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EVALUATION OF ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *Theriophonum minutum*

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ABSTRACT

The aim of the study was to evaluation of antimicrobial activity of *Theriophonum minutum* plant extracts against *Escherichia coli* and *staphylococcus aureus.* The dried powder of whole plant was extracted with various solvents in increasing order of their polarity using soxhlet extraction method. Out of the extracts ethyl acetate, ethanolic and hydro-alcoholic extracts were subjected to preliminary phytochemical screening for detection of chemical constituents present in them and showed the presence alkaloids, glycosides, flavonoid, tannin and phenol, Saponin, sterol, gum and mucilage. The antibacterial activities of extracts were evaluated using the cup and plate method and the inhibitory zones were recorded in millimeters. The effect of ethyl acetate plant extracts of *Theriophonum minutum* plant showed the better antibacterial activity than ethanolic and hydro-alcoholic extracts against *E.coli* and *S. aureus*. Zone of inhibition for ethanolic, ethyl acetate and hydroalcholic extract was found to be more for *S. aureus* than *E.coli*. Hence ethanolic extract can be further investigated for more study.

Keywords: *Theriophonum minutum,* Antibacterial activity, *Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

Ever since the birth of mankind there has been a relationship between life, disease and plants. Primitive men started studying diseases and treatments [1]. There is no record that people in prehistoric times used synthetic medicines for their aliments but they tried to make use of the things they could easily procure. The most common thing they could find was there in environment i.e. the plants and animals [2]. They started using plants and found that majority of plants were suitable as food, where as other were either poisonous or medicinally useful [3]. By their experience, this knowledge of herbal remedies was transferred to generation as folk medicine. So the history of herbal medicine is as old as human history.

Herbal medicine is still the mainstay of about 75–80% of the world's population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine are a few important examples of what medicinal plants have given us in the past.

An antimicrobial agent is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi or protozoan's. Antimicrobial drug either kill microbes (microbicides) or prevent the growth of microbes (micro biostatic).Disinfectants are antimicrobial substances used on non-living objects or outside the body [4].

Theriophonum is a genus of flowering plants in the family *Araceae*. It is found only in India and Sri Lanka. In India most found in central and southern India. Species of *Theriophonum: Theriophonum dalzellii, Theriophonum danielii, Theriphonumin faustum,Theriophonum minutum, Theriophonum sivaganganum.*

MATERIAL AND METHODS:

Material

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Plant sample collection

The plant material in this study consisted of *Theriophonum minutum* plants were collected from Gondia.

Bacteria

Strains of *Stryphylococcu saureus*, and *Escherichia coli* were obtained from microbiology department of Smt. Kishoritai Bhoyar College of pharmacy Kamptee.

Nutrient agar media formulated by Himedia laboratories Pvt. Ltd. Mumbai were obtained from microbiology department of Smt. Kishoritai Bhoyar College of Pharmacy Kamptee. Ethanol and DMSO (Di-methyl sulphoxide) were also obtained from microbiology department of same college.

Antibiotics

Streptomycin injection manufactured by Abbott Ltd. and was purchased from Shri. Local market of Nagpur.

Phytochemical screening

The extract where subjected to preliminary phytochemical screening for possible presence of bioactive antimicrobial compounds.

Test for alkaloids

Dragendorffs Test:To 2-3 ml test solution, few drops Dragendorff reagent were added. Formation of reddish brown precipitation indicates the presence of alkaloids.

Wagners test:- A few drop of wagners reagent were added to few ml of test solution. Areddish brown precipitation indicates the presence of alkaloid.

Hagers Test:-To 2-3 ml of test solution, few drops and Hagers reagent were added. Formation of yellow orange precipitate indicates the presence of alkaloid.

Test for Glycoside

For anthraquinone glycoside

Borntragers Test:-To 2 ml of test solution, 3ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Appearance of pink or red colour indicates presence of anthraquinone glycosides.

Modified borntragers test:-Test solution was boiled with 2ml of dilute sulphuric acid. To it 2ml of 5% aqueous ferric chloride solution was added and allowed to stand for 5 min, they it was shaken with equal volume of chloroform and chloroform layer was separated and 10% ammonia solution was added to it. Appearance of pink or red colour indicates presence of C type of anthraquinone glycoside.

For cardiac glycoside

Keller -killiani Test:-To 2ml extract, add glacial aceticacid, one drop 5% $FeCl_3$ and conc. H_2SO_4. Reddish brown colour appear at junction of the two liquid layer and upper layer appear bluish green.

Legal's test:-To aqueous or alcoholic extract, add 1ml pyridine and 1 ml sodium nitroprusside. Pink to red colour appear.

Coumarin glycoside

Take moistened dry powder in test tube. Cover test tube with filter paper soaked in dilute NaOH. Keep in water bath. After some time expose filter paper to ultra violet light. It show yellowish- green fluorescence.

Saponin glycoside

Foam Test:-Shake the drug extract or dry powder vigorously with water. Persistent stable foam observed.

Test for Flavonoids

Shinoda Test:-To dry powder or extract, add 5ml 95% ethanol/ t- butyl alcohol, few drop of conc. HCl and 0.5 g magnesium turnings. Orange, pink, red to purple colour appear.

Test for Steroids

Salkowski reaction:-To 2 ml extract, add 2ml chloroform and 2ml conc. H_2SO_4. Shake well. Chloroform layer appear red and acid layer show greenish- yellow fluroscence.

Liebermann Burchard reaction:-Mix 2ml extract with chloroform. Add 1-2ml acetic anhydride and 2 drops conc. H_2SO_4 from the side of test tube. First red, then blue and finally green colour appear.

Libermann's reaction:- Mix 3ml of extract with 3ml acetic anhydride. Heat and cool, add few drop of conc. H_2SO_4. Blue colour appear.

Test for Phenol and Tannin

Ferric chloride test:-The test solution was treated with ferric chloride solution, appearance of blue colour indicate presence of hydrolysable tannin and appearance of green colour indicate the presence of condensed tannin.

Lead Acetate Test:-To 2-3ml of aqueous test solution, few drop of lead acetate solution was added, formation of precipitate indicate the presence of tannin.

Potassium dichromate test:-To 2-3ml of aqueous test solution few drop of potassium dichromate was added, appearance of red precipitate indicate the presence of condensed tannin.

Test for Terpenoids

1ml of test solution was treated with 1% $CuSO_4$ solution; formation of emerald green colour indicate the presence of diterpine.

Test for Fixed oil

Presspowder of crude drug between two filter paper. Filter paper gets permanently stain due to oil.

Test for Gum and Mucilage

The test solution was dissolved in 10 ml of distilled water and to this 2ml of absolute alcohol was added with constant stirring. White or cloudy precipitate indicate the presence of gum and mucilage [5, 6].

Antimicrobial activity

Various methods have been used from time to time to evaluate the antimicrobial activity. The evaluation can be done by the following methods:

- Agar diffusion method
- Turbidimetric method
- Agar streak dilution method
- Serial dilution method

The following conditions must be met for the screening of antibacterial activity:

- There should be an intimate contact between the test organisms and substances to be evaluated.
- Required conditions should be provided for the growth of microorganisms.
- Conditions should be same throughout the study.
- Aseptic/sterile environment should be maintained.

In present study, the agar diffusion technique is used to assess antibacterial profile of new compounds. The methods generally used are given as follows:

- Agar cup-plate method
- Liquid dilution (Broth) method
- Solid (Agar) method
- Disc diffusion method.

The cup-plate agar diffusion method is used to evaluate the antibacterial activity of extracts of *T.Minutum*. It is one of the in-vitro bacterial susceptibility tests. This classic method yields a quantitative result for the amount of antibacterial agent needed to inhibit the growth of specific bacterial strains [7].

CUP-PLATE AGAR DIFFUSION METHOD

Preparation of solutions of compounds

Before testing of extraction for antibacterial activity, each compound was then dissolved in Di-Methyl Sulphoxide (DMSO) individually to obtained 1000 µg/mL concentration.

Standard

Streptomycin 1000 µg/mL concentration.

Test cultures

1. *Escherichia coli* (Gram-negative bacteria)
2. *Staphylococcus aureus* (Gram-positive bacteria)

Preparation of inoculums

The bacterial cultures were grown on nutrient agar slants and nutrient broth for 24h at 37ºC in incubator. They were stored at 4ºC and sub culturing was done after one week. For evaluation of antibacterial activity, 24h fresh culture of bacteria was suspended in nutrient broth. The culture of bacteria was obtained from Porwal College, New kamptee Nagpur, India.

Preparation of culture medium

The medium used throughout the experiment was Hi-media (India make) have the following composition.

The media for antibacterial activities were prepared by dissolving 28.0g of ingredients in 1 L of distilled water and sterilized in an autoclave at 121ºC at 15 lbs/inch pressure for 20min

Procedure for cup plate method

The agar well diffusion /cup plate method was used for the determination of antibacterial activity of various prepared extracts. Well were made by cork borer in petri plates containing solid nutrient agar medium previously seeded with test organisms and well were filed with samples. After allowing diffusion of solution for 20 min, the plates were incubated at 37° for 24 hr. The diameter of zone of inhibition was measured in each plate [8].

Method : Cup plate/Agar well diffusion
Media : Nutrient Agar Media
Solvent : DMSO
Conc of test sample: 50µg/ml,100µg/ml,150µg/ml, 200µg/ml and 1000µg/ml

MINIMUM INHIBITORY CONCENTRATION (MIC)

Minimum inhibitory concentration (MIC) is the minimum concentration of antimicrobial compound found to inhibit the growth of a particular test microorganism. It is applied to disinfectant, antiseptic, preservative and antibiotics. MIC values are usually expressed in terms of µg/ml or units/ml. MIC of different antimicrobial is determined by broth dilution method or solid dilution method. The advantages of solid dilution method are as follows.

1. Several microorganisms can be tested at the same time by using multipoint incubator.
2. Contaminations are easily detected on solid media.

Procedure for MIC

Broth dilution method

Prepare nutrient broth (double strength) test tubes and label them. In first test tube (UT), inoculum is not added which is used for checking the sterility of medium and as a negative control. Other all test tubes, inoculums (three to four drops) is added to reach the final concentration of microorganisms is 10^6 cells/mL in all test tubes, test antimicrobial compound is added ranging from 0.5 to 5 ml except un-inoculated (negative control) and

control (positive) tube. The positive control tube is used to check the suitability of the medium for growth of the test microorganism and the viability of the inoculum. Adjust the final volume (10 ml) in all test tubes by using sterile water. All test tubes are properly shaken and then incubated at 37ºC for two days [4].

$$\text{Working Conc } (\mu g/mL) = \frac{\text{Volume of stock taken (mL)} \times \text{Stock Conc}(\mu g/mL)}{\text{Working volume (mL)}}$$

Solid dilution method

In this method, first test chemical is mixed into molten agar and then poured into Petri plates. After solidification, inoculum is spread on the surface of agar medium. All plates are incubated at 37ºC for two to three days.

After incubation, all test tubes or petri plates are examined for the growth in the form of turbidity and colonies, respectively. Record the result and calculate the minimum inhibitory concentration by comparing all results with positive and negative control [4].

RESULT AND DISCUSSION

Quantitative phytochemical screening

The phytochemicals screening of *Theriophonum minutum* plant extract was assessed for different chemical tests and showed in table.

Antimicrobial study

The antimicrobial activity studied using *E.coli* and *S.aureus* was studied as shown in figure 1 and table 5.

All the above extracts were tested for antibacterial activity, and they shows effective zone of inhibition for antibacterial activity using *E.coli* and *S.aureus* when compared with streptomycin as standard. Activity against *S.aureus*is more as compare to *E.coli*.

MIC was appropriately calculated by broth dilution method. The solid dilution method was performed but, not given the proper interpretation of results. MIC against *E.coli* was found to be, 50 µg/ml for ethyl acetate extract. MIC against *S.aureus* was found to be, 70µg/ml for ethyl acetate extract.

Table 1. Culture Media and Chemicals

Sr. No.	CONTENT	INGREDIENT
1	Culture Media	Nutrient agar
2	Chemicals	DMSO, Ethanol.

Table 2. Preliminary Phytochemical Screening of *Theriophonum minutum* extract

Sr. No	Chemical Test	Observation	Test
1	Alkaloids	Orange red precipitate is observed	Positive
2	Glycoside	Red colour is observed	Positive
3	Flavonoids	Yellow coluor is produced	Positive
4	Saponins	Foam is produced	Positive
5	Tananin& Phenol	Brown colour is produced	Positive
6	Terpenoids	Reddish brown colour is produced	Positive
7	Steroid	Greenish yellow fluorescence is produced	Positive

Table 3. Nutrient Agar Medium

Ingredients	Quantity
Peptone	5.0g
Sodium chloride	5.0g
Beef extract	1.5g
Yeast extract	1.5g
Agar	15.0g
Water up to	1000g
Final pH (at 25ºC)	7.4±0.2

Table 4. Phytochemical screening of *Theriophonum minutum* plant extracts

Plant constituent	Test performed and reagent	Extract			
		EA	ET	PE	HA
Test for alkaloids	Dragendorffs reagent	-	+	-	+
	Wagers reagent	-	+	-	+
	Hagers reagent	-	+	-	+

Test for Glycosides	Killer killani test	+	+	+	+
	Legal test	+	+	+	+
	Borntrager test	+	+	-	+
	Modified borntrager test	+	+	-	+
	Coumarin Glycoside	-	+	-	+
	Saponin Glycosides	-	+	-	+
Test for Steroids	Salkowaski reaction	+	+	+	+
	Liebermann Burchard reaction	-	+	-	+
	Liebermann reaction	-	+	-	+
Test for Flavonoids	Shinoda test	+	+	-	+
Test for Fixed oil		+	+	-	+
Test for Tannin and Phenol	Ferric chloride test	-	+	-	+
	Potassium dichromate test	-	+	-	+
	Lead acetate test	-	+	-	+
Test for Terpenoids			-	-	+

Present= (+) Absents= (-)

Table 5. Antibacterial activity of Ethyl acetate extract of *Theriophonum minutum*

Sr. No.	Test culture	Zone of inhibition(mm)						
		DMSO	Positive control 1000(µg/ml)	Ethyl acetate extract				
				50(µg/ml)	100(µg/ml)	150(µg/ml)	200(µg/ml)	1000(µg/ml)
1	*E.Coli*	-	21.50	17.25	18.25	20	18	15
2	*S.aureus*	-	23.50	19.50	22.25	17	17	24.25

Ethyl acetate extract of *T.minutum* showed comparable antibacterial activity against all the selected test cultures at the conc. of 1000µg/ml

Table 6. Antibacterial activity of ethanolic extract of *Theriophonum minutum*

Sr. No.	Test culture	Zone of inhibition(mm)						
		DMSO	Positive control 1000(µg/ml)	Ethanol extract				
				50(µg/ml)	100(µg/ml)	150(µg/ml)	200(µg/ml)	1000(µg/ml)
1	*E.Coli*	-	21.50	17.75	-	-	16.25	17
2	*S.aureus*	-	23.50	18.25	16	20.25	-	16.25

Table 7. Antibacterial activity of Hydroalcholic extract of *Theriophonum minutum*

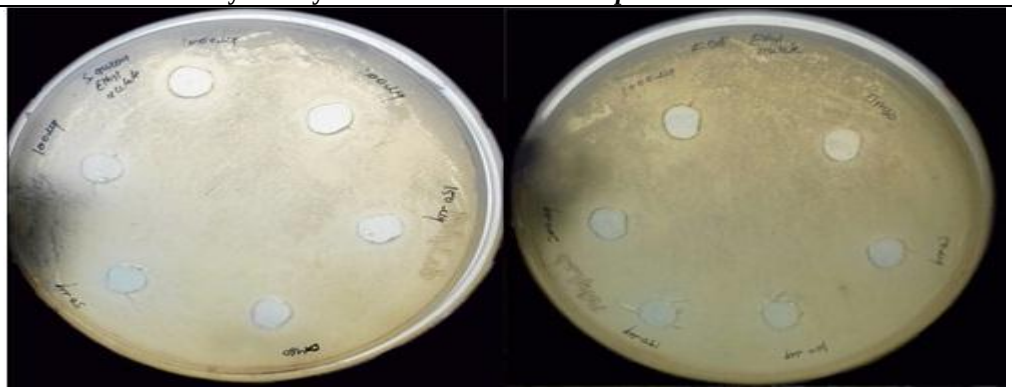
Sr.No.	Test culture	Zone of inhibition(mm)						
		DMSO	Positive control 1000(µg/ml)	Hydroalcholic extract				
				50(µg/ml)	100(µg/ml)	150(µg/ml)	200(µg/ml)	1000(µg/ml)
1	*E.Coli*	-	21.50	-	-	-	-	18.75
2	*S.aureus*	-	23.50	-	-	-	-	19.75

Table 8. Minimum Inhibitory Concentration (MIC) Study

Tube No.	Volume of double strength medium (ml)	Volume of test antimicrobial compound (ml)	Volume of sterile water (ml)	Growth	
				E.coli	*S.aureus*
Un-inoculated (UT)	5	0.0	5.0	-	**Un-inoculated (UT)**
Control (CT)	5	0.0	5.0	++	**Control (CT)**
1	5	0.5	4.5	+	**1**
2	5	1.0	4.0	+	**2**
3	5	1.5	3.5	+	**3**

4	5	2.0	3.0	+	**4**
5	5	2.5	2.5	+	**5**
6	5	3.0	2.0	-	**6**
7	5	3.5	1.5	-	**7**
8	5	4.0	1.0	-	**8**
9	5	4.5	0.5	-	**9**
10	5	5.0	0.0	-	**10**
MIC of compounds (µg/ml)				**50**	**70**

Fig 1. Antibacterial activity of ethyl acetate extract of *Theriophonum minutum* for *S.aureus* and *E.coli*

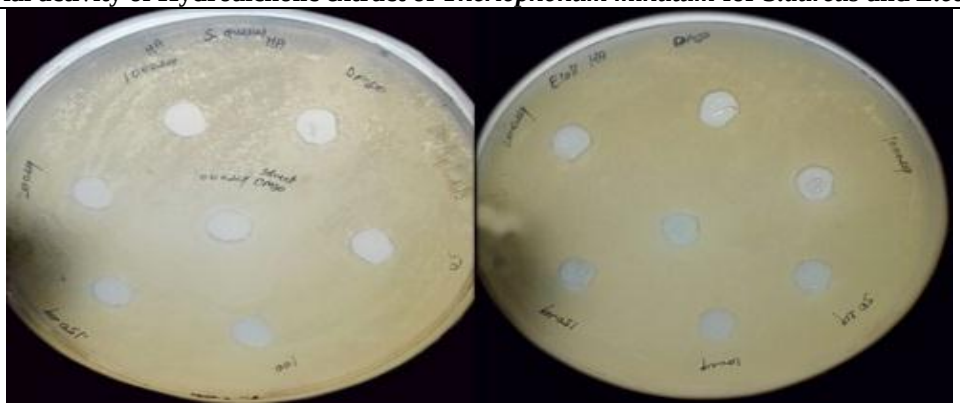


For *S.aureus* For *E.coli*

Fig 2. Antibacterial activity of ethanol extract of *Theriophonum minutum* for *S.aureus* and *E.col*

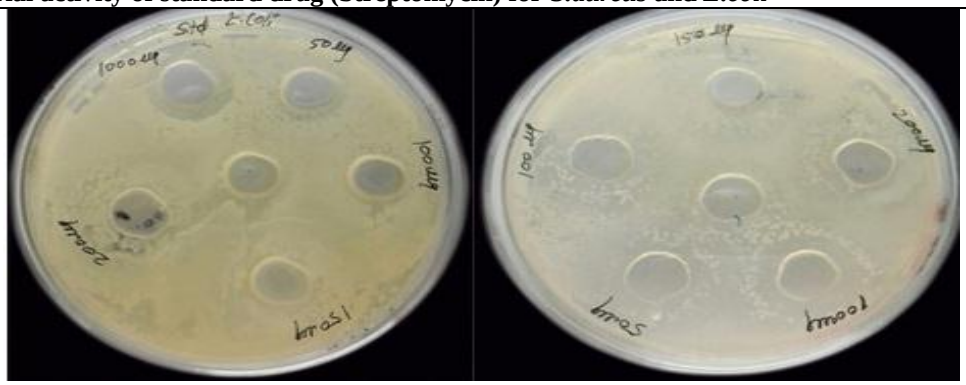
For *S.aureus*For *E.coli*

Fig 3. Antibacterial activity of Hydroalcholic extract of *Theriophonum minutum* for *S.aureus* and *E.coli*



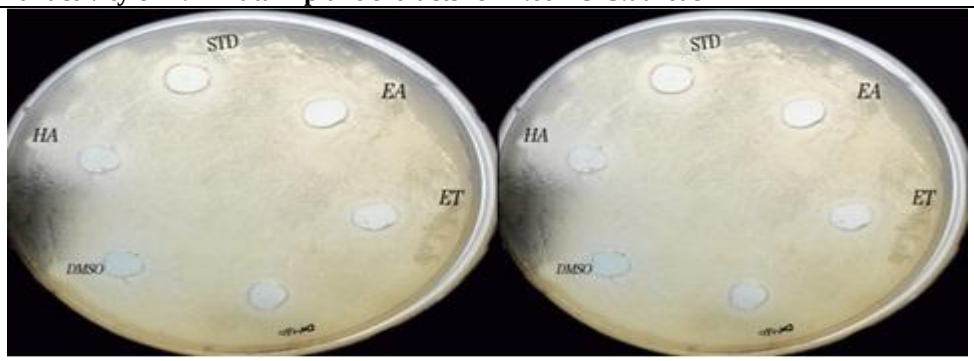
For *S.aureus*For *E.coli*

Fig 4. Antibacterial activity of standard drug (Streptomycin) for *S.aureus* and *E.coli*



For *S.aureus*For *E.coli*

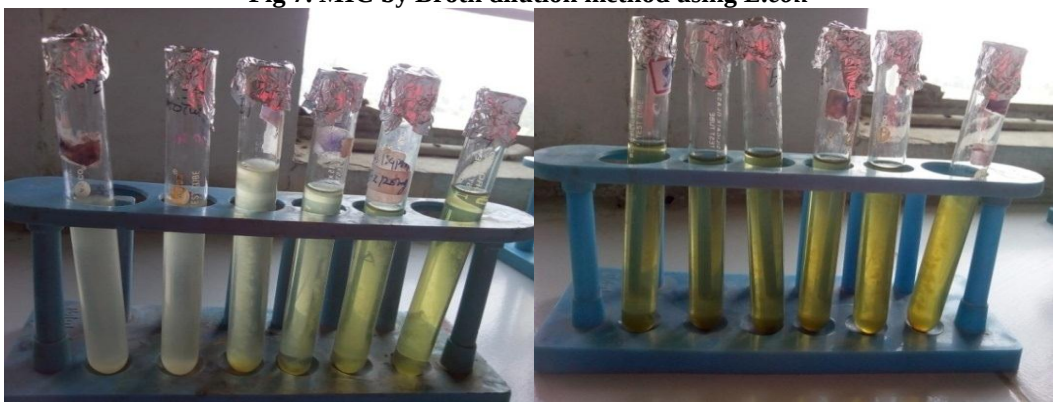
Fig 5. Antibacterial activity of *T. minutum* plant extracts for *E.coli* & *S.aureus*



For *S.aureus*For *E.coli*

Fig 6. MIC by broth dilution method using *S. aureus*

Fig 7. MIC by Broth dilution method using *E.coli*



-No growth (inhibition), Optimum + growth, ++ Maximum growth

CONCLUSION

Despite of significant antibacterial activity, ethyl acetate *Theriophonum minutum* extract exhibited antimicrobial activity. The phytoconstituents need to be isolated from the extracts and further screen for antimicrobial activity.

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