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Research Article

ANTICRYPTOCOCCAL ACTIVITY OF ALKALOID RICH FRACTION OF LEAVES OF *PROSOPIS JULIFLORA* - A FUTURE PROMISING SUPPLEMENTARY THERAPY FOR CRYPTOCOCCOSIS AND CRYPTOCOCCAL MENINGITIS?

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ABSTRACT

Objective: To study the antifungal activity of alkaloids from leaves of *Prosopis juliflora* against clinical and environmental isolates of *Cryptococcus neoformans* and to identify the bioactive compounds by GC-MS & FTIR.

Methods: The alkaloid rich fraction obtained from the leaves of *P. juliflora* was assessed for their antifungal activity against clinical and environmental isolates of *Cryptococcus neoformans*. Alkaloids were separated from the leaves by standard techniques and HPTLC profile of the alkaloids was performed. The alkaloids were identified by GC-MS analysis and the functional groups were identified by FTIR.

Results: Of the 10 isolates tested, the clinical isolates were more susceptible than the environmental isolates. Isolate C3 was found to be more susceptible with the zone of inhibition 17mm at a concentration of 200 µg. Mass spectrometric analysis revealed the presence of piperidine alkaloids. FTIR analysis was performed to identify the chemical groups constituting the bioactive compounds.

Conclusion: Alkaloids from leaf extract of *P. juliflora* was found to possess antifungal activity and have the potential to inhibit drug resistant fungal strains. GC-MS analysis revealed that leaves of *P. juliflora* are rich source of piperidine alkaloids.

Keywords: *Prosopis juliflora, Cryptococcus neoformans, Cryptococcal meningitis*, Antifungal, Piperidine.

INTRODUCTION

The spread of multidrug resistant strains of fungus resistant to the available antifungal drugs makes it necessary to discover new classes of antifungals and compounds that can inhibit these resistant mechanisms. This has led to a search for therapeutic alternatives particularly among medicinal plants and compounds isolated from them and empirically used for their antifungal properties. These plants provide a rich natural source of a series of molecules with antifungal activity against different strains of fungus.

The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections. The challenge has been to develop effective strategies for the treatment of candidiasis, cryptococcal meningitis and other fungal diseases, considering the increase in opportunistic fungal infections in HIV and other immune compromised patients. The majority of clinically used antifungal have various drawbacks in terms of toxicity, efficacy and cost and their frequent use has led to the emergence of resistant strains.

The emergence of antimicrobial resistance in microbes due to indiscriminate use of chemicals as antifungals requires the need to look for alternative sources of antimicrobial agents. One of the possible strategies towards achieving such a goal is identification of bioactive phytochemicals having antifungal activity.

Plants have always been a source of natural product for the treatment of various diseases. People in developing countries utilize traditional medicine for their primary health care needs but despite having a wide historical background, there are only a handful of plants that have been exhaustively studied for their potential value as a source of drug [1].

Many researchers reported that the concentration of secondary metabolites varies from one plant species to another and even in different parts of the same plant. Therefore it becomes imperative to analyze antifungal activities of different plants so that their pharmacological potential can be exploited for supplementary therapy in modern medicine.

Prosopis juliflora, a member of family leguminosae is found in arid and semiarid regions of India and other countries. It has been used as a folk remedy for catarrh, cold, sore throat and in healing of wounds. Leaf and seed extract is used as a disinfectant in wound healing and also in treating scurvy [2]. Tea made from *P. juliflora* is thought to be good for digestive disorders and skin lesions. *P. juliflora* is a rich source of piperidine alkaloids. Many alkaloids such as juliflorine, julifloricine, juliprosine, juliprosonine, juliflorinine, 3'-oxojuliprosine and 3'-oxojuliprosopine have been isolated from leaves and have proven to be pharmacologically active.

The present work aims at screening alkaloid rich fractions obtained from ethanolic leaf extract of *P. juliflora* for antifungal activity against clinical and environmental isolates of *C. neoformans* and its activity was compared with a standard antifungal agent. GC-MS & FTIR was carried out to identify the alkaloids present in the extract.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves of *P. juliflora* were collected from Sholinganallur, near Chennai, India. The plant was authenticated by a Botanist at the Research and Development Centre, Cholayil Private Limited, Chennai, India. All the samples were dried in shade for two weeks and pulverized using grinder. Powdered samples were taken for study.

Estimation of alkaloids

10g of powdered sample was mixed with 5ml of ammonia (25%) and extracted with 250 ml of ethanol in soxhlet apparatus for 6-8 hrs. The final extract was cooled, filtered and the filtrate was concentrated to 20ml on water-bath at 60°C and transferred to a separating funnel and diluted with distilled water. The aqueous ethanolic extract was acidified with diluted sulfuric acid to pH 3-4 and extracted with chloroform to remove lipophilic, acidic and neutral compounds. After basifying the aqueous solution to pH 9-10 with ammonia, it was extracted with chloroform and the combined chloroform extract was washed with distilled water to neutral pH.

The aqueous solution was then dried with sodium sulphate anhydrous and concentrated on water bath to obtain crude alkaloids. Total weight of the crude alkaloids was recorded. This was made up to 5ml and used for TLC profile comparison.

HPTLC profile of crude alkaloids of *P. juliflora*

High performance thin layer chromatography (HPTLC) was performed to check the profile of alkaloids extracted from *P. juliflora*. 10 µl of the above extract was spotted on pre-coated silica gel $60F_{254}$ (E.Merck) TLC after activation at 105°C. Then the spotted plates were developed in a pre-saturated chamber containing mobile phase Chloroform : Methanol : Ammonia (7.5 : 2.5 : 5) for separation. Developed plate was observed under UV 260 nm, 550 nm and the chromatogram was recorded. The plates were sprayed with Dragendroff's reagent to develop the color of the spots. After color development, the plate was observed and the chromatogram recorded.

Rf (Retention factor) = migration distance of the substance/ migration distance of the solvent

Anticryptococcal activity of alkaloid fraction extracted from ethanolic leaf extract of *P. juliflora*

The anticryptococcal activity of crude alkaloids was evaluated by disc diffusion method of NCCLS - National Committee for Clinical Laboratory Standards [3]**.** Activated cultures of 10 isolates of *C. neoformans* in Sabouraud Dextrose broth were adjusted to $1x10^8$ CFU/ml as per McFarland standard. 100 µl of the inoculum was introduced to molten Sabouraud dextrose agar and poured into sterile petri plates.

Sterile filter paper discs were impregnated with varying concentrations of alkaloids ranging from 50 µg to 200 µg per disc dissolved in DMSO and dried The discs were placed on yeast seeded plates and incubated at 37°C for 48hrs. Disc impregnated with only 100% DMSO served as negative control. Amphotericin B (100 units/disc) was used as positive control. Following an incubation period of 48hrs, plates were removed from the incubator and antifungal activity was evaluated by measuring zones of inhibition of fungal growth. Clear zone within which fungal growth was absent were measured and recorded as the diameter (mm) of complete inhibition. Experiments were conducted in triplicates.

GC-MS analysis of alkaloids from *P. juliflora*

The ethanolic extract was subjected to GC-MS analysis using the instrument GC-MS Shimadzu QP2010 with GC-MS solution version 2.53 software and Elite–DB-5M column. Initially oven temperature was maintained at 70°C for 2 minutes and the temperature was gradually increased up to 300°C at 10/35 minutes and 4µl of sample was injected for analysis. Helium gas of 99.995% purity was used as a carrier gas as well as eluent. The flow rate of helium gas was set to 1.5ml/minute, sample injector temperature was maintained at 260°C and the split ratio was 20 throughout the experiment period. Ionization mass spectroscopic analysis was done with 70eV. A mass spectrum was recorded for the mass range 40-1000 m/Z for about 30 minutes. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph, a fingerprint of the molecule [4]. The identification of compounds was based on the comparisons of their mass spectra with NIST library 2008, Wiley & Fame.

FTIR analysis was done to identify chemical groups present in the bioactive compounds. The samples were scanned using infrared in the range 5000-500 cm^{-1}. The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

RESULTS

HPTLC profile of crude alkaloids of leaves of *P. juliflora*

The leaf extracts were subjected to HPTLC for crude alkaloid separation in Chloroform: Methanol: Ammonia solvent system.

Ethanol extracts of *P. juliflora* showed band separation and their Rf values are tabulated in Table 1; Figure 1. The chromatogram of ethanol extracts recorded at 550nm showed 14 different peaks with different Rf values ranging from 0.05-0.86. Maximum peak height was shown by fraction 10 whose Rf valve was found to be 0.56 and the peak height 305.1with area 32.9 %. The chromatogram recorded at 260nm showed 9 bands with the last one showing an Rf value of 0.9 and maximum peak height 188.8 with area 51.4 %.

Table 1: HPTLC profile of crude alkaloids of *P. juliflora*

S. No.	Rf value at 550nm	Rf value at 260nm
1.	0.05	0.02
2.	0.08	0.15
3.	0.15	0.18
4.	0.19	0.20
5.	0.22	0.43
6.	0.26	0.58
7.	0.31	0.75
8.	0.42	0.85
9	0.48	0.90
10.	0.56	
11.	0.64	
12.	0.73	
13.	0.76	
14.	0.86	

Anticryptococcal activity of alkaloid fraction extracted from ethanolic leaf extract of *P. juliflora*

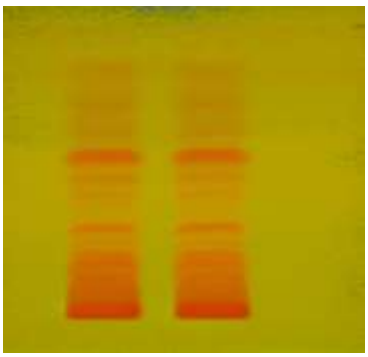

Fig. 1: It Shows HPTLC profile of crude alkaloids of *P. juliflora*

Fig. 2: Anticryptococcal activity of crude alkaloidfraction isolated from ethanolic leaf extract of P. juliflora against clinical isolates.

The alkaloid fraction obtained from ethanolic leaf extract of *P. juliflora* was tested for its antifungal activity against 5 clinical and 5 environmental isolates of *C. neoformans.* At the highest concentration tested (200µg), of all the 5 clinical isolates, maximum zone of inhibition was shown by C3 (17mm±0.24) followed by C4 (14mm±0.24), C2 (12±0.23) and C1 (11±0.3). C5 exhibited the least zone of inhibition (4±0.25) [Table 2; Figure 2].

Of all the 5 environmental isolates E5 showed maximum zone of inhibition (14±0.24) followed by E3 (13.5±0.24). The zone of inhibition for E1, E2 and E4 was less at (4±0.25), (4±0.25) and (3.5±0.25) respectively. [Table 3; Figure 3]

Table 2: Anticryptococcal activity of crude alkaloid fraction isolated from ethanolic leaf extract of *P. juliflora* against clinical isolates

S. No.	Isolates	Zone of inhibition in mm			
		50 µg	100 µg	150 µg	200 µg
1	C1	7±0.62	8±0.24	9±0.63	11±0.3
2	C2	6±0.63	9±0.63	11±0.25	12±0.23
3	C3	8±0.24	14±0.24	15±0.62	17±0.24
4	C4	9±0.63	11±0.25	12±0.23	14±0.24
5	C5	1±0.23	2±0.25	3±0.25	4±0.25

Note: Mean values of triplicates (zone of inhibition) ± SD

Table 3: Anticryptococcal activity of crude alkaloid fraction isolated from ethanolic leaf extract of *P. juliflora* against environmental isolates

S. No.	Isolates	Zone of inhibition in mm			
		50µg	100µg	150µg	200µg
1	E1	1±0.23	2.5±0.25	3±0.25	4±0.25
2	E2	2.5±0.25	3±0.25	3.5±0.25	4±0.25
3	E3	9±0.63	11±0.25	12.5±0.23	13.5±0.24
4	E4	1.5±0.62	2.5±0.25	3±0.25	3.5±0.25
5	E5	11±0.25	12±0.23	13±0.24	14±0.24

Note: Mean values of triplicates (zone of inhibition) ± S. D

Fig. 3: Anticryptococcal activity of crude alkaloid fraction isolated from ethanolic leaf extract of *P. juliflora* against environmental isolates.

GC-MS FTIR

GC-MS analysis was carried out on the alkaloid rich fraction separated from ethanolic leaf extract of *P. juliflora* and 23 different compounds were identified. The chromatogram showed 7 prominent peaks in the retention time range 5.014- 25.950 minutes. The largest peak at 21.420 retention time had a peak area 13.57% and the compound identified was 2,4-bis (1-phenylethyl) phenol. The second less prominent peak at 25.358 min retention time had the peak area 11.9% and the compound identified as 2,4,6 Tris-(1-phenylethyl) phenol.

The third less prominent peak at 20.940 min retention time with peak area 11.03% was also identified to be 2,4-bis (1-phenylethyl) phenol. The other compounds identified are dimethyl sulfone (9.87%), Benzothiazole (1.30%), AR-Ethylphenyl ether (6.00%), Zerombone (0.94%), 1,6,dimethyl-4-isopropylnaphthalene (1.65%) 7,9,-Di-tert-butyl-1-oxaspiro(4,5) deca - 6,8-diene-2,8-dione, Cassine (8.18%), 1,3,4,5-Tetraphenyl -1,3-dihydro-2H-imidazole-2-thione (0.56%),5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene 1,1',4,4'(10.81%). The results are presented in Table 4.The total ion chromatograph (TIC) showing the peak identities of the compounds identified have been given in Fig 4. FTIR results are presented in Table 5. The IR spectral qualities of the fraction indicated the presence of ketones, aldehydes carboxylic acids, amides, aromatic compounds and esters in their active components.

Table 4: **Identification of compounds present in alkaloid fraction of ethanolic leaf extract of *P. juliflora* by GC-MS analysis.**

S. No.	Peak No	Ret time	Area	Area %	Name of the compound
1	1	5.014	14637158	9.87	Dimethyl sulfone
2	2	8.268	1933796	1.30	Benzothiazole
3	3	10.200	12716421	8.57	1,1'Biphenyl
4	4	11.787	670128	0.45	2,4-DI-tert-butyl phenol
5	5	12.798	320559	0.22	Diethyl phthalate
6	6	14.272	8898909	6.00	AR-EthylPhenyl Phenyl Ether
7	7	14.506	1388598	0.94	Zerumbone
8	8	15.176	2449586	1.65	1,6-Dimethyl-4-isopropylnaphthalene
9	9	15.778	1203789	0.81	Diisobutylphthalate
10	10	16.332	609585	0.41	7,9,-Di-tert-butyl-1-oxaspiro(4,5)deca-6,8-diene-2,8-dione
11	11	16.722	2068532	1.39	Dibutyl Phthalate
12	12	20.811	16315674	11.00	2,4-bis(1-phenylethyl)phenol
13	13	20.940	16354024	11.03	2,4-bis(1-phenylethyl)phenol
14	14	21.016	886677	0.60	2,4-Diphenyl-6,7,8,9 –tetrahydro-5H-cyclohepta(d)pyrimidine
15	15	21.135	12140553	8.18	Cassine
16	16	21.420	20123317	13.57	2,4-bis(1-phenylethyl)phenol
17	17	21.792	904609	0.61	Monoethylhexyl Phthalate
18	18	25.358	16602425	11.19	2,4,6-Tris-(1-phenylethyl)phenol
19	19	25.482	16036011	10.81	5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'
20	20	25.631	831906	0.56	1,3,4,5-Tetraphenyl -1,3-dihydro-2H-imidazole-2-thione
21	21	25.846	362726	0.24	2,4-bis(1-phenylethyl)phenol
22	22	25.897	370982	0.25	2,4-bis(1-phenylethyl)phenol
23	23	25.950	505225	0.34	2,4-bis(1-phenylethyl)phenol

Table 5: It shows IR spectral qualities of fractions of ethanolic extract of *P. juliflora*

S. No.	Frequency Cm^{-1}	Nature of the structure
1	3005.15	Alkenyl C-H Stretch
2	2919.31	Alkyl C-H Stretch
3	2854.70	Alkyl C-H Stretch
4	1640.49	Amide C=O Stretch
5	1437.96	C-C stretch (in ring) aromatics
6	1408.06	C-C stretch (in ring) aromatics
7	1316.44	C–H wag (–CH_2X) alkyl halides
8	1021.33	C–N stretch aliphatic amines
9	953.82	=C–H bend alkenes
10	707.89	Aromatic C-H Bending
11	673.17	C(triple bond)C–H: C–H bend -alkynes
12	532.36	C–Br stretch - alkyl halides

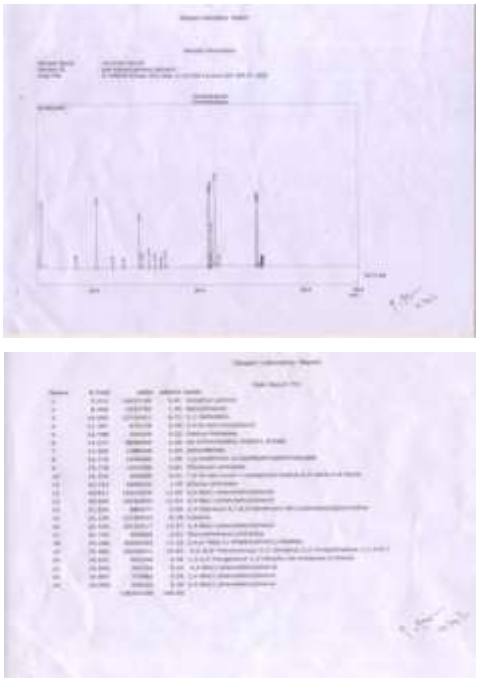

Fig. 4: It shows Gas chromatogram (TIC) of alkaloids present in ethanolic leaf extract of *Prosopis juliflora*

DISCUSSION

Cryptococcus neoformans has emerged as one of the leading fungal pathogen over the past 10 years mostly affecting individuals with compromised immune system. This may be due to increase in cases of AIDS, immunosuppression, prolonged steroid therapy and indiscriminate use of antimicrobial agents [5, 6]. Therefore, the discovery of newer antifungal agents effective against *C. neoformans* is an ongoing process.

Herbal remedies are known and used for many diseases for many thousand years. However, many such preparations are yet to be scientifically studied to establish their therapeutic importance.

Hence, the present study was undertaken to find out the antifungal activity of alkaloid rich fraction separated from the leaf extract of *P. juliflora*. The antifungal activity was determined against five clinical isolates and five environmental isolates of *C. neoformans* by disc diffusion assay. Of all the ten isolates, C3 was more susceptible to the alkaloid fraction with a zone of inhibition of 8 mm, 14 mm, 15 mm and 17 mm at a concentration ranging from 50-200 µg. The clinical isolates were more susceptible to the alkaloid fraction than the environmental isolates. The earlier reports have indicated antibacterial properties of leaf extract but the present investigation shows that the alkaloids also possess antifungal activity [7].

The HPTLC at 260 nm showed a total of nine alkaloids with the lowest Rf value of 0.02 and highest Rf value of 0.09 in the crude extract of the leaf of *P. juliflora*. At 550nm 14 band patterns with the lowest Rf value of 0.05 and highest Rf valve of 0.86 was observed. The antimicrobial activity of the leaf against *C. neoformans* can be attributed to these alkaloids. Earlier workers have reported on the antimicrobial activity of the alkaloids from the plant *P. juliflora* [8].

In large number of medicinal plants, the therapeutic value is due to the presence of alkaloids, which in certain aspect ranks among the most interesting of naturally occurring substances. The genus Prosopis (mesquite) are known to possess medicinal value. *P. juliflora* a shrub grows abundantly in Sindh and Punjab provinces of Pakistan. Juliflorine and julifloricine, the main alkaloids of *P. juliflora*, have been isolated for the first time and the antibacterial and antifungal activities were reported [8]. From *P. juliflora,* a benzene insoluble alkaloidal fraction (containing 2 major and 3 minor alkaloids) has also been isolated and reported to possess antibacterial and antifungal activities.

The alkaloids were identified by GC-MS analysis. Identification of compounds was based on comparison of their mass spectra. As individual compounds eluted from the gas chromatographic column, they entered the electron ionization detector where they were bombarded with a stream of electrons causing them to break into fragments. The fragments were actually charged with a certain mass. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph which is the fingerprint of the molecule. The identification of compounds was based on the comparisons of their mass spectra with NIST library 2008, Wiley & Fame.

Twenty three different compounds were identified. Major compounds were phenolic compounds 2,4-bis(1-phenylethyl)phenol, Cassine and Zerumbone. The phenolic compounds identified were antioxidants and also possess antimicrobial activity. The role of phenolic compounds as antioxidant, antifungal, analgesic, antiseptic and anticancer has been reported [9]. Cassine (8.18% area) with the molecular formula $C_{18}H_{35}NO_2$ was identified as one of the piperidine alkaloid**,** Prosafrinine Indolizidine ring was absent from this alkaloid. The presence of prosafrinine in the alkaloids extracted from leaves and pods of *P. juliflora* and proved its antibacterial activity [1]. The present investigation revealed the antifungal activity also of piperidine alkaloids from Prosopis leaf. Zerumbone which is a sesquiterpene phytochemical found in ginger was also identified among the alkaloids. Zerumbone suppresses free radical generation; is an antiinflammatory; suppresses cancer cell proliferation accompanied by apoptosis and counters HIV activity. Next to phenolic compounds Cassine (prosafrinine) one of the piperidine alkaloids is present in highest concentration. These alkaloids found to possess antifungal activity, were mainly responsible for the antifungal activity of the leaf extracts of *P. juliflora.*

According to World Health Report of Infectious Diseases 2000, overcoming antimicrobial resistance is the major issue of the WHO for the next millennium [10]. *P. juliflora* showed notable antifungal activity hence this plant can be used to discover bioactive natural products that may serve as leads for the development of new pharmaceutical products. Such screening of various natural organic

compounds and identifying active principles is the need of the hour because successful prediction of lead molecule and drug like properties at the onset of drug discovery will pay off later in drug development.

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