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ARTÍCULO DE INVESTIGACIÓN / ORIGINAL RESEARCH PAPER

ANTIFUNGAL ACTIVITY OF NEEM (*Azadirachta indica:* MELIACEAE) EXTRACTS AGAINST DERMATOPHYTES

Actividad antifúngica de extractos de neem (*Azadirachta indica:* Meliaceae) sobre hongos dermatofitos

Daniel Iván OSPINA SALAZAR[1], Rodrigo Alberto HOYOS SÁNCHEZ[1], Fernando OROZCO SÁNCHEZ[2], Myrtha ARANGO ARTEAGA[3], Luisa Fernanda GÓMEZ LONDOÑO[3].

[1] Departamento de Ciencias Agronómicas, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, sede Medellín. Calle 59A n°. 63-20. Medellín, Colombia.

[2] Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, sede Medellín. Calle 59A n°. 63-20. Medellín, Colombia.

[3] Laboratorio de Micología Médica, Facultad de Medicina, Universidad de Antioquia. Carrera 52 n°. 62-29. Medellín, Colombia.

For correspondence. diospinas@unal.edu.co

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ABSTRACT

In order to assess the antifungal activity of methanolic extracts from neem tree (*Azadirachta indica* A. Juss.), several bioassays were conducted following M38-A2 broth microdilution method on 14 isolates of the dermatophytes *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum canis* and *Epidermophyton floccosum*. Neem extracts were obtained through methanol-hexane partitioning of mature green leaves and seed oil. Furthermore, high performance liquid chromatography (HPLC) analyses were carried out to relate the chemical profile with their content of terpenoids, of widely known antifungal activity. The antimycotic Terbinafine served as a positive control. Results showed that there was total growth inhibition of the dermatophytes isolates at minimal inhibitory concentrations (MIC) between 50 µg/mL and 200 µg/mL for leaves extract, and between 625 µg/mL and 2500 µg/mL for seed oil extract. The MIC of positive control (Terbinafine) ranged between 0.0019 µg/mL and 0.0313 µg/mL. Both neem leaves and seed oil methanol extracts exhibited different chromatographic profiles by HPLC, which could explain the differences observed in their antifungal activity. This analysis revealed the possible presence of terpenoids in both extracts, which are known to have biological activity. The results of this research are a new report on the therapeutic potential of neem to the control of dermatophytosis.

Keywords: HPLC, neem, microbial sensitivity tests, minimum inhibitory concentration, terpenoids.

RESUMEN

Se determinó la actividad antifúngica de extractos metanólicos de la especie *Azadirachta indica* A. Juss. (Meliaceae), conocida comúnmente como neem, empleando el método de microdilución en caldo M38-A2 de referencia para hongos filamentosos y dermatofitos. Se evaluaron 14 aislamientos de los dermatofitos *Trichophyton mentagrophytes*, *Trichophyton rubrum, Microsporum canis* y *Epidermophyton floccosum*. Los extractos de neem fueron obtenidos mediante partición metanol-hexano a partir de aceite de semillas y hojas verdes maduras. Adicionalmente, se analizaron por cromatografía líquida de alta resolución (CLAR) con el fin de relacionar su perfil químico con el contenido de terpenoides, de conocida actividad antifúngica. Se empleó como control positivo el antimicótico Terbinafina. Los resultados mostraron inhibición total del crecimiento de los aislamientos de dermatofitos a concentraciones mínimas inhibitorias (CMI) entre 50 µg/mL y 200 µg/mL para el extracto de hojas y entre 625 µg/mL y 2500 µg/mL para el extracto de aceite de semillas. La CMI encontrada para el control positivo (Terbinafina) fluctuó entre 0,0078 µg/mL y 0,0313 µg/mL. Los extractos metanólicos de hojas y aceite de semillas de neem exhibieron diferentes perfiles cromatográficos en CLAR, lo cual podría explicar las diferencias observadas en su actividad antifúngica. Éste análisis químico reveló la posible presencia de compuestos

terpenoides en ambos extractos, los cuales se conocen por su actividad biológica. Los resultados de esta investigación son un nuevo aporte sobre el potencial terapéutico del neem para el control de dermatofitosis.
Palabras clave: concentración mínima inhibitoria, CLAR, neem, pruebas de sensibilidad microbiana, terpenoides.

INTRODUCTION

The research about neem has been focused not only on its known antifeedant activity on insects, but also on its antifungal potential (Govindachari *et al.*, 1998). Medical importance fungi, such as dermatophytes, are one of the most widespread causes of dermatology consultations. The nail lesions that produce are estimated to be 50 % of the whole infections on nails. Fungal infections of skin and nails affect 20-25 % of the world population, with a prevalence as high as 40 % in tropical areas (Nagabhushan *et al.*, 2013).

The genus *Trichophyton, Microsporum* and *Epidermophyton* are an important source of dermatophytic infections in several parts of the body. In a study carried out with 1105 patients in Pernambuco state, Brazil, it was found that *T. rubrum* was the species most often isolated in hands, feet and groin, followed by *T. mentagrophytes,* isolated from feet and nails. *E. floccosum* had lower prevalence than the above mentioned fungi, but it appeared also in groin, nails and feet. In scalp, *M. canis* was the prevalent fungi. The authors reported an increase in the prevalence of *T. rubrum*, which is a trend observed throughout the world (Rodrigues De Barros *et al.*, 2007). Dermatophytes can also be present in pets, as a study in Cali, Colombia revealed. Out of a sample of 165 street dogs, 23 were positive (13 %) to dermatophytes, and out of 86 pet dogs, 11 (12 %) were positive too. The main fungus isolate were *Microsporum gypseum,* followed by *M. canis, T. mentagrophytes* and *T. rubrum*; the two latter fungi were isolated only in pet dogs (Alvarez and Caicedo, 2001).

There is an increasingly interest on the isolation of natural compounds with antifungal activity, from which terpenoids have revealed to produce either fungistatic or fungicidal power on several pathogenic fungi.

To this respect, Ospina *et al*. (2011) evaluated the effect of essential oils from three chemotypes of *Lippia origanoides* (Verbenaceae) on the micelial growth and sclerotial formation of *Sclerotium cepivorum*. The results showed that the micelial growth and sclerotial formation of were totally inhibited at a minimal concentration of 120 µL/L by an essential oil rich in thymol, followed by trans-β-cariophylene (650 µL/L) and β-myrcene (1350 µL/L) chemotypes. Other terpenoids found to have antimicrobial activity are α-bisabolol, α-terpinene, cineole, nerolidol and terpinen-4-ol (Kurekci *et al.*, 2013).

Neem raw organic extracts and their components also inhibit the growth of several pathogenic fungi. Using a Sabouraud Dextrose broth dilution method, Natarajan *et al.* (2003) found a MIC of 31 µg/mL of a neem seed organic extract on *T. rubrum, T. mentagrophytes* and *Microsporum nanum*. The organic extract from neem leaves showed MICs between 150 µg/mL to 500 µg/mL in the above mentioned fungi. The authors also evaluated the growing pattern of each dermatophyte in Petri dishes and observed that the diameter of the colony was reduced during thirty days of evaluation, when mixing the Sabouraud medium with 15 µg/mL of neem seed extract.

Govindachari *et al.* (1998) has indicated that the active antifungal fraction of neem oil is a mixture of tetranortriterpenoids derived from methanol partitioning, when tested against *Drechslera oryzae, Alternaria tenius* and *Fusarium oxysporum* by measuring growth diameter in Petri dishes. Nevertheless, they noticed that the separated compounds, as azadiradione, nimbin and salanin, did not exhibit appreciable activity by itself, but the activity was recovered when mixed together again. Other authors have observed that pure azadirachtin was not highly effective as a fungicidal agent, when compared to azadirachtin rich raw neem seed extracts (Kavitha *et al.*, 2014). The previous studies suggest that there is a synergistic or additive effect of terpenoids in the extracts from neem.

Other authors support this hypothesis by evaluating different plant extracts or fractions obtained through chromatographic methods. Nagabhushan *et al.* (2013) found that only petroleum ether extract from *Eclipta prostrata* (Asteraceae) was active against *M. canis, M. gypseum* and *T. rubrum,* indicating a MIC of 15 mg/mL. Furthermore, they observed a loss of activity when separating the petroleum ether extract by thin layer chromatography, and determining the antifungal activity of each fraction. Similarly to the prior, several flavonoids present in methanol extracts from *Baccharis spp.* (Asteraceae) showed synergistic or additive effects when combined with the commercial antimycotic Terbinafine, enhancing the growth inhibition of *T. rubrum* by using M38 A2 broth microdilution method (Rodriguez *et al.*, 2013).

The neem tree is particularly rich in triterpenoids. It has been estimated that over 100 of this structurally related compounds have been isolated from various parts of the neem tree (Johnson *et al.*, 1996). The limonoids, also called tetranortriterpenoids, are a group of heterocyclic compounds highly oxygenated with alkoxy and hydroxyl groups, from which azadirachtin is the most known. Other similar compounds are salannin, nimbin, 3-desacetylsalannin and 6-desacetylnimbin (Jarvis *et al.*, 1999). These triterpenoids are often extracted by grinding the seed kernels and partitioning with hexane and alcohol to separate the oil from the terpenoids.

The action mechanism of plant extracts on dermatophytes are thought to be cell wall–related, since it has been observed that ether extracts of *Inula viscosa* (Asteraceae) inhibits chitin synthesis in dermatophytes and *Candida albicans* (Maoz *et al.*, 2000). It was reported that aqueous neem leaves extracts increase superficial hydrophobicity on cells of *Candida albicans* (Polaquini *et al.*, 2006). Neem seed methanolic extracts and pure azadirachtin were found to be inhibitive to ergosterol biosynthesis of *Aspergillus parasiticus;* this effect might be attributed to inhibition of enzyme (s), which is involved in the biosynthetic pathway of ergosterol (Kavitha *et al.*, 2014).

This study was planned to determine the antifungal activity of neem leaves and seed oil methanolic extracts, against 14 clinical isolated dermatophytes, by using a reference microdilution method. The possible relation of their antifungal activity with their chemical profile by HPLC of both extracts was also analysed.

MATERIALS AND METHODS

Neem extraction phase

Obtaining neem extracts

Neem green mature leaves were collected from healthy trees in the campus of the Universidad Nacional de Colombia, Medellín. The neem leaves extract was prepared according to Suresh *et al.* (1997). 500 mL of hexane were added to 24 g of fresh leaves leaving them overnight undisturbed. The resultant extract was filtered and concentrated in a rotary evaporator to 200 mL, then it was mixed with 100 mL of methanol (MeOH) 95 % three times, separated in a separatory funnel and discarded the hexane phase. The methanol phase was concentrated until give a residue, and kept sealed in darkness.

Neem seed oil, provided by Biotropical S.A., Colombia, was partitioned following Govindachari *et al.* (1998) method. The seed oil was partitioned with hexane – MeOH 95 % in a separatory funnel successively until total de-oiling. The hexane phase was discarded, whereas methanol phase was concentrated in rotary evaporator until give a residue, and kept sealed in darkness.

Analysis by high performance liquid chromatography of neem extracts

The HPLC analysis was done using a C18 column coupled to a system Agilent 1100 for LC and LC/MS equipped with auto sampler Agilent G1313A and a UV visible Agilent G1311B. Data were analyzed with the software LCMS chemStation Rev. A.09.03 [1417]. The separation was done with a C18 column LichroCART 125-4 LiChrosper 100 RP-18 (125 mm x 4.6 mm D.I, pore diameter 5 µm) (Merck, Germany).

The gradient program was started with a mobile phase flux of 1.0 mL/min, and varying the acetonitrile (ACN) ACN/H_2O ratio from 35:65 v/v at the beginning, to ACN/H_2O 45:55 (minute 10), ACN/H_2O 70:30 (minute 11), and ACN/H_2O 35:65 (minute 14 to 25). The injection volume was 50 µL, and the sample concentration was 4 mg/mL. It was registered at a wave length of 213 nm during 25 minutes (Orozco-Sanchez *et al.*, 2011). The resultant HPLC profiles were also compared with that of a methanol extract from neem cultured cell suspensions (Ospina *et al.*, 2014).

Determination of the antifungal activity

The dermatophytes fungi were isolated from patients remitted to the Medical Mycology Laboratory of the Medicine Faculty, Universidad de Antioquia, Medellín, Colombia. The classification was done by microscopic and macroscopic analysis of the colonies, according to criteria from Kane *et al.* (1997) and Rebell *et al.* (1979), as well as using biochemical testing (urea and agar-glucose-solids, bromocresol purple). The dermatophytes isolates were cultured repeatedly in Sabouraud-dextrose-agar medium in order to obtain pure colonies: *Trichophyton rubrum* and *Trichophyton mentagrophytes*: five isolates; *Epidermophyton floccosum:* three isolates; *Microsporum canis:* one isolate.

To prepare the conidial suspension inoculum, the isolates of *T. mentagrophytes, T. rubrum* and *E. floccosum* were first cultured in Potato-Dextrose-Agar (PDA), or boiled rice (*M. canis*) for up eight days to stimulate conidial formation; afterwards, the surface of the mycelium was covered with sterile saline solution 0.85 % p/v and scratched with a loop. The resultant conidial suspension was adjusted to 1.0 – $3.0x10^3$ CFU/mL (colony forming units per milliliter) by counting the conidia in Neubauer chamber and measuring their viability in Sabouraud's medium.

To proceed with the antifungal activity tests, M38-A2 broth microdilution method was followed (CLSI, 2008). Medium RPMI 1640 (GIBCO™) was previously prepared with twofold serial dilutions of each neem extract in MeOH:DMSO (dimethyl sulfoxide) 1:1, as well the commercial antimycotic Terbinafine (positive control) and MeOH:DMSO 1:1 (solvent control), whereas negative and sterility control had no additive. This prepared medium together with the conidial suspension inoculum were dispensed in 96-multiwell U-shaped microdilution plates (Falcon, USA) and incubated at 35 °C during seven days in an incubator (Centricol, Colombia), without neither light nor shaking. Each extract was evaluated in a concentration range from 0.0019 µg/mL to 7000 µg/mL. Only three isolates of *T. mentagrophytes,* four isolates of *T. rubrum* and no *E. floccosum* were tested with seed oil extract because of lack of inoculum. Each bioassay was carried out three times with each extract and dermatophytes isolate.

The Minimum Inhibition Concentration (MIC) was measured as that in which there was no turbidity in the well, i.e., no fungal growth (100 % of growth inhibition).

Statistical analysis

Response variables (mean inhibition percentage) were analyzed by SAS software (Statistical Analysis System) with

Duncan's multiple range test, in a completely randomized design, three replicates per treatment (each neem extract and their respective controls).

RESULTS

The antifungal activity assays showed different levels of growth inhibition between the 14 isolates of dermatophytes tested with the neem extracts. Table 1 shows significant differences between the leaves and seed oil extracts, in regard to their average fungal growth inhibition. This value was calculated as a mean percentage among all concentrations above and below the MIC of each extract. Therefore, it reflects the dimension of the activity range of each extract. Data on table 1 do not consider information from *E. floccosum,* in order to homogenate the output from both types of extracts.

The figure 1 shows actually how the growth of the fungi is inhibited by neem extracts. When comparing each well, the

Table 1. Average fungal growth inhibition of each extract.

Duncan Grouping*	Inhibition (%)	N	Extract
A	86.402	264	terbinafine
B	29.527	264	leaves
C	12.500	192	seed oil

*Means with the same letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple range test. Inhibition percentage was calculated among all the dermatophytes isolates tested.

Isolate	MIC
T. menta 1	100
T. menta 2	100
T. menta 3	200
T. menta 4	200
T. menta 5	100
T. rubrum 1	200
T. rubrum 2	100
T. rubrum 3	50
T. rubrum 4	100
E. floccos 1	100
E. floccos 2	100
E. floccos 3	100
M. canis 1	50

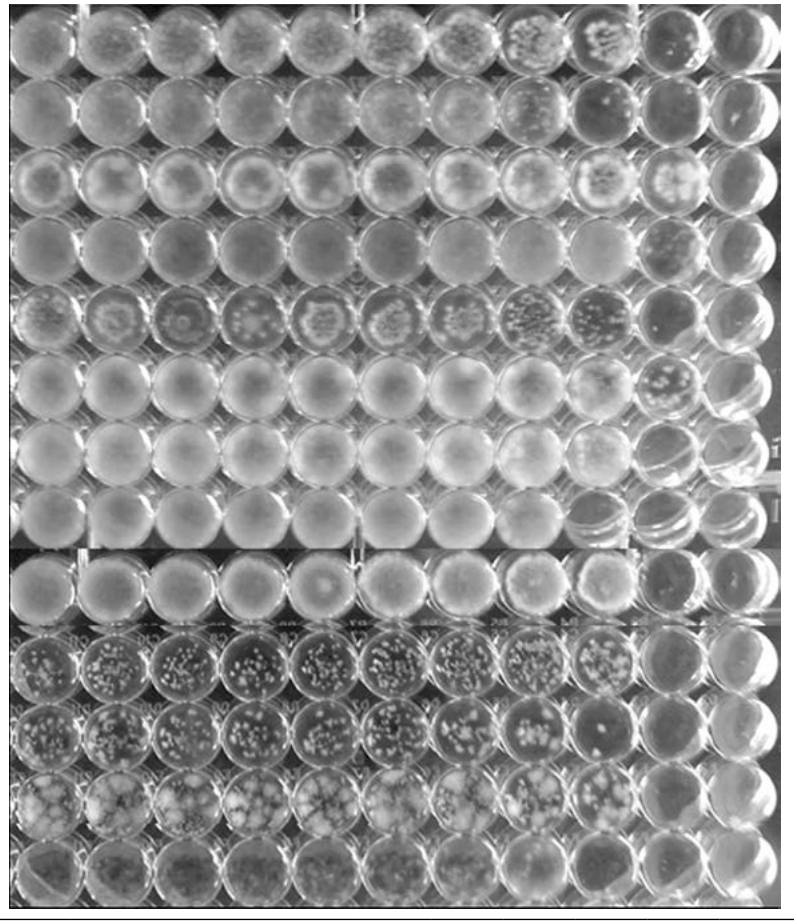

Figure 1. Aspect of the growth of dermatophytes in multiwell plates with leaves extract. Twofold serial dilutions from 0.39 µg/mL (1) to 200 µg/mL (10). SC. Solvent Control. Not all isolates are shown.

fungal growth is evinced by a white turbidity or little spots, whereas in the translucent wells there is no fungal growth. Note that solvent control (MeOH:DMSO 1:1) had no inhibitory effect on the growth of the tested dermatophytes; hence, solvent contribution to the activity of the extracts was negligible.

Between both neem extracts, leaves exhibited lower MICs (50 – 200 µg/mL) than seed oil (625 – 2500 µg/mL), in all the tested isolates (Table 2). However, positive control (Terbinafine) remains as the most effective antifungal compound, as their MICs (0.0313 – 0.0078 µg/mL) were the lowest of all.

In the chromatographic profile of the neem seed oil and leaves extracts, it was observed more than 25 peaks, which were more defined particularly between 14-18 minutes (Fig. 2). These peaks might represent at least 25 different

Table 2. Minimum Inhibitory Concentration (MIC) of neem extracts per isolate.

Isolate	MIC per extract (µg/mL)			
	Leaves	Oil	Terbinafine	SC
T. menta 1	100	-	0.0156	ND
T. menta 2	100	2500	0.0156	ND
T. menta 3	200	1250	0.0156	ND
T. menta 4	200	-	0.0156	ND
T. menta 5	100	1250	0.0078	ND
T. rubrum 1	200	2500	0.0156	ND
T. rubrum 2	100	625	0.0078	ND
T. rubrum 3	100	-	0.0156	ND
T. rubrum 4	100	1250	0.0078	ND
T. rubrum 5	100	1250	0.0156	ND
E. floccos 1	50	-	0.0313	ND
E. floccos 2	100	-	0.0156	ND
E. floccos 3	100	-	0.0313	ND
M. canis 1	50	625	0.0078	ND

Conventions: ND. Not Detected. SC. Solvent Control.

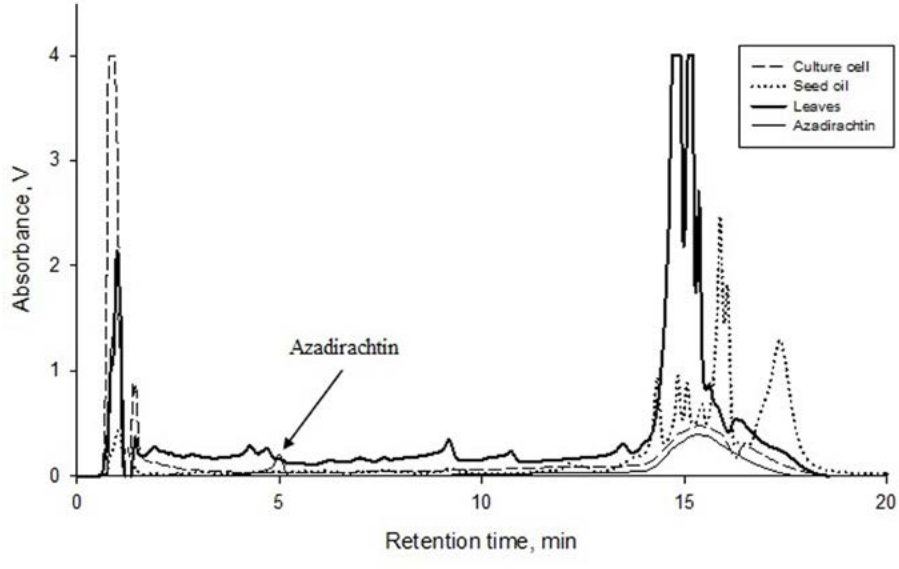

Figure 2. HPLC profile of leaves extract (gross solid line), seed oil extract (dotted line), cell culture extract (dashed line) and azadirachtin (thin line).

terpenoids. Leaves extract showed higher concentration around 15 minutes, nearly fourfold of that of the seed oil extract. However, this two extracts had higher peaks concentration than the culture cell extract, which could explain the differences in their antifungal activity (see discussion below). It is important to consider that all the extracts were injected at the same concentration of 4 mg/L. The peaks exhibited by neem seed oil, leaves and cell suspensions extracts above 14 minutes are less polar compounds than azadirachtin, whose retention time is five minutes.

DISCUSSION

Several authors have established the potential of neem extracts and their components as antifungal agents. Govindachari *et al.* (2000), by evaluating the tetranortriterpenoid azadiradione, isolated through preparative HPLC from neem seed oil, found a reduction of 76 % in the rust pustule count of *P. arachidis* at two concentrations of 1 and 10 µg/cm^2. This same author (Govindachari *et al.*, 1999) compared the effectiveness of neem leaf hexane extract against commercial fungicide Mancozeb on spore germination inhibition of *Fusarium oxysporum* and *Colletotrichum lindemuthianum*. They found that some fractions of the neem leaf extract were as effective as Mancozeb inhibiting the spore germination at concentrations of 400 – 2000 µg/cm^2. They attributed this effect to the presence in these fractions of the compound 10-undecyn-1-ol. Similarly, Song *et al.* (2011) report that several triterpenoids possess antifungal activity against *Colletotrichum gloeosporioides,* in particular one compound with a 27 carbon chain and carboxylic groups.

It is remarkable that the MICs of leaves (50 – 200 µg/mL), seed oil (650 – 2500 µg/mL), and cell suspension extracts (2500 – 5000 µg/mL; Ospina *et al.*, 2014) were progressively incrementing, unlike their absorbance peaks between 14 – 18 minutes were decreasing (Fig. 2). It could be inferred that the terpenoids present in this range of retention time are related to the antifungal activity of each extract.

Although neem seeds kernels are more widely used to obtain terpenoids from organic extracts, some reports show that the leaves can also yield this kind of compounds. Suresh *et al.* (1997) isolated various peaks and pure compounds (isomeldenin and nimonol) from neem green leaves subjected to hexane-methanol partitioning and semipreparative plus analytical HPLC separation. Then, by evaluating *in vivo* disease severity of *Puccinia arachidis* on *Arachis hipogaea* (Fabaceae) leaflets, they observed that the pustule formation was lower when applying different concentrations of two isolated peaks and a mixture of six of them. Isomeldenin, nimonol and the methanol fraction were effective to a lesser extent in controlling the disease severity than the other fractions. In this work, it was found that leaves methanolic extract had higher antifungal activity than seed oil extract.

As their HPLC profile reveals, neem extracts have compounds mainly present in retention time between 14 – 18 minutes; hence, regarding to their variable concentration in each of them, they are thought to be responsible of the different antifungal activity. In the case of leaves extract, its high concentration of terpenoids at this retention times is related with its lower MICs. It is possible that the seed oil extract ought its lower activity to a lower content of terpenoids in these retention times, which would be terpenoids with low polarity.

These peaks are complex mixtures of compounds, as other authors have suggested (Suresh *et al.*, 1997). The evidence pointed out that these peaks have by themselves antifungal activity, which is lost when separated compounds, as azadiradione, nimbin and salanin, are evaluated. The activity was recovered when mixed together again. The previous results indicated that there is a synergistic or additive effect of terpenoids in the methanolic extract from neem seed oil. The findings of Ospina *et al.* (2014), are also in agreement with the prior hypothesis, considering that a non-polar fraction of a neem cultured cell extract was more effective in inhibiting the growth of dermatophytes than the raw extract *per se*.

Although most of the previous works mention various microbial sensibility testings *in vitro*, one of the strengths of this study was to apply the reference broth microdilution method M38-A2 for filamentous fungi and dermatophytes, in order to determine the antifungal activity of neem extracts. This method is more suitable than others for evaluate the susceptibility of dermatophytes to antifungal compounds, since it is widely recommended to establish standard MICs of common antimycotics as terbinafine, fluconazole, voriconazol, or even others.

CONCLUSIONS

Both the neem leaves and seed oil extracts were capable of inhibit the growth of *T. mentagrophytes, T. rubrum, E. floccosum* and *M. canis*. The extract from neem leaves had the highest antifungal activity of both, perhaps due to a higher concentration of terpenoids with low polarity, as its HPLC profile revealed; the relation of HPLC profiles of each extract with their antifungal activity were consistent with previous results of these and other authors.

Although MICs of neem extracts were several magnitudes above those of the positive control Terbinafine, it is to consider that antagonistic effects could occurred between the different terpenoids present in them, as other authors have suggested. It is necessary to conduct further studies with pure isolated terpenoids of neem leaves and seed oil extracts employing the same bioassays methodology of this work.

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