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In vitro Dose Dependent Study on Anti-Dermatophytic Activity of *Annona squamosa* Linn. Leaf and *Annona reticulata* Linn. Bark Ethyl Acetate Extracts against *Trichophyton rubrum*

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This study was aimed to evaluate the anti dermatophytic activity of *Annona squamosa* leaf and *Annona reticulata* bark ethyl acetate extracts against Trichophyton *rubrum*. The study was carried out on clinical isolate of *T. rubrum* as well as *Standard T. rubrum* strain (MTCC3272) for comparison. Annona squamosa leaf and *Annona reticulata* bark was extracted with ethyl acetate and then used for the antidermatophytic study. Dose dependent study on anti dermatophytic activity was done by agar well diffusion method and minimum inhibitory concentration (MIC) studies were carried out by broth dilution assay. Isolated *T. rubrum* was identified by both microscopic and cultural characteristics followed by biochemical tests. *Annona squamosa* L. leaf and *Annona reticulata* L. bark ethyl acetate extracts showed significant inhibitory activity against clinical isolate of *T. rubrum* as well as standard T. rubrum(MTCC3272). Zone of inhibition is proportional to concentration of the extract. Minimum inhibitory concentration range was found to be between 10μg to 100μg/ml. The study concludes that further investigation is needed to identify the responsible antidermatophytic compound which is present in *Annona squamosa* leaf and *Annona reticulata* bark to develop a new novel drug.

Key words: *Trichophyton rubrum*, Anti dermatphytic activity, *Annona squamosa* L., *Annona reticulata* L., Agar well diffusion method, MIC.

Skin infections are common diseases in developing countries, of which dermatophytosis are of particular concern in the tropics. Dermatophytes are a group of closely related fungi known to cause human superficial mycoses in many tropical countries where their prevalence still remain a public health problem.

A wide variety of dermatophytes have been isolated from human beings, animals and soil. Human beings are the main or only host for anthrophilic dermatophytes like *Trichophyton rubrum*.

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Dermatophytosis is commonly caused by *T. rubrum*. Several antifungal agents including various azoles, tolnafate cream and allylamine derivatives have been introduced in the treatment. However, these antifungal agents are expensive and have varying degrees of toxicity[2,3]. Hence, there is the need to give greater attention to developing more anti fungal (anti-dermatophytic) drugs so as to effectively check the increasing prevalence of these infections, especially in the tropics and subtropics, where the climate makes people more susceptible to these infections. Coincidentally, natural materials especially of plant products have been found to possess one or more medicinal properties [4, 5]. Currently, several plants have been screened and discovered to possess significant antimycotic activities [6, 7].

Annona squamosa Linn, belonging to family Annonaceae is commonly found in India and cultivated in Thailand and originates from the West Indies and South America . It is known as custard apple, sugar apple, sweet après in English, and sharifa in Hindi and sitaphalam in Telugu in India and corossolier and cailleux, pommier cannelle in french [8, 9].It is considered beneficial for cardiac disease, diabetes, hyperthyroidism and cancer [10]. An infusion of the leaves is considered efficacious in prolapsusani of children, the crushed leaves are sniffed to overcome hysteria and fainting spells, they are also applied on ulcers and wounds. The ripe fruits of this plant are applied to malignant tumors to hasten suppuration. The dried unripe fruit powder is used to destroy vermin. A paste of seed powder can applied to the head to kill lice. It is also used for destroying worm in the wound of cattle[11].

Annona reticulata L. (Annonaceae) is commonly called as 'Ramphal'. It is a small tree with glabrous branches. Leaves are membranous, oblong-lanceolate, acute or obtuse, cuneate or rounded at the base. Upper surface is glabrous and the lower is with a few scattered hairs. Flowers are two to four on lateral pedicels. *Annona reticulata* fruits are subglobous or somewhat heart shaped, roughish form outside, become yellow or yellowish red when ripe. Seeds are smooth and blackish[8]. The plant is indigenous to the West Indies but now naturalized in India and occurring in Bengal, Burma, and South India. Traditionally the bark is a powerful astringent and used as a tonic. The powdered bark used for dysentery and diarrhoea. Unripe and dried fruits are astringent and used to treat diarrhoea. Leaves are used as an anthelmintic traditionally[12].

The present research sought to validate the ethno medicinal (anti dermatophytic) use of the plants, A. *squamosa* and A. reticulata.

MATERIALS AND METHODS

Collection of plant material

Annona squamosa leaf and *Annona reticulata* bark were used for this study (Fig. 1). These plants were collected from the forests of Paaderu, a tribal area near to Visakhapatnam. Plants are identified and authenticated by plant taxonomists, Department of Botany, Andhra University, Visakhapatnam.

Collection of test dermatophyte

Isolation of *Trichophyton rubrum* from patients

Skin scales, nail and hair Specimens were collected from patients with suspected dermatophytosis. Sample was obtained from patients attending the Dermatology outpatients department, King George Hospital of Andhra Medical College, Visakhapatnam (India). The affected area was thoroughly cleaned with 70% alcohol to remove the surface contaminants. Whatman no.1 filter paper was used for collecting specimens [13]. After disinfection with alcohol skin lesions was scraped with a scalpel to collect epidermal scales. From the scalp hair was epilated with sterile forceps. Nails sample collected using nail clip. 10% KOH solution was used for skin and hair and for nail scrapings 20% KOH was used. All preparations were examined under low power and confirmed under high power. Samples are also cultured on duplicate plates of Sabouraud Dextrose Agar (Himedia) and Dermatophyte Test Medium (DTM) prepared according to the manufacturer's instructions. The plates were inoculated with finely divided pieces of each sample and incubated at 28^0C in BOD incubator (Remi) for recovery of dermatophytes or moulds. The dermatophyte test medium (DTM) is an alternative culture medium that suggests the presence of dermatophyte pathogens, even though it does not identify specific organisms macroscopically [14], the ability to hydrolyze urea provides additional data that can be used to aid in the differentiation of *Trichophyton rubrum* (urease negative) from other *Trichophyton* sps [15]. *In-vitro* hair perforation test states that the inability of T. *rubrum* to penetrate the hair shaft. Similarly the cultures were identified on the basis of their macro and microscopic features [16].

Standard strain of *Trichophyton rubrum*

Standard strain of Trichophyton rubrum (MTCC 3272) was obtained from ITM, Chandigarh, India for comparative study.

Anti dermatophytic activity

Preparation of plant extracts

Annona squamosa leaf and *Annona reticulata* bark were removed and air dried then ground into powder which was dissolved in ethyl acetate so as to make 40% solvent extract. The extract was kept in orbital shaking incubator for

3 days and then centrifuged to remove the debris. Finally clear solvent extracts were collected and then the solvent was evaporated by using rotavapour (BUCHI, India) to get the concentrated residue of the solvent, which contains dissolved components of plant material. The concentrated residue of solvent extracts were appropriately dissolved in solvents and tested for anti dermatophytic assay.

Preparation of inoculum

21 days old grown culture of *T. rubrum* was scraped with sterile scalpel and dissolved in sterile saline solution to make different dilutions. One of diluted suspensions was used as inoculum which had absorbance of 0.600 at 450nm determined spectroscopically (Electronics India)[17,18].

Agar well diffusion method

Antifungal screening was carried out using the agar well diffusion assay. Twenty ml of sterilized Sabouraud dextrose agar medium poured into a 15 cm Petri dish. Twenty µl of inoculum suspension of the *T. rubrum* was distributed evenly over the surface. A 6mm well was cut in the centre of each plate using a sterilized cork borer. Different concentrations of Annona extracts and Griseofulvin (Dr.Reddy's Labs, India) were placed into the wells. The plates were incubated for 7 days at 28ºC. Pure ethyl acetate was used as control. Results were determined based on size of the inhibitory zone (mm) surrounding the wells containing the test solution. The diameter of zones of inhibition was measured in mm using HiMedia zone reader [19,20].

Determination of MIC by Broth Dilution Assay

The minimum inhibitory concentration (MIC) of the plant extract was determined using broth dilution assay. The medium containing different concentrations of plant extract viz., 100mg -1µg per ml prepared by serial dilution (10^{-1} dilution). After inoculation of culture, the tubes were incubated for 72 hours at 28^0 C. The MIC of each sample was determined by measuring the optical density in the spectrophotometer (Electronics India) at 520nm and compared the result with those of the non-inoculated broth used as blank. Control was prepared with media and inoculum only without plant extract. The experiment was conducted according to NCCLS standards (Now as CLSI) [21,22].

RESULTS

Isolation of *Trichophyton rubrum* from patients.

Samples from the patients (Fig. 2), were cultured on plates of Sabouraud Dextrose Agar (Himedia) and Dermatophyte Test Medium (DTM). *T. rubrum* colonies were white, velvety to fluffy, occasionally powdery to granular with diffuse wine red coloured pigmentation on reverse and microscopically which showed pyriform microconidia borne slightly along the hyphae and macrocondia which were pencil shaped. Urease test (Biochemical test) was negative; *in-vitro* hair perforation test was negative. *T. rubrum* was sub cultured to use in the study after its conformation.

Table 1. Anti dermatophytic activity of *Annona squamosa* L. leaf and *Annona reticulata L.* bark ethyl acetate extracts against *Trichophyton rubrum*

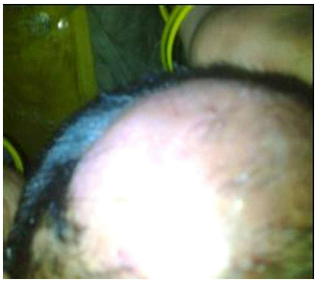
Concentrations of extracts (µg)	Zone of inhibition against Trichophyton rubrum			
	Clinical isolate		MTCC 3272	
	A. squamosa Leaf	*A. reticulata* Bark	*A. squamosa* Leaf	*A. reticulata* Bark
25	12	16	13	17
50	14	20	14	20
75	18	22	19	24
100	20	26	20	26
50(Griseofulvin)	26	35	28	35
Ethyl acetate (Control)	0	0	0	0

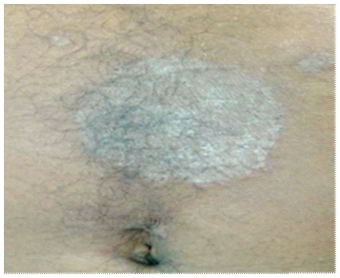
(a). *Annona reticulata L.*

1 (b). *Annona squamosa L.*

1(c). Bark of *A. reticulata*

1(d). Leaf *of A. squamosa*

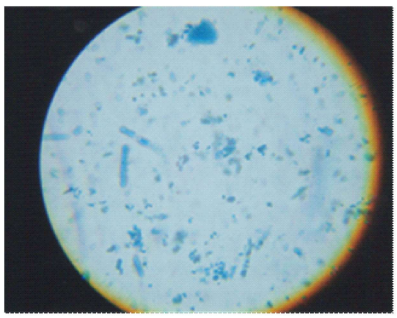




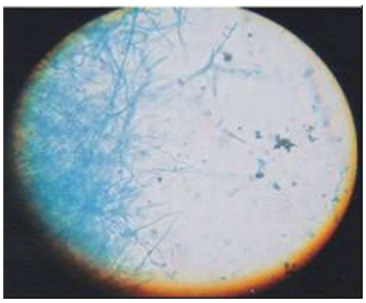
(a). The infected lesions of dermatophytosis patients caused by *T. rubrum*

2(b)*T. rubrum* on DTM

2(c). *T. rubrum* on SDA with pigmentation (reverse)
Cultural characteristics of *T. rubrum*



2(d). *T. rubrum* – Macroconidia



2(e). *T. rubrum* – Microconidia
Microscopic observation (LPCB mount)

Fig. 2. Isolation and identification of *T. rubrum* from dermatophtosis patients

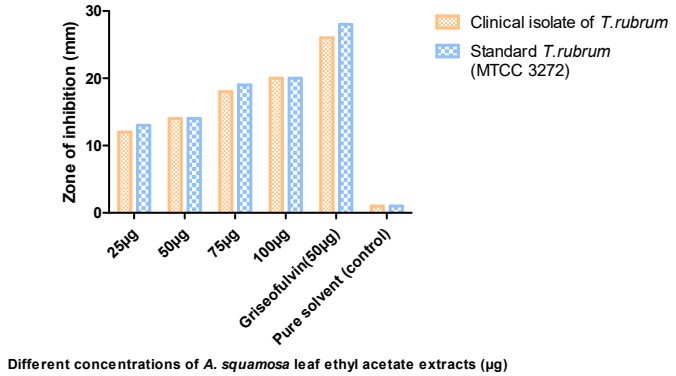


Fig. 3. Dose dependent effect of *A. squamosa* leaf ethyl acetate extract on *Tricophyton rubrum*

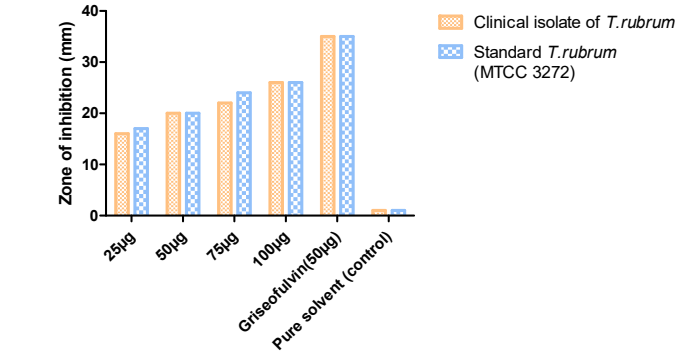


Fig. 4. Dose dependent effect of *A. reticulata bark* ethyl acetate extract on *Tricophyton rubrum*

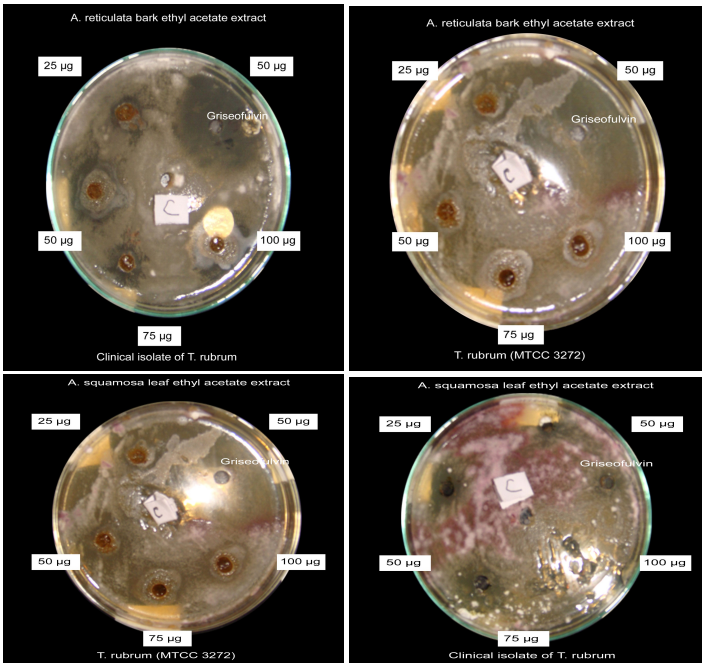


Fig. 5. Dose dependent inhibitory effect of Annona extracts against *T. rubrum*

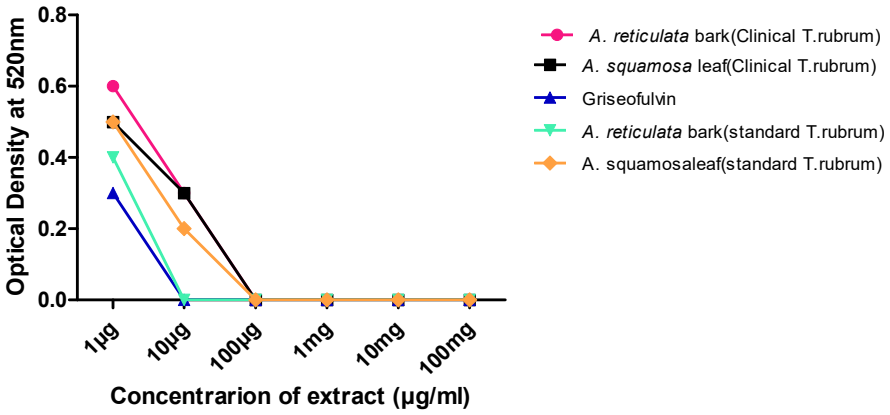


Fig. 6. Effect of *Annona squamosa* L. leaf and *Annona retuculata* L. bark ethyl acetate extracts concentration on growth pattern of *T. rubrum* determined spectroscopically

Anti dermatophytic activity

A. squamosa leaf and *A. reticulata* bark ethyl acetate extracts showed significant inhibitory activity against clinical isolate of *T. rubrum* as well as standard T. rubrum strain (MTCC3272). Table 1 shows dose dependent zone of inhibition by *Annona* extracts against *T. rubrum*. 12, 16mm were the smallest zones of inhibition showed by *A. squamosa* leaf, *A. reticulata* bark ethyl acetate extracts respectively against clinical isolate of *T. rubrum* while 13, 17mm were the smallest zones of inhibition against standard *T. rubrum* culture at 25µg concentrated extract. 20, 26 mm were the highest zones of inhibition showed by *A. squamosa* leaf, *A. reticulata* bark ethyl acetate extracts against clinical isolate of *T. rubrum* while 20, 26 mm were the highest zones of inhibition against standard strain of *T. rubrum* at 100µg concentrated extract. Small zone of inhibition was observed at low concentration while large zone of inhibition was observed at high concentration of the *Annona* extract. Zone of inhibition is proportional to concentration of the extract. Figure 3 and Figure 4 shows the dose dependent comparative inhibitory activity *A. squamosa* leaf, *A. reticulata* bark ethyl acetate extracts against clinical isolated as well as standard *T. rubrum*. Inhibitory activities of Annona extracts were comparable to Griseofulvin (standard). Pure ethyl acetate solvent (control) did not show inhibitory activity (Fig 5).

The absorbance at 520nm by the T. rubrum broth culture indicates the growth (Fig 6). Increasing concentration of *A. squamosa* extracts showed potent growth inhibitory activity. Minimum Inhibitory concentration (MIC) of *A. squamosa* leaf, *A. reticulata* bark ethyl acetate extracts against both clinical isolated and standard *T. rubrum* was showed in Table 2. The minimum inhibitory concentration of *Annona* extracts range was found to be between 10µg to 100µg/ml. 100µg/

Table 2. Minimum inhibitory concentration of *Annona squamosa* L. leaf and *Annona reticulata L.* bark ethyl acetate extracts against *Trichophyton rubrum*

Type of Extract	MIC against *Trichophyton rubrum* (µg/ml)	
	Clinical isolate	MTCC 3272
A. reticulata bark	100	10
A. squamosa leaf	100	100
Griseofulvin (Standard)	10	10

ml was MIC of bark extract against clinical isolate of *T. rubrum* while 10µg/ml was MIC bark extract against standard *T. rubrum.* 100µg/ml was MIC of leaf extract against both clinical isolate as well as standard *T. rubrum*. 10µg/ml was found to be the MIC of Griseofulvin(standard).

DISCUSSION

In this study we demonstrated that antidermatophytic activity of A. reticulata bark and A. squamosa leaf ethyl acetate extracts against both clinical isolate and standard *T. rubrum* culture. *A. squamosa* leaf extract and *A. reticulata* bark ethyl acetate extracts showed significant inhibitory activity comparable with the inhibitory activity showed by Griseofulvin.

Clinical isolate of *T. rubrum* showed slight resistance to *Annona* extracts when compared with standard *T. rubrum*(MTCC 3272) culture. Bark extract of *A. reticulata* showed potent inhibitory activity compared to *A. squamosa* leaf extract against *T. rubrum*.

Nowadays there is the need to give greater attention to developing more anti dermatophytic drugs so as to effectively check the increasing prevalence of these infections, especially in the tropics and subtropics, where the climate make people more susceptible to the infections. Coincidentally, natural materials especially of plant products have been found to possess one or more medicinal properties. Exploration of the chemical constituents of the plants and pharmacological screening may provide us the basis for developing the leads for development of novel agents. Currently, several plants have been screened and discovered to possess significant anti dermatophytic activities [23]. Annotemoyin-1, Annotemoyin-2, squamocin and cholesteryl glucopyranoside were isolated from the seeds of *Annona squamosa*. These compounds and plant extracts showed remarkable antimicrobial and cytotoxic activities [24]. A novel natural compound, 11-hydroxy-16-hentriacontanone, has been isolated from the leaf cuticular wax of Annona squamosa along with its known isomer 10-hydroxy-16-hentriacontanone [25]. Several bioactive compounds were isolated from A. *reticulata*. Anonaine, michelalbine, oxoushinsunine, and reticuline were isolated along with an unknown phenolic base from root bark [26, 27]. Dopamine, salsolinol, and coclaurine were isolated from leaves and stems. The stem bark contains the diterpenes (-)-kaur-16-en-19-oic acid, 16-hydroxy-(-)-kauran-19-oic acid, and methyl-17-hydroxy-16-(-)-kauran-19-oate. From the seeds a series of N-fatty acyl tryptamine, in which acyl portion ranged from hexadecanoyl to hexacosanoyl have been characterized [28]. Various cytotoxic acetogenins as squamocin, cis-/trans-isomurisolenin, annoreticuin, annoreticuin-9-one, bullatacin, cis-/trans-bullatacinone, cis-/trans-murisalinone, solamin, annomonicin, rolliniastatin-1 and 2,squamone, and isoannonareticin, were isolated [29]. Constituents of volatile oil viz., pinene, myrcene, limonene, terpinen-4-ol, and germacrene D were identified [12].

Finally study concludes that *Annona squamosa* leaf and *Annona reticulata* bark extracts showed potent inhibitory activity against *Trichophyton rubrum.* Further investigation is needed to identify the responsible antidermatophytic compound which is present in *A. squamosa* leaf and *A. reticulata* bark to develop a new novel drug.

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