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Efficacy of the *Rhinacanthus nasutus* Nees Leaf Extract on Dermatophytes with Special Reference to *Trichophyton mentagrophytes* var. *mentagrophytes* and *Microsporum canis*

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Abstract – The effect of *Rhinacanthus nasutus* leaf extract on the growth of dermatophytes had been investigated. *In vitro* the extract exhibited high activity against various species of dermatophytes (*Trichophyton mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *interdigitale*, *T. rubrum*, *Microsporum canis* and *M. gypseum*). The minimal inhibitory concentration (MIC) values of the extract revealed that all the dermatophytes tested had MIC values of 13.6 mg/ml. The extract exhibited fungistatic activity at lower concentrations (≤13.6 mg/ml or below the MIC value) and fungicidal activity at higher concentrations (≥13.6 mg/ml or above the MIC value). The results suggested that the extract acted on the cell wall of the dermatophytes which subsequently leading to the formation of cytopathological and membrane structural degeneration and finally leading to cell lysis and death.

Key words – *Rhinacanthus nasutus*, antifungal agent, fungistatic activity, fungicidal activity, minimum inhibitory concentration.

Introduction

Natural products from terrestrial plants have long been an important group of substances used as pharmaceutical compounds, and the significance of this study has been discussed extensively (Balandrin *et al.*, 1985; Labade, 1986; Kumar and Prasad, 1992). Furthermore, terrestrial plants have served as the primary source of useful natural products which currently constitute about 25% of the world market. Like most of the tropical countries, Malaysia's plants are also highly diversified with various medicinal values which of course differ from those present in the temperate countries (Soepadmo *et al.*, 1989). The biodiversity of Malaysias plant resources offer some 15,000 species of plants which possess medicinal values. About 1,000 of them have undergone simple chemical screening but much less have been subjected to chemical and pharmaceutical studies.

One of the popular indigenous plants of Malaysia is *Rhinacanthus nasutus* Nees (family Acanthaceae), and it has been reported to treat skin diseases such as ringworm infections (Lewis *et al.*, 1977) which are caused by dermatophytic fungi (dermatophytes). It was also reported to be able to treat *Herpes militaris* (Lindley, 1981). Previously, we also reported a preliminary study done on this plant extract (Darah and Annie-Clara, 1992). The extract inhibited the growth of several Gram positive bacteria (*Staphylococcus aureus* and *S. epidermidis*) and fungi, including dermatophytes. However, the extract did not show any inhibitory activity against yeast cells.

In this communication, we describe some of the results obtained from the evaluation of the antifungal activity of *R. nasutus* leaf extract on dermatophytes. We also described the effect of the extract on the alteration in the morphologies of dermatophytes after being exposed to it, with the special reference to *T. mentagrophytes* var. *mentagrophytes* and *M. canis*.

Experimental

Preparation of the *R. nasutus* leaf extract – Fresh leaves of *R. nasutus* were collected from a herbalist at Bukit Mertajam, Penang. Voucher specimens have been deposited at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Penang. The leaves were then washed thoroughly with running tap water and oven dried at 50°C for 2-3 days. The extraction was conducted as described

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previously (Darah and Annie-Clara, 1992). The stock solution containing 1,000 mg/ml (w/v) was prepared in 95% ethanol and further diluted in distilled water.

Dermatophytes – Clinical isolates of *T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *interdigitale*, *T. rubrum*, *M. canis* and *M. gypseum* were used throughout the study. The fungal cultures were grown and maintained on Sabouraud glucose agar slopes at room temperature (30±2°C) for 10 days.

Determination of the minimal inhibitory concentrations (MIC) – MICs were determined by the liquid dilution method. A series of dilution were set up between the concentrations of 0-500 mg/ml of extract in Sabouraud glucose broth medium. To each tube, 0.1 ml of standardized suspension of dermatophyte spores (4×10^5 spores/ml) was added and incubated at 30°C for 24-72 hours. The lowest concentration which did not show any growth of the tested microorganism after microscopic evaluation was determined as the MIC.

The effect of the extract concentrations on the growth of dermatophytes – One milliliter of standardized spore suspensions of dermatophytes was added into Sabouraud glucose broth containing extract to give a final concentration between 0-50 mg/ml. The mixtures were then incubated at 30°C for 72 hours, at the rotation of 200 rpm. The growth of the dermatophytes was determined by the dry weight basis after drying the samples at 60°C for 20 hours, until constant weight.

The viable cell method – One milliliter of standardized *T. mentagrophytes* var. *mentagrophytes* or *M. canis* spore suspension (4×10^5 spores/ml) was added into Sabouraud glucose broth containing extract to give final concentrations of 12.0, 13.6 and 50.0 mg/ml. The mixtures were incubated at 30°C and at every 6 hourly intervals, 0.1 ml of the mixture was spread onto two separate Sabouraud glucose agar plates. The inoculated plates were then incubated at 30°C for 72 hours. The mean number of colonies was enumerated and compared with that of controls (sterile distilled water).

The morphological study of the dermatophytes after exposure to the extract – One milliliter of standardized spore suspension of *T. mentagrophytes* var. *mentagrophytes* or *M. canis* (4×10^5 spores/ml) was added into Sabouraud glucose broth containing extract to give a final concentration of 13.6 mg/ml (equivalent to the MIC level). The mixtures were incubated at 30°C for 24 hours. A few drops of the test mixture were then taken out and fixed for scanning electron microscopy study (Borgers *et al.*, 1989).

Results and Discussion

The minimum inhibitory concentration (MIC) values of the *R. nasutus* leaf extract against dermatophytes were determined by liquid dilution method. The MIC analysis of the extract (Table 1) revealed that the MIC values were at 13.6 mg/ml for all the dermatophytes tested. Further investigation was performed to demonstrate the action of the extract on the growth of dermatophytes. Two of the isolates,

Table 1. The minimal inhibitory concentation (MIC) values of the *R. nasutus* leaf extract on dermatophytes

Dermatophytes	MIC values (mg/ml)
Trichophyton mentagrophytes var. mentagrophytes	13.6
T. mentagrophytes var. *interdigitale*	13.6
T. rubrum	13.6
Microsporum canis	13.6
M. gypseum	13.6

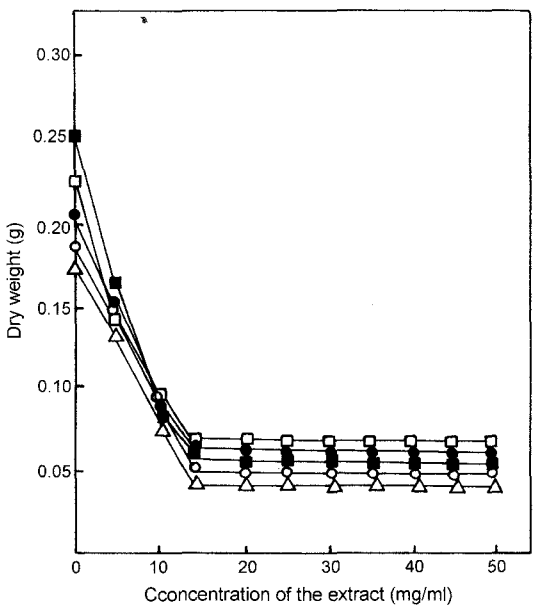

Fig. 1. The effect of the *Rhinacanthus nasutus* leaf extract on dermatophytes. Symbols: (■) *Trichophyton mentagrophytes* var. *interdigitale*, (□) *Microsporum gypseum*, (●) *M. canis*, (○) *T. mentagrophytes* var. *mentagrophytes*, (△) *T. rubrum*.

namely *T. mentagrophytes* var. *mentagrophytes* and *M. canis* were used to further study the growth inhibition in liquid medium. It seems that the growth of the dermatophytes was correspondingly decreased by increasing concentrations of the extract (Fig. 1), and the growth was completely inhibited at the MIC values (13.6 mg/ml). The reduction in growth was possibly due to interference by active compound (s) of the extract and such interference may be at the biosynthetic level.

The extract was then determined for its fungicidal or fungistatic activity. Both of the dermatophytes were tested against 12.0 mg/ml, 13.6 mg/ml (equivalent to MIC values) and 50.0 mg/ml of extracts by using the viable cell count method. The results revealed that the extract exhibited fungistatic activity at lower concentrations (12.0 mg/ml or below the MIC level) and fungicidal activity at higher concentrations (≥ 13.6 mg/ml or above the MIC level). As shown in Fig. 2a, the growth of *T. mentagrophytes* var. *mentagophytes* was slightly inhibited within the first 18 hours of exposure to the extract at the concentration of 12.0 mg/ml. However, the fungus started to recover and its growth continued again slowly after that fungistatic period of time. The results also revealed that the killing times for the fungus were shorter as the concentrations of the extract increased. The extract was found to kill *T. mentagrophytes* var. *mentagrophytes* within 72 hours of exposure at its MIC value (13.6 mg/ml) and within 36 hours at 50.0 mg/ml. The same pattern of activity was also exhibited by *M. canis* (Fig. 2b). It showed that the fungistatic activity occurred within the first 6 hours of contact with the extract at 12.0 mg/ml. The killing times of the extract were 72 hours at the concentration of 13.6 mg/ml (equivalent to MIC level), and 60 hours at the concentration of 50.0 mg/ml. The results also revealed that the extract needed a longer time to kill *M. canis* compared to *T. mentagrophytes* var. *mentagrophytes* at the same concentration. This phenomenon could be due to the morphological structures of the *M. canis* which possess thicker hyphal and coarsened conidial cell walls than *T. mentagrophytes* var. *mentagrophytes*.

Microscopic observations on the effect of the extract against *M. canis* and *T. mentagrophytes* var. *mentagrophytes* were performed by means of scanning electron microscope (SEM). The SEM of untreated *M. canis* culture exhibited many cylindrical, regular branching hypha and smooth-walled hyphal filaments

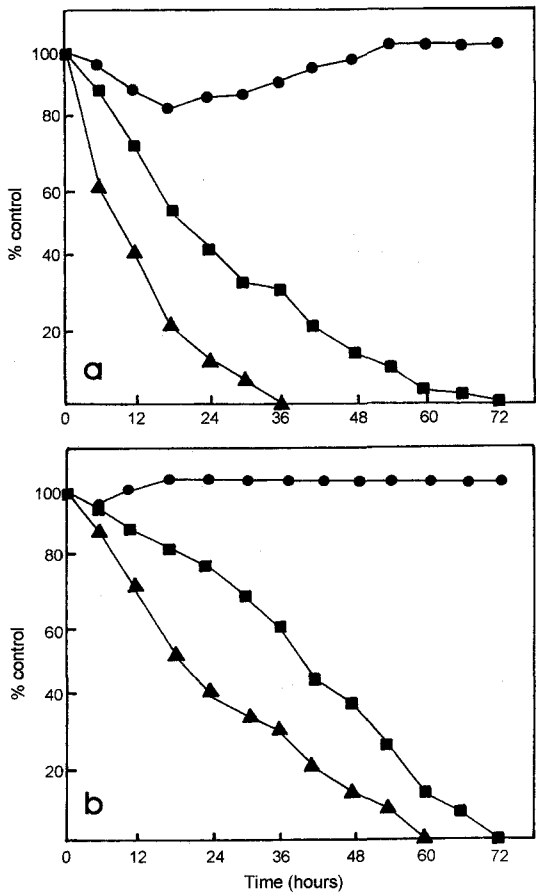

Fig. 2. Fungicidal and fungistatic effects of the extract on *T. mentagrophytes* var. *mentagrophytes* (a) and *M. canis* (b) The concentration of the extract were 12.0 mg/ml (●), 13.6 mg/ml (○) and 50.0 mg/ml (■).

(Fig. 3A), and also the regularly shaped macroconidia (Fig. 3B). However, after treatment with the extract for 24 hours at the concentration of 13.6 mg/ml, the hyphal filaments became shrunken and flat (Fig. 3C). The macroconidia also became irregularly shaped and collapsed (Fig. 3D). The same phenomenon was also shown by the culture of *T. mentagrophytes* var. *mentagrophytes* (Fig. 4). The untreated culture showed the presence of cylindrical, long branching and smooth-walled hyphal filaments (Fig. 4A). However, after 24 hours of treatment with the extract at 13.6 mg/ml, the hyphae became shrunken, distorted, flat and necrotic (Fig. 4B). Even though the mechanism of action of the extract has not been much studied and understood, the SEM studies suggested that the cells of both dermatophytes had undergone distinct

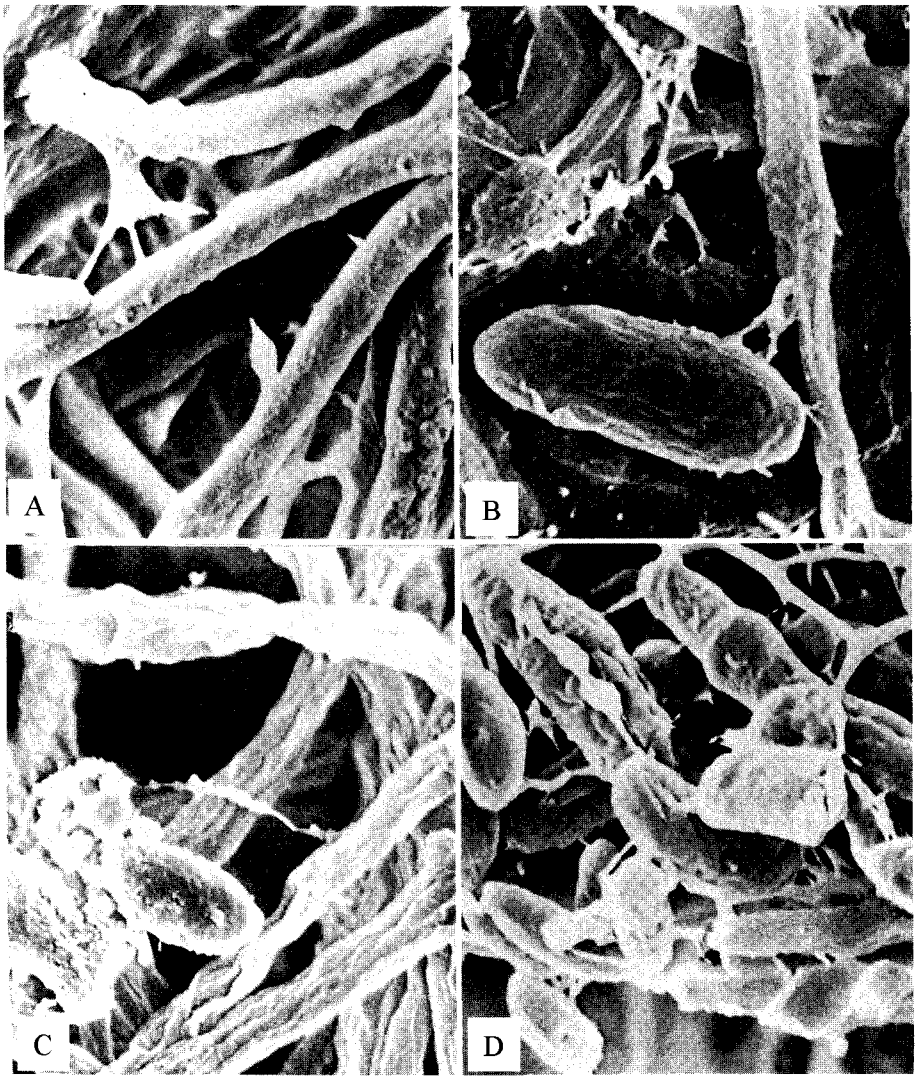

Fig. 3. Scanning electron micrographs of untreated culture of *M. canis*, showing cylindrical, smooth-walled hyphal filaments (A) and macroconidia (B), whereas the treated cultures showing irregularly shaped, flat and collapsed hyphal filaments (C) and also irregularly shaped and collapsed macroconidia (D) The culture was treated with 13.6 mg/ml of the extract for 24 hours.

morphological and perhaps cytological alterations. It is clear that the most interesting feature of the antifungal activities of the *R. nasutus* leaf extract is its ability to cause cell leakage or perhaps some alteration in the cell membrane permeability (Masperi *et al.*, 1989; Borger *et al.*, 1989; Darah *et al.*, 1996), which eventually resulting in the lysis of the growing cells of dermatophytes. Normally, lysis occurs during the growth of cells and does not occur when protein synthesis was blocked (Cassone *et al.*, 1979). However, based on the data presented here, it appeared that the extract studied affect the biosynthetic step (s) in cell wall synthesis of the dermatophytes. We suggest that the extract primarily impairs the structure of the cell membrane and its function. Cell membrane properties such as fluidity and permeability and also the activity of membrane bound enzyme (Cabib, 1981) might have been altered as a consequence of the impairment. Since there is compelling evidence that normal cell membrane activity is a prerequisite for regular synthesis and deposition of cell wall constituents, cell wall abnormalities also may be

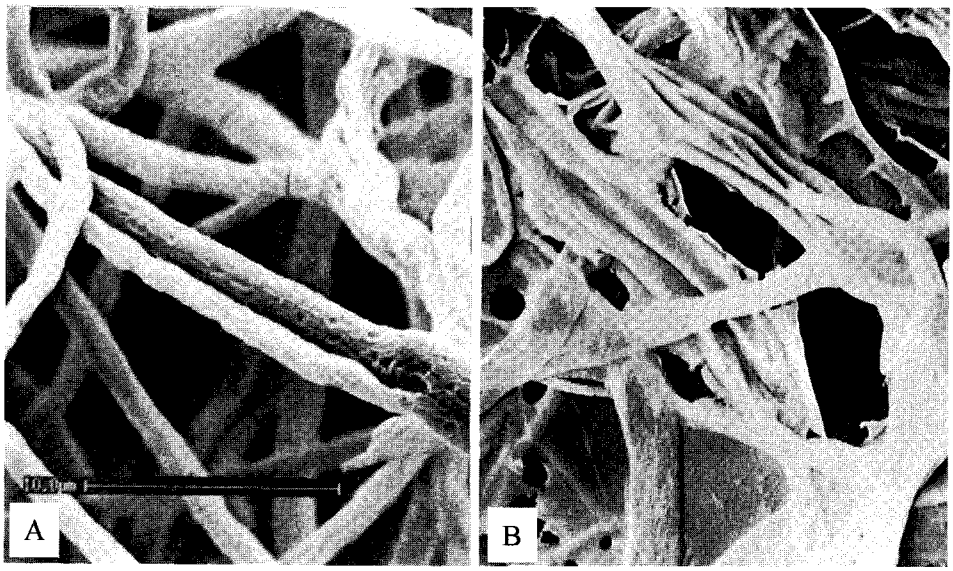

Fig 4. Scanning electron micrographs of untreated culture of *T. mentagrophytes* var. *mentagrophytes*, showing cylindrical and smooth-walled hyphal filaments (A). However, after 24 hours of exposure to 13.6 mg/ml of the extract, the hyphae became distorted, flat and collapsed (B).

attributable to cell membrane alterations. Changes in the permeability of the cell wall membrane could cause a decrease in cell size. In principle, cell lysis may be caused either by an interference with cell membrane function or by disturbance of the delicate balance between synthesis and degradation of compounds involved in the cell membrane construction which exist during the stage of cell multiplication (Meingassener and Sleytr, 1982).

The present investigation confirms that there are antifungal properties in the crude extract of *R. nasutus*. Whether these biological activities are different manifestation of the same compounds or are produced separately by different compound is not known. However, it is important to point out that crude extract such as this needs to be further processed to give pure compound (s) which can then be tested for antifungal activity.

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