IN VITRO ANTIDERMATOPHYTIC ACTIVITY OF ALLIUM SATIVUM L, NICOTINA TABACUM AND CADE OIL AGAINST TRICHOPHYTON RUBRUM

A. Aiboud1,2*, A. Moussaif2, N. El Abbadi2, A. Ettabia2, A. El hessni1, A. Ouichou1, M. Chakit1 and A. Mesfioui1

1Laboratory of Genetic, Neuroendocrinology and Biotechnology- Faculty of sciences, University Ibn Tofail. Kenitra – Morocco.
2National Center for Energy Sciences and Nuclear Techniques / Rabat- Morocco.

ABSTRACT

Onychomycosis is a disease that affects mainly the nails of the feet. It is caused by different fungi especially dermatophytes whose Trichophyton rubrum is the main cause in 80% of cases. The purpose of our study is to test the antifungal activity in vitro of different extracts of Allium sativum L, Nicotina tabacum and Oil of Cade usually used in traditional medicine for the treatment of skin disease in Morocco. The Minimum Inhibitory Concentration (MIC) was determined using a broth microdilution method and determined spectrophotometrically at 530 nm. T.rubrum has shown sensitivity against the various extracts of Allium sativum L, Nicotina tabacum and Cade Oil. The Nicotina tabacum extracts have shown the best inhibitor effect whose MICs were 0.1 µg / ml for hexanilic and chloroformic extract and 2µg/ml for the ethyl acetate extract followed by Allium sativum L whose the MICs were 10 µg / ml for the chloroformic extract and 100 µg / ml for hexanilic and ethyl acetate extracts. For the Cade Oil the MICs were 100 µg / ml for liquid phase washed with hexane and the liquid phase washed with water while the MIC was 40 µg / ml for the extract of water washing. Nicotina tabacum has shown the best antifungal activity against T.rubrum followed by Allium sativum L and Cade Oil also. The chloroformic extract was the most active in comparison with hexane and ethyl acetate extracts.

KEYWORDS: Dermatophytes; Trichophyton rubrum ; Allium sativum L; Nicotina tabacum; Cade Oil; Minimal Inhibitory Concentration.
INTRODUCTION
Dermatophytes affect keratinous tissue of humans and of other vertebrates, causing superficial infections. The organisms belong to three genera, Trichophyton, Epidermaphyton and Microsporum.\textsuperscript{[1,2]}

Among the dermatophytes can be distinguished the genus Trichophyton and within this, the species Trichophyton rubrum, one of the more adapted to humans, being the most common cause of tinea pedis, nail infection, tinea cruris and tinea corporis in the world-wide.\textsuperscript{[3]}

The increase of the incidence of fungi infections has become one of the main problems in the therapeutics. In the medical practice the availability of antifungal agents is relatively small, sometimes inefficient and most of them present certain toxicity. Besides the growth of those infections, the problem of the microbial resistance also showed an accentuated increase.\textsuperscript{[4]} All these reasons have motivated the research for a new antifungal or targets for this action.

Plants are a large source of new bioactive compounds with therapeutic potential. Medicinal plants have been used for several purposes including its use as antimicrobial agents and they have been presented an inhibition of fungi growth.\textsuperscript{[5,6]} Only a small percentage of living plants on earth have been phytochemically investigated. Thus, plants are an enormous reservoir of pharmaceutically valuable molecules to be discovered.\textsuperscript{[7,8]}

The World Health Organization estimated that 80% of the population of developing countries rely on plant drugs for their primary health care needs.\textsuperscript{[5]} Medicinal plants being natural, non-narcotic, having no side effects, safe, cost effective, preventive and curative therapies which could be useful in achieving the goal of "Health for all" in a cost effective manner in fact the traditional herbal remedies led the scientists to the development of numerous modern drugs.\textsuperscript{[9,10]}

Keeping these views in mind, in the present investigation, a scientific attempt has been made to explore the possibilities of anti-fungal activity of Allium sativum L, Nicotina tabacum and Cade Oil against T.rubrum.

MATERIAL AND METHODS
Plant Extract
Allium Sativum L Extract
600 g of plant material was extracted by maceration under moderate stirring for 48 hours separately with 600 ml of hexane, 600 ml of chloroform and 600 ml of ethyl acetate. The
various extracts were filtered, concentrated on a rotary evaporator, dried under reduced vacuum and stored at 4°C prior the essays. The extraction yields (w / w) was respectively 0.5%, 0.044% and 0.4%.

**Nicotina Tabacum Extract**

220 g of fine and dried powder of Nicotina tabacum, marketed by a tobacco company in Morocco was extracted by maceration under moderate stirring for 48 hours separately with 600 ml of hexane, 600 ml of chloroform and 600 ml of ethyl acetate. The various extracts were filtered, concentrated on a rotary evaporator, dried under reduced and stored at 4 °C prior the essays. The extraction yields (w / w) were 2.24%, 1.9% and 2.5% respectively.

**Cade Oil Extract**

**Washing of the Cade Oil with Water**

200g of cade oil obtained by dry distillation from Juniperus oxycedrus L was washed with 600 ml of purified water under stirring for 24 hours. This operation was repeated three times until the wash water became clear. Then the washing water was lyophilized to eliminate water, the extract obtained was stored at 4 °C prior the essays. The extraction yield (w / w) was 6%.

**Washing of the Cade Oil with Hexane**

200g of cade oil obtained by dry distillation from Juniperus oxycedrus L was washed with 600 ml of hexane, the mixture was allowed for a week, two phases were formed liquid and solid, the solid portion was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C until use.

**Preparation of Dilutions of the Extracts**

All the extracts were solubilized in DMSO and then the dilutions of each extract were prepared with demineralized water to obtain a concentration gradient ranging from 0.01 µg/ml to 100 µg/ml. the concentration of DMSO in each sample was less than 0.5%.

**Microorganism and Cultural Method**

**Insulation of the Fungal**

The Trichophyton rubrum strain was collected and identified from patients suspected for onychomycosis in a medical analysis laboratory. T.rubrum was preserved in an inclined tube of Sabouraud Chloramphenicol at 4 °C. Then the fungal was transplanted on Potato Dextrose
Agar (PDA) at 28°C for seven to 14 days to ensure the viability and the purity of the inoculum.[11]

Preparation of the Inoculum
The Inoculum suspension of T.rubrum was prepared from the 10 days cultures grown on PDA at 28 °C. The fungal colonies were covered with approximately 10 ml of sterile saline solution supplemented with Tween 80 of 1%, and the suspension was made by scraping the surface with the tip of a sterile loop. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to sterile tubes and left for 15 to 20 minutes at room temperature to sediment the heavy particles. The optical density of the suspensions containing conidia and hyphal fragments was read at 530 nm. The suspension is taken up by successive dilutions in phosphate buffered saline (PBS) to adjust the transmittance to 65 to 70% (∼10⁵-10⁶ cfu/mL).[12]

Determination of the Minimum Inhibitory Concentration (MIC)
Liquid dilution method was used to find out the extract concentration to be used for further analysis and find out the MIC. 20 µL of the suspension of T.rubrum was versed in a tube containing 10 ml of Sabouraud dextrose broth. Then, 100 µL of the inoculated culture medium of suspension was cast in each of well sterile microdilution plates 96 and simultaneously in the same well was added 100 µL of each extract with increasing concentration (from 0.01 to 100 µg / ml) . The first well does not contain antifungal and serves as a control. The microplates were incubated in an incubator in the dark at 28 ° C and the results were read at 72-96 hour.[13] The tests were performed in duplicate.

Further, The MIC was recorded spectrophotometrically at 530 nm using UNICAM UV 500 spectrophotometer. The MIC end-points for each antifungal agent was defined as the first concentration where spectrophotometrically 80% or more reduction was measured.[14]

RESULTS
The results of the antifungal activity of the extracts of Allium sativum L, Nicotina tabacum and Cade Oil are presented in (Table 1) and (Figure 1).
Table 1: Minimum inhibitory concentrations (MIC) of different extracts of Allium sativum L, Nicotina tabacum and Cade Oil against Trichophyton rubrum.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MIC</th>
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<tbody>
<tr>
<td>Cade Oil Extract of hexane wash</td>
<td>100µg/ml</td>
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<tr>
<td>Cade Oil Extract of water wash</td>
<td>40 µg/ml</td>
</tr>
<tr>
<td>Cade Oil Washed with water</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Allium sativum L Hexanilic extract</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Allium sativum L Ethyl acetate extract</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Allium sativum L Chloroformic extract</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Nicotina tabacum Hexanilic extract</td>
<td>0.1 µg/ml</td>
</tr>
<tr>
<td>Nicotina tabacum Ethyl acetate extract</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>Nicotina tabacum Chloroformic extract</td>
<td>0.1 µg/ml</td>
</tr>
</tbody>
</table>

MIC of antifungal activity of the different extracts against T. rubrum was determined after four days at 28°C by spectrophotometer at 530nm. For the Cade Oil the best antifungal activity was observed in the extract of water washing after lyophilzation (MIC: 40µg/ml), while the MIC was 100µg/ml for the Oil Cade washed with hexane and water both.

For Allium sativum L, all the extracts has shown a good inhibitory effect against the T. rubrum and the best antifungal activity was observed in chloroformic extarct (MIC: 10µg/ml), while for the hexanilic and ethyl acetate extracts the MIC was 100µg/ml for both. For Nicotina tabacum all the extracts has shown a higher inhibitory effect at small concentration against the T. rubrum compared to other extracts. The MIC was 0.1 µg/ml for hexanilic and chloroformic extract and 2µg/ml for the ethyl acetate extract.
DISCUSSION

Allium sativum L, Nicotina tabacum and Cade Oil have been used for long time in Morocco in the treatment of some superficial mycosis. The aim of our study is to investigate in vitro the antifungal activity of different extracts against T. rubrum which is the main cause of onychomycose Morocco.

A reference method for the antifungal susceptibility testing of dermatophytes is not available.\textsuperscript{[15]} In recent years, several studies on the in vitro susceptibility of dermatophytes to antifungal drugs have been done and the results have shown considerable variation.\textsuperscript{[16,17]} The results of antifungal susceptibility tests are influenced by type of medium, pH, inoculum size, incubation temperature. In this study, the broth dilution method was used and adapted according with the M 38-A document of filamentous fungi with some modifications. The medium used was Sabouraud broth.\textsuperscript{[18]} The incubation period is a point of discrepancy; we have used an incubation time of five days according to other studies.\textsuperscript{[19,20]}
Otherwise, visualization of growth inhibition could be confused with poor growth of the fungi in microdilution wells, indicating a false susceptibility profile. For that we chose to evaluate the inhibition of growth using the photospectrometric method by reading the optical density at 530 nm.\textsuperscript{[21]}

Many plant extracts have been shown a wide variety of biological activities like antifungal activity.\textsuperscript{[22,23,24]} Our results showed very interesting activity suggesting potential for development of new antifungal, especially to strains presenting multiple resistances.

By comparing the three products tested, T.rubrum has shown a high sensitivity to Nicotina tabacum; this can be explained by its chemical composition which contains different molecules known for their antifungal effect as pyridine similar to pyrimidine and alkaloids and acid oxalic.\textsuperscript{[25]}

Cade oil has also showed an interesting antifungal activity against T.rubrum and this can be explained by its chemical composition rich in $\alpha$-pinène (52,13 %).\textsuperscript{[26]} The most active part was observed in the extract of water wash who can be explained by the high solubility of phenols in water already known for it’s high antifungal property.\textsuperscript{[27]} The antifungal properties of Allium sativum L. are mainly attributed to its sulfur compounds, such as alliin, allicin and ajoene.\textsuperscript{[28,29]}

The chloroformic extract was the most active against T.rubrum in comparison with hexanilic and ethyl acetate extracts. This can be explained by it’s ability to dissolve the various compounds contained in the samples,\textsuperscript{[30]} and for its high content in molecules as sterols, triterpenes, steroids, and alkaloids bases.\textsuperscript{[31]}

**CONCLUSION**

The Nicotina tabacum, Allium sativum L and Cade Oil extracts showed important antifungal activity against T. rubrum. These results are very interesting suggesting the potential for development of new antifungal drugs including for resistant strains.

**REFERENCES**