, plant extracts were being used for human well-being to prevent themselves from various chronic and infectious diseases [1]. World health organization mentioned that about 80% of world population inextricably relies on traditional herbal medicine for their incipient healthcare settings [2, 3]. The new era of drug development process is searching for some natural compounds which have great medicinal value with zero side effects. Due to frequent use of synthetic drugs most of the bacteria are getting resistant to current drugs and making a potential future threat [4]. Currently more than 30 percent of plant materials are using as a source of modern drug [3].
Tridax procumbens L. is a common weed (12-24cm in length) found all over the world commonly known as Bhringraj in India and Coat buttons in English. The plant had its paradoxical use against malaria, hemorrhage, diarrhea, stomach ache, parasitic infection, liver disorder and inflammation [5-11]. Although, the plant is well studied but the current study was conducted repeatedly as geographical regions and environment may part critical role in plant growth and the structure of their phytochemicals. Inspiring from previous studies on in-vitro antibacterial and antifungal efficacy of different medicinal plants, the present study was designed with the methanolic extract of *T. procumbens* L to determine its potentiality against different pathogenic microorganisms [12-14]. In this study different phytochemical ingredients were screened to observe its chemical nature. In addition, antibacterial and antifungal study was obtained against *Escherichia coli, Vibrio cholera, Salmonella typhi, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Candida albicans, Aspergillus niger* and *Aspergillus fumigatus.* by disc diffusion, agar well diffusion, MIC and MFC methods and compared with commercially available antibiotic discs.

2. Methods and Materials
2.1 Collection of plant material
Tridax procumbens L. were selected and collected from Jessore University of Science and Technology (JUST), Bangladesh premises. The herb was identified and authenticated by Department of Botany, University of Rajshahi. Leaves, flowers and stems were separated and washed properly.

Further shade dried and grinded the leaves and flowers by mechanical grinder to form coarse powder. After grinding the powdered material was stored in an air tight container in cool, dry and dark place.

2.2 Extraction of leaf extract
150gm Dried powder leaf materials were taken in a 1000ml sterile Erlenmeyer flask and soaked with 400ml methanol properly. Then the mixture was accompanied with regular shaking and stirring for subsequent incubation period. After 10 days the leaf extract was separated from debris materials by filtration with sterile cotton plug. The residue was again soaked in 250 ml methanol for four days and filtrated for remaining extraction. The filtrate was then subjected to evaporation by using rotary evaporator. Then the crude extract was stored in an air tight container at 4 ^{0}C. The total yield of the crude methanol extract was 2.97% of dried plant materials.

2.3 Phytochemical analysis of plant materials
Standard protocols were used for the qualitative screening of phytochemical ingredients of methanol and ethanol extract. The presence of chemicals including Alkaloid, Flavonoid, Glycocides, Protein, Saponin, Steroid, Terpenoids and Tannin were tested according to standard method and confirmed [15].

2.4 Preparation of bacterial culture
Six bacterial strains (three gram positive and three gram negative) including *Escherichia coli, Vibrio cholerae, Salmonella typhi, Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* were obtained from stock culture in Department of Microbiology, Jessore University of Science and technology, Bangladesh. At first bacterial isolates were revived in nutrient broth (OXOID, UK) at 37 ^{0}C for 24 hours, then transferred into nutrient agar medium(OXOID, UK) and incubated at 37 ^{0}C for 24 hours.

2.5 Preparation of fungal culture
Three pathogenically important fungus including *Aspergillus flavus, Aspergillus fumigatus* and *Candida albicans* were taken from stock culture in Department of Microbiology, Jessore University of Science and Technology. They were first revived in potato dextrose broth (OXOID, UK) at 28 ^{0}C for 3 days and further transferred in potato dextrose agar (OXOID, UK) and incubated at 28 ^{0}C for 7 days. The fungal spores from mother culture was collected using sterile distilled water. 1ml spore solution was added in 9ml distilled water and serially diluted from 10^{-1} to 10^{-6}. Testing plate was prepated form 10^{-4} dilution.

2.6 Preparation of disc for antimicrobial assay
For determining antimicrobial activity of methanol extract of *Tridax procumbens* L, commercially available blank discs (6mm) were used. Discs were separated in two groups. One group contains 250µg/ml and another group contains 500µg/ml. Extract impregnated discs were stored at 4 ^{0}C for determination of disc diffusion assay.

2.7 Determination of antibacterial activity
Antimicrobial activity was performed by disc diffusion assay [16]. Tested bacterial cultures were swabbed over Mueller hinton agar media with sterile cotton bud and then two concentrated (500µg/ml and 250µg/ml) discs of plant extract were placed there. Ciprofloxacin 5 µg were used as standard and only methanol impregnated discs were used as negative control.

2.8 Determination of minimum inhibitory concentration (MIC)
The MIC of the extracted material was determined by two fold dilution method. Subjected bacterial strains were grown in Mueller Hinton (MH) broth (OXOID, UK) until it reaches to the exponential phase containing turbidity at *A*560 of 0.8 which represents 3×10^8 CFU/ml. *Tridax procumbens* L extract of different dilutions were prepared to give concentrations 20,10, 5, 2.5, 1.25, 0.625 and 0.312 mg/ml respectively. After that 0.5 ml extract of each concentration was added into separate test tubes containing 0.5ml MH broth with bacterial suspension at a final concentration 1ml in each tube and incubated at 37 ^{0}C for 24 hours. As negative control 7% 0.5 ml methanol was added with 0.5ml bacteria-broth solution. After proper incubation, 100µl of culture from each tube was transferred and spreaded over nutrient agar plate and incubated at 37 ^{0}C overnight for bacterial count.

2.9 Determination of antifungal activity by agar well diffusion method
Solidified potato dextrose agar plates were taken and spreaded with 100µl spores of tested fungi. In each plate 2 wells were prepared with sterile cork-borer and filled with 100 µl of 250µg/ml and 500µg/ml plant extract. Fluconazole discs were placed to another trident as standard. Plates were incubated at 28 ^{0}C for 4-5 days.

2.10 Determination of minimum fungicidal concentration (MFC)
Antifungal activity of *Tridax procumbens* L extract was also tested by minimum fungicidal concentration (MFC) assay. MFC was done by agar dilution method. Different concentrations of leaf extract including 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml were used. Using sterile screw capped test tube, 9ml PDA and 1 ml extract of different concentration was mixed and cooled. After solidification 2µl of spore of three fungi were transferred by sterile micropipette. Tube sets were incubated at 28 ^{0}C for 4 days.

3. Result
3.1 Results of phytochemical screening
After phytochemical test of *Tridax procumbens* L leaves methanol extract, the presence of alkaloids, flavonoids, glycocides, proteins, steroids and terpenoids were confirmed by visible observation of color change and chemical state change (Table 1).

Table 1: Determination of phyto-constituents of *Tridax procumbens* L. extract.

Phyto-constituents	Methanol extract
Alkaloid	+
Flavonoid	+
Glycocides	+
Protein	+
Saponin	-
Steroid	+
Terpenoids	+
Tannin	-

3.2 Determination of antimicrobial activity

Methanolic extract of *Tridax procumbens* L showed a wide range of antimicrobial activity against *E.coli*, *Vibrio cholera* and *Bacillus subtilis* at the concentration of 500µg/ml whereas at 250 µg/ml concentration most bacteria showed resistance except *B. cereus*. On the other hand *Salmonella typhi* was totally resistant to both concentrations of the plant extract (Figure 1). Range of the zone of inhibition was within 8-14mm (Table 2). In case of fungus, every tested strains including *Aspergillus niger*, *Aspergillus fumigatus* and *Candida albicans* showd susceptibility to tested plant at both 250 and 500 µg/ml concentration. Fungal range of inhibition was within 9-15mm in diameter (Table 3). Among three fungi *Aspergillus niger* and *Candida albicans* showed highest susceptibility to the extracted material (Figure 2).

Table 2: Disc diffusion assay of different bacteria.

Bacterial species	Zone of inhibition in diameter (mm)			
	Met. Extract 250 µg/ml	Met. Extract 500 µg/ml	Positive Control Cip. 5µg	Negative control (methanol)
E. coli	-	14	27	0
Vibrio cholera	-	11	26	0
Staphylococcus aureus	-	9	19	0
Salmonella typhi	-	-	30	0
Bacillus cereus	-	8	25	0
Bacillus subtilis	8	10	27	0

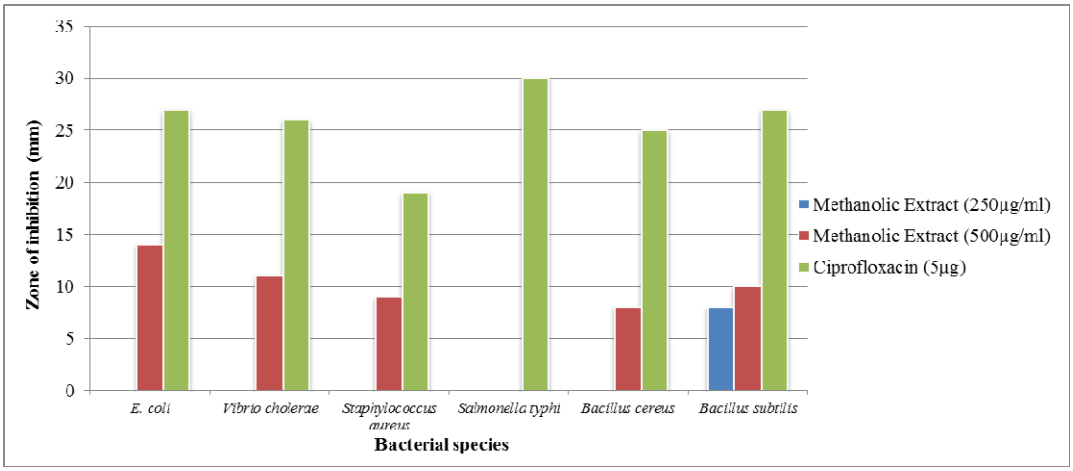

Fig 1: Zone of inhibition of methanolic extract of *T. procumbens* L. against different bacteria.

Table 3: Determination of anti-fungal activity by agar well diffusion method.

Fungal species	Zone of inhibition in diameter (mm)			
	Met. extract 250 µg/ml	Met. Extract 500 µg/ml	Positive Control Fluconazole	Negative control (Methanol)
Aspergillus niger	12	15	23	0
Aspergillus fumigatus	09	14	22	0
Candida albicans	11	15	24	0

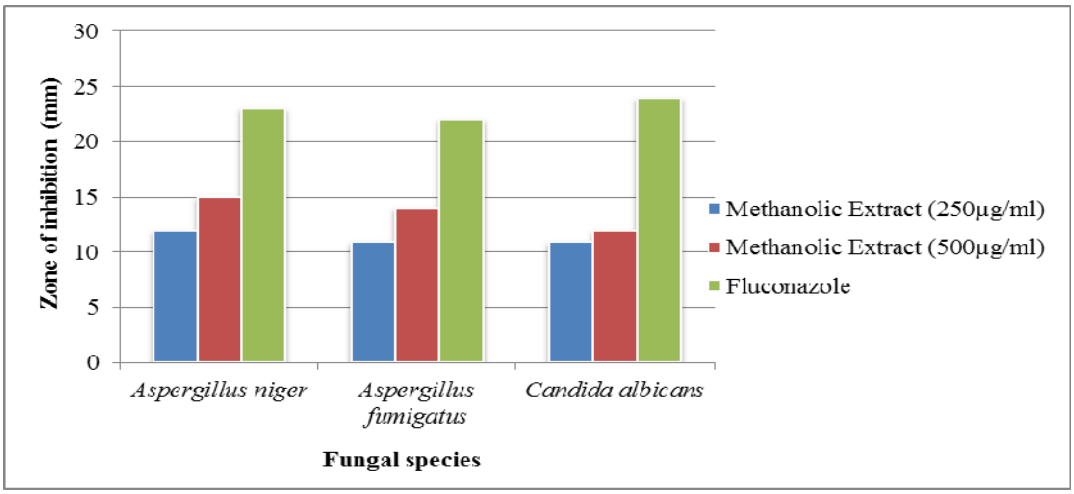

Fig 2: Zone of inhibition of different methanolic extract of *T. procumbens* L. against different fungi.

3.3 Determination of MIC and MFC

Minimum inhibitory concentration and Minimum fungicidal concentration is defined as the growth of microorganisms inhibited at the lowest concentration of plant extract used. In MIC test all types of bacteria were inhibited at the concentration 10-20mg/ml concentration of *Tridax procumbens* methanol extract except *Salmonella typhi* which was able to survive at 10mg/ml concentration (Table 4). On the other hand the MIC value of *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* was within the range of 5-20mg/ml concentration. In case of MFC, growth inhibition of *Candida albicans* and *Aspergillus niger* starts at 200 µg/ml and 250 µg/ml concentration of *T. procumbens* extract, so, their MFC ranges were within 200-250 µg/ml whereas the MFC of *Aspergillus fumigatus* was at 400 µg/ml (Table 5).

Table 4: Minimum inhibitory concentration of the methanolic extract of different bacteria.

Bacterial Isolates (Number of colony)	Minimum inhibitory concentration (MIC)						
	Concentration of methanol extract (mg/ml)						
	20	10	5	2.5	1.25	0.625	0.312
E. coli	0	0	0	13	27	74	131
Vibrio cholera	0	0	4	10	36	89	147
Staphylococcus aureus	0	0	0	06	22	68	112
Salmonella typhi	0	2	7	22	49	71	124
Bacillus cereus	0	0	0	07	38	92	157
Bacillus subtilis	0	0	2	11	41	113	187

Table 5: Minimum fungicidal concentration (MFC) of the methanolic extract of different fungus.

Fungal species	Concentration of leaf extract(µg/ml)										
	50	100	150	200	250	300	350	400	450	500	Negative control
Aspergillus niger	-	-	-	-	+	-	+	+	+	+	-
Aspergillus fumigatus	-	-	-	-	-	-	-	+	+	+	-
Candida albicans	-	-	-	+	+	+	+	+	+	+	-

(-) denotes no inhibition by plant extract; (+) denotes growth inhibition by plant extract.

4. Discussion

In present days, the therapeutic value of medicinal plant is increasing at significant rate for developing new drugs and combatting emerging diseases. Drug developers are targeting new sources of active materials to cope up with multi drug resistance (MDR) of different microorganisms. *Tridax procumbens* L. was targeted in this study as it is widely spreaded as a common weed in Indian subcontinent as well as all over the world [17]. Bioactive compounds of the methanolic extract of *T. procumbens* L. were tested as they have front role in halting bacterial and fungal growth, preventing infection of human body and many other uses medically [13, 22]. There are also a lot of evidences that alkaloids, flavonoids and tannin compounds have great impact on antimicrobial potentiality. In this experiment the leaves extract showed great potency against *E.coli, Vibrio cholera, Staphylococcus aureus, Bacillus subtilis, Candida albicans* and *Aspergillus flavus.* In case of *Salmonella typhi,* the resistance pattern to the extract was severe and it may be due to presence of their active compound blocker gene or their outer LPS layer which inhibits the hydrophobic essential oils to enter into the cell wall. In previous study the *T. procumbens* L. extract showed antiplasmodial and antileishmanial activity [18-22] But, we have tested only antimicrobial activity as it is the prime concern for MDR in today's world. In our study, the MIC value of *E.coli, S. aureus and B subtilis*. has showed vibrant significance. From this finding it can be predicted that the methanolic extract can inhibit gram positive bacteria properly comparing to the gram negative *S. typhi*. By observing MFC value it was also surprising that medically important opportunistic fungi *Candida albicans* showed great sensitivity on methanolic extract of *T. procumbens* L. suggesting ayurvedic use against candida infection mainly occurs in diabetic patient. Moreover, comparing to ciprofloxacin (5 µg) for bacteria and Fluconazole for fungi it can be assumed that the zone of inhibition (ranges from 8-15mm) of tested extract showed satisfactory result against both microorganisms. So, *T. procumbens* L. can be used undoubtedly as a potential source for drug development in future.

5. Conclusion

The findings of current study literally describe the antimicrobial potentiality of active bio-ingredients present in the methanolic extract of *T.procumbens* L. The extract validates the primitive use of the plant as a curative medicine in many diseases. Further immune-toxicological and in-vivo studies are needed with model animal to validate the stability and dose of the phytochemicals to design drugs against bacterial and fungal infection.

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