COMPARATIVE ANALYSIS OF ANTIDERMATOPHYTIC ACTIVITY OF CRUDE ACETONE LEAF EXTRACT OF *UVARIA NARUM* AND ITS MOST ACTIVE PARTIALLY PURIFIED FRACTION.

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Abstract : *Uvaria narum* has been used for gastrointestinal problems, constipation, low backache, jaundice, fever and for skin diseases (eczema, pityriasis) in traditional and ethnomedical practices. Our preliminary antifungal screening of various leaf extracts of *U. narum* revealed antifungal activity for its acetone extract. The extract was subjected to fractionation studies and the most active antifungal fractions were determined by studying their activities against 3 dermatophytes, *Trichophyton rubrum*, *Microsporum canis* and *Microsporum gypseum* that are human pathogens. The most active fraction was Fraction 8 of Partially Purified acetone extract of Uvaria narum leaves {PPAc-UnAc (8)} that was determined according to Clinical and Laboratory Standards Institute (CLSI) method M38-A2. A comparison of MIC and MFC of the extracts at three level of purity (Crude extract UnAC; Charcoal treated extract, CH-Ac and Partially Purified fraction 8 of PPAc-UnAc) was done with the same (CLSI) method M38-A2. It was found that the activity of the extracts increased with the purity of the fractions obtained. The Partially Purified fraction 8 showed the maximum antifungal activity and the crude extract exhibited minimum antifungal activity among the three. *Minimum Inhibitory Concentrations* (MIC)and *Minimum Fungicidal Concentrations* (MFC)were determined for all the three extracts at three levels of purification
The most active fraction showed very good activity against the three human dermatophytes *Microsporum canis*, *Microsporum gypseum* and *Trichophyton rubrum.*
This study justifies the ethnomedicinal uses of *U. narum* against skin conditions.

IndexTerms **- *Uvaria narum*, dermatophytes, CLSI method, MIC, MFC, Partially Purified fractions**

I. INTRODUCTION

Uvaria narum Dunal (Wall) (Annonaceae), known as narumpanal in local languages, is a woody climbing shrub found in low lying areas in the foothills of the southern Western Ghats in India. *U. narum* has been traditionally known for gastrointestinal uses in Tamil Nadu and for skin diseases in the Dakshina Kannada district of Karnataka in India (Prabhu *et al*., 2014). *U narum* is popularly used in ethnomedicine for the treatment of eczema and pityriasis (Reddy *et al*., 2012; Padyana *et al*., 2013; Florence *et al.*, 2014). *U. narum* is also used to treat constipation, low backache, jaundice and fever (Padyana *et al*., 2011). A decoction of the root bark of *U. narum* is given for women to control fits at the time of delivery (Khare, 2007). Genus *Uvaria* was spotted when Jolad and co-workers accidently isolated a new class of compounds, acetogenins, with significant anticancer activity from the roots of *U. acuminata* in 1982 (Jolad *et al.*, 1982). This report was followed by a surge of activities focused on acetogenins, primarily from Annonaceae (Rupprecht *et al.*, 1990), and now about 400 acetogenins have been isolated with new ones being added regularly. Acetogenins and other phytoconstituents were also isolated from *U. narum* by various authors (Hisham *et al.*, 1990; Hisham *et al.*, 1991a; Hisham *et al*., 1991b; Parmar *et al*., 1994; Parmar *et al*., 1995). Acetogenins isolated from the root bark of *U. narum* showed anifungal properties against *Candida albicans, Aspergillus niger, Penicillium notatum, Trichophyton mentagrophytes, Microsporum gypseum and Epidermophyton floccosum*, of which the last three are dermatophytes (Padmaja *et al.*, 1993). Aqueous extract of *U. narum* leaves inhibited the growth of the foot rot pathogen of black pepper, *Phytophthora capsici* (Bindu *et al.*, 1998). Essential oils from different parts of *U. narum* were also investigated by various authors (Hisham *et al*., 1989).

Our preliminary antifungal screening of the petroleum ether, acetone and methanol extracts of *U. narum* leaves revealed excellent antifungal activity for the acetone extract (UnAc)(Varghese AE *et al* ,2017(a); Varghese Alka E. *et al* 2017 *(b)*) Our objective was to purify the active crude extract and find out the most active fraction from the partially purified extract by analysing its antifungal activities against three dermatophytes, *Trichophyton rubrum, Microsporum canis* and *Microsporum gypseum.*

2. Material and methods

2.1. General

Silica gel (60-120, 200-400), hexane, acetone, chloroform, agar and activated charcoal were purchased from Merck, India. TLC was carried out using pre-coated silica gel (60 F254, 20 x 10 cm, 0.2 mm thickness) plates (E. Merck, Germany). Potato Dextrose Broth (PDB) was purchased from HiMedia, Mumbai. Distilled solvents were used for chromatographic separations.

2.2. Plant material

U. narum leaves were collected from the Vazhappally Temple grounds at Changanacherry, Kottayam district in Kerala in November 2017, washed thoroughly, shade dried and powdered.

2.3. Test organism

The following dermatophytes were procured from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh.

• Microsporum canis (MTCC 2820)
• Microsporum gypseum (MTCC 2819)
• Trichophyton rubrum (MTCC 296)

Cultures of *M. gypseum*, *M. canis* and *T. rubrum* were maintained in Sabouraud dextrose agar in HiMedia, (HiMedia Mo 33). The purity of standard cultures was tested by examining the morphology of macroconidia and shape and disposition of microconidia by lactophenol cotton blue staining and by macroscopic appearance and pigmentation of the colony on Sabouraud dextrose agar, (Hoog G.S.*et al*,)

2.4. Preparation of extracts

Dried *U. narum* leaf powder (100 g) was extracted with 400 ml acetone in a Soxhlet apparatus for 18 hours. The extract was filtered using ordinary filter paper, then with Whatman's filter paper no. 1 and finally concentrated using a rotary evaporator. Acetone extract (UnAc) was passed through activated charcoal to remove chlorophyll (Evans, 2009), and the resultant yellow coloured solution was concentrated to obtain the charcoal treated acetone extract (CT-UnAc).

2.5. Bioactivity guided fractionation and column chromatography:

CT-UnAc was subjected to silica gel column chromatography. Briefly, CT-UnAc (4 g) was pre-adsorbed onto 10 g of silica gel 200-400 and was loaded onto the column which was pre-packed with 100 g of silica gel 200-400. The column was run isocratically with 9.8:0.2 chloroform-methanol and a total of 18 fractions (25 ml each) were collected. These fractions were spotted on TLC with 7:3 toluene-ethyl acetate as the mobile phase, the plates were derivatized using anisaldehyde-H_2SO_4 reagent and heated for 5 minutes at 110°C. Similar fractions were pooled into 10 main fractions based on their TLC profiles. These 10 major fractions (PPCt-UnAC1-10) were concentrated, completely dried, weighed and subjected to antifungal activity screening) (Alka Elizabeth Varghese *et al* 2018)

2.6 Antifungal susceptibility testing of the Charcoal treated acetone extract, followed by 10 fractions of PPCT-UnAC (1-10) using only *Trichophyton rubrum*

The Charcoal treated acetone extract followed by all the fractions were subjected to antifungal susceptibility test. All antifungal tests were determined by broth dilution method. Acetone dissolved the most extracts from acetone and corresponding fractions and was determined to be non toxic at 2% of the final volume of the solvent. (Varghese A E, 2017)

Sabouraoud Dextrose broth (PDB) was kept as the assay medium and *Trichophyton rubrum* conidia and hyphal fragments (1-3x10 ^{4} CFU/mL) were taken as the inoculum. SDB was added in adequate amounts into different test tubes, along with the inoculum. Into these, small amounts (200µg) of the fraction residues (treatment) dissolved in permissible limits (2%of final volume) of acetone were added. Control tubes were also set with same volume of solvent in SDB and inoculum but without the treatment. The final volume of the media in the test tubes were maintained at 4mL and the final inoculum level was also maintained at (1-3x10 ^{4} CFU/mL). The results were collected when the fungal growth became visible as turbidity in the control tubes. Fractions in the tubes that inhibited the fungal growth remained clear even after one week as opposed to the control tubes and non active fraction tubes, which grew murkier and more turbid with rich fungal mycelial

growth. The fractions that exhibited fungal growth were discarded while the fractions that remained clear were considered to be inhibitory in nature.

2.7. Antifungal susceptibility testing, determination of MIC, MFC using all the three dermatophytes:
MIC and MFC of UnAc, CT-UnAc, PPCT-UnAc(8)and antifungal standards were tested against the dermatophytes taken in the study. MIC was determined by the method M38 A2 (with slight modifications) that has been adopted as a standardized procedure by the Clinical and Laboratory Standards Institute (CLSI, 2008) (CLSI 2008). Sabouraoud Dextrose Agar and Griseofulvin were used as the medium (instead of RPMI 1640) and reference antidermatophytic positive control, respectively (Jyothilakshmi *et al* 2017). All the extracts were dissolved in acetone and dilutions were prepared in sterile broth (doubling dilutions) so that after subsequent addition of the inoculums the final drug dilutions were in the range of 2-512 µg/mL. (doubling dilutions). Griseofulvin (Sigma) was used as the reference drug. Conidia were harvested from 21 days old cultures of all the three dermatophytes taken on Sabouraud dextrose agar slants, counted in Neubauer's haemocytometer and adjusted to 1-3 x 10^4 CFU/mL with Sabouraud dextrose broth. Required amounts of inoculums were drawn and added to each tube such that the final volume was 2 mL (final inoculum level 1-3 x 10^3 CFU/mL) and incubated at 30ºC for 3-6 days (till growth was visible in the control). Growth was read visually after the incubation and that concentration was noted as the MIC at which no visible growth was observed. The fungicidal activity was determined for those tubes that exhibited no visible growth or showed any sign of turbidity. After mixing the contents well, small volumes (aprrox.100 µL) of the samples were drawn from each tube and spread evenly on the plate by rotating the petri plate. The plates were incubated at 30ºC for 7 days. The minimum fungicidal concentration (MFC) was determined as the lowest concentration of the compound at which all the subcultures were negative.

III. RESULTS AND DISCUSSION

3.1 Charcoal treated Acetone extract:

The extract after purification with charcoal lost its green color and yet retained its antifungal activity.

3.2 Most active fraction :

Among the ten fractions of fractions 1-10, **Fraction 8** was the active fraction that inhibited the growth of the fungus *Trichophyton rubrum*. Hence this fraction was deemed the active fraction; it weighed **830 mg** and was given the abbreviation PPCAc-UnAc (8)

MIC and MFC values for UnAc, CT-Ac and PPCAc-UnAc (8) and the standard drug Griseofulvin for the antidermatophytic studies are given in the table 1. All the three extracts tested at different levels of purity were fungicidal towards all the three tested dermatophytes, but MFC values were higher than the MIC values. It was similar to the observation made in previous studies that more conidia germinated in the solid media than in the liquid media, as the contact with a solid surface substratum induced the gemination of the conidia of the tested fungus. The tested organisms were sensitive to the reference drug Griseofulvin. The MFC values for *Microsporum gypseum* and *Microsporum canis* were similar to Griseofulvin but MFC for *Trichophyton* was higher than the standard drug taken. One striking observation was the decrease in MIC and MFC values as the purity of the extract increased from *crude extract* to *charcoal treated extract* and finally in the *Partially Purified Acetone extract fraction 8*. Crude extracts are usually a mixture of several active and non-active compounds (Webster *et al*.) that may inhibit the activity of the active molecule. The inhibitory activities of the other non active molecules in the above extract might have been the cause for its higher MIC and MFC values, than the purer fraction.

Thus the above data revealed the strong antifungal potential of the Partially Purified Acetone Extract fraction 8 of the acetone leaf extract.

IV. Conclusion:

The **fraction 8** of the partially purified crude acetone leaf extract of *Uvaria narum* showed the presence of an active component that made the fraction -8 to exhibit antifungal activity towards the three dermatophytes taken for the study This study is adding to the discovery of antifungal acetogenins and other metabolites (Padmaja *et al*., 1993) from *U. narum*, and justifying its ethnomedicinal uses against skin conditions (Reddy *et al.*, 2012; Padyana *et al.*, 2013; Florence *et al*., 2014).

Figures andTables

Table 1. Table showing comparative MIC and MFC of the three dermatophytes against extracts of different levels of purity.

	Test type	Organism taken		
Compound		*Microsporum canis*	*Microsporum gypseum*	*Trichophyton rubrum*
UnAc*	MIC	64	64	128
	MFC	512	512	1024
CH-Ac **	MIC	32	16	64
	MFC	256	256	512
PPAc-UNAc#	MIC	16	8	32
	MFC	64	128	256
Ac Griseofulvin	MIC	4	2	4
	MFC	64	128	128

* *U.narum* Acetone crude Extract
** Charcoal purifiedAcetone extract
#: Partially Purified Acetone extract fraction 8

II. ACKNOWLEDGMENT

The author obtained no grants from any funding agency for conducting the above research work on checking antidermatophytic activity. Fractions were partially purified and antidermatophytic works were conducted in the lab of the affiliated institution and department using the proper set methods and they have been properly acknowledged.

REFERENCES

[1] Alka Elizabeth Varghese, Balaji Govindan, Jyothilakshmi Madhavankutty, Antony Thomas Valiyaveetil, Madhusudhanan Karadka & Sabulal Baby (2018) A new antifungal benzoic acid ester from *Uvaria narum*, Natural Product Research, 2:22, 2657-2662, DOI: 10.1080/14786419.2017.1375925

[2]. Bindu, T.K., Shafi, P.M., Rajan, P.P., Sarma, Y.R., 1998. Antifungal activity of *Uvaria narum* extracts. Allelopathy Journal 5, 67-76.

[3] CLSI., 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard-Second Edition, CLSI document M38-A2, Wayne, PA: Clinical and Laboratory Standards Institute.

[4] Florence, A.R., Joselin, J., Shynin Brintha, T.S., Sukumaran, S., Jeeva, S., 2014. Preliminary phytochemical studies of select members of the family Annonaceae for bioactive constituents. Bioscience Discovery 5, 85-96.

[5] Hisham, A., Janssens, J.J., Schepens, P.C., Vlietinck, A.J., 1989. Preliminary study of the essential oil isolated from *Uvaria narum*. Planta Medica 55, 636.

[6] Hisham, A., Pieters, L.A.C., Claeys, M., Esmans,E, Dommisse, R., Vlietinck, A.J., 1990. Uvariamicin-I, II and III: three novel acetogenins from Uvaria narum. Tetrahedron Letters 31, 4649-4652.

[7] Hisham, A., Pieters, L.A.C., Claeys, M., Esmans, E., Dommisse, R., Vlietinck, A.J., 1991a. Squamocin-28-one and panalicin, two acetogenins from Uvaria narum. Phytochemistry 30, 545-548.

[8] Hisham, A., Pieters, L.A.C., Claeys, M., van den Heuvel, H., Esmans, E., Dommisse, R., Vlietinck, A.J., 1991b. Acetogenins from root bark of Uvaria narum. Phytochemistry 30, 2372-2377.

[9] Jolad, S., Hoffmann, J.J., Schram, K.H., Cole, J.R., Tempesta, M.S., Kriek, G.R., Bates, R.B., 1982. Uvaricin, a new antitumor agent from Uvaria acuminata (Annonaceae). Journal of Organic Chemistry 47, 3151-3153.

[10] Jyothilakshmi, M., Jyothis, M., Narayanan, G.N., Latha, M.S., 2017. Antidermatophytic and protease-inhibiting activities

of zerumbone: a natural sesquiterpene from the rhizome of Zingiber zerumbet (L.) Roscoe ex J.E; Smith. Pharmacognosy Magazine 13, 2-6.

[11] Khare, C., 2007. Indian Medicinal Plants, Springer, New York.

[12] Parmar, V.S., Tyagi, O.D., Malhotra, A., Singh, S.K., Bisht, K.S., Jain, R., 1994. Novel constituents of Uvaria species. Natural Product Reports 11, 219-224.

[13] Parmar, V.S., Bisht, K.S., Malhotra, A., Jha, A., Errington, W., Howarth, O. W., Tyagi, O.D., Stein, P.C., Jensen, S., Boll, P.M., Olsen, C.E., 1995. A benzoic acid ester from Uvaria narum. Phytochemistry 38, 951-955.

[14] Prabhu, S., Vijaykumar, S., Yaabesh, J. E., Ravichandran, K., Sakthivel, B., 2014. Documentation and quantitative analysis of the local knowledge in medicinal plants in Kalarayan Hills of Villupuram district of Tamil Nadu, India. Journal of Ethnopharmacology 157, 7-20.

[15] Padmaja, V., Thankamany, V., Hisham, A., 1993. Antibacterial, antifungal and antihelminthic activities of root barks of Uvaria hookeri and Uvaria narum. Journal of Ethnopharmacology 40, 181-186.

[16] Padyana, S., Ashalatha, M., Rao, R., Saraswathi, S., 2013. Pharmacognostic evaluation of an ethnomedicine - Uvaria narum (Dunal) Wall. International Journal of Chemical and Pharmaceutical Analysis 1, 5-8.

[17] Padyana, S., Ashalatha, M., Yende, A., Bhat, R., Prajna, P., 2011. Evaluation of antibacterial and antioxidant properties of Uvaria narum (Dunal) Wall. International Research Journal of Pharmacy 2, 142-144.

[18] Reddy, J., Jose, B., Jalli, R.D., Gopu, S., 2012. Evaluation of antibacterial and DPPH radical scavenging activities of the leaf essential oil and leaf extracts of Uvaria narum (Dunal) Wall L. International Journal of Research in Pure and Applied Microbiology 2, 13-19.

[19] Rupprecht, J., Hui, Y.-H., McLaughlin, J.L., 1990. Annonaceous acetogenins: A review Journal of Natural Products 53, 237- 238.

[20] Varghese, A.E. 2017 : Antifungal activity guided phytochemical analysis of a few selected species of Annonanceae: Thesis submitted to Mahatma Gandhi University for award of Doctoral degree.

[21] Varghese, A.E.; Antony, V.T.; Madhusudhanan, K. **2017**(a) Comparative Analysis of the Antifungal Activity of Different Solvent extracts of *Uvaria narum* (Dunal) Wall. Against *Fusarium moniliforme* and *Corynespora cassiicola*. Int. J. Pure App. Biosci. Vol. 5, 224–228.

[22] Varghese E Alka, VT Antony, K Madhusudhanan 2017 (b) Efficacy of four acetogenin containing members of Annonaceae against *Colletotrichum gloeosporioides* , International Journal of Recent Advances in Multidisciplinary Research,4 (8), 2745-2750

[23] Webster, D., Taschereau, P., Belland, R., Sand, C., & Rennie, R. (2008). Antifungal activity of medicinal plant extracts: Preliminary screening studies. *Journal of Ethnopharmacology* , Vol.115: 140-146.