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PHARMACOGNOSTICAL AND PHYTOCHEMICAL EVALUATION OF BARK EXTRACT OF ZMA AND ZME *ZIZIPHUS MAURITIANA* AND *ZIZIPHUS NUMMULARIA* (RHAMNACEAE)

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ABSTRACT

Medicinal plants are used in traditional system of medicine to treat various diseases and many of these plants have been evaluated for their different pharmacological activities. *Ziziphus mauritiana* (ZM) (Rhamnaceae) is found wild and cultivated in many parts of India, Burma and Srilanka. Seeds of *Ziziphus mauritiana* have been reported to have sedative-hypnotic, hypotensive, antihypoxic, antihyperlipidemic and hypothermic activities. It is reported to contain minerals, alkaloids, flavonoids, sterols, tannins and saponins. Traditional medicine using herbal drugs exists in every part of the world. The major areas are Chinese, Indian and European traditions. The philosophies of these traditional medicines have some resemblance to each other but differ widely from modern western medicine. In view of the progress of western medicine not only new synthetic drugs but also herbal drugs have to fulfill the international requirements on quality, safety and efficacy. Herbal drugs have the advantage of being available for patients in the geographical area of the special traditional medicine. The development procedure of herbal drugs for world-wide use has to be different from that of synthetic drugs.

Keywords: *Ziziphus mauritiana*, Antioxidant, Anti-inflammatory, Nephroprotective, *Ziziphus nummularia*, Rhamnaceae.

INTRODUCTION

The bark of *Ziziphus mauritiana* and *Ziziphus nummularia* used for the present studies were collected from local area of Jaipur district of Rajasthan. The plants were identified, confirmed and authenticated by comparing with voucher

specimen available by taxonomist. A copy of certificate is attached. The bark was cut into small pieces and shade dried. The dried material was then pulverized separately into coarse powder by a mechanical grinder. The resulting powder was then used for extraction.

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MATERIAL & METHODS

Drugs: Indomethacin (Microcid from MicroLabs), Cisplatin (Kemoplat from Dabur)

Plant: The bark of *Ziziphus mauritiana* was procured and authenticated from Regional Research Institute (Ay.) (Central Council of Research in Ayurveda and Sidha, Dept of AYUSH, Ministry of Health and Family Welfare, Govt. of India, New Delhi), Government Central Pharmacy, Annexe, Ashoka Pillar, Jayanagar, Bangalore-560011.

Reagents: Benedict's reagent, Barfoed's reagent, Millon's reagent, Dragendorff's reagent. Hager's reagent, Mayer's reagent. Wagner's reagent.

Chemicals: All chemicals used were of analytical grade.

Collection of plant materials

Bark of the two plants (*Z. mauritiana* Lam. and *Z. nummularia*) were collected in polythene bags from in and around local area of Jaipur-Rajasthan and air dried for two weeks in the Microbiology Laboratory. The dried leaf material was then ground into powder using blender (Monlinex 530, 240V) and packed in polythene bags for further use.

Phytochemical Screening

i. Preparation of Methanolic Extract:

The powdered drugs were dried and packed well in separate Soxhlet apparatus and extracted with 1500 ml of methanol for seven days. The extracts were concentrated and dried using Rotary flash evaporator. Both were kept in desiccators until used.

ii. Qualitative phytochemical screening:⁷⁶

The following tests were carried out on the standardized herbal extracts to detect various phyto-constituents present in them.

iii. Detection of carbohydrates

Small quantity of the extract was dissolved in distilled water and filtered. The filtrate was subjected to

1. Molisch's test
2. Fehling's test
3. Barfoed's test

iv. Molisch's test

To the filtrate few drops of alcoholic α -naphthol was added and 2ml of conc. sulphuric acid was added slowly through the slides of the test tube. No purple-colored ring was formed at junction of the two layers, which indicates absence of carbohydrates in both the extracts.

Fehling's test

Small portion of the extract was treated with Fehling's solution I and II and then heated on water bath. No brick red colored precipitate was formed, which indicates absence of carbohydrates in both the extracts.

v. Barfoed's test

Small portion of the extract was treated with Barfoed's reagent. No red precipitate formed, which indicates absence of carbohydrates in both the extracts.

vi. Test of starch

A small amount of powdered drug was treated with diluted iodine solution. No blue color was observed, which indicates absence of starch in both the drugs.

vii. Detection of proteins and amino acids

Small quantity of extract was dissolved in few ml of water and was subjected to Millon's, biuret and ninhydrin test.

viii. Millon's test:

The extract was treated with Millon's reagent. No white precipitate was produced, shows the absence of proteins and free amino acids in both the extracts.

ix. Biuret test:

To the extract equal volume of 5%w/v NaOH and four drops of 1%w/v CuSO_4 solution were added. No pink or purple color was formed indicating the absence of proteins in both the extracts.

x. Ninhydrin test:

The extract was treated with ninhydrin reagent. No purple color was produced, indicating the absence of proteins in both the extracts.

xi. Detection of phenolic compounds and tannins

The decoction was diluted with distilled

water and filtered. The filtrates were treated with following reagent.

xii. Ferric chloride test:

The filtrate was treated with 5% of ferric chloride solution. No black precipitate was found in the decoction of the plant, indicating the absence of tannins and phenolic compounds in both the filtrates.

Test with Lead acetate Solution:

Few ml of filtrate was treated with lead acetate solution. No white precipitate was produced in the decoction of both the plants.

Gelatin test:

To the filtrate of decoction, add 1ml of 1% solution of gelatin. No white precipitate was seen, which indicates absence of tannin in both the plants.

xiii. Test for phytosterols

Small quantity of decoction were dissolved in 5ml of chloroform separately.

Then these chloroform layer subjected to,

- a) Salkowskistest
- b) Libermann – Burchards test

xiv. Salkowski test:

To 1ml of the above prepared chloroform solutions, few drops of conc H_2SO_4 was added. Red color produced in the lower layer, shows the presence of phytosterols in both the samples.

xv. Libermann – Burchardstest:

The above chloroform solution was treated with few drops of conc. H_2SO_4 followed by 1ml of acetic anhydride solution. Green color was produced, shows the presence of phytosterols in both the solutions.

xvi. Test for fixed oils and fats

3Spot test:

A small quantity of extracts was pressed between two filter papers, oil stain was observed in both the extracts, show presence of fixed oils in both the samples.

xvii. Saponification:

Few drops of 0.5N alcoholic potassium hydroxide was added to extracts along with a few drops of phenolphthalein. The

mixture were heated on a water bath for about 1 – 2 hours. Formation of soap or a partial neutralization of alkali indicated the presence of fixed oils and fats in both the samples.

xviii. Test for alkaloids

Small amount of extracts were stirred with a few ml of dil. HCl and filtered. The filtrates were tested with various alkaloidal reagents such as Mayer's, Dragendroff's, Wagner's and Hager's reagent.

xix. Mayer's test:

To the small amount of filtrates few drops of Mayer's reagent was added. A white color precipitate was formed, indicating the presence of alkaloids in both.

xx. Dragendroff's test:

(potassium bismuthiodide)

To the small amount of filtrates few drops of Dragendroff's reagent was added. An orange red color precipitate was formed in both the samples, indicating the presence of alkaloids in both the samples.

xxi. Wagner's test:

To the small amount of filtrate of both the drugs few drops of Wagner's reagent was added. A brown color precipitate was formed in both, indicating the presence of alkaloids in both the samples.

xxii. Hager's test: (picric acid)

To the small amount of filtrate of both drugs few drops of Hager's reagent was added. A yellow crystalline precipitate was formed in both samples, indicating the presence of alkaloids in both the samples.

Test for glycosides

Small amount of the extracts were hydrolyzed separately with hydrochloric acid for one hour on separate water baths and hydrolysates were subjected to

Legal's test

Balget's test

Borntrager's test

Modified borntrager's test.

xxiii. Legal's test:

To the hydrolysate 1ml pyridine few drops of sodium nitroprusside solution was added and then made alkaline with NaOH solution. Pink color was obtained showing the presence of glycosides in both the samples.

Balget's test:

To a solution of extract sodium picrate solution was added. Yellowish orange color was obtained showing, the presence of glycosides in both the samples.

xxiv. Borntrager's test:

Hydrolysate was treated with chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Pink color was observed in ammoniacal layer, confirms the presence of glycosides in both the samples.

xxv. Modified borntrager's test:

The extracts were boiled with few ml of dil. HCl and 5ml of ferric chloride solution. The contents are cooled and shaken with organic solvent. Organic layer was separated and to this equal volume of ammoniacal solution was added. The ammoniacal layer showed pink color. In this test, addition of ferric chloride was added to break the C – C linking of glycosides which is a stronger than C = O linkage in both the samples.

Test for flavonoids

The extracts were dissolved in ethanol separately and then subjected to the following tests.

xxvi. Ferric chloridetest:

To a small quantity of Methanolic solution of extract few drops of neutral ferric chloride was added. Blackish red color was observed, showing the presence of flavonoids in both the samples.

xxvii. Shinoida's test:

To the alcoholic solution a small piece of magnesium ribbon was added along with conc. HCl. Magenta color was formed, showing the presence of flavonoids in both the samples.

xxviii. Fluorescence test:

Alcoholic solution was seen under ultra violet light. Green color fluorescence was observed, indicating the presence of flavanoids. Reaction with alkali and acid: With sodium hydroxide solution the extracts gave yellow color. Extract gave orange color with conc H₂SO₄ indicating the presence of flavonoids in both the samples.

xxix. Zinc, HCl reduction test:

To a small quantity of extract, a pinch of zinc dust was added. Then add few drops of conc. HCl. Magenta color was produced, the presence of flavonoids in both the samples.

xxx. Lead acetatesolution:

To a small quantity of extract a few drops of 10% lead acetate solution was added. Yellow precipitate was produced, shows presence of flavonoids in both the samples.

xxxi. Detection of saponins

The extracts were diluted, with 20ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. A one centimeter layer of foam was formed, indicating the presence of saponins in both the samples.

xxxii. Detection of coumarins

To a small quantity of extract were dissolved in alcohol and exposed to UV light, shows green fluorescence.

To small quantity of extract were dissolved in alcohol and add ferric chloride solution, shows green color, indicating the presence of coumarins in both the samples.

Extraction of active compounds using ethanol as solvent for extraction

Ten grams (10g) of the ground barks samples were separately soaked in 200 ml of ethanol and allowed to stand for about 72 h for extraction. After the 72 h, it was then filtered using No.1 Whatman filter paper. The filtered samples were sterilized by passing through Millipore filter and

later evaporated to dryness (Mann et al., 2008).

Preparation of test organisms

Clinical specimens of *Staphylococcus aureus*, *E. coli*, *S. pyogenes*, *C. albicans* and *A. niger* were obtained from Advance Labs, Mansarovar, Jaipur. The organisms were sub-cultured on agar slants prior to use. 18 h liquid culture of each of the organisms was used for sensitivity testing.

Sensitivity testing

Ethanol extract (preparation is shown above) of each plant sample was tested against each of the organisms using agar cup well method as described by Okeke et

al. (2001). After making holes with No. 4 cork borer, the surface of the agar was lawned with 18 h culture of the test organism which has been previously standardized to 10^6 . Same volume (0.1 ml) of different concentrations of the extract (500, 50, 5 and 1 mg) was dropped with the aid of dropper pipette into each well. The plates were incubated at 37°C for 24 h and 72 h at 25°C for bacteria and fungi respectively.

Control experiment

Standard antibiotics were used as control and the experiment procedure is as described above.

RESULT

Phytochemical constituents present in Methanolic extract of bark of *Ziziphus moutiana*

Table 1.1: Details of qualitative phytochemical tests.

S.No.	Test	Pet-ether Extract	Chloroform Extract	Methanolic Extract
1	Carbohydrates			
	Mohlish's test	-	-	+
	Fehling's test	-	-	-
2	Proteins and amino acids			
	Ninhydrin test	-	-	-
	Biuret test	-	-	-
3	Alkaloids			
	Mayer's test	-	+	+
	Wagner's test	-	-	+
4	Fixed oils and fats			
	Spot test	+	-	-
5	Glycosides			
	Borntrager's test	-	-	+
	Legals test	-	+	+
6	Triterpenoids			
	Tin + thionyl chloride	+	-	-
7	Phenolics and tannins			
	Ferric chloride test	-	-	-
	Gelatin test	-	-	-
	Lead acetate test	-	-	-
	Alkaline reagent test	-	-	-
	Dilute HNO ₃ test	-	-	-
8	Saponins			
	Foam test	-	+	+
	Haemolysis test	-	+	+
9	Flavones and Flavonoids			
	Caddy's test	-	+	+
	Shinoda test	-	+	+

s(+) Indicates positive result (–) Indicates negative result.

In, preliminary phytochemical studies of extracts of *Ziziphus mauritiana* confirmed the strong presence of desired phytochemicals in methanolic extracts when compared to pet-ether and chloroform extracts. Hence, for the further studies Methanolic extract of *Ziziphus mauritiana* (MEAL) have been selected.

Phytochemical constituents present in Methanolic extract of bark of *Ziziphus nummularia*

Table 1.2: Details of qualitative phytochemical tests.

S.No.	Test	Pet ether Extract	Chloroform Extract	Methanolic Extract
1	Carbohydrates			
	Mohlish's test	-	-	+
	Fehling's test	-	-	-
2	Proteins and amino acids			
	Ninhydrin test	-	-	-
	Biuret test	-	-	-
3	Alkaloids			
	Mayer's test	-	+	+
	Wagner's test	-	-	+
4	Fixed oils and fats			
	Spot test	+	-	-
5	Glycosides			
	Borntrager's test	-	-	+
	Legals test	-	+	+
6	Triterpenoids			
	Tin + thionyl chloride	+	-	-
7	Phenolics and tannins			
	Ferric chloride test	-	-	-
	Gelatin test	-	-	-
	Lead acetate test	-	-	-
	Alkaline reagent test	-	-	-
	Dilute HNO ₃ test	-	-	-
8	Saponins			
	Foam test	-	+	+
	Haemolysis test	-	+	+
9	Flavones			
	Caddy's test	-	+	+
	Shinoda test	-	+	+

(+) Indicates positive result (–) Indicates negative result.

In, preliminary phytochemical studies of extracts of *Ziziphus nummularia* confirmed the strong presence of desired phytochemicals in methanolic extracts when compared to pet-ether and chloroform extracts. Hence, for the further studies methanolic extract of *Ziziphus nummularia* (MEAL) have been selected.

Table 1.3: Sensitivity analysis showing zones of inhibition (mm) around crude extracts at varying concentrations.

Conc. of extracts(mg/ml)	<i>S.a</i>	<i>E.c</i>	<i>S.p</i>	<i>A.n</i>	<i>C.a</i>	<i>S.a</i>	<i>E.c</i>	<i>S.p</i>	<i>A.n</i>	<i>C.a</i>
1	0	0	9 ± 3	0	0	0	0	0	0	0
5	0	9 ± 1	14 ± 3	0	0	0	10 ± 4	13 ± 1	0	0
50	11 ± 2	12 ± 2	16 ± 3	0	0	12 ± 3	14 ± 0	18 ± 0	0	0
500	13 ± 1	16 ± 3	26 ± 2	0	0	15 ± 2	17 ± 1	28 ± 2	0	0

Key: *S.a* = *Staphylococcus aureus*, *E.c* = *Escherichia coli*, *S.p* = *Streptococcus pyogenes*, *A.n* = *Aspergillus niger*, *C.a* = *Candida albicans*.

Table 1.4. Zones of clearing (mm) of susceptibility testing with standard antibiotics.

Organisms	Antibiotics									
	CH	CPX	E	LC	GM	APX	RP	FLX	S	NB
<i>E. coli</i>	9 ± 0.1	8 ± 0.1	9 ± 0.1	0	12 ± 0.1	0	0	9 ± 0.1	8 ± 0.1	12 ±
<i>S. pyogenes</i>	12 ± 0.1	10 ± 0.1	9 ± 0.1	8 ± 0.1	11 ± 0.1	8 ± 0.1	8 ± 0.1	9 ± 0.1	9 ± 0.1	14 ±
<i>S. aureus</i>	18 ± 0.1	18 ± 0.1	16 ± 0.1	15 ± 0.1	16 ± 0.1	12 ± 0.1	14 ±	12 ±	16 ±	16 ±
<i>A. niger</i>	19 ± 0.1	0	0	22 ± 0.1	22 ± 0.1	18 ± 0.1	22 ±	0	16 ±	0
<i>C. albicans</i>	0	0	0	0	0	0	0	0	0	0

Key: CH - Chloramphenicol (10 mg), CPX - Ciprofloxacin (10 mg), E - Erythromycin (20 mg), LC - Lincocin (30 mg), GM - Gentamycin (10 mg), APX - Ampiclox (10 mg), RP - Rimbaprim (10 mg), FLX - Floxapin (30 mg), S - Streptomycin (30 mg), NB - Narbactin (10 mg).

Table 1.5 Minimum inhibitory concentration (MIC) of plant extracts against test organisms.

Plantparts	Organisms	Concentration of extract(mg/ml)							
		50	40	30	20	5	1	0	MIC
<i>Z. mauritiana</i>	<i>E. coli</i>	-	-	-	-	-	+	+	5
	<i>S. aureus</i>	-	-	+	+	+	+	+	40
	<i>S. pyogenes</i>	-	-	-	-	-	-	+	1
<i>Z. nummularia</i>	<i>E. coli</i>	-	-	-	-	+	+	+	20
	<i>S. aureus</i>	-	+	+	+	+	+	+	50
	<i>S. pyogenes</i>	-	-	-	-	-	+	+	5

Key: + = Growth, - = No growth.

Minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration was determined using tube dilution technique. Varying concentrations of each extract were prepared and 1 ml introduced into 9 ml of nutrient broth in test tubes. About 0.1 ml of the 18 h culture diluted to 10^6 cell ml^{-1} was added and incubated accordingly. The least concentration of the extract that did not permit turbidity in the broth was taken as the minimum inhibitory concentration.

Minimum bactericidal concentration (MBC)

Spread plate technique was employed. A fresh solid medium was inoculated with inoculum from the least concentration that showed no visible growth and incubated for 24 h at 37°C. The lowest concentration in which no growth occurs on the solid medium was accepted as the minimum bactericidal concentration.

Table 1.3 shows that *Z. mauritiana* was active against *S. pyogenes* at 1 mgml^{-1} , but were active against *E. coli* at 5 mgml^{-1} but active against *S. aureus* only at 50 mgml^{-1} . The two fungal isolates *A. niger* and *C. albicans* were resistant.

From Table 1.4 all the organisms were

susceptible to the antibiotics except *C. albicans* which was resistant. *Z. mauritiana* showed an MIC of 1 mgml^{-1} against *S. pyogenes*, 5 mgml^{-1} against *E. coli* and 40 mgml^{-1} against *S. aureus* while *Z. spinachristi* showed the MIC of 5 mgml^{-1} against *S. pyogenes*, 20 mgml^{-1} against *E. coli* and 50 mgml^{-1} against *S. aureus*.

Table 1.5 *Z. mauritiana* showed an MBC of 20 mgml^{-1} against *S. pyogenes*, 30 mgml^{-1} against *E. coli* and 50 mgml^{-1} against *S. aureus* while *Z. nummularia* showed the MBC of 30 mgml^{-1} against *S. pyogenes*, 40 mgml^{-1} against *E. coli* and 50 mgml^{-1} against *S. aureus* (Table 4).

CONCLUSION

The findings of this research work have shown clearly that the plants extracts are probably inactive against fungi and may not be useful in treating diseases of fungal origin. The extracts were active against the clinical isolates employed for this analysis. All the plants extracts were active against *S. pyogenes* an indication that the plant can be used to cure acute tonsillitis and sore throat caused by this bacterium. *Z. mauritiana* was active against *E.*

coli, *S. pyogenes* and *S. aureus* while *Z. nummularia* was very active only against *S. pyogenes* but moderately active against the rest test organisms. *Z. mauritiana* showed stronger activity against the organisms compared with *Z. nummularia*.

The standard antibiotics used as control showed higher activity on the organisms than the extracts (Tables 1.3 and 1.4). This is not surprising because standard antibiotics are well refined industrial products so there is no doubt their activity will be more compared to crude extracts. If the extracts used in the present work are refined, more and better activity could be observed. The Minimum Inhibitory Concentration of the extracts against the organisms was 1 mgml⁻¹ against *S. pyogenes* and 5 mgml⁻¹ against *E. coli* while the Minimum Bactericidal Concentration was 5 and 20 mgml⁻¹, respectively against the organisms had similar results in their experiments involving some of these organisms. A cidal drug kills pathogens at levels only two or four times the MIC whereas a static drug kills pathogens at much higher concentrations. Some of the organic compounds

detected in the extracts include tannins, saponins, resins, polyphenols and cardiac glycosides. These compounds have variously been reported to have antimicrobial activity and could be the reason for the activities recorded against these test organisms. Plants chemicals are thought to have the potentiality of useful drugs if properly harnessed.

The plants extracts were found to be inactive against the test organisms even at very high concentration which means they may not be useful against in the treatment of fungal infections such as dermatophycoses. The cell wall components of bacteria are quite different from those of fungi. While the cell wall of bacteria are either made up of acetyl muramic acid (AMA) or acetyl glucose amine (AGA) fungal cell wall is made up of fungal cellulose. This may explain the reason for the differences in their susceptibility to the plants extracts in this experiment. On the basis of these results therefore, we would like to state that constituents of these plants extracts may serve as a source of industrial drugs useful in the chemotherapy of some bacterial infections.

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