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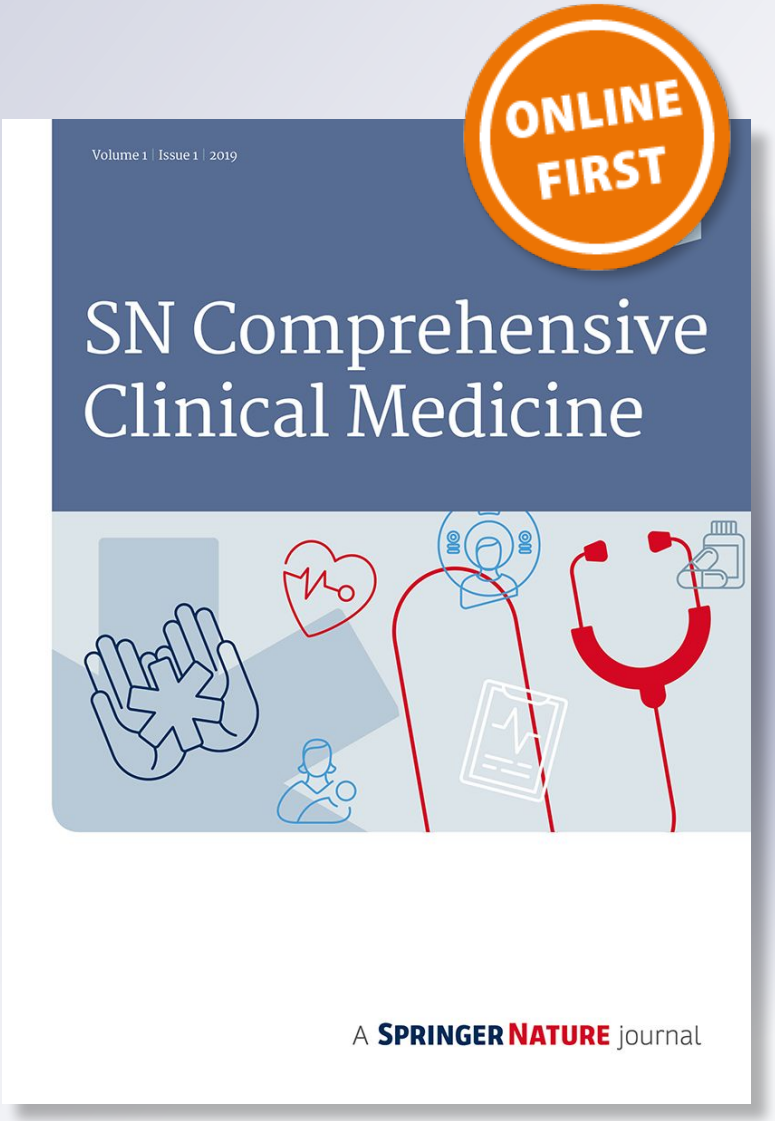

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MEDICINE

Bioactive Compound in *Urginea indica* (Kunth.) from Bastar and its Spectral Analysis by HPLC, UV-Vis, FT-IR, NMR, and ESI-MS

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Abstract

The integration of traditional and modern medicine has gained increased recognition globally. In light of vast potentiality of medicinal plants as therapeutics, the present endeavor deals with the assessment of antifungal and synergistic activity of *Urginea indica* (Kunth.) (Family: Liliaceae) commonly known as wild onion against human pathogenic fungi viz., *Aspergillu niger* (MTCC 872) and *Candida albicans* (MTCC 183) procured from IMTECH, Chandigarh, India. The antifungal and synergistic activity was assessed by agar well diffusion method. The results were analyzed statistically using ANOVA with DMRT. The qualitative phytochemical analysis, column chromatography, TLC, MIC, and MFC was performed following standard protocols. Finally, the purified fraction was chemically characterized by HPLC, UV-Vis, FT-IR, (^{1}H & ^{13}C) NMR, and ESI-MS. The antifungal activity profile showed that acetone root extract exhibited broad-spectrum antifungal activity. However, in case of *C. albicans*, the extract exhibited significantly higher activity index. The qualitative analysis revealed the presence of varied phytochemicals. The extract exhibiting the highest activity was purified and tested for its synergistic or antagonistic potentiality against fungi revealed that the bioactive compound exhibited statistically significant synergism with three different commercially available antifungals viz., clotrimazole, ketoconazole, and fluconazole. Finally, the purified fraction was chemically characterized. The combination therapy against human pathogenic fungi seems to be a boon for patients with severe infections caused by multidrug-resistant pathogens. Thus, in current scenario, there is an urgent need to target new dimensions towards alternative antifungal therapy to preserve better human health for generations ahead.

Keywords Antifungal · Bastar · Bioactive · Synergism · Spectral analysis · Human health

Introduction

The integration of traditional and modern medicine is gaining increased recognition globally [1]. The extensive use of the antibiotics to combat the diseases has led to the emergence of multidrug resistance [2]. One strategy employed to overcome these resistant mechanisms is the use of combination of drugs. Combination therapy is often profitable for patients with serious infections caused by drug-resistant pathogens [3]. It can be used to expand the antimicrobial spectrum, to minimize toxicity and to obtain synergistic antimicrobial activity [4]. Sometimes synergistic behavior delays the emergence of microbial resistance [5]. However, there is an urgent need of extensive research for the novel bioactive compound in combination with standard antibiotics which will contribute for the better, safer, and cost-effective novel drug development. Thus, in the current scenario, the need of the hour is to target new dimensions towards alternative antimicrobial therapy along with its chemistry using sophisticated analytical instruments to preserve better human health for generations ahead.

Urginea indica (Kunth.) is polytypic genus endemic to India, Africa, and Mediterranean regions [6]. It is an Indian medicinal plant belonging to family Liliaceae [7, 8]. It is a glabrous herb commonly known as "Indian squill" and locally as "Jungli piyaz." Although, all parts of this plant are reported to have therapeutic potentiality; but recently, the bioactive compounds of bulbs have received much attention due to its anticancer properties [9]. Its bulb is used as anthelmintic, digestive, expectorant, stomachic, diuretic, emmenagogue,

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purgative, cures paralysis, rheumatism, leprosy, skin diseases, and infectious wound [10], antimicrobial [11], antioxidant, antiangiogenic, and pro-apoptotic [12], laxative, and spasmodic [13]. The bulbs are used in whooping cough, arthritis, tumors, edema, male sterility, gout, chronic cough, psoriasis, swellings, pulmonary troubles, expectorant, diuretic properties, and cardiac tonic [14, 15]. It is a perennial geophyte and the flowers bloom in April and May after the first shower: the round conical, pear-shaped bulbs with transparent white outer scales consisting of fleshy coats. It has fibrous roots of about 6–10 in. in length, starting from the base of the bulb with dark-green leaves. It exhibits phyllotaxy which is whorled hysteranthus or synanthus [16]. The general morphology of *U. indica* (Kunth.) is presented (Fig. 1).

Chhattisgarh is identified as an "Herbal State" due to its rich repository of medicinal and aromatic plants in many tribal districts including Bastar [17]. Bastar on the southern part of Chhattisgarh is known for its unique blend of distinctive tribal cultures and traditional knowledge of medicinal plants all over the world. About 70% of the total population of Bastar comprises tribals, which is 26.76% of the total tribal population of Chhattisgarh. The tribes of Bastar region are traditionally dependent on plants for curing their ailments since long. Despite of rich abundance of medicinal flora, the region is relatively less explored with reference to the antimicrobial properties of medicinal plants. Thus, in light of vast potentiality of medicinal plants as novel sources of bioactive compounds, *U. indica* (Kunth.) was investigated for the assessment of antifungal and synergistic activity based on its usage by the tribal community of Bastar as therapeutics in their daily life.

Materials and Methods

Bastar district (19.1071° N, 81.9535° E) is located in the southern part of Chhattisgarh and has an area of 4029.98 km^2 (Fig. 2). It is surrounded by Bijapur, Dantewada, Kondagaon, Narayanpur, and Sukma districts of the state. Bastar, the land of tribals and

Fig. 1 General morphology of *Urginea indica* (Kunth.)

Fig. 2 Map of Bastar area showing its position in Chhattisgarh, India

natural resources, is also surrounded with dense forests, hilly mountains, natural caves, waterfalls, and streams. Jagdalpur is both district and divisional headquarter of Bastar district. The city lies on the southern bank of river Indravati with an average elevation of 562 m. It has a total forest area of 292,130 ha which is more than 19% of the total land area of the district. The region has an annual temperature of 10.6 °C in winter to 46 °C in summer with annual rainfall of 1386.77 mm. Lateritic and alluvial are main soil types of the area. The texture of the soil varies from sandy to fine-textured clayey soils. The relative humidity is about 90% in rainy season to 30–40% during winter.

Selection and Identification of Medicinal Plant

U. indica (Kunth.) (Family: Liliaceae) was selected for study from Bastar region based on the ethnomedicinal importance as herbal drug and therapeutic usage by the tribal community as the source of food, medicine, and cosmetics [18]. Traditionally, wild plants consisting of several bioactive phytochemicals are used as a source of herbal preparations possessing therapeutic properties. The *U. indica* (Kunth.) was collected and identified at SGCARS, Jagdalpur, Chhattisgarh, India (Fig. 3).

Extraction Method

The extraction of phytochemicals was done through Soxhlet apparatus as hot extraction could extract completely the phytochemicals from the materials used for extraction. However, the selection of extraction methods mainly depends on the objectives, nature of samples and target compounds [19]. The powdered material was placed in a thimble of filter paper.

(a) Root Sample (b) Stem Sample (c) Leaf Sample

Fig. 3 Different parts of *Urginea indica* (Kunth.), **a** root sample, **b** stem sample, **c** leaf sample

The powdered plant material was extracted sequentially in four different solvents based on their polarity index [20, 21] viz., chloroform (nonpolar), acetone (dipolar), methanol (polar), and aqueous (polar). Fifteen grams of powdered material was extracted in 150 ml of chloroform, acetone, methanol, and in aqueous according to their increasing polarity index in the Soxhlet apparatus (Tempo) for 8–10 h at a temperature not exceeding the boiling point of the respective solvents. The extracted material was dried to the residue. The sample was dissolved in 50% dimethyl sulphoxide to prepare a 10% stock solution (*w*/*v*) and stored in a refrigerator at 4 °C in small sterile glass tubes until use.

Microorganism for Antifungal Activity

Antifungal activity was assessed against microbial cultures procured from Institute of Microbial Technology, Chandigarh, India. The culture details as provided by the IMTECH, Chandigarh, India included the following:

(i) *Aspergillu niger* (MTCC 872): growth medium: potato dextrose agar; growth conditions: aerobic; temperature: 30 °C; incubation time: 48 h; subculture: 30 days
(ii) *Candida albicans* (MTCC 183): growth medium: potato dextrose agar; growth conditions: aerobic; temperature: 30 °C; incubation time: 48 h; subculture: 60 days; special feature: used for the assay of sterility testing and fungicide tests

Preparation of Fungal Inoculums

The test fungal organisms were maintained on potato dextrose agar slants. One loop full of each fungal culture was inoculated in a 25-ml potato dextrose broth and incubated at 28–30 °C in incubator. Stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar (Hi-media) following National Committee for Clinical Laboratory Standards [22]. Stock suspensions were adjusted to optical densities that ranged from 0.09–0.11 at 530 nm using a spectrophotometer which was equivalent to 0.9×10^4 to 4.7×10^4 cfu/ml.

Antifungal Activity

The antifungal activity of the plant extract was evaluated by agar well diffusion method [23]. Two hundred microliters of the standardized cell suspension were spread on potato dextrose agar (Hi-media) plate using a sterile swab and air-dried to remove the surface moisture. Wells were bored into the agar using a sterile 6-mm diameter cork borer. The crude extract was aseptically introduced into the well at a concentration of 2 mg/20 µl, allowed to stand at room temperature for about 1 h as a period of pre-incubation diffusion to minimize the effect of variation in time between the application of different solutions and later the plates were incubated at 28–30 °C for 48 h. Controls were also set up in parallel and effects were compared with clotrimazole as standard antifungal at a concentration of 10 µg/20 µl. The plates were observed for the zone of inhibition after 48 h. The experiment was conducted in triplicates and the values are expressed as mean ± SE.

Activity Index

The activity index was determined using clotrimazole as a standard antifungal. The activity index was expressed as zone of inhibition of test sample/zone of inhibition of standard antimicrobial [24].

Phytochemical Analysis

Phytochemical analysis of the extracts was carried out following Harborne [25] & Trease and Evans [26].

Silica Gel Column Chromatography

The column chromatography was performed in a glass column (50 cm × 2 cm). The column was erected straight on a stand. In the setting up of column, the lower part of the column was packed with glass wool. The slurry was prepared by mixing 20 g of silica gel (60–120 mesh) in 100 ml chloroform. The slurry was poured carefully into the column taking care that no air traps in and was allowed to settle for 2 h. The column was tapped from outside to eliminate the air bubbles if any. The tap of the glass column was open to allow free flow of solvent into a conical flask. The set up was considered to be in order when the solvent drained freely without carrying either silica gel or glass wool. At the end of the packing process, the tap was closed. The column was washed with 150 ml eluent and allowed to stabilize for 24 h. The flow rate of the solvent was kept at 2 ml/ min. Five millimeters of crude sample was loaded in the column and elution of the extract was done with solvent systems of gradually increasing polarity using chloroform (nonpolar), acetone (dipolar), and methanol (polar). The following ratios of solvent combinations were sequentially used in the elution process viz., chloroform: acetone 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. Acetone: methanol was used in a ratio of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. The eluted fractions were collected in aliquots of 5 ml in test tubes for further analysis.

Thin-Layer Chromatography (TLC)

The thin-layer plate (20 cm × 20 cm) was prepared by spreading aqueous slurry of the silica gel G. (8 g in 100 ml distilled water) on a clean surface of a glass to obtain a thickness of 0.25 mm. The plate so prepared was activated in an oven at 110–120 °C for 30 min prior to sample loading. The crude and purified acetone root extracts of *U. indica* was applied as a spot using a capillary tube, 3 cm above the edge of the plate. The plate was air-dried for evaporating sample solvent. The plate was placed in the solvent system, toluene: diethyl ether: ethyl acetate: acetic acid [80:10:10:0.2 *v*/*v*]. After completion of the run-up to two-thirds of the length of the plate, the plate was examined in the ultraviolet chamber, photographed, and the bands were identified and their corresponding Rf values were determined.

Minimum Inhibitory Concentration (MIC)

The broth macrodilution sensitivity test was performed following [27] with some modifications to determine minimum inhibitory concentration (MIC) of the extracts. The test was performed in clean and sterile glass test tubes (12 mm × 75 mm) using potato dextrose broth (Hi-media). The *U. indica* extracts were taken in the first tube and serial two-fold dilutions of the extracts were prepared in successive test tubes and mixed thoroughly to give final concentrations ranging from 2 mg/ml–0.0625 mg/ml for fungal cultures. Of fungal inoculum (previously adjusted with the optical density of the culture broth using a spectrophotometer), 0.5 ml was added in all the tubes. An appropriate solvent blanks and standard antifungal were also incubated as negative and positive controls respectively. Tests were carried out in duplicates. The cultured tubes were sealed with parafilm and incubated at 28–30 °C for 48 h. The MIC of sample was detected following addition of 50 µl of 0.5% TTC (2,3,5 triphenyl tetrazolium chloride, Hi-media) prepared in 2% NaOH solution in all the test tubes and incubated at 37 °C for 30 min. Microbial growth

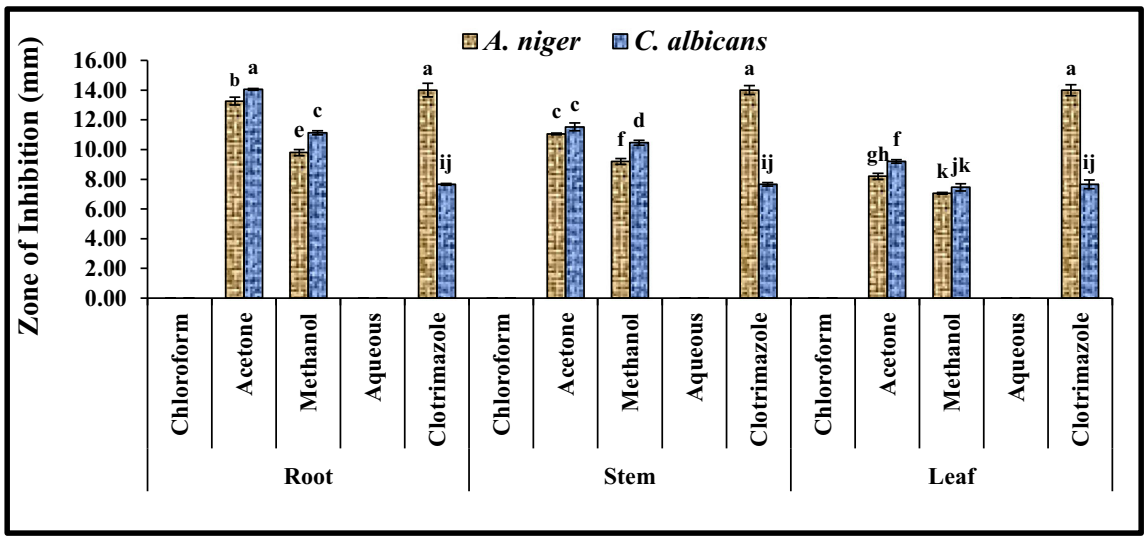

Fig. 4 Antifungal activity of root, stem, and leaf extracts of *U. indica vis-à-vis* clotrimazole (ANOVA summary: $F_{23,\ 48} = 217.213$, $p < 0.001$, means having different alphabets, as superscripts, are statistically significant from each other at $p < 0.001$) (based on Duncan's multiple-range test)

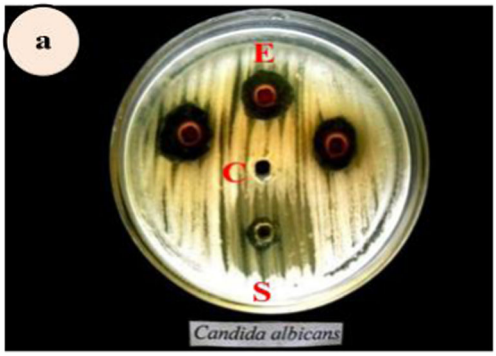

Fig. 5 Antifungal activity of crude acetone root extracts of *U. indica* against **a** *C. albicans* (E, crude extract; C, control; and S, standard antifungal)

was determined by observing the change in color of TTC in the tubes (pinkish-red formazan when there is growth and clear solution when there is no growth) without shaking [28]. The lowest concentration of the extract that inhibited the growth was taken as MIC. MIC value < 0.5 mg/ml was defined as a potential strong activity.

Minimum Fungicidal Concentration (MFC)

Minimum fungicidal concentration (MFC) was determined by spot inoculation method. A sample was taken from each test tube along with the control tube and spotted on potato dextrose agar (Hi-media) plates with the help of sterile cotton swabs. Plates were incubated at 28–30 °C for 48 h. All the experiments were performed in duplicates. The MFC value was considered as the lowest concentration of *U. indica* extracts which exhibited no growth after incubation.

Synergistic/Antagonistic Activity

Antimicrobial activity was measured using agar well diffusion method according to the National Committee for Clinical Laboratory Standard [29]. Briefly, petri plates containing approximately 25–30 ml of potato dextrose agar was inoculated using a sterile cotton swab with the standardized fungal cultures. The combination effect was evaluated for purified acetone root fraction of *U. indica* at the concentration 2 mg/ml with standard antifungal clotrimazole, ketoconazole, and fluconazole at the concentration 20 µg/ ml. Wells (6 mm diameter) were punched in the agar and filled with 10 µl of purified fraction or antifungal and in case of synergistic/antagonistic activity, the purified fraction and antifungal was added into the well. The fungal plates were incubated at 28–30 °C for 48–72 h. The antifungal activity was assessed by measuring the inhibition zone diameter (mm) around the well. The average of three replicates for each purified fraction, antifungal, and combination were recorded. Synergistic activity was considered when combinations exhibited with enlargement of combined inhibition zone size by 0.5 mm [30].

Chemical Characterization of Bioactive Compound

The bioactive fraction from *U. indica* possessing highest antifungal activity was chemically characterized by spectral analysis using HPLC, UV-Visible spectroscopy, FT-IR, (^{1}H and ^{13}C) NMR, and ESI-MS spectrometry.

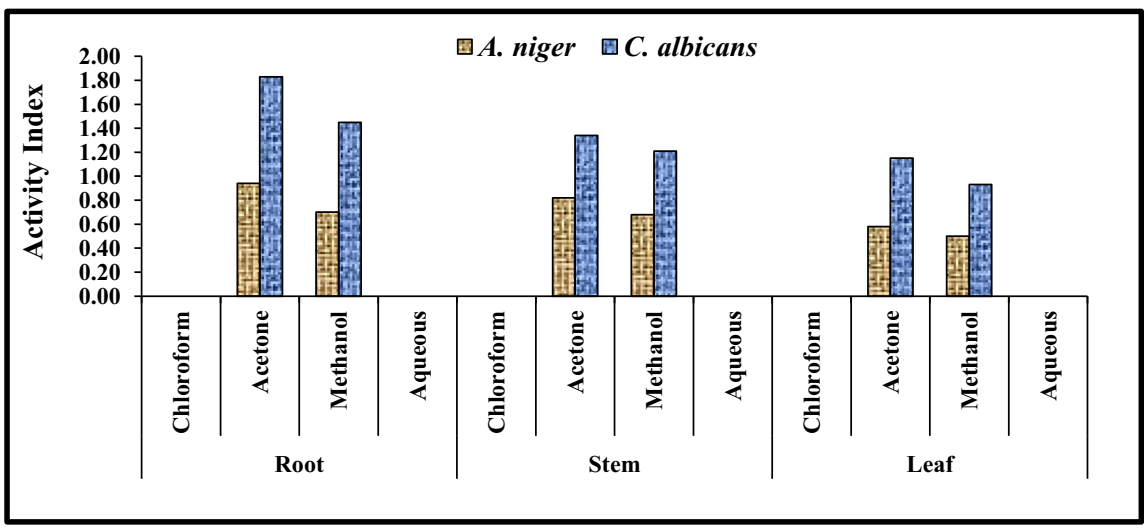

Fig. 6 Activity index of root, stem, and leaf extracts of *U. indica* with reference to clotrimazole

Table 1 Phytochemical analysis of root, stem and leaf extracts of *U. indica*

Tests	Aqueous			Methanol			Acetone			Chloroform		
	R	S	L	R	S	L	R	S	L	R	S	L
Alkaloids												
Mayer's	−	−	−	−	−	−	+	+	−	−	−	−
Wagner's	−	−	−	−	−	−	+	+	−	−	−	−
Hager's	−	−	−	−	−	−	+	+	−	−	−	−
Flavonoids												
Alkaline reagent	+	+	+	++	++	−	+++	+++	−	+	−	−
Lead acetate	+	+	+	++	++	−	+++	+++	−	+	−	−
Phytosterols												
Salkowski	+	++	+	++	++	+	+++	+++	++	++	+	++
Libermann-Burchard	+	++	+	++	++	+	+++	+++	++	++	+	++
Tannins												
Ferric chloride	−	−	−	−	−	−	++	++	−	−	−	−
Gelatin	−	−	−	−	−	−	++	++	−	−	−	−
Saponins												
Foam test	−	−	++	−	−	−	+	+	−	−	−	−
Quinones	−	−	−	−	−	−	+	−	−	−	−	−
Resins	−	−	−	−	−	−	++	++	−	+++	+++	−
Glycosides	+	+	+	++	++	−	+++	+++	−	−	−	−

+++, strongly positive; ++, moderately positive; +, positive; −, negative; R, root; S, stem; L, leaf

Results

The antifungal activity of crude extracts of *U. indica* is presented (Figs. 4 and 5). The acetone root extract was found to be potent against both the fungal cultures. The study revealed that the acetone root extract showed the highest zone of inhibition against *C. albicans* (14.06 ± 0.06 mm) followed by *A. niger* (13.26 ± 0.26 mm), whereas methanol root extract exhibited zone of inhibition of 11.13 ± 0.13 and 09.80 ± 0.20 mm against *C. albicans* and *A. niger* respectively. However, chloroform and aqueous extract did not show a statistically significant zone of inhibition against both the fungal cultures under investigation.

The activity index of root, stem, and leaf extracts of *U. indica* with respect to clotrimazole revealed that the acetone root extract had highest activity index of 1.83 in case of *C. albicans*, whereas for *A. niger*, the activity index recorded was 0.94 (Fig. 6). The phytochemical analysis of *U. indica* is presented (Table 1).

Active crude acetone root extract exhibiting high antifungal activity was purified by column chromatography for further characterization. The 10th, 11th, and 12th fractions gave the highest antifungal activity against *A. niger* and *C. albicans*. They were pooled and subjected to TLC along with crude acetone root extract. The active crude extract

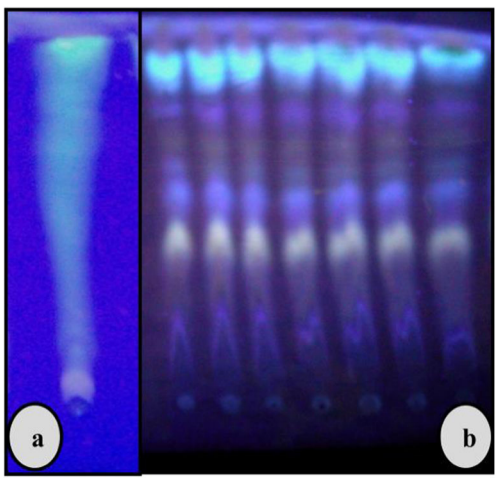

Fig. 7 TLC profile of acetone root extract of *U. indica*. **a** Band pattern in purified pooled fraction; **b** band pattern in crude acetone root extract

Table 2 Rf value of crude acetone root extract and purified fraction of *U. indica* by TLC

Bands	Rf values	Color of bands
a. Purified pooled fraction	0.90	Blue flourescent
b. Crude acetone extract		
Band 1	0.90	Blue flourescent
Band 2	0.88	Blue
Band 3	0.79	Blue
Band 4	0.67	Light orange
Band 5	0.64	Light blue
Band 6	0.55	Dark blue
Band 7	0.46	Yellowish

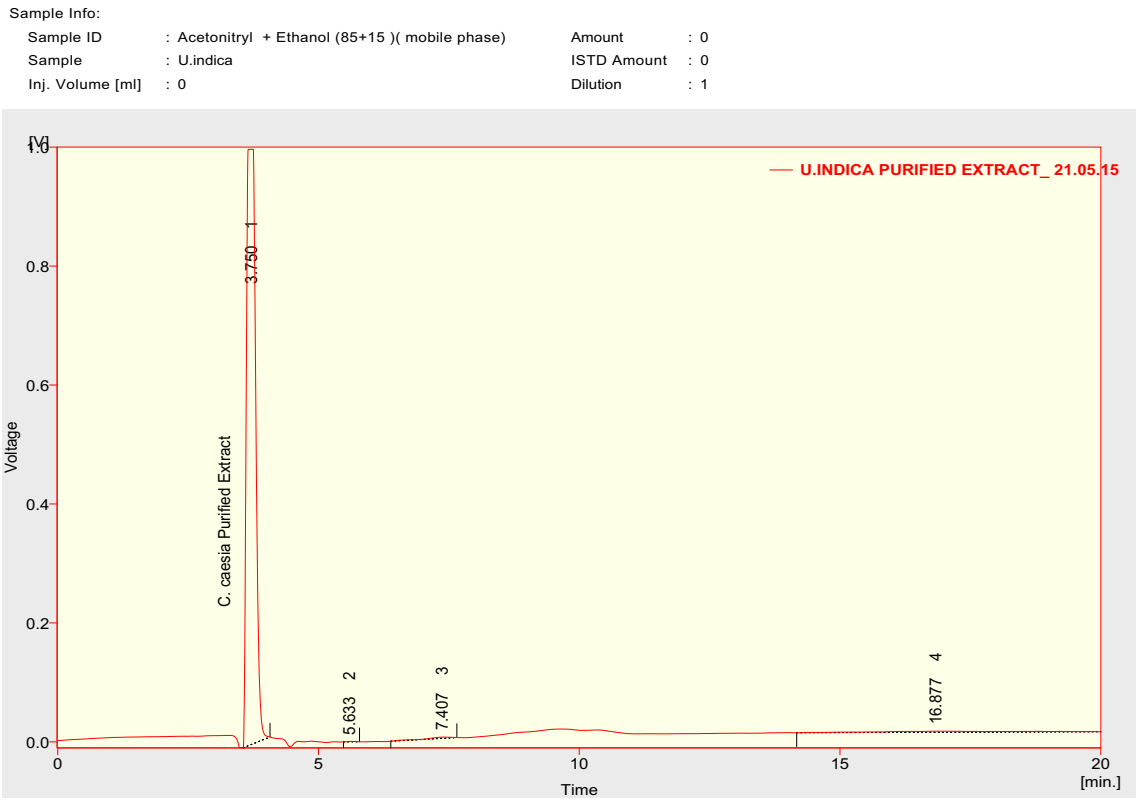

Fig. 8 HPLC chromatogram of the purified acetone root fraction of *U. indica*

showed seven prominent bands on TLC plate having Rf values 0.90, 0.88, 0.79, 0.67, 0.64, 0.55, and 0.46 respectively. The pooled fractions showed a single band with Rf value of 0.90 (Fig. 7 and Table 2). The pooled fractions were further analyzed for its purity by HPLC and the purified product (96.61%) was obtained with the retention time of 3.750 min (Fig. 8).

The MIC and MFC of the crude extract and purified fraction were evaluated for human pathogenic fungi. The crude acetone root extracts of *U. indica* showed the MIC and MFC value of 1 and 0.5 mg/ml for *A. niger* and *C. albicans* respectively. However, the corresponding purified fraction exhibited significantly higher inhibition at 0.0625 mg/ml with *C. albicans*. The results revealed that the purified fraction exhibited broad spectrum and significantly greater antifungal activity as compared to its corresponding crude extracts. The results of the MIC and MFC are presented (Table 3).

The synergistic or antagonistic activity of purified fraction of *U. indica* was assessed with three antifungals viz., clotrimazole, ketoconazole, and fluconazole against two pathogenic fungi viz., *A. niger* and *C. albicans*. The clotrimazole with the purified fraction of *U. indica* exhibited synergistic activity with a zone of inhibition of 16.20 ± 0.11 mm for *A. niger*. However, the antagonistic activity was recorded for *C. albicans* (14.06 ± 0.17 mm). The findings illustrated that the combination of purified fraction of *U. indica* with clotrimazole exhibited significant synergistic activity for *A. niger* but antagonistic activity for *C. albicans* (Figs. 9 and 10).

The synergistic or antagonistic activity of purified fraction of *U. indica* with ketoconazole showed significantly higher synergistic activity for *C. albicans* with a zone of inhibition of 19.60 ± 0.11 mm, whereas the antagonistic activity (11.33 ± 0.06 mm) was recorded for *A. niger*. The results revealed that the combination of purified fraction of *U. indica* with ketoconazole was found to be synergistic for *C. albicans* but antagonistic for *A. niger* (Fig. 11).

The synergistic or antagonistic activity of purified fraction of *U. indica* with fluconazole exhibited maximum zone of inhibition of 19.33 ± 0.13 mm for *C. albicans* showing significantly higher synergistic antifungal activity but antagonistic activity with an zone of inhibition of 12.40 ± 0.11 mm was recorded in case of *A. niger* respectively. (Fig. 12).

Table 3 MIC and MFC of the crude acetone root extract and purified fraction of *U. indica* for fungal isolates

Fungal cultures	Crude extract (2 mg/ml)	Purified fraction (2 mg/ml)	Positive control (20 µg/ml)
A. niger	1	0.25	1.25
C. albicans	0.5	0.0625	20

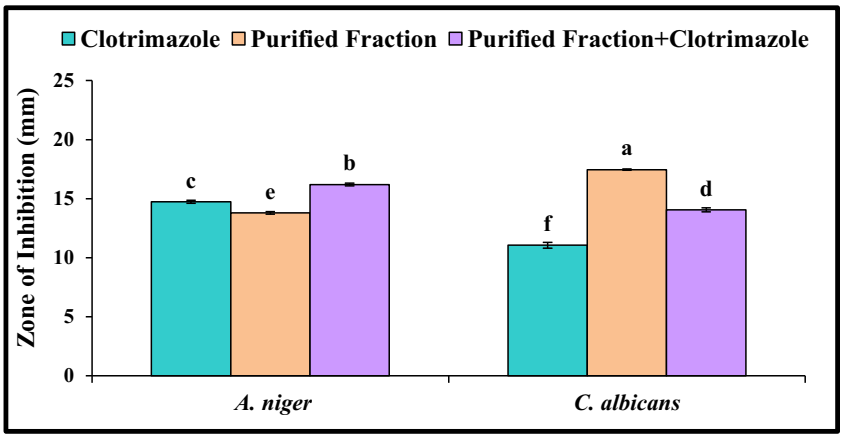

Fig. 9 Synergistic/antagonistic activity of purified acetone root fraction of *U. indica* with clotrimazole (ANOVA summary: $F_{5,42} = 448.259$, $p < 0.001$, means having different alphabets, as superscripts, are statistically significant from each other at $p < 0.001$) (based on Duncan's multiple-range test)

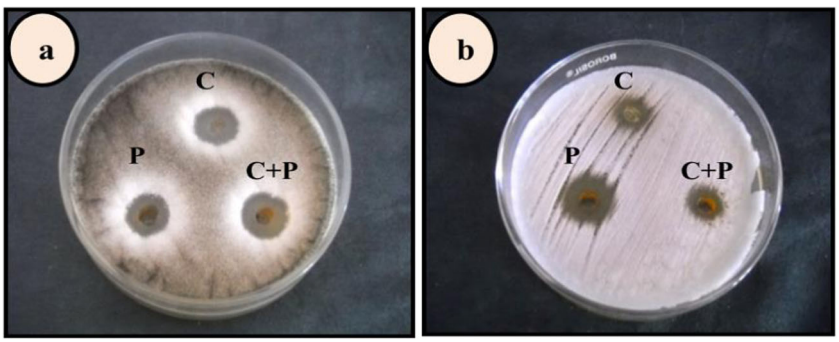

Fig. 10 Synergistic/antagonistic potential of purified acetone root fraction of *U. indica* with clotrimazole: **a** *A. niger*, **b** *C. albicans* (C, clotrimazole; P, purified fraction; and P+C, purified fraction + clotrimazole)

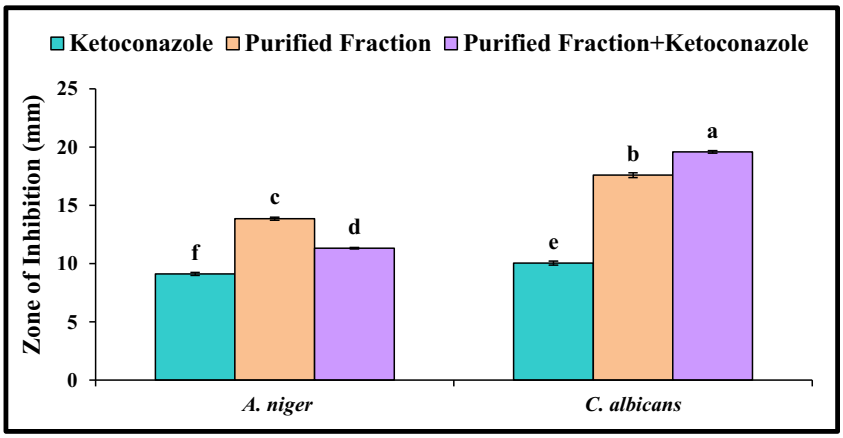

Fig. 11 Synergistic/antagonistic activity of purified acetone root fraction of *U. indica* with ketoconazole (ANOVA summary: $F_{5,42} = 1236.00$, $p < 0.001$, means having different alphabets, as superscripts, are statistically significant from each other at $p < 0.001$) (based on Duncan's multiple-range test)

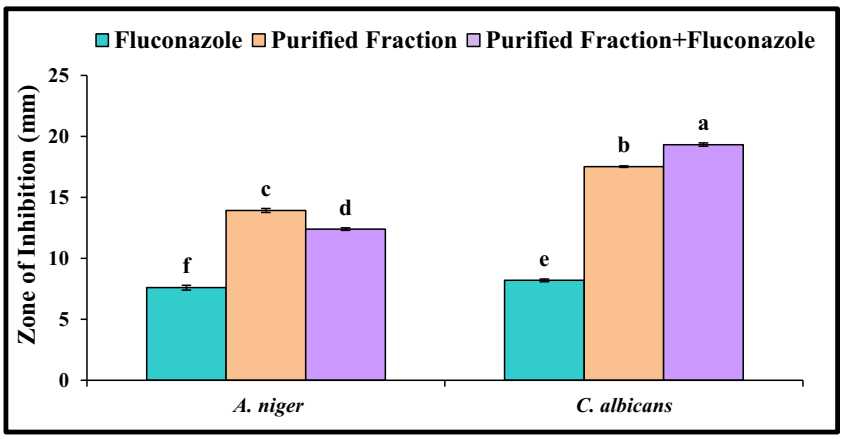

Fig. 12 Synergistic/antagonistic activity of purified acetone root fraction of *U. indica* with fluconazole (ANOVA summary: $F_{5,42} = 1916.00$, $p < 0.001$, means having different alphabets, as superscripts are statistically significant from each other at $p < 0.001$) (based on Duncan's multiple-range test)

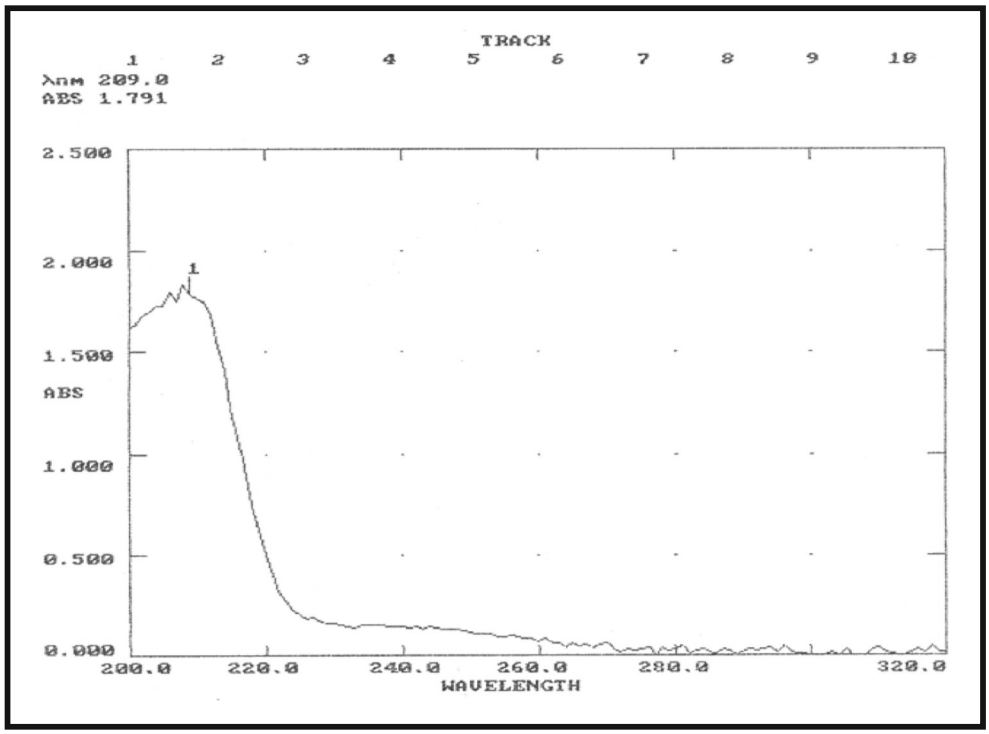

Fig. 13 Ultraviolet visible spectrum of the purified acetone root fraction of *U. indica*

Chemical Characterization of Purified Fraction

The bioactive purified acetone root fraction from *U. indica* was chemically characterized by advanced spectral studies, i.e., UV-Visible spectroscopy, FT-IR, (^{1}H and ^{13}C) NMR, and ESI-MS.

UV-Visible Spectroscopy

The UV-Visible spectrum of acetone root fraction of *U. indica* indicates the presence of an aromatic ring with a maximum absorption at λ_{max} (acetonitrile) of 209 nm and absorption of 1.791 (Fig. 13).

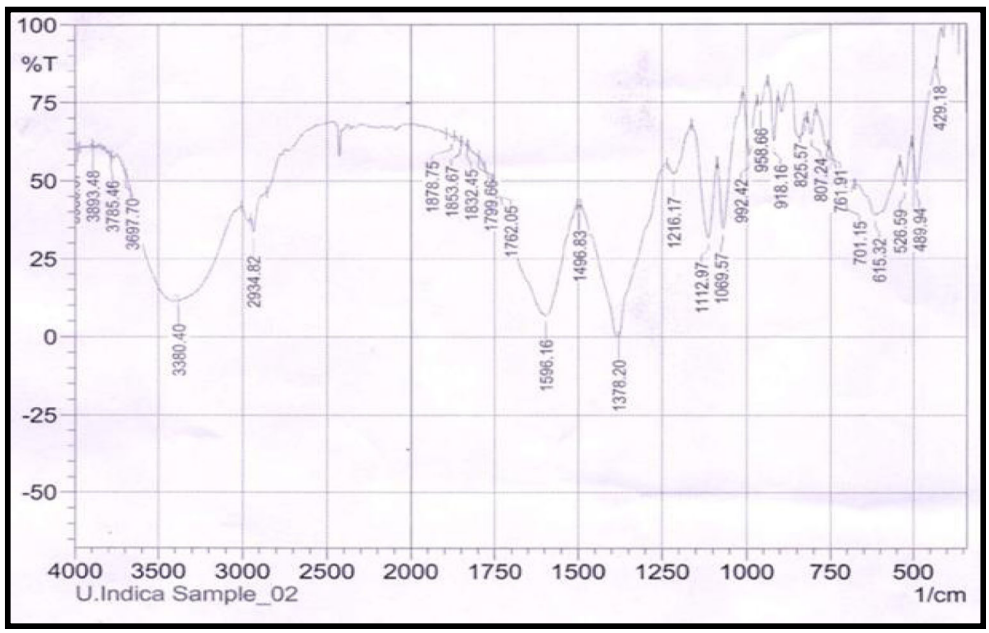

Fig. 14 Infrared (IR) spectrum of the purified acetone root fraction of *U. indica*

Fourier-Transmission Infrared (FT-IR) Spectroscopy

FT-IR spectrum of the purified acetone root fraction of *U. indica* using KBr pellet technique showed peaks at 500–4000 cm^{-1} at different wavenumber range. The characteristic peaks were observed at 3393.90 (O-H stretch, H-bonded), 2934.82 (C-H stretch), 1596.16 (C-C stretch in ring), 1495.86 (C-C stretch in a ring), and 1112.97, 1069.57 (C-O stretch) respectively (Fig. 14).

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the purified acetone root fraction in deuterated water (D_2O) were assessed with 300 MHz Bruker DR X.

^{1}H NMR (D2O Exchangeable) δ

The ^{1}H NMR spectra of the purified fraction of *U. indica* and the corresponding chemical shift values (δ) are expressed in ppm, intensity (Int) in percent, frequency (Freq), and coupling constant (J) in Hertz. The spectrum of the compound revealed the presence of different types of neighboring H atoms and their splitting pattern (Fig. 15).

^{13}C NMR (D2O Exchangeable) δ

^{13}C NMR showed different peaks at 11.921–21.0 (C-CH_3 group), 22.993 (C-CH group), 25.998 (C-H group of cyclopentane ring), 31.498–31.827 (C-H of cyclohexane ring), 33.861 (C-H group), 36.092 (C-CH_3 group), 37.211 (C-H of cyclohexane ring), 42.242 (C-CH_3 group of cyclohexane ring), 42.159 (C-H group of cyclohexane ring), 45.732 (C-H group), 50.063 (C-H of cyclohexane ring), 56.435 (C-H of cyclopentane ring), 56.698 (C-H of cyclohexane ring), 71.591 (C-OH group of cyclohexane ring), 121.580 (-C = of cyclohexane ring), and 140.713 (C-H of cyclohexane ring) respectively (Fig. 16).

Electrospray Ionization Mass Spectrometry (ESI-MS)

The molecular weight of the purified fraction from *U. indica* was confirmed by ESI-MS analysis. The presence of M+H (415)

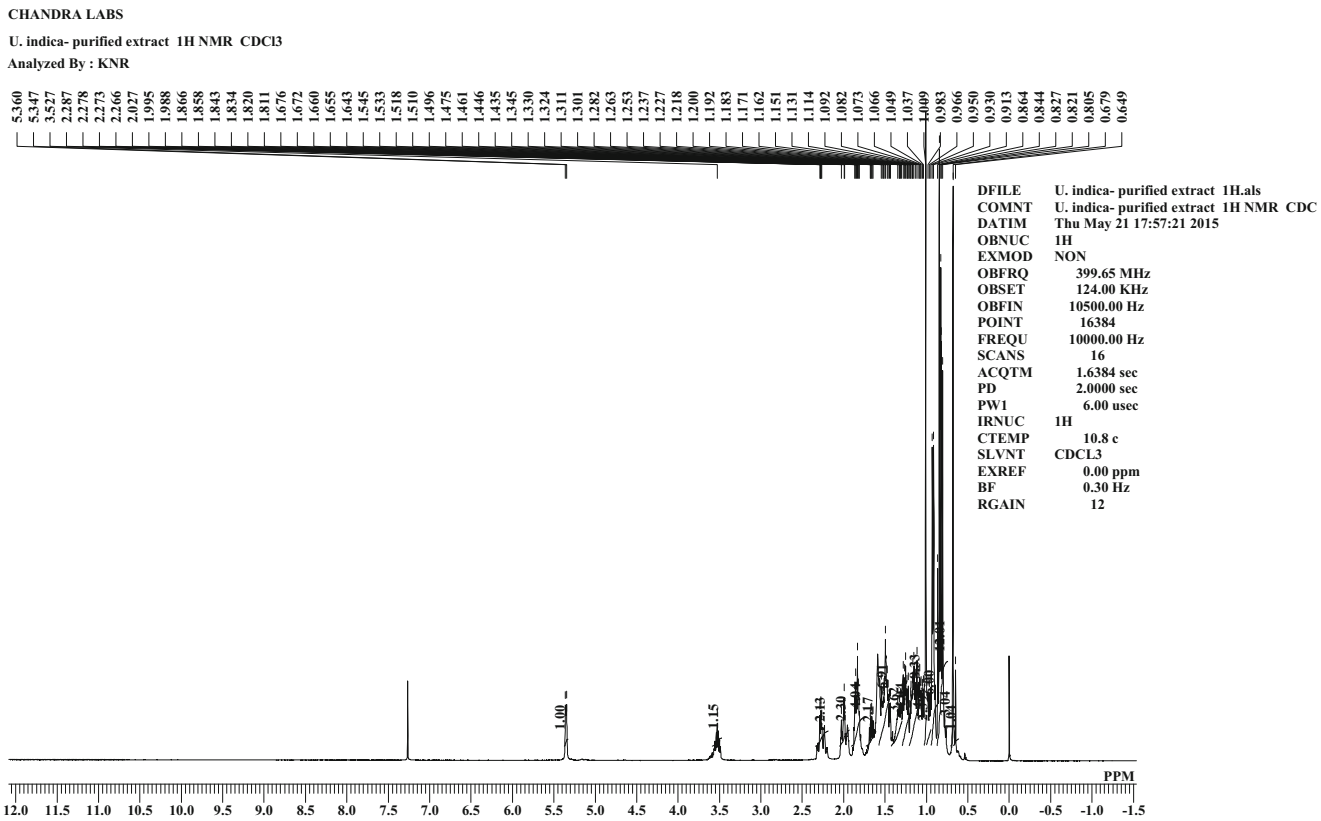

Fig. 15 ^{1}H NMR spectrum of the purified acetone root fraction of *U. indica*

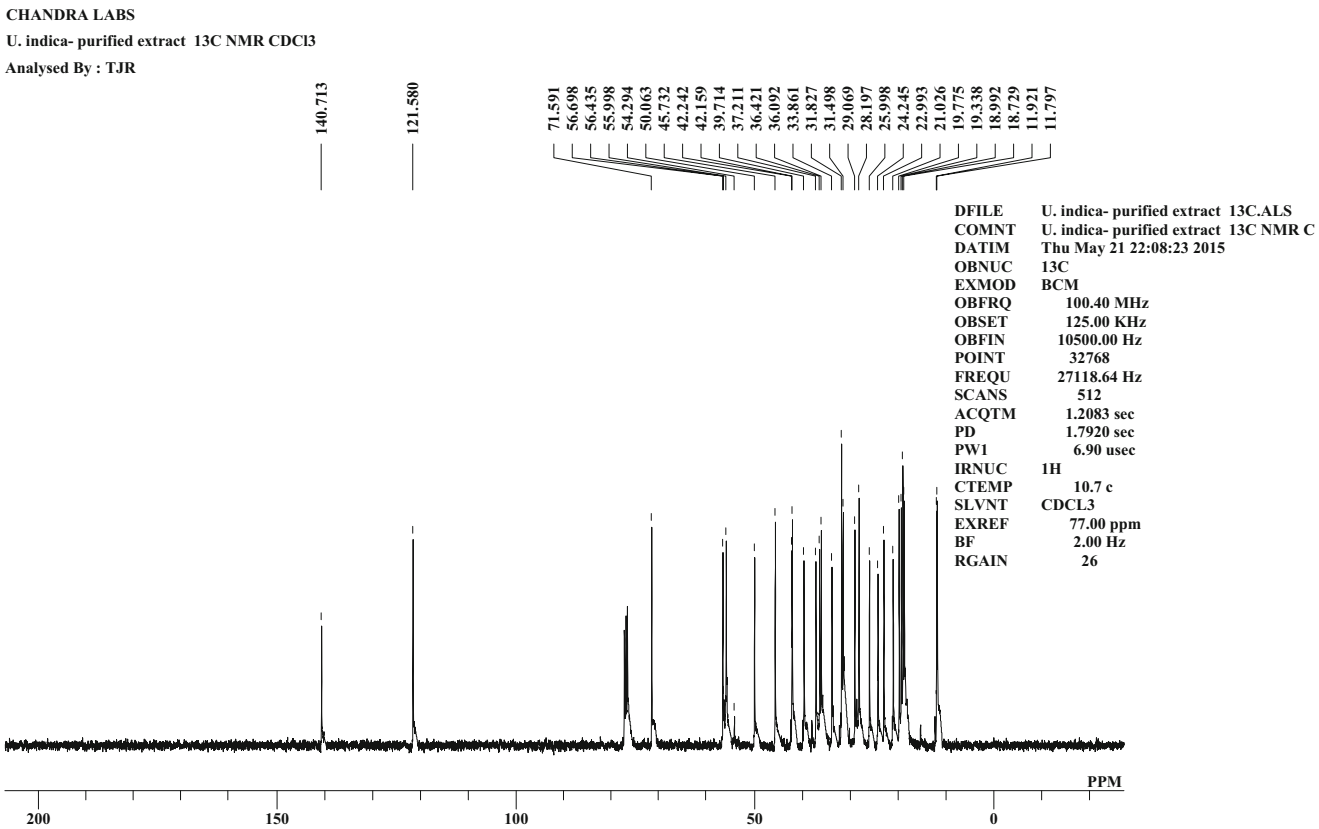

Fig. 16 ^{13}C NMR spectrum of the purified acetone root fraction of *U. indica*

confirmed the molecular weight of the purified compound as 414; molecular formula as $C_{29}H_{50}O$ with different functional groups. Its chemical characteristics and structural properties showed conformity with that of (17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol). The fragmented ions of the purified fraction showed mass/charge values at 201.1, 337.4, 390.0, and 573.5 respectively (Fig. 17).

Discussion

Traditionally wild plants consisting of several bioactive phytochemicals are extensively used as a source of herbal preparations possessing therapeutic properties [31, 32]. The antifungal activity and activity index assessment revealed that acetone root extract was found to exhibit higher efficacy against *C. albicans* and *A. niger*. The findings revealed that the acetone root extract was potent against both the fungal cultures as compared from stem and leaf extracts. High antimicrobial activity in root extracts of *Chrysocoma ciliate* was reported [33]. However, similar reports were also documented in root extracts of *Andrographis ovata*, *Aristolochia indica*, *Eclipta prostrata*, and *Gloriosa superb* [34]. Since root is exposed to numerous soil microbes, so the plant upon recognizing the invading pathogen synthesize and stores varied phytochemicals to activate its defense mechanism [35–38].

The phytochemical analysis of *U. indica* extract revealed the presence of several bioactive phytochemicals in the crude acetone root extract. Similar findings reporting the presence of varied bioactive phytochemicals in crude extracts of several medicinal plants conferring antimicrobial activity was well-documented [39, 40].

The highest antifungal activity was recorded in 10th, 11th, and 12th fraction against *A. niger* and *C. albicans*. The active crude acetone root extract of *U. indica* showed seven prominent bands on TLC. The pooled acetone root fraction showed a single band with a Rf value of 0.90 suggesting the presence of a single compound. Similar results were also documented with Rf value of 0.90 from *Helicteres isora* with the similar solvent system, later identified as β-sitosterol [41].

The MIC and MFC of the crude acetone root extract and purified pooled fraction were evaluated against *A. niger* and *C. albicans*. The crude acetone root extracts of *U. indica* showed the MIC and MFC value of 1 mg/ml for *A. niger* and 0.5 mg/ml for *C. albicans*. The corresponding purified fraction exhibited significantly higher inhibition at 0.0625 mg/ml for *C. albicans*. The results revealed that the purified pooled fraction exhibited broad spectrum and significantly greater antifungal efficacy against both the fungal

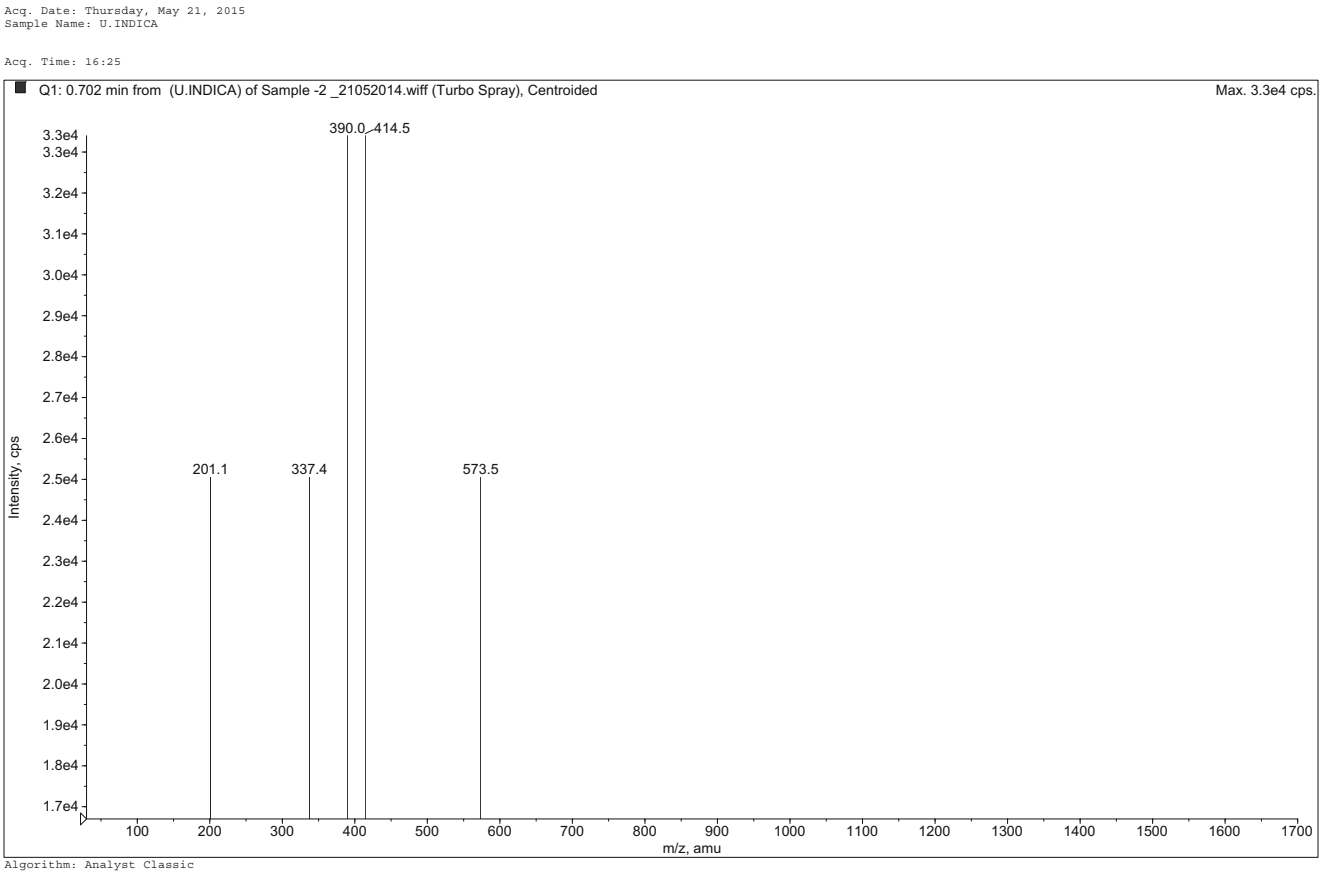

Fig. 17 Mass spectrum of the purified acetone root fraction of *U. indica*

cultures under investigation. The current observations corroborate with the work documented for better efficacy in the purified fraction as compared to their corresponding crude extract [42].

The purified fraction was further assessed for its synergistic or antagonistic potentiality against standard antifungals viz., clotrimazole, ketoconazole, and fluconazole and the results revealed that clotrimazole in combination with the purified fraction of *U. indica* exhibited synergistic activity against *A. niger* and antagonistic activity against *C. albicans*. Reports documenting synergistic/antagonistic activity of plant extract with antifungals against fungal species are available [43]. The purified fraction in combination with ketoconazole showed significantly higher synergistic antifungal activity for *C. albicans*, whereas antagonistic activity against *A. niger*. There are considerable reports documenting the use of ketoconazole as the drug of choice to produce significant synergistic activity against fungal pathogens [44, 45]. Fluconazole in combination with the purified fraction showed significant synergy against *C. albicans* but antagonistic activity in case of *A. niger*. Several researchers have documented the combination of fluconazole with different solvent crude extracts to produce significantly higher synergistic activity against human pathogenic fungal species [46–48].

The spectral analysis of the purified compound from crude acetone root extract of *U. indica* indicates that molecular weight of the purified compound is 414, molecular formula is $C_{29}H_{50}O$ with different functional groups. Its chemical characteristics and structural properties are similar to that of (17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol). β-sitosterol has been reported in literature [49–52] possessing antimicrobial activity from different plant species viz., *Flemingia strobilifera*, *Dillenia indica*, *Momordica charantia*, *Caylusea absyssinica*. However, the presence of this phytosterol in *U. indica* from Bastar possessing antifungal activity is the pioneer report of its kind. It has also demonstrated significantly higher synergy with known antifungal as well.

Conclusions

The combination therapy against human pathogenic fungi is a novel concept gaining increased recognition globally. Although, antibiotics are considered to be the most powerful weapons in combating microbial infections, but over the past few decades, they have become quite less effective against certain diseases due to the increased emergence of multidrug

resistance among human pathogens. The current situation is alarming and has turned the attention of scientists worldwide to combat this great problem by screening of a good number of medicinal plants for novel bioactive compounds for the amelioration of several dreadful diseases. Therefore, the combination therapy seems to be a boon for several patients with severe infections caused by multidrug-resistant human pathogenic microorganisms. However, in vitro testing and clinical trials are the prerequisite for the successful implication of this alternative therapy. Thus, keeping in view the above specific prospects there is an urgent need of extensive research in the area of medicinal plants with the aim of isolation, purification, and chemical characterization of the bioactive compounds using advanced spectral analysis viz., HPLC, UV-Vis, FT-IR, NMR (^{1}H & ^{13}C), ESI-MS, and subsequently exploring the synergistic antimicrobial activity along with its mode of action will definitely contribute for the better, safer, and cost-effective novel drug development for future generations.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Statement of Human Rights and Animal Welfare This article does not contain any studies with human participants or animals performed by any of the authors.

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