

Investigations into the antibacterial activity of *Ziziphus mauritiana* Lam. and *Ziziphus xylopyra* (Retz.) Willd.

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Abstract

Ziziphus plants (Rhamnaceae family) are reported to possess bioactive compounds, recognized for traditional use and medicinal importance. Present work aims to evaluate antibacterial activity of stem extracts of *Ziziphus mauritiana* Lam. and *Ziziphus xylopyra* (Retz.) Willd. Stem barks were extracted in different solvents and analyzed by agar well diffusion assay. *Z. mauritiana* methanol extract (ZMM-A) had greatest antibacterial activity against *S. aureus* (22 mm) which was identical to positive control Gentamycin (22 mm), while the Gram negative test organisms were resistant to *Z. xylopyra* aqueous-dioxane extract (ZXAD). Minimum inhibitory concentration (MIC) to suppress most sensitive *S. aureus* exemplified ZMM-A extract at 1.5 mg/ml and *Z. xylopyra* methanol extract (ZXM-A) at 2.5 mg/ml. Gas Chromatography – Mass Spectrometry (GC-MS) study of potent ZMM-A and ZXM-A extracts reflected presence of variety of compounds responsible for antibacterial activity and require further intensive study. This research finding signifies methanolic *Ziziphus* stem bark extracts as possible and reliable natural source of pharmaceutical drug with antibacterial potential.

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Introduction

A major disaster for the mortality and morbidity amongst humans and animals are infectious diseases. Emergence of multi-drug resistance, existing antimicrobials with undesirable effects and restricted anti-microbial spectrum creates further annoyance diverting attention towards natural antimicrobials (Ngoci *et al.*, 2014). Traditionally from prehistoric times, the use of different parts of medicinal plants was practiced to cure specific ailments evidently due to presence of some bioactive compounds like alkaloids, flavonoids, essential oil, glycosides, tannins, terpenoids, steroids and others. Many of the drugs, currently in use have been isolated from natural sources based on information about curative agent in folklore medicine. United States Food and Drug Administration (FDA) have approved unmodified natural products or partly-synthetic drugs derived from natural source for 78% of new drugs between 1983 and 1994 (Sharmin *et al.*, 2014; Ngoci *et al.*, 2014).

Ziziphus species comprises about 40 species distributed in warm-temperate and subtropical regions. *Ziziphus* plants are traditionally used as medicine for the treatment of various diseases such as digestive disorders, urinary troubles, diabetes, skin infections, diarrhea, fever, bronchitis, liver complaints, anaemia, etc. (Mishra and Bhatia, 2014;

Goyal *et al.*, 2012). Screening of plant extracts for antimicrobial activity has demonstrated that higher plants epitomize a potential source of novel antibiotic prototypes. Antimicrobial activity of some members of genus *Ziziphus* has already been reported in the literature (Abalaka *et al.*, 2010; Ahmad *et al.*, 2011; Dangoggo *et al.*, 2012; Dhunmati *et al.*, 2013). For instance, the seed extract of *Z. mauritiana* was stated to demonstrate antiplasmodial effects (Mishra and Bhatia, 2014). Further, the fruit extract of *Z. rugosa* Lam. was determined to undertake cytotoxic, antibacterial and free radical scavenging activity (Hossain *et al.*, 2013). Present study was aimed to investigate the antibacterial potential of different solvent extracts of the barks of *Z. mauritiana* and *Z. xylopyra* for its ethnomedical use in the treatment of infectious diseases.

Materials and Methods

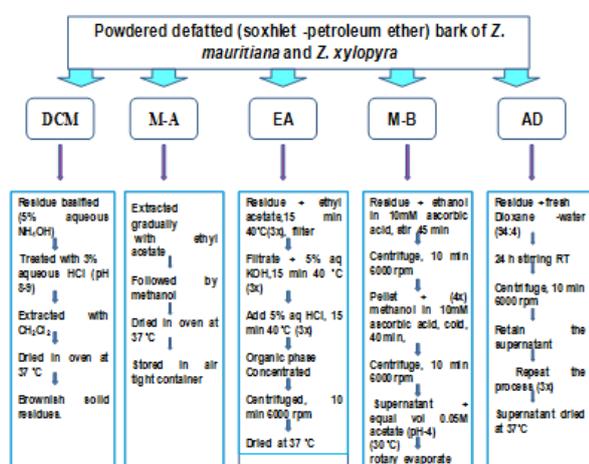
Authentication of plant materials

Ziziphus mauritiana Lam. and *Ziziphus xylopyra* (Retz.) Willd. were identified through ethnobotanical approach by the taxonomist of Department of Botany, RTM Nagpur University, Nagpur, Maharashtra, India. A sample specimen was conserved with the voucher number as RTMNU BD 9138 and 9139, respectively.

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Preparation of extract

Bioactive compounds from the bark of above referred species were processed using different solvent (Fig 1) to isolate various extracts.



Crude solvent extracts as DCM – Dichloromethane, EA – Ethyl acetate, MA – methanol, MB – methanol containing ascorbic acid, AD – aqueous-dioxane

Figure 1. Outlines of extraction procedure followed for different *Ziziphus* species extract. Solvent extracts of DCM- dichloromethane, M-A- methanol, EA- ethyl acetate, M-B- methanol containing ascorbic acid, AD- aqueous-dioxane

A method directing acid-base treatment was followed to achieve ethyl acetate solvent extract of *Z. mauritiana* (ZMEA) and *Z. xylopyra* (ZXEA) as mentioned by Cordeiro *et al.* (1999). Another procedure track successive soxhlet extraction as cited by Uddin *et al.* (2003) to with ethyl acetate, concluding to get methanol solvent extract of *Z. mauritiana* (ZMM-A) and *Z. xylopyra* (ZXM-A). Isolates were obtained as brownish crude residue with dichloromethane solvent extract of *Z. mauritiana* (ZMDCM) and *Z. xylopyra* (ZXDCM), as revealed by Perez *et al.* (2004). Extraction method (Hagerman, 2002) started with ethanol containing ascorbic acid (10 mM) at low temperature followed by methanol containing ascorbic acid (10 mM) as solvent extract of *Z. mauritiana* (ZMM-B) and *Z. xylopyra* (ZXM-B). A solvent mixture of dioxane-water (96:4, v/v) was also pursued to isolate aqueous-dioxane solvent extract of *Z. mauritiana* (ZMAD) and *Z. xylopyra* (ZXAD), by the method of Ramasamy (1982).

Phytochemical screening

Phytochemical screening of *Z. mauritiana* and *Z. xylopyra* extracts was performed by standard methods (Harborne, 1973; Trease and Evans, 1978).

Test isolates

Antibacterial activity of *Z. mauritiana* and *Z. xylopyra* extracts was determined against Gram positive microorganisms using *Bacillus megatherium* (NCIM 2087), *Bacillus subtilis* (NCIM 2479), *Klebsiella aerogenes* (NCIM 2239), *Staphylococcus aureus* (NCIM 2079) and Gram negative microorganisms as *Escherichia coli* (NCIM2572), *Proteus vulgaris* (NCIM 2027), procured from National Collection of Industrial Microorganisms, Pune (India).

Determination of antimicrobial activity

Agar well bioassay and even disc diffusion assay was performed to investigate antibacterial activity of *Z. mauritiana* and *Z. xylopyra* extracts, as mentioned by Berghe with slight modification (Berghe and Vlietinck, 1991; McFarland, 1907). Filter sterilized samples solubilized in dimethylsulphoxide (DMSO, 0.05%) were loaded onto the test strain seeded in Mueller Hinton II agar plates and incubated at 37°C for 24 h with DMSO (0.05%) as negative control and standard antibiotic amoxycillin and gentamycin as positive control. Antibacterial activity was determined by measuring the diameter of the zone of inhibition. Experiments were performed in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined for *Ziziphus* extracts showing efficacy with the sensitive bacterial isolates by a modified agar well diffusion technique (Okeke *et al.*, 2001). A dilution of extracts with a decreasing concentration range from 10 to 1 mg/ml in DMSO was loaded onto Mueller Hinton Agar plates seeded with test bacterial cells for least concentration of extract showing a clear zone of inhibition, in triplicate.

Gas chromatography – Mass spectrometry

A Varian 4500 GC coupled with Varian MS240 ion trap mass spectrometer (Varian, Walnut Creek, USA) was employed for the determination of analytes using electron ionization (EI) mode. Split less injections of 1 μl volume were carried out with a split promable temperature injection (STI) Type 1079 kept at 270°C . The Ion trap, manifold and the transfer line were kept at 240, 40 and 250°C , respectively. Separations were performed on Varian Chrompack Capillary column WCOT Fused Silica (30 m long, 0.25 mm ID) CP-Sil 8CB, helium (Ultra pure 99.99%) was employed as a carrier gas. Compounds were identified by direct comparison of their MS (Mass Spectrum) with data from the NIST library.

Table 1. Antibacterial activity of different *Ziziphus mauritiana* extracts evaluated against the test organisms

Test organisms	ZMDCM extract	ZMEA extract	ZMM-A extract	ZMM-B extract	ZMAD extract	Gentamycin (10mcg)	Amoxicillin (30 mcg)
<i>Bacillus megatherium</i>	11	11	12	12	7	23	24
<i>Bacillus subtilis</i>	12	13	14	14	9	22	18
<i>Escherichia coli</i>	12	12	16	15	10	22	24
<i>Klebsiella aerogenes</i>	8	9	11	9	7	22	14
<i>Proteus vulgaris</i>	10	9	12	11	9	14	12
<i>Staphylococcus aureus</i>	13	14	22	16	11	22	36

The diameters of zone of inhibition (mm) are expressed as mean (n=3). Solvent extracts of ZM- *Z. mauritiana* as DCM- dichloromethane, M-A- methanol, EA- ethyl acetate, M-B- methanol containing ascorbic acid, AD- aqueous-dioxane

Table 2. Antibacterial activity of different *Ziziphus xylopyra* extracts evaluated against the test organisms

Test organisms	ZXDCM extract	ZXEa extract	ZXM-A extract	ZXM-B extract	ZXAD extract	Gentamycin (10mcg)	Amoxicillin (30 mcg)
<i>Bacillus megatherium</i>	10	9	12	11	7	23	24
<i>Bacillus subtilis</i>	11	9	16	14	9	22	18
<i>Escherichia coli</i>	8	10	15	12	--	22	24
<i>Klebsiella aerogenes</i>	10	8	11	11	8	22	14
<i>Proteus vulgaris</i>	8	8	11	10	--	14	12
<i>Staphylococcus aureus</i>	12	13	18	13	11	22	36

The diameters of zone of inhibition (mm) are expressed as mean (n=3). Solvent extracts of ZX- *Z. xylopyra* as DCM- dichloromethane, M-A- methanol, EA- ethyl acetate, M-B- methanol containing ascorbic acid, AD- aqueous-dioxane

Results

Phytochemical analysis revealed existence of alkaloids, flavonoids, triterpenoids and tannins in the bark extract of *Z. mauritiana* Lam. and *Z. xylopyra* (Retz.) Willd. Investigation showed that almost all the *Ziziphus* bark extracts retain significantly diverse antibacterial activity against the test organisms compared to standard antibiotics (Table 1 and 2) with significant activity towards Gram positive bacteria than the Gram negative test strains.

ZMM-A and ZXM-A extracts exhibited greater activity against Gram positive *S. aureus* (22mm and 18mm, respectively), whereas ZMAD extract witnessed nil action against Gram negative bacteria. Overall study noticeably illustrates *S. aureus* with relatively higher susceptibility against all the extracts and lowest MIC as demonstrated by ZMM-A (1.5 mg/ml), as presented in Table 3.

Z. mauritiana (ZMM-A extract- 22mm) gave fairly equivalent and convincing results to that of standard antibiotics (gentamycin-22 mm), demonstrating identically susceptibility potential on *S. aureus*. Advance study would certainly expose facts

between the potential of actual bioactive compounds involved and a synergistic outcome of the extracts.

GC-MS performed for the most potent ZMM-A illustrated presence of compounds as 2H-1-Benzopyran-2-one, 9,10-Anthracenedione, 4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-, 3,4-Dihydroisoquinolin-7-ol, stigmasterol, Olean-12-ene-3 β ,15 α ,16 α ,21 β ,22 α ,28-hexol,-methoxy-3,3-dimethyl-1-methylsulfanyl-, Stigmastane-3,6-dione, (5 α -), Olean-12-ene-3 β ,15 α ,16 α ,21 β ,22 α ,28-hexol, 4H-1-Benzopyran-4-one,2-(3,4-dihydroxyphenyl)-7-(β -D-glucopyranosyloxy)-5-hydroxy- known for its medicinal properties. Analysis of ZXM-A extract by GC-MS showed presence of few reported therapeutic compounds as Lup-20(29)-en-3 β -ol, Stigmastane-3,6-dione, (5 α -), 4H-1-Benzopyran-4-one, 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-, 3,22-Dihydroxy-28-oxoolean-12-en-16-yl acetate, β - Sitosterol, 4H-1-Benzopyran-4-one, 7-(β -D-glucopyranosyloxy)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl).

Discussion

Existence of bioactive compounds in plants has made its extensive use in pharmacological research and drug development (Newman and Cragg, 2012). Our study witnessed *Z. mauritiana* and *Z. xylopyra*

Table 3. Minimum inhibitory concentration (MIC) of different *Z. mauritiana* and *Z. xylopyra* bark extracts against the most susceptible experimental microorganisms

<i>Ziziphus</i> species	<i>Ziziphus</i> extracts	Experimental microorganisms	MIC values (mg/ml)
<i>Z. mauritiana</i>	ZMM-A	<i>S aureus</i>	1.5
	ZMM-A	<i>E coli</i>	2.5
	ZMM-B	<i>E coli</i>	3
	ZMM-B	<i>S aureus</i>	2
<i>Z. xylopyra</i>	ZXM-A	<i>S aureus</i>	2.5
	ZXM-A	<i>B subtilis</i>	3.5
	ZXM-A	<i>E coli</i>	5
	ZXM-B	<i>B subtilis</i>	3.5
	ZXEA	<i>S aureus</i>	3

The diameters of zone of inhibition are expressed as mean \pm SEM (n=3)

bark extracts illustrating competent inhibitory effects against the test isolates (Table 1 and 2). This is in agreement with the work showing secondary metabolites from *Ziziphus* species with broad spectrum of antibacterial activity (Goyal *et al.*, 2012; Najafi, 2013).

Major facet influencing hinderance in the treatment of multi-factorial infection involves association of *Escherichia coli*, *Staphylococcus*, *Bacillus*, *Pseudomonas* and *Proteus* species. Bacterial test organism which includes *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella aerogenes* are recognized to be associated with gastro-intestinal diseases, infections of the skin, urinary tract and respiratory tract. Hence these test organisms were opted pertaining to its pathogenic and clinical encountered aspect (Adetutu *et al.*, 2011; Dangoggo *et al.*, 2012).

ZMM-A and ZXM-A extracts have demonstrated higher antibacterial activity than any other extracts tested corroborating effective methanol extract. Our results concur with literature evidence pertaining to alcohol as more consistent extraction solvent of antimicrobial substances from medicinal plants (Abalaka *et al.*, 2011). Difference in activities observed amongst species can be associated with diversity of bioactive compounds under influence of genetic features and environmental aspects (Ngoci *et al.*, 2014). Some extracts illustrated less susceptibility against Gram negative bacteria might be due to the fact convincing the presence of barrier against various antibiotic molecules and the enzymes able to degrade exogenous molecules in its membrane (Monte *et al.*, 2014). Successive isolation of compounds from plant material is largely dependent on the type of solvent used and the extraction procedure followed. GC-MS observations with potent extracts showed existence

of documented bioactive compounds predominantly in *Z. mauritiana* than *Z. xylopyra* extracts possessing therapeutic properties (Al Muqarrabun *et al.*, 2013; Sabandar *et al.*, 2013).

Conclusion

Present study extends the potential value of *Ziziphus* stem bark, predominantly *Z. mauritiana* for the treatment of wounds, stomach distress and urinary tract infections, exemplified to inhibit causative isolates. Antimicrobial potency could be evidently allied with existing bioactive phytochemicals of plants. We therefore propose further analysis with purified samples to validate our claim.

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