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Antifungal Activity of Nettle (*Urtica dioica* L.), Colocynth (*Citrullus colocynthis* L. Schrad), Oleander (*Nerium oleander* L.) and Konar (*Ziziphus spina-christi* L.) Extracts on Plants Pathogenic Fungi

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Abstract: Anti-mycotic activity of the ethanol extracts from Nettle (*Urtica dioica* L.), Colocynth (*Citrullus colocynthis* L. Schrad), Konar (*Ziziphus spina-christi* L.) and Oleander (*Nerium oleander* L.) floral parts were screened *in vitro* against four important plant pathogenic fungi viz., *Alternaria alternate*, *Fusarium oxysporum*, *Fusarium solani* and *Rizoctonia solani* using agar dilution bioassay. Extracts showed antifungal activity against all the tested fungi. Among the plants, Nettle and Colocynth were the most effective against *A. alternate* and *R. solani* while Oleander possesses the best inhibition on *F. oxysporum* and *F. solani*. Konar was the most effective extract by reducing the growth of *Rizoctonia solani* than other fungi. These results showed that extracts could be considered suitable alternatives to chemical additives for the control of fungal diseases in plants.

Key words: Ethanol extract, *Fusarium oxysporum*, *Rizoctonia solani*, *Fusarium solani*, *Alternaria alternate*

INTRODUCTION

The harvest losses due to fungal disease in world crop protection may amount to 12% or even higher in developing countries (Montesinos, 2003; Agrios, 1997). Many pathogens including *F. oxysporum* (vascular wild), *R. solani* (Damping off), *F. solani* (fruit root) and *A. alternate* (black mold) are causing severe damage to agriculture in pre- and post-harvest (Fletcher *et al*., 2006).

The increasing social and economic implications caused by fungi means there is a constant striving to produce safer food crops and to develop new antifungal agents. In addition, resistance to fungicides is one of critical causes of poor disease control of agriculture (Steffens *et al*., 1996; Aquino *et al*., 2006). Therefore, there is a need to develop alternative agents for the control of pathogenic fungal diseases in plants. There is a good reason to suppose that the secondary metabolites of plants have evolved to protect them from attack by microbial pathogens (Benner, 1993). So, natural products from plants have great potential as novel fungicide sources for controlling pathogenic fungi (Prabavathy *et al*., 2006; Chang *et al*., 2007). In general, plant-derived natural substances are considered as non-phytotoxic compounds and potentially effective against plant pathogenic fungi. In recent years, interests have been generated in the development of safer antifungal agents such as plant-based essential oils and extracts to control phytopathogens in agriculture (Ojala *et al*., 2000; Kordali *et al*., 2003; Nunez *et al*., 2006; Field *et al*., 2006; Lee, 2007). Historically, many plant oils and extracts have been reported to have antimicrobial properties (Lawless, 1995). It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds (Ojala *et al*., 2000). Also, the resurgence of interest in natural control of plant pathogens and increasing consumer demand for effective, safe, natural products means that quantitative data on plant oils and extracts are required (Bajpai *et al*., 2008).

Konar (*Ziziphus spina-christi* L. Desf.) belongs to the family Rhamnaceae is a tropical evergreen tree of southern of Iran. It is suggested that this is the only tree species considered holy by Muslims. This species is well soaked in the local folklore as well as the ethno medicine of almost all the ethnic groups living in the Land of Southern Iran. This tree has been widely used as a fruit plant and as a medicinal plant since antiquity and is still in use at present (Dafni *et al*., 2005). The Colocynth (*Citrullus colocynthis* L. Schrad) belongs to the family Cucurbitaceae is a viny plant native to the Mediterranean Basin and Asia, especially Turkey and Iran. It is used for

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amenorrhea, ascites, bilious disorders, cancer, fever, jaundice, leukemia, rheumatism, snakebite, tumors and urogenital disorders. Oleander (*Nerium oleander* L.), is an evergreen shrub or small tree in the dogbane family Apocynaceae. Oleander grows well in warm subtropical regions It is tolerant of a variety of poor soils and drought tolerant. Despite its toxicity it was an effective snakebite cure. Nettle (*Urtica dioica* L.) belongs to the family Urticaceae is a perennial plant growing in temperate and tropical wasteland areas around the world. In folk medicine Nettle plants have been used as a diuretic, antiasthmatic, arthritis, antidandruff, galactogogue, haemostatic, hypoglycemic, stings, tonic and rheumatism. Externally it has been used to improve the appearance of the hair and is said to be a remedy against oily hair and dandruff. (Rechinger, 1963). However, there is no report available on antifungal properties of these plants in the literature. Hence, in the current study, efforts have been made to investigate the *in vitro* antifungal potential of ethanol extracts from these semiarid plants against certain important plant pathogenic fungi causing severe destruction to crop, vegetable and ornamental plants in Iran.

MATERIALS AND METHODS

Collection and identification of plant materials: The plant materials, Konar, Oleander and Nettle leaves and fruit of Colocynth were collected from Research Institute of Forests and Ranglands, Ahvaz, Iran during 2007. Voucher specimen was deposited in the Botany Department of Science College, Shahid Chamran University. Plant material were freed from foreign materials and carefully rubbed between soft cloths to remove dust.

Screening of crude extracts for *in vitro* antifungal activity

Preparation of crude extracts: Plant material was dried in the shade at room temperature and made into powdered form by using a cutting mill, soaked in 95% ethanol (w/v) at a ratio of 2 mL dry weight on a roller mill overnight and the supernatant subsequently decanted. This was repeated three times. The combined suspensions were filtered twice, first under vacuum through a double layer of Whatman filter paper (No. 3 and 1) and then by gravity through a single sheet of Whatman No. 1 filter paper. The ethanol was removed from the clear supernatant by means of vacuum distillation at 30-35°C using a Rotary Evaporator. The remaining aqueous solution was referred to as the crude extract (Tegegne *et al*., 2008).

Plant pathogenic fungi: Four fungal pathogens used in this study, selected from different taxonomic groups, were provided by the Plant Pathogenic Laboratory Culture Collection (PPLCC), Plant Protection Department, Agriculture College of Shahid Chamran University in Iran including the following (strain numbers and taxonomic groups in brackets): *Fusarium oxysporum* Schlechtend.: Fr. (PPCC1048; Hyphomycetes), *Rizoctonia solani* Kűhn ((PPCC2031; Agonomycetes), *Fusarium solani* (Mart.): Sacc. (PPCC1320; Hyphomycetes) and *Alternaria alternata* (Fr.: Fr.) Keissler; (PPCC 19; Deuteromycetes).Cultures of each fungal species were maintained on Potato-Dextrose-Agar (PDA) slants and stored at 4°C.

Agar dilution method: The agar dilution method described by Sistia *et al*. (2008), with slight modification, was used for determining the inhibition of mycelial radial growth of the test organisms by the plant extracts. All plant pathogenic test fungi were cultured on PDA, prepared according to the specifications of the manufacturers and autoclaved for 20 min at 121°C. On cooling to 45°C in a water bath, 300 mL of a 33% (m/v) streptomycin solution was added to the basal medium for controlling bacterial growth.

Dried material of each plant extract was dissolved in 100 mL sterile distilled water and amended in the agar to obtain different final concentrations. Concentrations 0.3, 0.5, 0.7 and 0.9% were tested for Colocynth, Konar, Oleander and Nettle extracts. Working in a laminar flow cabinet, the medium was poured into 90 mm sterile plastic Petri dishes, at a temperature of 40-45°C and allowed to set. The centre of each test plate was subsequently inoculated with a 6 mm size plug of 5-7 days old cultures, for each of the pathogens separately. A plate containing only the basal medium served as control.

Plates were incubated for 4 days at 25±2°C in a growth cabinet. Each assay was replicated three times and the screening procedure repeated twice. Radial mycelial growth was evaluated each day during nine days by calculating the mean of two perpendicular colony diameters for each replicate. The values were expressed in millimeters diameter/day and was calculated as percentage mycelial growth inhibition according to the formula:

$$(dc-dt)/dc\times100$$

Where:

dc = Average diameter of the fungal colony of the control

dt = Average diameter of the fungal colony treated with the extracts

The data from two screening experiments were pooled and averaged.

Statistical analysis: Each parameter was tested in triplicate. Conventional statistical methods were used to calculated means and standard deviations. Statistical analysis (ANOVA) was applied to the data to determine differences ($p<0.05$). To ascertain significant differences between the levels of the main factor, Tukey's test was applied between means. Statistical data analysis was undertaken using the statistical package Stat graphics plus 2.0 (version). A plate containing only the basal medium served as control.

RESULTS

The extracts of Konar, Oleander and Nettle leaves and Colocynth fruits at the different concentrations showed the capacity to reduce or inhibit the growth of the *R. solani*, *F. oxysporum*, *F. solani* and *A. alternaria*. Table 1 shows percentage mycelial growth inhibition obtained with the extracts at day 9. The extract of Nettle exhibited a moderate to high antifungal activity against all the plant pathogens tested.

In the case of *A. alternata*, its growth was completely inhibited when a concentration of 0.9% of Nettle extracts was used. This extract produced the greatest reduction in mycelium growth with this fungus at 0.3, 0.5 and 0.7% with percentage reduction of 30.5, 39.4 and 58.1%, respectively. The second most effective extract was Colocynth extract, with reductions in mycelia growth of 21.6, 26.0 and 48%, respectively, at the same concentrations. The Konar, Oleander extracts caused the lowest percentage of mycelium reduction in *A. alternata*, although the reduction obtained with the latter at 0.5% and 0.7% were close to those obtained at the same concentrations with Colocynth extract (15.6 and 30.9%, respectively), while at 0.3% the reduction provoked by this extract and Konar extract (4.1 and 8.7%, respectively) were much below those obtained with Nettle and Colocynth (30.5 and 21.6%, respectively).

In the case of *R. solani*, though, the Konar extract showed the highest inhibitions of mycelium growth with values of 54.5, 61.8 and 64.8% at 0.3, 0.5 and 0.7%, respectively. This extract was followed in order of effectiveness by Colocynth and Oleander extracts, while Nettle obtained approximately half of those obtained with the most effective (Konar) 15.3 and 27.2 and 42%, respectively for the same concentrations.

In so far as *F. oxysporum* is concerned, Oleander extract produced the best reduction figures, with values of 35.2, 49.5 and 58.3% at concentrations of 0.3, 0.5 and 0.7%, respectively. This was followed by colocynth extract with slightly lower reductions of 21.5, 45 and 56.2% at the same concentrations. Nettle and Konar showed the lowest reductions, although it must be pointed out that at 0.9% all four extracts produced the total inhibition of this fungus, along with *A. alternata* and *R. solani*.

Oleander was also the most effective extract in reducing the growth of *F. solani* (reduction percentages of 36.1, 41.2 and 53.6%), which is similar to the reductions obtained with *F. oxysporum*. As with this fungus, too, colocynth was the next best extract, showing values similar to those obtained with Oleander when used at concentrations of 0.3 and 0.7% (48.4 and 66.7%, respectively). As mentioned earlier all four extracts produced approximately inhibition at 0.9%.

Table 1: Impact of plant extracts on the per cent of growth reduction of *Alternaria alternate*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium solani*

		Growth reduction (Mean and SD n = 3) (%)			
Plant species	Concentration (%)	*A. alternata*	*R. solani*	*F. oxysporum*	*F. solani*
Nettle	0.3	30.5±0.04aA	15.3±0.03bA	29.1±0.6cA	24.3±0.07dA
	0.5	39.4±0.72aF	27.2±0.07bF	40.7±0.7cF	40.7±0.08dC
	0.7	58.1±0.13aJ	42.0±0.01bJ	47.7±0.06cJ	58.8±0.1dJ
	0.9	100.0±0.00aR	97.3±0.03aR	80.2±0.01aR	80.0±0.01aR
Colocynth	0.3	21.6±0.05aB	51.1±0.14bB	21.5±0.01cB	22.3±0.07dB
	0.5	26.0±0.09aG	52.9±0.11bC	45.0±0.02cG	48.4±0.06dG
	0.7	48.0±0.12aR	67.2±0.03bk	56.2±0.16cK	66.7±0.02dK
	0.9	100.5±0.12aR	100.0±0.00aR	89.9±0.1bR	75.5±0.13dR
Konar	0.3	4.1±0.11aC	54.5±0.02bC	17.2±0.07cC	19.2±0.09dC
	0.5	15.6± 0.08aA	61.8±0.07bH	31.2±0.11cH	35.7±0.01dH
	0.7	30.9±0.06aL	64.8±0.08bL	42.6±0.12cL	46.3±0.04dL
	0.9	58.7±0.1aR	100.0±0.00aR	73.9±0.03aR	79.3±0.02aR
Oleander	0.3	8.7±0.03aD	49.6±0.10bD	35.2±0.03cD	36.1±0.18dD
	0.5	25.7±0.09aI	54.4±0.12bI	49.5±0.17cI	41.2±0.08dI
	0.7	35.0±0.11aM	67.4±0.06bM	58.3±0.12cM	53.6±0.07dM
	0.9	46.3±0.02aR	70.3±0.05aR	90.3±0.01aR	89.2±0.13aR

Values followed by the same small letter(s) within the same line are not significantly different ($p>0.05$) according to Tukey's multiple range tests. Values followed by the same letter(s) (A-D), (F-I), (J-M) and (R-U) within the same column are not significantly different ($p>0.05$) according to Tukey's multiple range tests

DISCUSSION

In this study, the present results of antifungal screening indicated that ethanolic extracts derived from various plants markedly inhibited the mycelial growth of all tested fungi at a concentration of 0.9%. Nettle and Colocynth extracts completely (100%) or almost completely (>97%) inhibited the mycelial growth of *A. alternaria* and *R. solani* and showed a relatively high degree of control against *F. oxysporum* (80-90%) and *F. solani* (75-80%). In the case of the later to fungal pathogens, the Oleander extract showed a higher degree of control (>89%) than did extracts from the other plants. Konar extract possess the best activity only against *R. solani*. This difference in antifungi efficacy is a result of higher concentrations of the same chemical or a result of different chemicals composition between plants.

Several studies have been conducted to understand the mechanism of action of plant extracts and essential oils, however it is still unclear. Several researchers attributed this function to the phenolic compounds: the amphipathicity of these compounds can explain their interactions with biomembrane and thus the antimicrobial activity (Veldhuizen *et al*., 2006). Possible action mechanisms by which mycelial growth may be reduced or totally inhibited have been proposed. It is commonly accepted that it is the toxic effects of essential oils components and extracts on the functionality and structure of the cell membrane that is responsible for the aforesaid activity (Sikkema *et al*., 1995). Omidbeygi *et al*. (2007) suggested that components of the essential oils and extracts cross the cell membrane, interacting with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death. Cristani *et al*. (2007) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. Lucini *et al*. (2006) indicated that mycelial growth inhibition is caused by the monoterpenes present in essential oils. These components would increase the concentration of lipidic peroxides such as hydroxyl, alkoxyl and alkoperoxyl radicals and so bring about cell death. For Sharma and Tripathi (2006), essential oils components and extracts would act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hypha cell wall, resulting in its collapse and death of the mycelium.

The extract of Nettle (*U. dioica*) showed remarkable antifungal effect against all the plant pathogens tested. However, one of the fungal pathogen *F. solani* was found slight resistant to the extract at the used concentrations with 80.0% of fungal mycelial growth inhibition.

Earlier papers on the analysis and antifungal properties of different natural substances, such as essential oils and extracts of some species of various genuses have shown that they have a varying degree of growth inhibitory effects against some *Fusarium*, *Botrytis*, *Rhizoctonia* and other fungi species due to their different chemical compositions. Bajpai *et al*. (2008) reported that essential oil and the leaf extracts revealed remarkable antifungal effect against *F. oxysporum*, *F. solani*, *Phytophthora capsici*, *Colletotrichum capsici*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *R. solani*, in the growth inhibition range of 39.6-67.6 and 9.3-61.3%, respectively. Crude extracts of various *Agapanthus africanus* plant parts completely or almost completely inhibited the mycelial growth of *B. cinerea*, *Sclerotinia rolfsii*, *R. solani*, *Botryosphaeria dothidea* and *Mycosphaerella pinodes* and showed a relatively high degree of control against *F. oxysporum* (77%), *Pythium ultimum* (64%) and *A. alternate* (60-80%) (Tegegne *et al*., 2008). Among the 37 potentially pathogenic fungi screened with crude methanolic extract from micropropagated shoots of Rubus ulmifolius Schott, showed that the 70% of the species resulted variably sensitive to methanolic extract (Sistia *et al*., 2008). Ethanol extracts from three endemic species in Coahuila state: Flourensia microphylla, *F. cernua* and *F. retinophylla* showed highly antifungal activity against three pathogens attacking commercial crops: *A. alternata*, *R. solani* and *F. oxysporum* (Rodriguez *et al*., 2007).

From some plant extract and oils, such as wintergreen, eucalyptus, clove, sage, Oleander and Colocynth there has been much research and reporting of toxic and irritant properties (Lawless, 1995; Newall *et al*., 1996; Rechinger, 1963). In spite of this, most of these substances are available for purchase as whole oils or as a part of pharmaceutical or cosmetic products, indicating that toxic properties do not prohibit their use. However, the ongoing investigation of toxic or irritant properties is imperative, especially when considering any new products for human use, by them medicinal or otherwise.

Certain plant extracts and phytochemicals act in many ways on various types of disease complex and may be applied to the crop in the same way as other agricultural chemicals. *U. dioica* may also be used as a leading factor in a wide range of activities against many phytopathogens, where these pathogens have developed

resistance against the specific fungicides (benzimidazoles, dicarboximides, diethofuncarband and the sterol biosynthesis inhibitors) (Elad, 1991).

In the current study, organic extracts showed varying antifungal activities against various plant pathogenic fungi. It would also be interesting to study the effects extract of Nettle on medically important fungi and bacteria for development of new antimicrobial agents for preventive treatment of serious disease infections in animals and human beings along with plant bacteria and fungal diseases. In this regard, we have started a program aimed at the evaluation of antifungal activity of essential oil and various extracts of Nettle, in hope to find out new natural products to be used in the biocontrol of certain important plant pathogenic fungi.

In conclusion, organic extract of Nettle (*U. dioica*) and Colocynth (*C. colocynthis*), Konar (*Z. spina-christi*) and Oleander (*N. Oleander*) could be applied as alternative industrial products to synthetic fungicides for using in agro-industries and also to screen and develop such novel types of selective and natural fungicides in the biocontrol of many agricultural plant pathogens causing drastic losses to crops.

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