

Full Length Research Paper

Overview on *Echinophora platyloba*, a synergistic anti-fungal agent candidate

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Echinophora Platyloba DC. is one of the four native species of this plant in Iran. The aim of this five-step study was to investigate the antimicrobial properties of this plant. Dried aerial parts of the plant were extracted by ethanol 70% in percolator. The antimicrobial activity of ethanolic extract was evaluated against dermatophytes, *Candida (C) albicans* and gram positive bacteria by agar dilution method and microbroth dilution assay. Finally, the synergistic effect of Amphotericin B plus 5% ethanolic extract against *C. albicans* was determined by measuring MIC (minimum inhibitory concentration) and MLC (minimum lethal concentration) values. Gram positive bacteria were resistant to the extract according to measurement of zones of inhibition; *Trichophyton schenlaini* and *Trichophyton verucosum* were sensitive to concentrations ≥ 35 mg/ml, while other dermatophytes showed various susceptibilities to extract. MIC value of 5% ethanolic extract was 2 mg/ml against *C. albicans* using broth micro dilution method. In synergism assay, there was a 50% reduction in MIC and a 75% reduction in MLC values of the mixture of Amphotericin B and 5% ethanolic extract against *C. albicans* in comparison to Amphotericin B alone. Regarding this study, some degrees of synergy was recorded in the combination of Amphotericin B plus *E. platyloba* extract covering *C. albicans* which represented promising finding in antifungal therapy.

Key words: Amphotericin B, *Candida albicans*, *Echinophora platyloba*, ethanolic extract, traditional medicine.

INTRODUCTION

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being (El Astal et al., 2005). Natural products have served as a major source of drugs for centuries and about half of the pharmaceuticals in use today are derived from natural products (Clark, 1996). Also, another study reports that 25 - 50% of current pharmaceuticals are derived from plants (Cowan, 1999). Microbiologists are combing the earth for phytochemicals which could be developed for treatment of infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as

tannins, terpenoids, alkaloids and flavonoids, which have been reported to have *in vitro* antimicrobial properties (Cowan, 1999). A study demonstrates the antifungal activity of extracts of some Thai medicinal plants which can be excellent candidates for the development of remedy for opportunistic fungal infections in AIDS sufferers (Phongpaichit et al., 2005). Another study reports the activity of traditional medicinal herbs from Balochistan, Pakistan against *C. albicans*, *Bacillus subtilis* and *Bacillus cereus* (Zaidi et al., 2005).

In Iran, traditional medicine has a major therapeutic role and for thousands of years, traditional healers have been using different plants to treat patients. *E. platyloba* DC. is one species of *Echinophora* genus (Rechinger, 1987). The 10 different species of this plant has been defined as: *Echinophora tenuifolia*, *E. platyloba* DC., *Echinophora sibthorpiana* Guss, *Echinophora anatolica* Boiss, *Echinophora cinera*, *Echinophora vadiaus* Boiss,

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Figure 1. Picture of *Echinophora platyloba* in the field.

Echinophora orientalis Hedge and Lamond, *Echinophora tournefortii* Joub, *Echinophora trichophylla* Sm, *E. spinosa*. Four of these species are native to Iran: *Echinophora orientalis*, *Echinophora sibthorpiana*, *Echinophora cinerea* and *E. platyloba* (Vanden et al., 2002). *E. platyloba* is mainly used for food seasoning in Iran (Chaharmahal va Bakhteyari province) (Sadrai et al., 2002), rather than preventing tomato paste and pickles from mold. The hypothesis of these serial studies was based on the plant's specific characteristic as food preserver which might have been due to its antimicrobial properties. During past 7 years, the authors did several studies on antimicrobial activity of *E. platyloba* ethanolic extract. This article is a review of all previous studies indicating the effectiveness of ethanolic extract of *E. platyloba* against *C. albicans*, dermatophytes and some gram positive bacteria, in addition to its significant synergy with Amphotericin B against *C. albicans* (Figure 1).

MATERIALS AND METHODS

Plant material

The plant was collected from the southwestern parts of Iran (Shahr-e-kord). A voucher specimen of plant was deposited in the Herbarium at the Faculty of Sciences, Isfahan University, Isfahan, Iran. The aerial parts of the plant were separated, shade dried and grinded into powder using mortar and pestle. The prepared powder was kept in tight containers protected completely from light.

Method for preparation of plant extract

Extraction of ethanolic extract was carried out by macerating 100 g of powdered dry plant in 500 ml of 70% ethanol (Istelak, Iran) for 48 h at room temperature. Then, the macerated plant material was extracted with 70% ethanol solvent using percolator apparatus (2 liter volume) at room temperature. The plant extract was removed from percolator, filtered through Whatman filter paper (NO.4) and dried under reduced pressure at 37°C with rotator evaporator.

before being added to ethanol as the solvent. Three different concentrations of ethanolic extracts (4, 5.2, and 11%) were prepared according to amount of evaporation.

Microorganisms

Cultures of *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* and *Streptococcus pyogenes* ATCC 19615 were obtained from Biotechnology Research Center of Iran, Tehran, Iran. *C. albicans* ATCC 10231 and dermatophytes (*T. schenlaini*, *T. verucosum*, *Trichophyton rubrum*, *Trichophyton mentagrophyte*, *Trichophyton violaceum*, *Epidermophyton flucosum*, *Microsporum gypsum*, *Microsporum canis*) were obtained from Fungi and Parasitological department, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Bacterial suspensions were made in Brain Heart Infusion (BHI) broth to a concentration of approximately 10^8 CFU/ml using standard routine spectrophotometrical methods. Suspensions of fungi were made in Sabouraud dextrose broth. Subsequent dilutions were prepared from the above suspensions, to be used in the tests.

4- Antimicrobial screening

Study No.1

This initial study was performed to evaluate the probability of any antimicrobial potency of the plant against ethanolic extracts (5 and 11%) in addition to the plant which derived essential oil. Essential oil was obtained by hydrodistillation method using a Clevenger apparatus. The yield was 0.3%. In brief, microbial suspension containing 10^8 CFU/ml of bacteria was swabbed and spread on Muller- Hinton agar. Three sterile blank discs (Padtan Tab Co, Tehran, Iran), impregnated with 20 μ l of 5 and 11% ethanolic extracts, and essential oil and were placed on the inoculated agar. Kefline 30 mcg/disc, Cloxaciline 30 mcg/disc and Penicillin G 100 u/disc (Himedia Company in Iran) were used as positive control standards to determine the susceptibility of gram positive bacteria. The inoculated plates were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the diameter of zone of inhibition against the test microorganisms (Mahboobi et al., 2006).

The minimal inhibitory concentrations (MICs) of extracts against different bacteria were determined by micro broth dilution assay. The extract was twofold serially diluted with 10% Dimethylsulfoxide (DMSO) which contains 25 - 0.39 mg of extract per testing well. Muller Hinton broth was used as broth media exception of *S. pyogenes* that was used as MHB supplemented with 3% horse lysed blood. After shaking, 100 μ l of the extract was added to each well. The suspension of each organism was adjusted to 10^4 - 10^5 CFU ml⁻¹ and then 100 μ l was added to each well and cultivated at 37°C. MIC was defined as the first well with no visible growth after 24 h. Minimal bactericidal concentration (MLCs) were determined as the lowest concentration resulting in no growth on subculture (M7-A7, NCCLS, Wayne, PA, 2006).

Study No.2

The activity of 0, 35, 50, 150 and 250 mg/ml of ethanolic extract of *E. platyloba* was tested against dermatophytes by agar dilution method using sabouraud dextrose agar (SDA). Inocula were prepared by growing isolates on SDA slopes. All dermatophytes were incubated for 7 days at 30°C. Slopes were flooded by normal saline. The final concentrations of dermatophytes were 2.5×10^3 - 2.5×10^4 Cfu ml⁻¹. 20 μ l of inoculums were spread onto each plate.

The plates were incubated at 30°C for 5 - 7 days. The MIC was the lowest concentration at which there was no visible fungal growth after incubation (Mustafa NK et al., 1999).

Study No. 3

The antifungal activity of ethanolic extract of *E. platyloba* ATCC 10231 was evaluated against *C. albicans*. The activity of 0, 35, 50, 150 and 250 mg extract ml⁻¹ was tested against *C. albicans* ATCC 10231 by agar dilution assay using sabouraud dextrose agar (SDA) and Inocula 0.4×10^4 - 5×10^4 CFU ml⁻¹. The MIC was defined after 48 h.

Study No.4

A single colony of the strain of *C. albicans* ATCC10231 to be tested was grown overnight at 35°C in sabouraud dextrose broth. The inoculum was prepared by diluting the overnight growth with 0.9% NaCl to obtain a turbidity of 0.5 McFarland (1×10^5 to 1×10^6 CFU/ml). The suspension was swabbed on sabouraud dextrose agar. To test the antifungal activity of extract, 20 μ l of ethanolic extract (5, 4 and 11%) and 5, 10, 15 and 20 μ l of essential oil were placed onto the inoculated plates. Disk containing Amphotericin B (Himedia Mumbai, India) and disk impregnated with DMSO were used as controls. The plates were incubated at 35°C for 48 and the diameter of inhibitory zones (mm) was measured (Griggs et al., 2001).

Minimal inhibitory concentration: The minimal inhibitory concentrations (MICs) of extract and Amphotericin B against *C. albicans* ATCC 10231 were determined by micro broth dilution method. The *E. platyloba* extract and Amphotericin B were two fold serially diluted separately with 10% DMSO which contain 25 - 0.39 mg/ml extract and 256 - 1 μ g/ml Amphotericin B per testing well. Sabouraud dextrose broth was used as broth media. After shaking, 100 μ l of agent solutions was added to each well. The suspension of *C. albicans* was adjusted to 10^4 - 10^5 CFU ml⁻¹ and then 100 μ l was added to each well and cultivated at 35°C. MIC was defined after 24 h for yeast (Griggs et al., 2001).

Study No.5

The synergy between ethanolic extract of *E. platyloba* and Amphotericin B: The Amphotericin B was two fold serially diluted with 10% DMSO which contain 16 - 0.125 μ g/ml Amphotericin B per testing well also each well contain 0.78 mg/ml of ethanolic extract (< MIC of *E. platyloba* extract against *C. albicans* found in study no 4). The inoculum was adjusted to 10^4 - 10^5 CFU ml⁻¹ and was added to each well and cultivated at 35°C. MIC was defined after 24 h.

RESULTS

Result of study NO 1 (Avijgan et al., 2005)

MIC and MLC values for 5% extract against *S. aureus*, *S. epidermidis*, and *S. Pyogenes* are shown in Table 1. The diameter of zone of inhibition for antibiotics varies from 30 - 56 mm and the average measurements for 5% ethanolic extract, 11% ethanolic extract and the essential oil were 8, 8 and 12 mm, respectively.

Table 1. MIC and MLC values of 5% ethanolic extract of *E. platyloba* against gram positive bacteria.

Names measurements (mg/ml)	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. pyogones</i>
MIC	3.1	1.5	3.1
MLC	12.5	6.2	6.1

Table 2. The susceptibility for some dermatophytes to 5% ethanolic extract of *Echinophora platyloba*.

Concentrations of extract (mg/ml) fungi	35	50	150	250
<i>T. schenlaini</i>	S	S	S	S
<i>T. verucosum</i>	S	S	S	S
<i>T. rubrum</i>	R	R	R	R
<i>M. canis</i>	R	R	R	S
<i>M. gypsum</i>	R	R	R	R
<i>T. violaceum</i>	R	R	S	S
<i>T. mentagrophyte</i>	R	R	R	S
<i>E. flucosum</i>	R	R	R	S

S = Sensitive; R = Resistant.

Table 3. The susceptibility of *C. albicans* ACTT 10231 to different types and concentrations of *E. platyloba* and Amphotericin B by disc diffusion method.

DISC content	Ethanolic extract 4%	Ethanolic extract 5%	Ethanolic extract 11%	Amphotericin B	Ethanolic extract 5% + Amphotericin B	Aqueous extract	ETHANOL 70%
Zone of Inhibition (mm)	8	13	12	18	22	0	7
MIC (mg/ml)	NC	1569	NC	2	1	NC	NC
MLC(mg/ml)	NC	3125	NC	8	2	NC	NC

NC= not checked.

Results of study NO.2: (Avijgan et al., 2006)

The susceptibility of dermatophytes to 5% ethanolic extract are shown in Table 2.

Result of study NO 3 (Avijgan et al., 2006)

In this study the authors tested the susceptibility of *C. albicans* to 5 different concentrations (0, 35, 50, 150 and 250 mg/ml) of 5% ethanolic extract. After 24 h of incubation period, the yeast grew only in the tube used as the control; while no growth was recorded in the other 4 tube containing 35, 50, 150 and 250 mg/ml of extract.

Result of study NO 4 (Avijgan et al., 2006)

C. albicans grew in two tubes in this study, the one used as control tube and the one containing 1 mg/ml of the

extract. No growth was recorded in tubes containing 2, 4, 8, 16, 32, 64, 128 and 256 mg/ml concentrations of extract.

Result of study NO.5 (Mahbobi et al., 2009)

The results of this part of the study are shown in Table 3.

DISCUSSION

The medicinal properties of plant species have made an outstanding contribution in the origin and evolution of many traditional herbal therapies. These traditional knowledge systems have started to disappear with the passage of time due to scarcity of written documents and relatively low income in these traditions. Over the past few years, medicinal plants have regained a wide recognition due to an escalating faith in herbal medicine

in view of its lesser side effects compared to allopathic medicine in addition, the necessity of meeting the requirements of medicine for an increasing human population (Chandra et al., 2006).

E. platyloba is one of the four species of the plant native to Iran (Rechinger, 1987). As a traditional herb, it is used for food seasoning (Sadrai et al., 2002), rather than preventing the pickles and tomato past from mold. Various studies have expressed the anti fungi properties of plants (Na et al., 2003; Quiroga et al., 2004; Shin et al., 2003; Akagawa et al., 1996; Nwosu et al., 1998; Phongpaichit et al., 2005; Zaidi et al., 2005). According to previously done surveys, *E. platyloba* is an enriched source of saponin, alkaloid and flavonoid (Nourozi, 1989), while there are studies demonstrating that, these three substances have significant antifungal activity (Renault et al., 2003; Mel'nichenko et al., 2003; Kariba et al., 2002; Quiroga et al., 2004). The hypothesis of these serial studies was based on the plant's specific characteristic as food preserver which might have been due to its antimicrobial properties.

In the first study, the 11 and 5% ethanolic extracts and the essential oil, 3% did not exhibit any inhibitory activity against *S. aureus*, *S. epidermidis* and *S. pyogenes*. According to Table 1, MIC and MLC values of 5% extract against bacteria were much higher than MIC and MLC values of antibiotics used in this test. Also, diameters of zones of inhibition of 5% extract, 11% extract, and essential oil of plant in comparison to diameters of zones of inhibition of used antibiotics, revealed the fact that the plant dose not have effective antibacterial properties. In the second trial, as it is apparent in Table 2, *T. schenlaini* and *T. verucosum* were sensitive to concentrations ≥ 35 mg/ml and they grew only in the plate used as control. *T. rubrum* and *M. gypsum* showed resistance to all concentrations. *T. mentagrophyte*, *M. canis* and *E. flucosum* were resistant to 35, 50 and 150 mg/ml but sensitive to 250 mg/ml. *T. violaceum* was resistant to 35 and 50 mg/ml but sensitive to 150 and 250 mg/ml. according to this result, the 5% ethanolic extract showed antifungal activity against *T. schenlaini* and *T. verucosum*. Further studies are definitely needed to evaluate the susceptibility of *T. schenlaini* and *T. verucosum* to lower concentrations of the extract. In the third study, the 5% ethanolic extract showed antifungal activity against *C. albicans*, in concentrations ≥ 35 mg/ml (35, 50, 150 and 250 mg/ml). Yeast grew only in the tube used as control.

In the forth study *C. albicans* growth was inhibited by concentrations ≥ 2 mg/ml of extract (2, 4, 8, 16, 32, 64, 128 and 256 mg/ml). The last study was done on the base of anticandidal activity of the plant that was revealed in the third study. According to the results in Table 3, there was a 50% reduction in MIC and a 75% reduction in MLC values of the mixture of Amphotericin B and 5% ethanolic extract against *C. albicans* in comparison to Amphotericin B alone. The zone of inhibition of the mixture showed 22% increase in diameter in compa-

ison to that of Amphotericin B alone. In this test, the most potent antifungal agent was the mixture of ethanolic extract 5% plus Amphotericin B, followed by Amphotericin B, ethanolic extract 5%, ethanolic extract 11%, ethanolic extract 4% and ethanol 70% in order. Aqueous extract did not show any antifungal activity. Also, the results showed that 5% ethanolic extract was slightly stronger than 11% ethanolic extract.

A series of dose dependent side effects are related to usage of Amphotericin B. A report highlights hypokalemia due to usage of Amphotericin B as a rare cause of rhabdomyolysis. So that patients under treatment with Amphotericin B should be checked for this life-threatening complication regularly (da Silva et al., 2007). Amphotericin B is widely used for severe life threatening fungal infections. Its use is limited by a dose-dependent nephrotoxicity manifested by a reduction in glomerular filtration rate and tubular dysfunction. An elevated creatinine associated with Amphotericin B is not only a marker for renal dysfunction but is also links to a substantial risk for the use of hemodialysis and a higher mortality rate. Several manipulations have been proposed to try and minimize Amphotericin B induced nephrotoxicity (Deray et al., 2002). Mechanisms to prevent nephrotoxicity include the use of lipid formulations such as Amphotericin B lipid complex, Amphotericin B colloidal dispersion and liposomal Amphotericin B and the concurrent use of volume repletion (Goldman et al., 2004).

Antifungal combination may increase the magnitude and rate of microbial killing *in vitro*, shorten the total duration of therapy prevent the emergence of drug resistance, expand the spectrum of activity and decrease the drug related toxicities by allowing the use of lower doses of antifungal.

Multiple compound therapies along the disease pathway may need to be manipulated simultaneously from an effective treatment. When one drug is used ,the required high dosage for efficacy often produce bioavailability problems and unwanted side effects in addition to drug resistance (Zhang et al., 2007). There are several arguments that justify the strategy of combining anti fungal drugs to optimize therapy such as the *in vitro* data showing the potential for a synergistic effect, broader spectrum of activity and decreased risk of emergence of resistant strains and absence of a negative or harmful effects of monotherapy (Kontoyiannis et al., 2004; Marr, 2004; Ramesh et al., 2008; Chendrasekur et al., 2002; Steinbach, 2005; Baddely et al., 2005). *E. platyloba* has been used for ages traditionally and effectively in Iran, so, it is presumed that side effects should be less. The synergistic combined mixture of Amphotericin B and the extract detected in this *in vitro* study need further *in vivo* studies to evaluate its actual effect. This will represent promising finding in antifungal therapy and enable the use of the local, rich plant heritage as an effective medicine with probably fewer

side effects.

Conclusions

Regarding this study, it is clear that *E. platyloba* indeed exhibits a potent antifungal and a weak antibacterial activity. Its inhibitory action against *C. albicans* was the highest followed by *T. schenlaini* and *T. verucosum*. Some degrees of synergy was recorded in combination of Amphotericin B plus *E. platyloba* 5% ethanolic extract covering *C. albicans*. The synergistic combined mixture in this *in vitro* study need further *in vivo* studies to evaluate its actual effect.

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