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IN – VITRO FUNGITOXIC EVALUATION AND GCMS ANALYSIS OF *CALOTROPIS PROCERA*

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ABSTRACT

The present investigation was carried out to find the fungitoxic potential of different extracts of *Calotropis procera*, and GCMS analysis of the best extract. Four different extracts of *Calotropis procera* (leaves) were prepared using four different solvents viz aqueous, methanol, acetone and ethanol. These extracts were analyzed for antifungal activity against different isolates of *Alternaria alternata*. Ethanolic extract completely inhibited the growth while methanol inhibited 76% - 81%, acetone 86% - 91% and aqueous gave least inhibition i.e. less than 3%. As ethanol showed best result. MIC and MFC was carried out for ethanolic extract and MIC for all isolated was noted to be 6.25 mg/ml and MFC for all isolates was 12.5 mg/ ml. Phytochemical analysis was also carried out which showed the presence of different secondary metabolites in different extracts. GCMS of ethanolic extract of *Calotropis procera* was also carried out which revealed the presence of Phytol (9.77%), n-Hexadecanoic acid (7.71%), Methyl commate A (7.71%), Lup - 20 (29) - EN - 3- YL acetate (5.73), as major compounds. The present study provides the insight that *Calotropis procera* ethanolic extract can serve as biofungicide for controlling disease caused by *Alternaria alternata*. Thus there will be natural origin of the fungicide which will have no side effects.

KEYWORDS: *Calotropis procera*, *Alternaria alternata*, MIC, MFC, GCMS.

INTRODUCTION

Calotropis procera belongs to family Asclepiadaceae and has a very wide range of ecological amplitude. It is a drought resistant, salt tolerant weed found along degraded roadside, lagoon

edges and in overgrazed pastures. It is commonly known as calotrope, rubber tree, akando, usher, madar, arka, swallowed wort, Sodom apple etc. All parts of plant exude white latex when cut or broken, which act as a defence strategy against insects, viruses and fungi.[1] A large number of secondary metabolites have been isolated from this plant that include many flavonoids,[2] cardiac glycosides,[3] Triterpenes[4] and sterols.[5] The whole plant contains a and b amyrin, teraxasterol, gigantin, giganteol, isogiganteol, b-sitosterol. In Ayurveda, the Indian system of medicine, this plant is reported for the treatment of several infections. It also serve as a source of renewable energy, hydrocarbons,[6] green manure, sulphur hydroxide emission and an indication of exhaust soil.[7]

The objective of the present investigation was to acertain the antifungal potential of different leaf extracts of *Calotropis procera* to control the phytopathogenic fungi *Alternaria alternata*.

MATERIALS AND METHODS

Collection of leaves: Fresh and healthy leaves of *Calotropis procera* were collected from BHEL area, Haridwar for screening their antifungal activity. Identity of plant species was authenticated by referring taxonomic literature.

Isolation of test fungus: Different isolates of the phytopathogenic fungi *Alternaria alternata* were isolated from diseased leaf of Potato (*Solanum tuberosum*), Tomato (*Lycopersicon esculentum*), Onion (*Allium cepa*) and Mustard (*Brassica campestris*). The standard tissue isolation procedure was followed to isolate the pathogen. The infected leaf bits were surface sterilized with 1:1000 mercuric chloride ($HgCl_2$) solution for 30 sec and repeatedly washed separately in sterilized distilled water to remove the traces of mercury if any and then transferred to sterilized petri plate (4 leaf bits per petri plate) containing potato dextrose agar.The petriplates were incubated at 28±2°C and observed periodically for the growth. As a bit of fungal growth developed from infected tissue, it was transferred to PDA slants and incubated at 28±2°C for 7 days. Then these slants with pure culture were used for further studies. The pathogens were subcultured on PDA slants and allowed to grow at 28±2°C for 7 days and such slants were preserved in refrigerator at 5°C and renewed once in 30 days.

Identification of pathogen: Identification of pathogen was done by lactophenol cotton blue staining and further observing them under microscope (40X). The conidial body, beak and septa were observed. The observation was compared with those of the standard

characteristics given by Barnett to identify the pathogen.[8] Further, the identity of the pathogenic isolates was confirmed by the division of pathology, IARI, New Delhi.

Pathogenicity test: Pathogenicity test was conducted to identify the abilities of the fungi to infect the host plant. Pathogenicity test was performed by spraying the spore suspension on healthy leaves of one month old potted host plant. Control plants were sprayed with sterile water. The plants were covered with plastic bags for 10 days. The pathogenicity test was repeated once. The first lesion appeared after a period of 12±2 days. The pathogens were consistently reisolated from the lesion. The isolates obtained were compared with the original culture for confirmation of fungus under study.[9]

Extract preparation: Extract was prepared by cold soxhlet extraction method. The leaves were thoroughly washed 2-3 times with tap water and then with distilled water and were shade dried. After getting dried up they were converted into powdered form with the help of mixer grinder. 200 mg/ml concentration was selected, so 20 g of powdered leaf material was used for extraction using 100 ml of different solvents (aqueous, methanol, acetone and ethanol). The obtained extracts were further filtered through Whatmann filter paper no.1. The supernatant was collected and was evaporated in vacuum rotavapour to make final volume 1/5th of the original volume. Then the final extract was stored at 4˚C in airtight bottles for further study.[10]

Antifungal Activity: The effect of phytoextracts of different weeds was tested in vitro by food poison technique to know their antifungal effect on the growth of different isolates of *Alternaria alternata.*[11, 12, 13 & 14] 3 ml of extracts was mixed with 50 ml of PDA and then autoclaved. The autoclaved media along with the extract were transferred into sterilized petri plates aseptically. The petri plates were inoculated after solidification by placing 3 mm mycelium disc from 7 days old culture of fungal isolates at the center which was cut aseptically with flamed cork borer. Three replicates of each treatment were maintained. The plate with distilled water served as negative control and plate along with synthetic fungicide mancozeb (0.2%) served as positive control. The petri plates were incubated at 28±2°C for 7 days. Radial mycelium growth was recorded and further converted to percentage inhibition by using following formula.[15]

$$\text{PERCENTAGE INHIBITION} = (G_c - G_t / G_c) \times 100$$

G_c = Radial diameter of control – diameter of inoculums disc

G_t = Radial diameter of plate with plant extract – diameter of inoculums disc

Minimum inhoibitoy concentration (MIC): The minimum inhibitory concentration (MIC) was determined by microdilution method using serially diluted plant extracts according to the NCCLS protocol.[16] As the ethanolic extract of all the three weeds gave good antifungal result, the ethanolic extracts of all the three weeds were selected for the MIC. These ethanolic extracts were diluted to get a series of concentration from 0.39 mg/ ml to 200 mg/ ml, in sterile PD broth in 96 well microtiter plate. Mancozeb Fungicide used was also diluted. The fungal suspension of 50 µl was added to the broth dilutions. These were incubated for 7 days at 28±2°C. MIC of each extract was taken as the lowest concentration that did not give any visible fungal growth.

Minimum fungicidal concentration (MFC): Minimum fungicidal concentration is the lowest concentration of antifungal agent that will prevent the growth of an organism after subculture on an antifungal free media. To determine MFC 50 µl mixture from the well showing MIC and from wells having extract concentration more than MIC was placed on PDA plate without extract and the plates were incubated at 28±2°C for 7 days. After incubation the plates were examined for the growth of fungal isolates to determine the concentration of the extract at which no growth occurred.

Chemical analysis: Phytochemical screening was done by standard procedures and for Chromatographic analysis Gas Chromatography Mass spectroscopy (GCMS) was selected. The GCMS analysis was done by using GC-MS-QP 2010 Plus (Shimadzu Mass Spectrometer- 2010 series system) from AIRF, JNU, New Delhi equipped with Rtx_5Ms column (30 m x 0.25 mm id), 0.25 µm film thickness. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1.21 ml/min. Injection temperature was set at 260°C. The oven temperature was programmed at 60°C with 2 min hold time, 250°C with 5 min hold time and 310°C with 14 min hold time. 2.5 µl of sample was injected through autoinjector with split mode. Identification of the compounds of the sample was based on GC retention time on Rtx_5Ms column. The total running time was 65 minutes. The software used for analyzing the compounds was GCMS solution software by the help of Nist-08 and wiley-08 libraries. The relative percentage amount of each component was calculated by comparing its average peak area to the total area.

RESULTS

Four different isolates of *Alternaria alternata* were isolated from selected diseased plants of potato (*Solanum tuberosum*), tomato (*Lycospersicon esculentum*), Onion (*Allium cepa*) and mustard (*Brassica campestris*) denoted as A1, A2, A3 & A4 respectively. The cultures were grown on PDA. The pure culture of all the four fungal isolates appeared to be grayish white at first and became black later on. Microscopic study revealed that fungus produced abundant conidia having 3-8 transverse septations and 1-2 longitudinal septation. Conidia were solitary and also in short chains, mostly ovoid with a short conical or cylindrical apical beak and smooth walled. Hyphae were branched, and septate. Septate conidiphores were present that were variable in length. Based on these characters the isolated fungus were identified as *Alternaria alternata*.[8] The fungal isolates were further confirmed as *Alternaria alternata* from pathology department, IARI (Indian Agricultural Research Institute) New Delhi, they also assigned laboratory code numbers to these fungal isolates. A standard ITCC culture *Alternaria alternata* (6203) was also procured from pathology department, IARI, New Delhi. Pathogenicity test revealed that the spores of fungal isolates (A1, A2, A3, A4), which were sprayed to their respective host lead to cause disease which confirmed the pathogenicity of the fungal isolates and later on when these pathogens were reisolated from the diseased lesions and identified, their characters matched with characters of *Alternaria alternata*. Thus the pathogenicity confirmed the pathogenesis of fungal isolates (A1, A2, A3 & A4) to their respective host.

Aqueous extract gave very little activity for all the fungal isolates which were recorded as 2.19%, 2.86%, 1.89% and 2.81% for A1, A2, A3 and A4 respectively. Methanolic extract showed 76%- 83% percentage inhibition for A1, A2, A3 and A4 respectively. For acetonic extract the percentage inhibition was recorded 86.43% for A1, 89.71% for A2, 90.79% for A3, 91.57% for A4. Ethanolic extract showed 100% inhibition for all the fungal isolates (**Table-1).** For A1, A2, A3 and A4 the MIC of ethanolic extract *Calotropis procera* was recorded as 6.25 mg/ml and MFC was recorded 12.5 mg/ml for all isolates.

Phytochemical tests revealed that alkaloid, flavanoid and terpenoid was present in all the extracts, saponin, glycoside, steroid was present in all except aqueous extract. Tannin was reported in acetone and ethanol extract. Phenol was observed only in ethanol extract. Anthraquinone and reducing sugar was absent in all extracts.

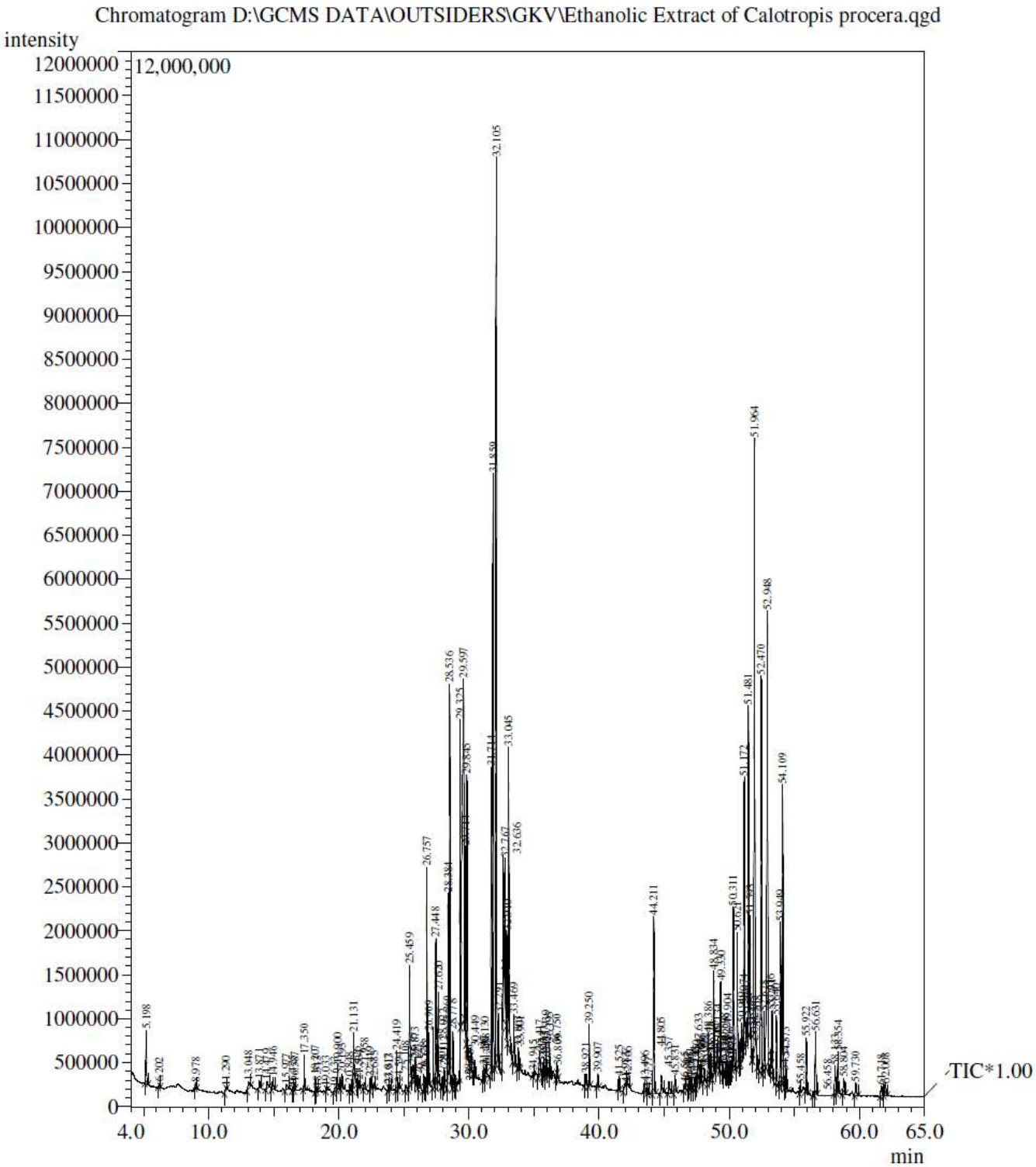
Interpretation on mass spectrum GC-MS was conducted using the database of National Institute standard and technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test material was ascertained. The analysis of the organic compounds present in the ethanolic extract of leaf of *Calotropis procera* by GC-MS analysis revealed the presence of 147 compounds. The 25 major compounds with their retention time (RT), Molecular weight (MW) and Concentration (%) are presented in **Table-2** and the chromatogram is presented in **graph-1**. The identified compounds belong to different phytochemical groups such as terpenoids, fatty acid, sterols, glycosides, hydrocarbons, phenolics and heterocyclic compounds. The major compounds belong to terpenoid group followed by fatty acid, sterols and others.

Table 1: Antifungal activity of *Calotropis procera* extracts.

S.No	Extracts	A1		A2		A3		A4	
		Mean ±S.D	Percentage inhibition	Mean± S.D	Percentage inhibition	Mean± S.D	Percentage inhibition	Mean ±S.D	Percentage inhibition
1	**C.Aq**	59.66± 0.57	2.19	56.66± 0.57	2.86	53.33± 0.57	1.89	57.66± 0.57	2.81
2	**C.Me**	14.33± 1.52	76.5	11.33± 1.15	80.57	9.33±0. 57	83.63	11±0	81.45
3	**C.Ac**	8.33±0 .57	86.34	6±0	89.71	5±0	90.79	5±0	91.57
4	**C.Eth**	0±0	**100**	0±0	**100**	0±0	**100**	0±0	**100**
5	**Mancozeb**	18.0±0	70.49	17±0	72.85	16.66± 0.57	69.7	18±0	69.66
6	**Control**	61±1	0	58.33± 1.15	0	54.33± 0.57	0	59.33± 0.57	0

C.Aq = *C.procera* aqueous extract; C.Me = *C.procera* methanolic extract

C.Ac = *C.procera* acetonic extract; C.Eth = *C.procera* ethanolic extract



Graph 1: GCMS analysis of ethanolic extract of *Calotropis procera*

Table- 2: GCMS analysis of ethanolic extract of *Calotropis procera*

S.No.	Name	R.Time	Area	Area%
1	Phytol	32.105	44479440	9.77
2	n-Hexadecanoic acid	29.597	35098539	7.71
3	METHYL COMMATE A	51.964	35127002	7.71
4	LUP-20(29)-EN-3-YL ACETATE	52.948	26099122	5.73
5	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	31.859	26022260	5.72
6	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,	52.47	20249048	4.45
7	ETHYL (9Z,12Z)-9,12-OCTADECADIENOATE #	33.045	13942361	3.06
8	HEXADECANOIC ACID, METHYL ESTER	28.536	13087550	2.87
9	LUPENYL ACETATE	54.109	12840622	2.82
10	NOROLEAN-12-ENE	51.481	11313183	2.48
11	Dibutyl phthalate	29.325	11262941	2.47
12	STIGMAST-5-EN-3-OL, (3.BETA.)-	51.172	11034890	2.42
13	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	31.714	10494565	2.3
14	Squalene	44.211	9557899	2.1
15	HEXADECANOIC ACID, ETHYL ESTER	29.845	8654201	1.9
16	ERGOST-5-EN-3-OL, (3.BETA.,24R)-	50.311	8634372	1.9
17	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE	26.757	7156517	1.57
18	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	32.767	7010420	1.54
19	Tetradecanoic acid	25.459	6744275	1.48
20	Lupan-3-ol, acetate	53.949	6728355	1.48
21	Butyl octyl Phtalate	29.714	6242611	1.37
22	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	28.384	5705474	1.25
23	Stigmasterol	50.621	5575980	1.22
24	Pthalic acid , isobutyl ester	27.448	5457573	1.2
25	Methyl 7,11,14-eicosatrienoate	32.636	5374844	1.18

DISCUSSION

The result of this conceptual study clearly reflects that this weed has inherent ability to control the growth of this phytopathogenic fungi. Antifungal activity of different extracts of leaves *C. procera* against *A. alternata* revealed complete inhibition of *A. alternata* by ethanolic extract followed by acetone and methanol and negligible inhibition was noticed in case of aqueous extract which revealed the presence of antifungal bioactive compounds in *C. procera* extracts. The present finding was in agreement with several previous reports. Sharma & Trivedi, 2002 found that *C. procera* showed good antifungal activity as compared to *P. hysterophorus* which was in agreement with present finding. Chand and Singh, 2005 found that *C. procera* have significant effect on fungus. Hassan *et al*., 2006 screened aqueous and other organic solvent extracts of leaves in *C. procera* and found that it has antifungal

potency. Similarly Kareem *et al*., 2008 found the antimicrobial activity ethanolic extracts of leaf of *C. procera*. Vadlapudi and Naidu, 2009 also found that *C. procera* has antifungal potency against *A. alternata*, which supported the present study. Vadlapaudi & Naidu, 2010 examined bioassays for antimicrobial activities using stem, leaves, and flowers of *C. procera*. The antimicrobial activities of the organic solvent extracts were tested on various test organism (*Alternaria alternata, Aspergillus flavus, Aspergillus niger, Bipolaris bicolor, Curvularia lunata, Penicillium expensum, Pseudomonas margenales, Rhizoctonia solani* & *Ustilago maydis*). The results of *C. procera* showed highest antimicrobial activity against *A. alternata* with all concerned.[17, 18, 19, 20, 21 & 22]

Neenah and Ahmad, 2011 found antimicrobial activity of *C. procera* extract and MIC value reported by them was 0.25 mg/ml and 0.75 mg/ml. Goyal and Mathur, 2011 also found that extracts of *C. procera* have antimicrobial potency. In an investigation performed by Vadlapudi *et al.*, 2012 antimicrobial activity was reported by methanolic extract of aerial parts of *C. procera*. It gave moderate effect against tested bacteria and fungus.[23, 24 & 25]

Komathi *et al.*, 2012 confirmed the antifungal potency of ethanolic extract of leaves of *C. procera* which supports the present finding. Vadlapudi *et al.*, 2012 showed significant to moderate antibacterial activity. The result of MIC values were lowest at 66 mg/ml and highest 152 mg/ml. The synthetic fungicide used for the comparison was Mancozeb (0.2%). It gave 68-77% inhibition against *A. alternata*.[25 & 26] Mancozeb has been reported as effective measure by several workers against *A. alternata*.[27, 28, 29, 30 & 31]

In *C. procera* extracts it was observed that alkaloid, flavanoid and terpenoid was present in all the extracts. saponin, glycoside, steroid was present in all except aqueous extract. Tannin was reported in acetone and ethanol extract. Phenol was observed only in ethanol extract. Anthraquinone and reducing sugar was absent in all extracts. Doshi *et al.*, 2011 performed phytochemical screening of the ethanol extracts of flowers, young bud, mature leaves and stems of *C. procera* (Ait) R. Br. (Asclepiadaceae) and found that alkaloid, cardiac glycoside, saponin, phenolics, triterpenoids and tannins were present in almost parts which was in agreement with present result.[19 & 32]

147 compounds were identified by GCMS analysis. The major percentage of the compounds was terpenoids followed by fatty acid, sterols and others. The major compounds found in GCMS were Phytol (9.77%), Methyl commate A (7.71%), n-Hexadecanoic acid (7.71%),

Lup-20(29) En-3-yl acetate (5.73%), 9,12,15-Octadecatrienoic acid, methyl ester (5.72). The present result clearly indicates that antifungal activity shown by extracts of *C. procera* is due to these secondary metabolites. There are several reports presenting the antifungal potential of these secondary metabolites.

Singh and Singh, 2003 found the antimicrobial activity of terpenoids. Walters *et al*., 2004 that fatty acids such as linolenic acid, linoleic acid and oleic acid were effective against plant pathogenic fungi *Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avanae* and *Crinipellis perniciosa*. Altieri *et al*., 2009 found that lauric acid exerted strong bioactivity against moulds. Similarly Gupta *et al*., 2011 also found that the terpenoids have antimicrobial potency. Thornton, 2011 performed antifungal activity of fatty acids to *Pithomyces chartarcum* (Berk. And Curt) M. B. Ellis and found good antifungal potency of fatty acid.[33, 34, 35, 36 & 37]

The above reports supports that the antifungal potency shown by the weeds is due to the combined effect of different secondary metabolites. For specification each of the identified secondary metabolites has to be studied individually for detecting their antifungal activity.

CONCLUSION

The present study concludes that the disease caused by *Alternaria alternata* can be controlled by use of weed extracts. In this way the weeds which are considered as undesirable plant, will become significant as they will be exploited against fungal disease. Thus one problem itself will be the solution of other problem and best out of worst can be used.

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