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Antiviral and Antidermatophytic Activity of a Compound Extracted from *Cuminum cyminum* Seeds

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ABSTRACT

In vitro activity of EHP [1-(2-Ethyl, 6-Heptyl) Phenol], a natural compound purified from cumin seeds extract, was investigated against ten dermatophytic isolates; *Trichophyton* (n=5), *Microsporum* (n=4), and *Epidermophyton* (n=1), as well as three viruses; HAV, Cox B4, and HSV-1. The highest antidermatophtic activity was against *T. fulvum* and *E. floccosum* while *T. terrestre* exhibited the most resistant isolate followed by *T. schoenleinii*. MIC of EHP ranged from 2.5 to 25 µg/ml, however owing to cytotoxicity results, EHP concentrations up to 10 µg/ml are preferably used. EHP possessed antiviral activity against the investigated viruses with HAV being the most affected followed by Cox B4 and then HSV1 with a plaque reduction percentage of 70, 65, and 60, respectively.

Key words: *Cuminum cyminum ,* Viruses, Dermatophytes, EHP.

INTRODUCTION

Plants are safe to human and the ecosystem, and can easily be used by the public who used them for thousands of years to enhance flavor and aroma of foods as well as its economic value (Aly *et al*., 2000). These natural plants involve garlic, lemon grass, cumin, datura, acacia, a triplex, ginger, black seed, neem, basil, eucalyptus, alfalfa and basil (Aly and Bafiel, 2008).

Additionally, spices and herbs have been used traditionally for thousands of years by many cultures not only as flavouring agents but also as food preservatives. They are generally recognised as safe because of their traditional use without any documented detrimental impact. They are also inexpensive, show better patient tolerance and are readily available for low socioeconomic population (Bag, A. and Chattopadhyay, R.R., 2015)

A popular spice in world, cumin is rich in iron, an important mineral for immune health. Cumin has also been used traditionally to improve digestion, and preliminary scientific evidence suggests that its traditional reputation may be justified. Animal studies indicate that cumin may have anti-carcinogenic properties as a result of its antioxidant content and ability to enhance detoxification enzymes in the liver, protecting against the formation of liver and stomach tumors.Cumin seeds were also reported to possess antimicrobial activities for different microorganisms, including bacterial strains, yeasts and fungi (De *et al*., 2003).

Dermatophytes are fungi that can cause infections of the skin, hair, and nails due to their ability to utilize keratin. The organisms colonize the keratin tissues and inflammation is caused by host response to metabolic by-products. The dermatophytes are included in three fungal genera viz,:1. Epidermophyton: This genus consists of 2 species, one of which is a pathogen 2. Microsporum: There are 19 described species but only 9 are involved in human or animal infections. 3. Trichophyton: There are 22 species, most causing infections in humans or animals (Indira,G. 2014)

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some fungal species to different fungicidals used in medicinal practice. Fungi are one of the most neglected pathogens, as demonstrated by the fact that the amphotericin B, a polyene antibiotic discovered 1956, is still used as a "gold standard" for antifungal therapy. The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections (Abad *et al*., 2007).

Unlike the search for antibiotics, which took root from the discovery of penicillin late 1930s, the search for antiviral agents began in the 1950s but had a breakthrough in 1964. Early success in this direction included the use of methisazone for the prophylaxis of small pox and the use of idoxuridine for the treatment of herpes keratitis (Kinchington *et al*., 1995).

Two major obstacles to the development and use of effective antiviral chemotherapy are the close relationship that exists between the multiplying virus and the host cell, and that viral diseases can only be diagnosed and recognized after it is too late for effective treatment. In the first case, an effective antiviral agent must prevent completion of the viral growth cycle in the infected cell without being toxic to the surrounding normal cells (Dessel berger, 1995). One encouraging development is the discovery that some virus specific enzymes are elaborated during multiplication of the virus particles and this may be a point of attack by a specific inhibitor. However, recognition of the disease state too late for effective treatment would render that antiviral agent useless even if they were available.

Until early recognition of the disease state is provided, most antiviral chemotherapeutic agents will have their value as prophylactic agents. The reason for the apparent lack of progress in antiviral chemotherapy as compared with the field of antibacterials has been a problem of selectivity (Kinchington, 1995). Any antiviral agent must selectively kill the pathogenic organism in the presence of other living cells. Sufficient biochemical differences exist between the metabolism of prokaryotic bacterial cells and mammalian cells to enable selectivity to be achieved, hence the early development of antibacterial agents, which were safe for human use. Viruses on the other hand, despite their apparent simplicity present a bigger problem. This is because during their replicative cycle, they become physically and functionally incorporated into the host cells and it is therefore difficult to distinguish unique biochemical features suitable for selective attack.

Some viruses also persist in a latent infection (Cann, 1993), in which case, antiviral drugs are less likely to be effective. However increased understanding of the molecular events of virus infections has meant that the search for antiviral drugs against specific targets can be conducted on a more rational basis(Abonyi *et al*., 2009).

Early cultures also recognized the value of plant materials in medicine. Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria, fungi, protozoa and viruses (Soylu *et al*., 2005). In the recent years, researches on medicinal plants have attracted a lot of attention globally. Large body of evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary and alternate systems of treatment of human diseases. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc, which have been found *in vitro* to have antimicrobial properties (Yoshida *et al*., 2005).

Medicinal plant products have been used as folk remedies for different kinds of ailments including viral diseases (Field and Biron, 1994). Thereis a need to search for new compounds for treatment of viral infections since there is an increasing resistance to antiviral drugs .Traditional plant extracts having anti-infective properties, have been screened for their antiviral activity (Chiang et al., 2003).

Also, several antiviral compounds have been tried as therapeutic use in earlier decades (Kinchington et al., 1995). Nucleoside derivative drugs such as acyclovir (AVC), gancyclovir(GCV)

and pencyclovir have been widely approved drugs for the treatment of HSV infections. However, wide spread use of these drugs has shown resistance especially inimmunocom promised and bone marrow transplant recipients. (Van den et al., 19986 and Vijayan et al., 2004). In order to circumvent the problem of viral resistance, development of new antiviral products with different mechanism of action is crucial. The activity of the Indian medicinal plant extract, *Swertiachirata* agains therpes simplex virus type-1 (HSV-1) using multiple approaches.

Previously, EHP (1-(2-ethyl,6-heptyl)phenol) compound which extract from *Cuminum cyminum* (cumin) seeds by benzene solvent had possessed antifungal activity in an *in vitro* study against ten pathogenic fungal isolates (Mekawey *et al*., 2008). Also, high activities were recorded against and seven cell lines of tumor (Mekawey *et al*., 2009). The present study was designed to evaluate the antiviral and antidermatophyte activity of EHP (1-(2-ethyl,6-heptyl)phenol) compound, the antifungal drugs activities at the same concentrations and organic solvent are compared. The percentage of inhibition and MIC are also recorded.

MATERIALS AND METHODS

Plant Material

The present study deals with the screening of 1-(2-Ethyl, 6-Heptyl) Phenol (EHP)extracted from seed of *Cuminum cyminum* (cumin) for anti-dermatophytic and antiviral activities.

Fungal Specimen Collection

The specimens were collected from different parts of the body (skin scrapings, nails, hairs) of patients suffering from dermatophycoses. Forty (40) individuals of different ages, many childeren were sampled. This procedure was carried out using forty (40) new surgical blades for each individual. Specimens were collected by scraping affected spots into clean sheets of paper which were then transferred into sterile containers that had been properly labeled with respect to each individual's data; these were brought to the laboratory for inoculation.

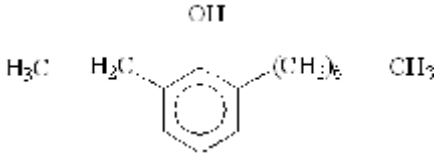

Fig. 1: EHP compound

Identification of fungal isolates

An Image analysis system, soft imaging system GmbH software (analySIS ® pro ver. 3.0) at the fungal identification unit of The Regional Center for Mycology and Biotechnology AL- Azhar University was used for identifying the isolated dermatophytes.

Culture Media

Sabouraud Dextrose Agar (SDA) [Dextrose, 20.0g; Bacteriological Peptone, 10.0g; Agar, 20.0 g; 0.1 g/L cyclohexamide was used for isolation]. The pH of the media was adjusted at 5.6 ± 0.2 at 25 (±2) °C. Other media were used for maintenance of fungal isolates; Malt Extract Agar (MEA) [Malt extract, 20.0g; Bacteriological Peptone, 5.0g; Agar, 20.0g]. The pH was 5.4 ± 0.2 at 25 (±2)°C. Each medium was prepared by dissolving the solid ingredients in 1 liter of cold distilled water and then heating to 60-70 °C with stirring. Media were sterilized by autoclaving at 121°C (1.5 atm.) for 15-20 minutes (Atlas, 1993). For maintenance of stock cultures, 15 ml of cooled molten agar were poured into test tubes, autoclaved at 121 °C for 15 minutes, and then tilted to provide slopes for stock cultures.

Screening of Antidermatophytic Activity

The filter paper disc method was used for screening the antifungal activity of EHP. Rinsed Petri plates were sterilized in an oven at 110°C overnight. Each of the sterilized plates was half filled with Sabouraud's Dextrose Agar medium. The thickness of the agar medium was kept equal in all the Petriplates. Standard size Whatman filter paper discs (6.0 mm in diameter) were sterilized in an oven at 140°C for one hour, saturated with different concentrations of EHP and airdriedat room temperature under aseptic condition to remove any residual solvent that might interfere with the determination. The discs were then placed on the surface of sterilized Sabouraud's Dextrose Agar Medium that had been inoculated with the investigated dermatophytes.

Each of the plates was homogenized to ensureuniform distribution of the inoculum and airdried toremove surface moisture. Petri plates containing thepaper discs (6 mm) dipped in benzene, methanol and water were then run ascontrol. Before incubation, all the test and control Petriplates were kept at 5°C for one hour to allow the diffusion of the substance from the disc into the agar medium plate. Plates were incubated at 37 °C for 48–72 hours, after which the zone of inhibition was measured. All the experiments were done in five replicates.

Determination of minimal inhibitory concentration of EHP compound on human pathogenic fungal growth

Determination of the Minimum Inhibitory Concentration (MIC) of EHp compound was carried out by broth dilution assay. Different standard discs (Griseofulvin; Itraconazole; Terbinafin, fluconazole and Ketoconazole) dissolved in methanol/dis. water for different fungi were used for determining the activity of EHP (compared to the highest standard value). Two-fold serial dilutions of the extracts were prepared in Sabouraud Dextrose broth to give concentrations ranging from 100- 1.56 mg/ml. 0.2 ml of each of the microorganisms suspension was inoculated into the different concentrations of the test compound in test tubes. The tubes were incubated at 37±2 °C for 4-5 days. The concentration of the extract which exhibited no visible growth of the fungus was considered as the MIC.

Screening of Antiviral Activity

Determination of extract cytotoxicity

The ctotoxcity of EHP was performed according to(Van den Berghe *et al.*, 1978) at the virology center , Faculty of medicine, Al-Azhar university.

Titration of HAV - H10, HSV – I, COX – B4 infectivity

Titration of HAV - H10, HSV – I, COX – B4 infectivity isolated was performed by plaque formation method according to (Dulbecco and Vogt, 1954). The virus infectivity titer represented as a number of plaque formation unit (P.F.U.) / ml of the stock virus suspension and it was calculated from the following equation:

P.F.U./ml = No. of plaque X reciprocal of dilution X reciprocal of volume in ml.

Determination of anti-infectivity effect of EHP

Anti-infectivity of EHP was achieved according to (Kaul *et al.*, 1985).

RESULTS

Evaluation of the Antifungal Activity of EHP

Table (1) and figure (2) shows the antidermatophytic activities of different EHP concentrations against the investigated dermatophytes. *Trichophyton terrestre* was the most resistant strain to EHP, exhibiting an inhibition zone of 3mm at 25 µg/ml, followed by *Trichophyton*

Table 1: Antidermatophytic activities of different concentrations of (EHP) (100 µl)

Fungal isplates	Zones of Inhibition (mm) of different concentration of cumin compound (EHP) (µg/ml)					
	25	**20**	**15**	**10**	**5**	**2.5**
Microsporum gypseum	20	16	10	6	-	-
Microsporum fulvum	30	25	20	14	8	2
Microsporum ferrugineum	20	15	10	7	4	-
Microsporum canis	22	16	12	8	5	-
Epidermophyton floccosum	34	30	25	12	8	2
Trichophyton interdigitale	35	26	20	6	-	-
Trichophyton mentagrophytes	26	20	10	5	-	-
Trichophyton schoenleinii	10	4	-	-	-	-
Trichophyton terrestre	3	-	-	-	-	-
Trichophyton tonsurans	29	15	8	3	-	-

schoenleinii with an inhibition zone of 4mm at 20µg/ml.

The most sensitive isolates were *E. floccosum* and *M. fulvum*, each exhibiting an inhibition zone of 2 mm at the least studied concentration of 2.5 µg/ml.

The *in vitro* susceptibility of the clinical dermatophyte isolates against EHP and frequently used 5 antifungal agents was investigated (Table 2 and Figure 2).

MIC for EHP ranged from 2.5 to 25 µg/ml. It exhibited the minimum MIC value of 2.5µg/ml for *E. floccosum* and *M. fulvum* while the maximum one of 25µg/ml for *T. terrestre*. Table (2) and figure (2) reveals the competing capability of EHP regarding the investigated isolates with the exception of *T. terrestre* and *T. schoenleinii* as their MICs, 25 and 20 µg/ml respectively, greatly exceeded the cytotoxic concentration of EHP (10 µg/ml).

Evaluation of the Antifungal Activity of EHP

Two EHP concentration, 6.25, and 12.5 ug/ml, were used to investigate its effect on viral activity using three widespread viruses (CoxB4, HSV1 and HAV). The percentage of viral plaque reduction was not greatly affected by EHP concentrations (figure 3).

Table 2: MICs (µg/mL)of EHP compared to antifungal drugs

	EHP	Griseofulvin	Itraconazole	Terbinafin	fluconazole	Ketoconazole
M. gypseum	8	6.25	2.5	3.125	3.125	3.125
M. fulvum	2.5	3.125	2.5	1.25	6.25	3.125
M. ferrugineum	5	6	2.5	2.25	5	3
M. canis	4	5	4	1	2	1.5
E. floccosum	2.5	3	1.5	1	5	2
T. interdigitale	7.5	5	6.5	2	6.25	3.5
T. mentagrophytes	10	6.25	2.5	1.5	17.5	1
T. schoenleinii	20	4	2	1	5	1.5
T. terrestre	25	7.5	4	2	10	3
T. tonsurans	10	5	7	1	9	1.7

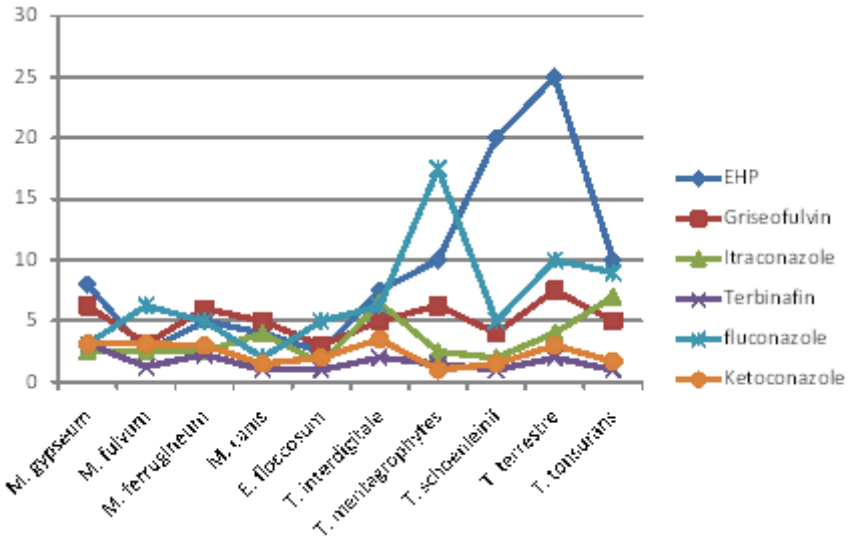

Fig. 2: MIC (µg/ml) of EHP and a number of commercially available antifungal drugs against fungal pathogens

Maximum activity was observed against HAV resulting in a plaque reduction percentage of 70 regarding both concentrations. HAV was followed by CoxB4 and then HSV1 with reduction percentages of 65 and 60, respectively.

No cytotoxicity was observed for EHP as ensured from the negative effect of EHP on vero cells (Fig. 3).

Compounds extracted from plants can provide an alternative approach to new therapies. They present characteristics such as high chemical diversity, lower cost of production and milder or inexistent side effects compared with conventional treatment (jardim et al., 2014).

In the current study, EHP, a compound extracted from cumin and was proved to possess antibacterial, antifungal and antitumor activities (Mekawy et al., 2007 & 2009), was found to possess antidermatophytic as well as antiviral activities.

Regarding dermatophytes, EHP exhibited activity against the ten investigated species; 5 species belonging to *Tricophyton*, 4 species to *Microsporum*, and one to *Epidermophton*, with *E. flocossum* and *M. fulvum* exhibiting inhibition zones at all the investigated concentrations even the lowest one (2.5µg/ml). According to the available research, a few studied the effect of cumin on dermatophytes; Romagnoli et al. (2010) studied the antidermatophytic and antifungal activity of essential oil from fruits of indian *Cuminum cyminum* which proved active in general on all investigated fungi but in particular on the dermatophytes, where *Trichophytonrubrum* was the most inhibited fungus also at the lowest dose of 5 microL. Other studies concerned the in vitro antifungal activities of essential oil from *Cuminum cyminum*, however against different pathogenic fungi, not dermatophytes; Naeini et al. (2014) studied the effect of essential oil from *Cuminum cyminum on Candida* strains where the results suggested the potential substitution of the antifungal chemicals by *C. cyminum* essential oil as natural inhibitors to control the growth of the most important pathogenic *Candida* species and alternative therapies for candidiasis.

Generally, the antimicrobial activity of essential oils of cumin seeds was reported by other investigators. Ozkan (2003) reported that essential oils of cumin potentially might be used as antibacterial agents to prevent the spoilage of food products. Iacobellis et al. (2005) also suggested using cumin oils to control bacterial diseases. Ani et al. (2006) reported the inhibitory effect of cumin extract on the growth of some food-borne pathogenic and spoilage bacteria. The antibacterial effect of cumin extract was also shown by Bonjar (2004) and Damasius et al. (2007).

The current study compared the MIC of EHP to that of a number of commercially available antidermatophtic agents against dermatophytic isolates where EHP proved efficient against all the investigated isolates except for *T. terrestre* and *T.*

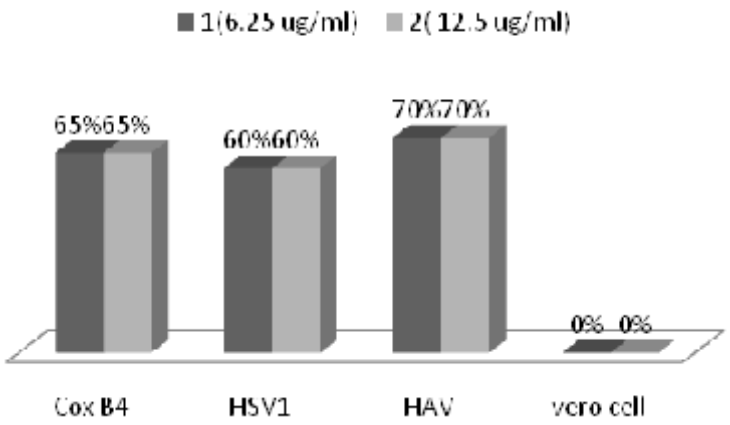

Fig. 3: Cytotoxicity and effect of EHP investigated concentrations on the percentage of plaque reduction of Cox B4, HSV1, and HAV viruses

schoenleinii whose MICs exceeded the cytotoxic EHP concentration of 10 mg/ml. The current MIC results agreed with those of Indira (2014) who reported that terbina fine had the lowest MIC range of 0.001 to 0.64 ìg/ml followed by ketoconazole at a MIC range of 0.01-3.84 ìg/ml. The itraconazole showed a MIC range of 0.082-20.45 ìg/ml whereas the griseofulvin and fluconazole showed a highest MIC range of 0.32-5.12 ìg/ml. Hence, in the current study, with the exception of *T. terrestre* and *T. schoenleinii*, EHP proved to compete with frequently-used, commercial antifungal agents

The present study also indicated antiviral activity of EHP against Cox B4, HSV1, and HAV, with HAV being the most affected followed by Cox B4 and then HSV1.

According to the available research, only one studied the antiviral activity of cumin; Motamedifar et al. (2010) reported that the methanolic extract of cumin seeds has inhibitory effect on HSV-1. They also reported that the exact mechanism of cumin methanolic extract antiviral activity has not been studied yet and might be due to the interaction of some components of extract including phenolics with Vero cell membrane and/ or HSV-1 envelope. This agrees and reinforces the current results as the structure of EHP [1-(2-Ethyl, 6-Heptyl) Phenol] possess a phenolic ring. Additionally, the concentrations used by Motamedifar et al. (0.01, 0.1, 0.25, 0.5 and 1 mg/ml) were much more than those used in the current study (6.25, and 12.5 µg/ml) which used the pure active compound extracted from cumin seed extract. Other research concerned the activity of various plant extracts on viruses; e.g., Jardim et al. (2015) who reported that natural alkaloids and lignans isolated from Brazilian plants dramatically inhibited HCV replication in vitro.

Conclusively, the antimicrobial and antiviral activity of the natural, purified, phenolic compound extracted from cumin seeds can be extended for future investigation and application into the field pharmacology, phytochemistry, or food chemistry for the development of better medicinal or preservative preparation.

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