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Antimicrobial Efficiency of Phytochemical Constituents of *zornia gibbosa* (L.) Pers.

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Abstract: *The phytochemical investigation of the whole plant of Zornia gibbosa revealed the presence of secondary metabolites. The antimicrobial efficiency and minimum inhibition concentration values were determined for these phytochemical constituents as crude extracts using the agar well diffusion and two-fold serial dilution methods, respectively. The results indicated that Klebsiella pneumoniae was the most susceptible bacterium with high inhibition zones for the methanol and chloroform extracts of 30 mm and 24 mm, respectively. The minimum inhibitory concentration values indicated that extracts possess good phytochemicals with significant minimum inhibitory concentration value against Klebsiella pneumoniae and Escherichia coli at 15.6 µg/ml concentration. Gram-negative bacteria were more susceptible than the Gram-positive. The present study suggests that Zornia gibbosa whole plant possess potent antimicrobial efficiency and can be a good source for the development of new antibiotics.*

Keywords: *Zornia gibbosa, Solvent extract. Phytochemicals. Antimicrobial efficiency.*

1. INTRODUCTION

Medicinal plants have been identified and used throughout human history. They have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to depend against attack from predators such as insects, fungi and bacteria. The world health organization (WHO) estimates 80 percent of the population of Asia and African countries presently use herbal medicine for some aspects of primary health care. Studies in United States and Europe have shown that their use is less common in clinical settings, but has become increasingly more common in recent years as scientific evidence about the effectiveness of herbal medicine has become more widely available. The annual global export value of pharmaceutical plants in 2011 accounted for over US$2.2 billion [1]. India is rich in medicinal plant diversity because of agro climatic, ecological and edaphic conditions are found. In India, from ancient times, different parts of plants have been used to cure specific ailments. The increased interest in medicinal plant cures is because, primarily plants as medicines are safe, less rigorous and more affordable than synthetic drugs. Thus, there is a constant and urgent need to develop new antimicrobial drugs from medicinal plants for the treatment of infectious.

Zornia gibbosa L. (Fabaceae) is an annual herb, branching or straggling to 30 cm high, of dry sandy locations, commonly throughout the region from Senegal to N and S Nigeria, and widespread over tropical and southern Africa and Madagascar (Fig. 1). It is a common in open meadows on gravelly soil and in waste lands. Leaves are taken as a laxative and cooked leaves are given to children suffering from kwashiorkor. In Cango sap from the plant used as eye drops against epilepsy and the root is eaten as an aphrodisiac. In Zimbabwe the roots are used to treat venereal diseases, to prevent abortion and to ease children [2].Therefore, the present study is focused to assess the *in vitro* antimicrobial efficiency and minimum inhibitory concentration (MIC) of *Z. gibbosa* against selected microorganisms followed by preliminary phytochemicals in the whole plant extracts responsible for antimicrobial efficiency.

2. MATERIAL AND METHODS

2.1. Plant material

The whole plant of *Z. gibbosa* was collected from Kambalaakonda forest, Visakhapatnam district, Andhra Pradesh, India. The specimen was authenticated by Prof. Vatsavaya S. Raju, Plant Systematics Lab, Kakatiya University, Warangal and voucher specimen (L. Mutyala Naidu –3672) was deposited in the Herbarium of Botany Department (BDH), Andhra University, Visakhapatnam, India. The collected whole plant made into small pieces and dried in the shadow until it gets dried completely. Then it was powder in the mixture grinder and stored in airtight bottles.

2.2. Extraction of plant

The shade dried and powdered material (10 g) was extracted with hexane, chloroform, followed by methanol by using sequential extraction method [3]. Thereafter it was filtered by rotary evaporator at 40 ^{0}C and to give the crude dried extract. Simultaneously, the aqueous extract of the whole plant was prepared by adding boiled water to the powdered in a beaker on water bath, with occasional stirring for 4 hrs. The aqueous extract was then filtered and centrifuged at 5000 rpm for 15 min. The supernatant was collected and evaporated to dryness to give the crude dried extract. The extracts were dissolved in DMSO to get the known concentrations of 25 mg/ml, 50 mg/ml and 100 mg/ml.

2.3. Microbial strains

The tested microbial strains used in the study procured from Microbial type culture and collection (MTCC), Chandigarh, India. The bacterial and fungal strains were *Bacillus subtilis* MTCC B2274, *Enterococcus faecalis* MTCC B3159, *Escherichia coli* MTCC B1560, *Klebsiella pneumoniae* MTCC B4030, *Micrococcus luteus* MTCC B1538, *Pseudomonas aeruginosa* MTCC B22927, *Proteus vulgaris* MTCC B7299, *Staphylococcus aureus* MTCC B3160, *Streptococcus pneumoniae* MTCC B2672, *Aspergillus niger* MTCC F4325, *Candida albicans* MTCC F7315 and *Saccharomyces cerevisiae* MTCC F2567. The bacterial strains were grown in the nutrient broth and maintained on nutrient agar slants at 4 ^{0}C while fungal strains were grown in Sabouraud broth and maintained on Sabouraud agar slants (*C. albicans* and *S. cerevisiae*) and potato dextrose agar slants (*A. niger*) at 4 ^{0}C.

2.4. Antimicrobial efficiency

The antimicrobial efficiency of hexane, chloroform, methanol and aqueous extracts of whole plant of *Z. gibbosa* was determined by agar well diffusion method [4] and agar disc diffusion method for standard antibiotics. The lyophilized culture was sub cultured and concentration of working stock culture was assessed as 10^{-6} CFU/ml. For susceptibility test, 100 µl of inoculum, was mixed with 6 ml of sterilized nutrient agar and poured immediately into the sterile petridishes. The petridishes were left to solidify for 10 minutes. A sterilized 6 mm metal borer was used to make wells in the centre of the divided areas. About 50 µl of each extract was then pipette into the wells. The petridishes were incubated at 28 ^{0}C for 24 hrs. The experiment was done three times to minimize error. After incubation period the antimicrobial efficiency was evaluated by measuring the inhibition zones by using an antibiotic zone reader scale (HiAntibiotic Zonescale-c).

For the fungal strains, the same method as for bacteria was adopted of nutrient agar, Sabouraud agar was used. The inoculated petridishes were incubated at 25 ^{0}C for two days for the *C. albicans, S. cerevisiae* and three days for *A. niger*. About 500 µg of Nystatin was dissolved in 1 ml of sterile deionized water. About 10 µl of 0.5 mg/ml of nystatin (equivalent to 5 µg dose) pipette into the wells for comparison with fungal inhibition zones. The bacterial inhibition zones were compared with tetracycline disc (5 µg /disc) of multidrug disc (Axiom Laboratories Ltd. India). About 50 µl of DMSO was pipette into each well for bacteria and fungi as a negative control.

The extracts that exhibited inhibition zones were subjected to MIC assay by using two-fold serial dilution [5]. A quantity of 0.6 g of each extract was dissolved in 300 ml sterile nutrient broth which yields initial concentration of 2000 µg/ml. Subsequently, two-fold serial dilution was made from the stock to obtain 1000, 500, 250, 125, 62.5, 31.2, 15.6 µg/ml concentrations. One ml of standardized inoculums of each test organism was introduced into each extract nutrient broth mixture and then incubated at 37 ^{0}C. The lowest concentration inhibiting growth was regarded as the MIC of the extracts. For the fungi, the inoculated medium was incubated at 25 ^{0}C for two (*C. albicans* and, *S. cerevisiae*) to three (*A. niger*) days.

2.5. Statistical analysis

Each experimental data from triplicates of standard error was subjected to one way ANOVA using Minitab version 15. A significant level of $p < 0.001$ was used for all statistical analyses.

3. RESULTS AND DISCUSSION

The antimicrobial efficiency of the three different concentrations of hexane, chloroform, methanol and aqueous extracts of whole plant of *Z. gibbosa* revealed that significant antimicrobial efficiency against tested nine bacterial and three fungal strains (Table 1). Methanol extract exhibited the highest, while aqueous and chloroform showed moderate; and least antimicrobial efficiency for hexane extracts of whole plant of *Z. gibbosa* (Fig. 2 to 5). The antimicrobial efficiency was increased with increasing concentration of extracts. The results of the present study were significant at level of $p>0.05$.

The zone of inhibitions indicated that *Klebsiella pnemoniae* was the most susceptible bacterium with high inhibition zones for the methanol and chloroform extracts of 30 mm and 24 mm, respectively. Hexane extract showed high zone of inhibition against *Staphylococcus aureus* and *C. Albicans* whereas aqueous extract against *S. Cerevisiae* followed by *S. Pneumonia* and *C. Albicans* . The aqueous extract did not shown inhibition zones against *B. Subtilis, Aspergillus niger* and *P. vulgaris*. The fungal strain *Aspergillus niger* also resistant to hexane, chloroform and aqueous extracts. Methanol and chloroform extracts exhibited zone of inhibition values more or similar to standard antibiotics (Fig. 6), whereas DMSO, a negative, control did not shown growth inhibition of bacteria and fungi.

From the MIC values (Table 2), it was observed that *K. pneumonia,* and *S. aureus* exhibited the least MIC value for methanol extract, while chloroform extract showed the against least MIC value against *B. subtilis*; and hexane extract against *S. aureus* and aqueous extract against *C. albicans*. The preliminary phytochemical analysis revealed the presence of alkaloids, aminoacids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, steroids, tannins and terpenoids in the hexane, chloroform, methanol and aqueous extracts of whole plant of *Z. gibbosa* (Table 3).

Preliminary phytochemical analysis was noticed that in some cases chemical constituents fail to answer due to trace amount or other reason. The extract obtained from successive solvents is subjected to phytochemical tests to reveal presence of different phytochemicals especially the primary and secondary metabolites present in the extract. The phytochemicals present in extracts responsible for the antimicrobial efficiency by inhibiting the growth of microorganisms. Different solvents have been reported to have the capacity to extract different phtochemical constituents depending on their solubility or polarity and property of the solvent.

Whole plant of Z. *gibbosa* exhibited varying degree of broad spectrum antimicrobial efficiency against tested microorganisms. Plants have a wide range of compounds viz. alkaloids, steroids, saponins, tannins, phenols, flavonoids, quinines, terpenes, terpenoids, glycosides, carbohydrates, amino acids etc. which are responsibility for bioefficiency. Antimicrobial efficiency of aqueous, hexane and chloroform extracts showed variable antimicrobial efficiency and that could be attributed to the presence of phenols and sterols as such activities with these compounds are reported [6,7]. The antimicrobial efficiency of methanol extract may be due to the presence of tannins, triterpenoids and flavonoids. Tannins have been known to form irreversible complexes with proline rich protein resulting in the inhibition of cell wall synthesis [8]. Triterpenoids are known to weaken the membranous tissue, which results in dissolving cell wall of microorganism [9]. Flavonoids have ability to bind with extracellular and soluble proteins and complexes with microbial cell walls. Antimicrobial efficiency of steroids is specifically associated with membrane lipids and cause leakage from liposoomes [10]. The antifungal efficiency may be contributed due to the presence of coumarins and this is supported by earlier work [11] showing antifungal efficiency of herbal plants containing coumarins.

The results revealed that solvent and aqueous extracts possessed good antimicrobial efficiency. Among extracts methanol extract showed the higher degree of inhibition zones against tested microorganisms. This is accordance with the previous study reported that methanol is the most effective solvent for plant than hexane and chloroform [12]. All the extracts root extracts worked in a dose dependent manner, as the concentration of the extract was increased the efficiency also increased. This is due to susceptibility of the microorganisms towards concentration of the extracts, after which the extract damages that microbe which is not tolerable for it [13].

It is clear from the present study that the Gram positive bacteria have found to be the most resistant than Gram negative bacteria. Generally Gram positive bacteria are more susceptible to commercial antibiotics, crude extracts and isolated compounds from natural sources, which may be related to cell wall structure. According to Tortora et al [14] the cell wall of Gram negative bacteria acts as a barrier to a number of substances, including antibiotics.

4. CONCLUSIONS

From the results obtained, the present study highlights the significant antimicrobial efficiency of methanol extracts of whole plant of Z. *gibbosa* against selected bacterial and fungal strains. The study serves as a preliminary scientific validation of Z. *gibbosa* methanol extract as an important source for development of therapeutic antimicrobial compounds against infectious pathogenic microbial strains. However the exact structure of chemical components and

mode of action of phytochemicals are currently not clear. Our next line of further investigations is fractionation, purification and characterization of the bioactive components in the methanol extracts of whole plant of Z. *gibbosa*.

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Table 1. Antimicrobial efficiency of whole plant of *Z. gibbosa*

Organisms	Zone of inhibition (mm)[a]												S	D
	Hexane extract (mg)			Chloroform extract (mg)			Methanol extract (mg)			Aqueous extract (mg)				
	25	50	100	25	50	100	25	50	100	25	50	100		
B. subtilis	-	12±0.50	15±0.90	22±0.50	24±0.50	26±0.44	24±0.90	24±0.64	26±0.28	-	-	-	18^{T}	–
E. coli	-	10±0.28	13±0.28	11±0.10	12±0.60	18±0.16	15±0.50	20±0.90	24±0.45	11±0.52	12±0.52	14±0.28	22^{T}	–
K. pneumoniae	10±0.20	12±0.50	12±0.90	15±0.76	19±0.50	24±0.44	24±0.90	26±0.90	30±0.50	16±0.50	18±0.50	20±0.50	24^{T}	–
P. aeruginosa	11±0.45	13±0.44	15±0.19	16±0.19	18±0.90	20±0.16	15±0.28	17±0.19	19±0.45	10±0.90	12±0.90	14±0.44	25^{T}	–
P. vulgaris	-	10±0.08	12±0.28	12±0.76	14±0.19	16±0.50	13±0.52	17±0.76	24±0.52	-	-	-	22^{T}	–
S. aureus	10±0.20	12±0.50	16±0.20	12±0.20	15±0.20	18±0.90	14±0.45	21±0.50	25±0.50	10±0.28	12±0.19	14±0.50	24^{T}	–
S. pneumoniae	-	-	-	11±0.22	13±0.22	19±0.22	14±0.90	19±0.90	24±0.22	12±0.20	16±0.20	21±0.20	22^{T}	–
E. fecalis	-	-	10±0.40	10±0.50	11±0.22	13±0.90	12±0.64	15±0.28	16±0.90	10±0.44	11±0.52	13±0.50	22^{T}	–
M. luteus	-	-	-	10±0.20	12±0.20	14±0.20	12±0.28	14±0.76	16±0.28	10±0.20	12±0.20	14±0.20	22^{T}	–
A. niger	-	-	-	-	-	-	12±0.20	16±0.20	22±0.20	-	-	-	18^{N}	–
C. albicans	10±0.16	12±0.10	16±0.46	12±0.90	13±0.44	19±0.44	17±0.64	21±0.76	25±0.45	18±0.44	20±0.45	21±0.44	23^{N}	–
S. cerevisiae	-	10±0.60	12±0.16	10±0.20	10±0.16	12±0.22	15±0.19	18±0.22	21±0.45	20±0.22	22±0.45	24±0.19	20^{N}	–

a: Each value is the mean of three replicates with standard deviation; P value is <0.05 extremely significant when compared with standard; S: Standard antibiotics; T-Tetracycline; N- Nystatin; D: DMSO; –: No efficiency.

Table 2: MIC values of different extracts of whole plant of *Z. gibbosa*

Microorganisms	Hexane extract	Chloroform extract	Methanol extract	Aqueous extract
B. subtilis	500	31.2	31.2	1000
E. coli	>1000	>1000	31.2	>1000
K. pneumoniae	>1000	125	15.6	250
P. aeruginosa	1000	62.5	250	1000
P. vulgaris	1000	1000	500	>1000
S. aureus	62.5	62.5	15.6	>1000
S. pneumoniae	>1000	1000	125	>1000
E. fecalis	1000	1000	250	1000
M. luteus	1000	>1000	500	>1000
A. niger	>1000	>1000	1000	>1000
C. albicans	125	1000	125	125
S. cerevisiae	>1000	>1000	500	>1000

Table 3: Phytochemical constituents of whole plant of *Z. gibbosa*

Phytochemical constituents	Hexane extract	Chloroform extract	Methanol extract	Aqueous extract
Alkaloids	+	+	+	+
Amino acids	+	+	+	+
Anthraquinones	-	-	+	-
Carbohydrates	-	+	+	+
Cardiac glycosides	-	+	+	-
Flavonoids	-	-	+	+
Glycosides	-	+	+	-
Phenols	-	-	+	+
Saponins	-	+	+	+
Steroids	+	+	+	-
Tannins	-	-	+	+
Terpeniods	+	+	+	-

Fig. 1. *Zornia gibbosa*

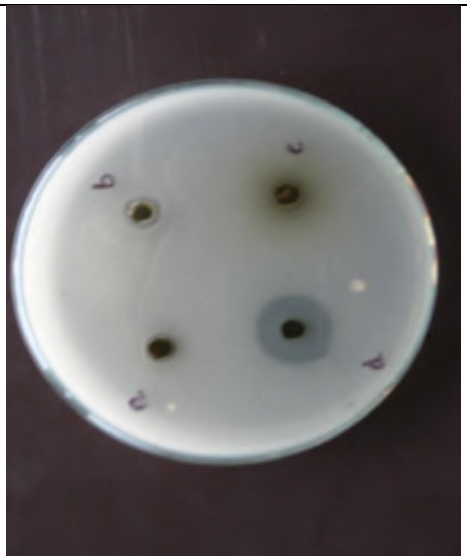

Fig. 2. Inhibition zones against *E. Coli*

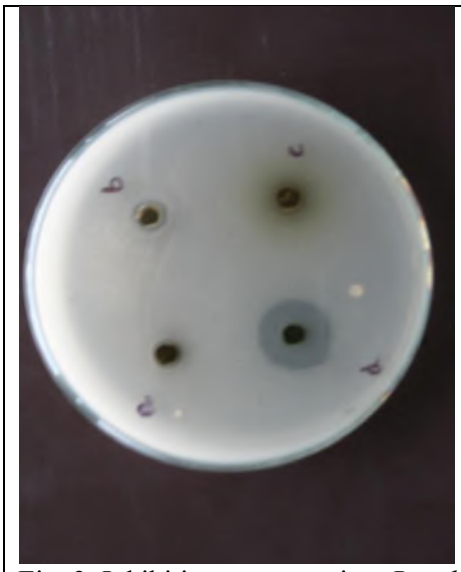

Fig. 3. Inhibition zones against *B. subtilis*

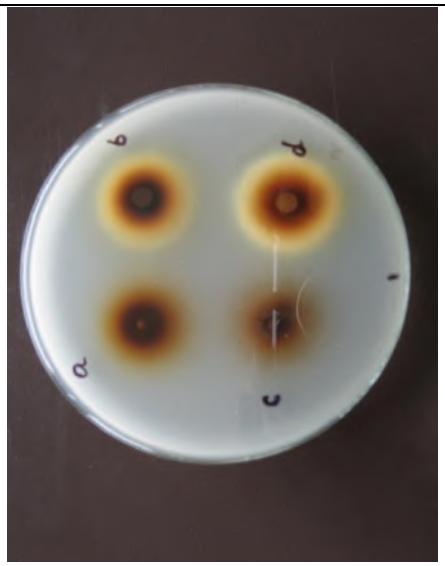

Fig. 4. Inhibition zones against *S. aureus*

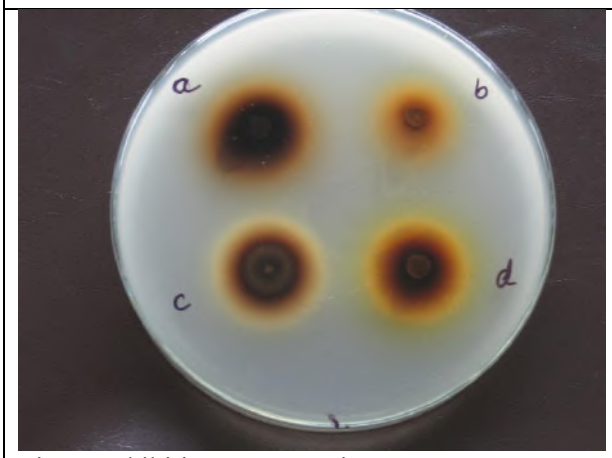

Fig. 5. Inhibition zones against *K. pneumoniae*

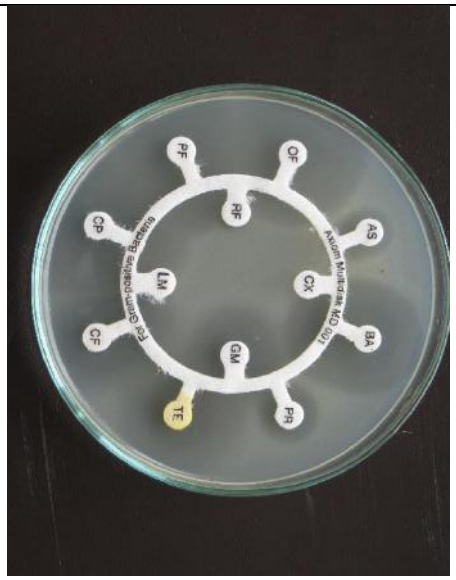

Fig. 6. Inhibition zones against multi drug strip