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Antidermatophytic activity of ethanolic leaves extract of *Limonia acidissima* Groff.

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Key words: Antidermatophytic activity; *Limonia acidissima* Groff. Leaves extract; MIC; MFC; MBC

ABSTRACT

In the present study *Limonia acidissima* Groff. ethanolic leaves extract was used for the detection of its antidermatophytic assay. It results broad spectrum of antifungals and antibacterial. Where the antimycotic activity against *Trichophyton rubrum*, *Trichophyton tonsurans, Trichophyton mentagrophytes, Microsporium gypseum, Candida albicans* and four *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* were more pronounced. The effective antidermatophytic activity observed against *M. gypseum, T. tonsurans, E. coli* and *B. subtilis.* Minimum Inhibitory Concentration of all the test microbes was determined using broth dilution technique. MFC, MBC also standardized. Preliminary Phytochemical tests were carried for the revealing basics antimicrobial responsible molecules of secondary metabolites.

1. INTRODUCTION

Plant existence is necessary for the continued existence of all animals on earth planet, together with human being (TS Jasmine et al., 2007, S Jeeva et al., 2007). Man has forever been reliant upon plants for the requirements for his endurance, not only for foodstuff, feed and energy, but also for remedy, fibers, natural products and other goods S Kiruba et al., 2008, RC Laloo et al., 2006). The reason for the rising consciousness among the population towards natural products, phytochemistry, natural medicine is magnetize more concentration than allopathic system. Moreover this system of medicine ecofriendly and free and less toxic without side effects (Malviya R et al., 2012).

Limonia acidissima belongs to family Rutaceae. The deferent parts of this plant like leaves, bark and fruits have been using medicinal purposes from ancient era (Khewkhom N 2008, Adikaram NKB 2007, Khare CP 2007, Kirtikar KR 2005, Tarakeswara Naidu M 2012, Kamat CD 2003, Kim KH 2009, Bandara BMR 1990, Gupta R 2009, Khewkhom N 2008). As the plant is reported to have various medicinal uses, but there was no reports on antidermatophytic studies so that in the present report the antidermatophytic activity was aimed, revealed using ethanolic leaves extract.

2. MATERIALS AND METHODS

Collection of plant materials

The leaves of *Limonia acidissima* were collected in fresh bags from different places of Hyderabad Karnataka, identified with the help of Flora of Gulbarga district (Seetharam Y N 2000). The voucher specimen (HGUG-443) deposited in herbarium centre, department of Botany, Gulbarga University, Karnataka, India. The collected plant materials were initially rinsed with distilled water to remove soil and other contaminants and dried on tissue papers in laboratory at 37 ± 2ºC for week.

Extraction of crude using soxhlet technique

The leaves after drying were ground in a grinding machine in the laboratory. 25 g of shade dried powder was weighed and extracted with ethanolic in soxhlet extractor for 48h. The extracts were concentrated under reduced pressure using oven and preserved in refrigerator in airtight bottles for further use.

Safeguarding of Dermatophytes for the experiments

Five fungal cultures strains, *Trichophyton rubrum*, *Trichophyton tonsurans, Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and five bacterial strains, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* obtained from MRMC, Gulbarga, Karnataka, India were used in the present study. Bacterial cultures were grown in nutrient broth (Himedia, M002) at 37ºC and maintained on nutrient agar slants at 4ºC fungal cultures were grown in potato dextrose broth at 28 ºC and maintained on potato dextrose agar slants at 4ºC.

Standardized antidermatophytic experimental cup plate technique (Magaldi S 2004)

The assay was conducted by agar well diffusion method. Using PDA, fungal lawn was prepared using 6 days old culture strains. The fungal strains were suspended in 0.85% NaCl solution and adjusted to a turbidity of 0.5 Mac Farland standards (108 CFU/ml). 1 ml of fungal strain was spread over the medium using a sterilized glass spreader. Using flamed sterile borer, wells of 4 mm diameter were punctured in the culture medium and required concentrations of serially diluted extract (0.62-40mg/ml) was added to the 20µl to each wells. The plates thus prepared were left for diffusion of extracts into media for one hour in the refrigerator and then incubated at 37^{o}C. After incubation for 48h, the plates were observed for zone of inhibition. Diameter zone of inhibition was measured and expressed in millimetres. Dimethyl formamide (DMF) was used as a negative control. The experiments were conducted in triplicates. The same method was followed for testing antibacterial activity using nutrient agar medium incubated at 37^{o}C for 18h.

Determination of minimum crude drug dose concentrations viz., MIC, MFC, MBC's (NCCLS 1997)

One ml of sterile liquid Sabouraud medium was added to 11 sterile capped tubes, 1 ml of each solvent extracts suspension was added to tube 1. The contents were mixed and 1 ml was transferred to tube 2. This serial dilution was repeated through to tube nine and 1 ml was discarded from tube 9. Fifty µl of inoculum was added to tubes 1-10 and the contents were mixed. Medium control (no inoculum and no drug) and inoculum control (no drug) tubes were prepared. The final concentrations of each plant solvent extracts ranged from 05mg/ml to 0.02 mg/ml. The tubes were incubated at 30^{o} C for 96 h. The fungal growth in each tube was evaluated visually depending up on the turbidity in the tubes. MIC was defined as the drug concentration at which the turbidity of the medium was the same as the medium control.

Ten µl aliquot of cell suspension from the tube without observed growth of dermatophytes was inoculated on to Sabouraud dextrose agar. Minimum fungicidal concentration (MFC), Minimum bactericidal concentration (MBC) of test compound were determined as the lowest concentration of the agent at which no colonies were seen after 4 days at 30^{o}C. Triplicate sets were maintained for each experiment.

Occurrence techniques of Secondary metabolites in ethanolic crude extract of *Limonia acidissima*

Preliminary tests, for the detection of secondary metabolites, were carried out using ethanolic leaves extracts of *Limonia acidissima* by adopting standard methods (Horborne JB 1998).

Statistical analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of difference $p \sim 0.05$ was considered to denote a statistically significance All data were presented as mean values ± standard deviation (SD).

3. RESULTS AND DISCUSSION

Antidermatophytic fungal experimental results by cup plate technique of leaf ethanolic extracts of *Limonia acidissima* showed against in order to *M. gypseum, T. tonsurans, T.*

mentagrophytes, *C. albicans* and least *T. rubrum*. The zone of inhibition was partially linked with concentration of crude (Table 1). The Minimum Inhibitory Concentrations of MFC were given in figure 2.

Figure 1. *Limonia acidissima* Twig and fruit.

Table 1. Antidermatophytic fungal experimental results by cup plate technique of leaf ethanolic extracts of *Limonia acidissima.*

leaf ethanolic extracts of *Limonia acidissima*	Diff. Concentrations in mg/ml	Test organisms & inhibition of zones in mm				
		Fungal strains				
		T. rubrum	*T.tonsurans*	*T.mentagrop-hytes*	*M .gypseum*	*C .albicans*
	40	06.33±1.52	10.33±0.57	08.00±0.00	12.66±0.57	08.33±0.57
	20	05.66±1.15	08.66±1.52	05.66±1.52	07.00±1.00	06.66±1.15
	10	NA	06.33±0.57	NA	05.33±1.52	03.66±0.57
	5	NA	04.00±0.00	NA	04.66±0.57	NA
	2.5	NA	NA	NA	NA	NA
	1.25	NA	NA	NA	NA	NA
	0.62	NA	NA	NA	NA	NA
K	01	20.00±0.00	24.00±0.00	18.00±0.00	21.00±0.00	23.00±0.00

Tr - Trichophyton rubrum, Tt – Trichophyton tonsurans, Tm- Trichophyton mentagrophytes, Mg- Microsporium gypseum, Ca - Candida albicans NA-Not Active, K-Ketoconazole,

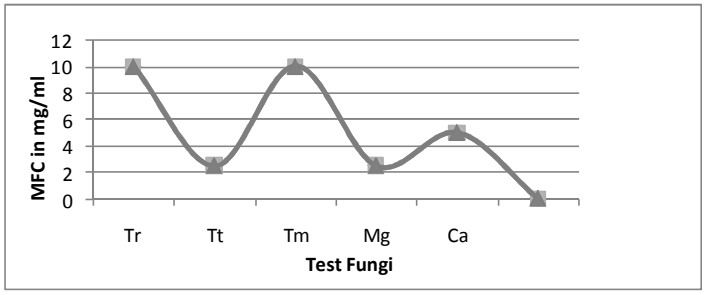

Figure 2. Determination of minimum crude drug dose concentrations viz., MFC, (mg/ml) of *Limonia acidissima*

Tr - Trichophyton rubrum, Mg- Microsporium gypseum, Ca - Candida albicans, Tt -Trichophyton tonsurans, Tm -Trichophyton mentagrophytes.

Table 3, Antidermatophytic bacterial experimental results by cup plate technique of leaf ethanolic extracts of *Limonia acidissima*

leaf ethanolic extracts of *Limonia acidissima*	Diff. Concentra-tions in mg/ml	Test organisms & inhibition of zones in mm			
		Bacterial strains			
		S. aureus	*P. aeruginosa*	*B. subtilis*	*E. coli*
	40	09.66±0.52	10.33±0.57	16.66±1.15	18.66±0.00
	20	06.00±2.00	07.00±1.00	14.33±0.00	14.00±1.00
	10	05.66±2.57	06.66±0.00	09.00±1.00	10.00±0.00
	5	04.00±0.00	04.00±1.00	06.00±0.00	07.00±2.00
	2.5	NA	NA	05.33±0.15	05.00±0.00
	1.25	NA	NA	NA	04.00±0.00
	0.62	NA	NA	NA	NA
S	01	25.00±0.00	24.00±0.00	24.00±0.00	26.00±0.00

Sa- Staphylococcus aureus, P a- Psudomonas aeruginosa, Bs- Bacillus subtilis, *Ec- Escherichia coli, NA-Not Active, S-Streptomycin Sulphate.*

Antidermatophytic bacterial experimental results by cup plate technique of leaf ethanolic extracts of *Limonia acidissima* showed against in order to *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Psudomonas aeruginosa.*

The zone of inhibition was purely linked with concentration of crude (Table1). The Minimum Inhibitory Concentrations of MBC were given in figure 3.

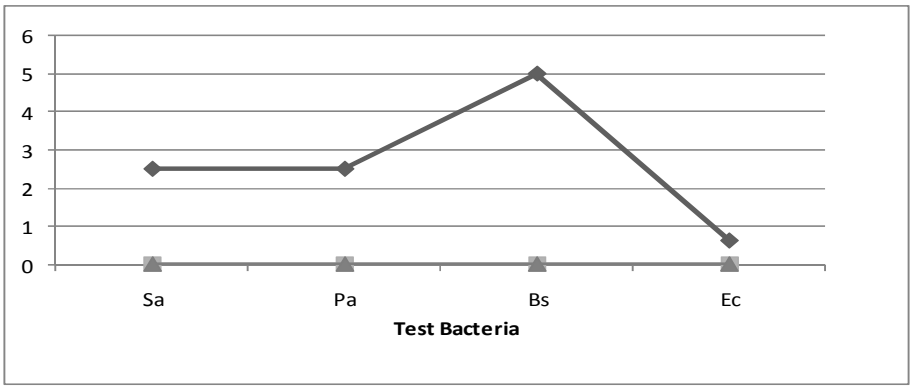

Figure 3. Determination of minimum crude drug dose concentrations viz., MBC, (mg/ml) of *Limonia acidissima*.

Sa- Staphylococcus aureus, Bs- Bacillus subtilis, *Ec- Escherichia coli*, *Pa- Pseudomonas aeruginosa.*

The negative control (DMF) was not shown inhibition against all the tested fungal and bacterial strains. Ketoconazole used as a positive control at conc. 2 mg/ml showed 18 to 24 mm against antifungal activity, whereas the streptomycin sulphate standard antibacterial drug showed 24 to 26 mm inhibition zone.

Table 2. Occurrences of Secondary metabolites in ethanolic crude extract of *Limonia acidissima*

Phytochemical	Inference
	Ethanolic leaf extract
Alkaloids	++
Phenol	+
Flavonoids	++
Tannins	-
Triterpenoids	+
Steroids	-
Saponins	+
Glycosides	++

+Present, ++Strongly Present, -Absent.

In the present report the ethanolic extract used for the antidermatophytic activity its first report, whereas earlier with the same extract the antimicrobial activity was reported by Neelamadhab Panda et *al.,* in (2013), but they were not screened dermatophytes. From the earlier period the fruit have been using so that the maximum researchers viz., Jayashree VH, Ramesh Londonka (2014), Prasanta kumar Mahapatra and Debasis pradhan (2014) were concentrating on fruit, somewhat especially the present report revealed antidermatophytic activity using leaves extracts. Ethanolic solvent one of the polar solvent so its having capability to dissolve a maximum solvents, so that in the present study ethanolic extract used for the tests, while previously G. Keshava naidu *et al.,* (2014) used low polar solvents for antibacterial activity.

The preliminary phytochemical results of the present report reveals the strongly presence of phytochemicals such as alkaloids, flavonoids and glycosides. Slightly positive response shown the tests of phenols, triterpenoids, saponins. Where the steroids, Tannins were absent. It may defers with earlier studies of Saima Y , Ghosh P , Parthasarathi G , Swapnadeep Parial *et al.,* (Saima Y 2000 , Ghosh P 1982, Parthasarathi G 1991, Swapnadeep Parial 2009, G. Kishor Naidu 2014) used methanolic extract. Therefore, further detailed studies to be desirable in isolation, purification and identification of pure compounds responsible for antidermatophytic activity.

4. CONCLUSION

The antidermatophytic action of ethanolic leaf extract of *Limonia acidissima* recorded very good results against fungi and bacteria but where dose depended activity observed against bacterial dermatophytes, this may due to secondary metabolites different reactions with mycotic fungi and bacteria. The purify fragment should given the detailed data of mode of reaction. Supplementary study on the fine-tuning of targeted groups to fungi and bacteria to be found.

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