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Antimicrobial (Screening) properties of Various Plant Extracts from Ocimum basilicum L. and Nerium oleander L. against Fungal Common Rots of Potato *In vitro Bioassay*

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ABSTRACT

Developments of more effective and less toxic antimicrobial agents were required for the biological control of yam tuber rots. A study was undertaken to investigate the qualitative phytochemicals and antimicrobial properties of different solvent crude extracts of two Iranian plants namely, Nerium oleander and O.basilicum against various fungal and bacterial phytopathogens. Leaves were extracted with different organic solvents to investigate their microbial activities *in vitro bioassay*. Results of the phytochemical screening indicated the presence of Triterpenoids, flavonoids, Saponins, tannins, alkaloids and phenolic compounds in the Nerium Basil extracts. The extracts were tested against four bacterial and fungal isolates using the disc diffusion and broth macro dilution methods. The maximal inhibition zones and MIC values for the bacterial and fungal isolates to N.oleander and O.basilicum solvent extracts were in the range of 11-24 mm and 0.026-0.33 µg/ml; and 9-22 mm and 0.12-1.25 µg/ml, respectively. MBC (MFC) values for microbial isolates to N.oleander and O.basilicum solvent extracts were in the range of 0.024-0.095 µg/ml; and 0.24-0.098 µg/ml, respectively. The ethanol extract of Nerium showed the highest activities against the tested bacteria. While, the maximal inhibition zones, MIC and MFC values for fungal isolates were more sensitive to the Basil ethanol extract comparing with Nerium extracts. The *in vitro* antimicrobial assay and preliminary phytochemical analysis may open way for complementary future investigations in identifying potentially useful properties of chemical and pharmacological importance.
KEY WORDS: Antimicrobial activity, Basil, Basilicum, Nerium, Extracts, phytochemical

INTRODUCTION

Losses in potatoes in storage due to rot are considered heavy in Iran. The evaluation of rot in different parts of Iran showed that extent of rotting ranged from 5 to 10% at harvesting while storage rot ranged from 10 to 20% .Microbial rotting of potato tubers accounts for a substantial proportion of the annual losses in potato production in Iran. Preharvest rots is due to infection by microorganisms in the soil.Okigbo and Ikediugwu associated the different forms of tuber rotting they observed in the storage barn to microbial attacks that probably took place in the field and increased in storage. Stored potatoes may suffer from fungal diseases, causing rot which quickly spreads. For example, fungi which are associated with storage losses are Ralstonia solanacearum, Fusarium sp., Pythium sp., Verticillium sp. In the world, conventional potato production is not possible without fungicides. However, these increase production costs, and those commonly used are considered as environmental and human health hazards. Fusarium species possessing the genetic base for mycotoxin production can biosynthesize zearalenons, trichothecenes, fumonisins, monilioformin, fusarin C, etc. (Thrane, 2001). The consumption of food contaminated with mycotoxins has been associated with various diseases in humans, livestock, and domestic animals. They have been recognized as causes of cytotoxicity, hepatotoxicity, teratogenicity, mutagenicity, neurotoxicity, etc. (Joffe, 1974; Marasas et al., 1984). A few antifungal agents are available and licensed for use in veterinary practice or human being treatment. Development of more effective and less toxic antifungal agents is required for the treatment of dermatophytosis. The investigated basil extract, in addition to the growth inhibitory effect, caused changes in the macro and micro morphology of fungi. Antibacterial activity of essential oil extracted from Nerium oleander flowers indicated their antibacterial potential. This study supports the traditional use of different plant extracts in bio-control of potatoes' rot caused by pathogenic fungi in Iran either by using a single or combined extracts.

MATERIALS AND METHODS

Collection of yam

Yams with symptoms of soft rot were obtained from the traditional yam barn of National Root Crop Research Institute, Lahijan, Iran. The yams with softness of tissues were identified as being rotted. Fresh yams were also collected from the barn.

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Collection of plant materials

Ocimum basilicum and *Nerium oleander* were collected in April 2011 from forest of Guilan province, Iran.A voucher number is deposited at Botanical Research Institute of Iran, Lahijan, herbarium.

Preparation of Plant Extracts

Plant materials were washed separately under running tap water, followed by sterilized distilled water. Then plant material was air dried in the shade and grind to a fine powder and stored. Dried powdered leaves were defatted with petroleum ether (60-80^{0}C) for 4 hrs. Again soaked and extracted with 500 ml (our desired solvent in 1:8 ratio) for 6-8 hours continuous hot percolation. Clear Saturation is the indicating point in siphon tube than extract was concentrated on water bath at 25^{0}C. Concentrated product was evaporated on a water bath to a syrupy consistency and then evaporated to dryness. Concentrated extracts was stored at cold temperature until further study. The dried extracts and fractions were weighed and their percentage in terms of the dry weight of the plant material was estimated by the following formula and given in below:

$$\text{Percent Extractive} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant}} \times 100$$

Preparation of Solvent Extracts:

20 g of each powdered leaf plant material was extracted separately in the ratio of 1:6 at room temperature using various solvents namely hexane, petroleum ether, ethyl acetate, chloroform, ethanol and methanol with gentle stirring for seven days(three times within this period)till colorless extract obtained on the top of the extractor. Extract from each solvent (extract) was concentrated separately under reduced pressure and preserved at 4°C in airtight bottle for further Investigations.

Isolation of spoilage fungi from rotted yam

Pieces of yam tuber 3 x 3 x 2 mm in dimension cut from advancing edge of a rot were surface sterilized in 70% alcohol for 1 min, dried on sterile tissue paper and plated out on PDA amended with moisture of penicillin and streptomycin. A minimum of five replicates pieces from each of the rots were plated out. The plates were incubated at room temperature for up to 5 days and fungal growth associated with rot affected tissue identified and their frequency of occurrence determined (Okigbo and Ikediugwu, 2000).

Isolation of pathogens: Rotted yam tubers were rinsed in distilled water, surface sterilized with 70% ethanol and cut open (Fig. 1) with a sterilized knife. About 10 pieces (5mm in diameter) of the infected yam tissues were picked from the point of advancement of rot with a flamed sterilized forceps and inoculated on a solidified Potato Dextrose Agar (PDA) medium. Two replicates were made for each of the 10 yam tuber samples and the 10 plates inoculated on. The inoculated plates were incubated at room temperature (28°C) and observations made daily for possible fungal growth. Subculturing was done to obtain pure cultures of the isolates. Stock cultures were prepared using slants of PotatoDextrose Agar (PDA) in Biju bottles and stored in a refrigerator at 4°C. Cultural characteristics of the fungi were observed and recorded. Occurrence of the organisms was recorded as follows:

$$\frac{\textbf{Total number of figures}}{\textbf{Total number of fungal occurrence} \times 100}$$

Identification of organisms: The identification of the isolates was done by examining the isolates macroscopically and microscopically. The colony characteristics, spores, mycelium either septate or not, conidium were taken note of. These structural features were matched with standards in Barnet and Barry (1972) and Booth (1971). To confirm pathogenicity of the isolate, the pure culture was reinoculated onto related plants. When actively growing on PDA plates, mycelium-agar plugs were excised from the margin of the fungal colony and used to inoculate leaves of these plants. Inoculated leaves were then incubated in a growth chamber at 23~25 °C with 100% relative humidity until observation.

Anti-fungal activity of the extracts: The effect of the extract was determined by measuring the mycelial dry weight.Fifty ml of potato dextrose broth (PDB) was poured into each flask containing different concentrations (10, 25, 50 and 100) of the respective extracts (2ml each). With a sterile cork borer (3mm) mycelia disc of 7 day old cultures of the isolates were inoculated in the flask and incubated at 28± 2°C. After 7 days the different fungi from the different broths, were taken on dried and weighted filtered papers in a desiccator. After this, fungal mycelia were dried at 70°C for 24 hours and the weight was recorded.Inhibition of fungi by various concentrations was calculated as:

$$\frac{100 - \text{Weight of fungus in extract}}{\text{Weight of fungus in PDB}} \times 100$$

DATA ANALYSIS

Two way-Analysis of Variance (ANOVA) was applied in comparing the different isolates and concentrations.

Preparation of leaf extracts

Fresh leaves of *O.Basilicum* and *N.oleander* were washed thoroughly under running that water and sterile distilled water and air dried at 28ºC for 2 h. This was further homogenized into a paste for each leaf with a blender (mixer model 830 L, Taiwan).An aqueous extract of the ground leaves was prepared by adding 100 g of the dried leaf to 100 ml of cold sterile water in a 500 ml beaker, stirring vigorously, allowing 1 h for settling, and then filtering the extract through folds of sterile cheese cloth. Hot water extracts were obtained by infusing the ground or paste of leaf materials from each plant separately with 100 ml sterile distilled water using 500 ml conical flasks in water bath at 90^{o}C for 45 minutes. Thereafter, the suspension was filtered 5 times through sterile muslin cloth. An ethanol extract was prepared by mixing 15 g of ground leaf with 100 ml of 70% ethanol in a 1L beaker. The extract was filtered through 5 folds of sterile cheese cloth. Different concentrations of 10, 25, 50 and 100% were prepared. 5 ml from each of the concentrations was dispensed into 12 cm diameter Petri dishes after which 25 ml of melted PDA were poured into the plate, shaken together and allowed to solidity.

Determination of MIC and MBC for various extracts using organic solvents

MIC assay of the extracts were also determined using the same method by macro dilution method accepted that the paper discs were soaked in different concentrations of crude extract dispersed in water (10 - 2000 µL). After incubating at 24 h at 37°C, the MIC of each sample was determined by measuring the optical density in the spectrophotometer (620 nm), and comparing the result with those of the non inoculated MHB (oxoid 2009). For determination of MBC , 1 ml (was) pipette from the mixture obtained in the determination of MIC tubes which did not showed any growth and streaked on MHA and incubated for 24 h .The least concentration of the extract with no visible growth after incubation was taken as the minimum bactericidal concentration.

RESULTS AND DISCUSSION

The percentage extract yields obtained from of Plant (*O.basilicum* and *N.oleander*) Leave Extracts varied from 1.7 to 5.3 %, with the greatest concentration found in Petroleum ether fraction among solvent extracts of two plants Results of the phytochemical screening revealed the presence of Triterpenoids, flavonoids, Saponins, tannins, alkaloids and Reducing Sugars in the Nerium ad Basil extracts (Table 2). The result showed phenolic compounds considerably in Nerium extract particularly. Phenolic compounds have received increasing attention because of their biological activities. They constitute a major group of compounds that acts as antioxidants. Most of the *Basil* phenolics were derivatives of caffeic acid. The maximal inhibition zones and MIC values for the bacterial and fungal isolates to *N.oleander* and *O.basilicum* solvent extracts were in the range of 11-24 mm and 0.026-0.33 µg/ml; and 9-22 mm and 0.12-1.25 µg/ml, respectively (Tables 4-7).MBC (MFC) values for microbial isolates to *N.oleander* and *O.basilicum* solvent extracts were in the range of 0.024-0.095 µg/ml; and 0.24-0.098 µg/ml, respectively (Tables 6-7). Based on these results, the ethanol extract of *Nerium* has a stronger and broader spectrum of antimicrobial activities compared with the *Basil* extracts. These results did not confirm the previous studies which reported that hexane was a better solvent for the more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents such as water, methanol, and ethanol .This conflict can be explained that the better extraction of antimicrobial compounds from various medicinal plants may require different solvents.

The organisms associated with the rot of white yam in the present study were *R.solani*, *Phytophthora* sp., *Pythium sp*., *Fusarium sp.* and *Sclerotinia* .These organisms have been associated with post-harvest rot (Ogundana et al., 1970; Okigbo, 2002, 2005). Rotting in storage probably starts in the soil and progress in storage. This may happen when infected tubers do not show perceptible external symptoms (Ogundana et al., 1970). Each type of rot is characteristic of causal organism (Ekundayo and Naqvi, 1972).

In conclusion, our study demonstrated that many plant extracts, *O. basilicum* and *N. oleander*, can be used for the biocontrol of the early blight disease. Thus, this method of control an contribute to minimizing the risks and hazards of toxic fungicides, especially on vegetables produced for fresh consumption. Further research into these extracts will identify the active compounds esponsible for their fungicidal activity. 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 .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. It was 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. Also,it was 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. 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. Earlier papers on the analysis and antifungal properties of different natural substances, such as essential oils and extracts of some species of various genera 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. 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. In the current study, organic extracts showed varying antifungal activities against various plant pathogenic fungi. It would also be interesting to study the effects of Basil and Oleander extract 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 bacterial and fungal diseases. In this regard, we have started a program aimed at the evaluation of antifungal activity of various extracts of Basil and Oleander, in hope to find out new natural products to be used in the biocontrol of certain important plant pathogenic fungi.In conclusion, organic extracts 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.

In our present investigation, the leaves extracts of *O.basilicum* were possess the highest inhibition zone against fungal phytopathogens.*N.oleander* indicated more activity against bacterial phytopathogens .It has been proved that ethanol extract of *N. oleander* and *O.basilicum* exhibited the highest activity microbial phytopathogens. This may be attributed to two reasons; firstly, the nature and potentiality of biological active components (alkaloids, flavonoids, phenols, Terpenoids etc.), which could be enhanced in the presence of ethanol. Secondly, the stronger extraction capacity of ethanol could have produced greater number/amount of active constituents responsible for antibacterial activity. *Basil* ethanol extract showed excellent antifungal properties compared to other plant extract which may be due to free and bound flavonoid fractions and phenolic compounds, showed the greatest fungicidal properties. Nwachukwu and Umechuruba (2006) found that Leaf extracts of Neem, Basil, Bitter leaf and paw-paw, which are cheap and environmentally safe, are promising for protecting African yam bean seeds against major seed-borne fungi. Many of the herbs properties can be traced back to the flavonoids that plant contains.Based on disk diffusion method, the gram-negative bacteria isolates*: P.syringae* pvs. And X.campestris pvs. tested were found to be more sensitive to *Nerium* ethanol extract compared to *Basil* ethanol extract (24 and 17 mm vs. 16-17 mm) .Our investigation revealed an effective antibacterial activity for *Nerium* with MIC and MBC ranging from 0.026 to 0.33 µg/ml and 0.224 to 0.73 µg/ml compared to ciprofloxacin using by macro dilution broth method. This may be attributed to two reasons; firstly, the nature and potentiality of biological active components (alkaloids, flavonoids, biterpenoids etc.), which could be enhanced in the presence of ethanol. Secondly, the stronger extraction capacity of ethanol could have produced greater number/amount of active constituents responsible for antibacterial activity. This gives an indication of the presence of promising antibacterial compounds. Most of the identified components with antimicrobial activity extracted from plants are aromatic or saturated organic compounds and they are more soluble in methanol and ethanol. The maximal inhibition zones, MIC and MFC values for fungal isolates which were more sensitive to the *Basil* ethanol extract comparing with *Nerium* extracts (*Verticillium sp.* and *Pythium sp*.) were in the range of 22-21 mm, 0.12-1.25 µg/ml and 0.486 to 2.5 mg/ml, respectively. There is a need to explore its maximum potential in the field of agricultural and pharmaceutical sciences for novel application. This study has shown that that the use of plant extracts *N.oleander* and *O.basilicum* to control rot of yam in storage has potential as a substitute for chemical pesticide. This approach to plant disease management is economically viable and poses little risk environmental.

Table1. Percent Extractive of Different Partially Purified Fractions of Plant Leave Extracts (*O.basilicum* and *N.oleander*)

Percent extractive value of Basil leaves	Percent extractive value of Nerium leaves	Solvent	S.No.
5.3%	3.6%	Petroleum ether fraction	**1**
2.4%	1.7%	Ethyl acetate fraction	**2**
3.1%	3.1%	Chloroform fraction	**3**
4.7%	3.5%	Methanol fraction	**5**
4.2%	4.2%	Ethanol fraction	**6**
3.8%	4.7%	Hexane fraction	**7**

Table2. Preliminary photochemical screening of leaf extracts of *Nerium*

Various Extracts						
Hexane	Ethanol	Methanol	Chloroform	Ethyl acetate	Petroleum ether	**Chemical Compounds**
+	+	+	+	+	+	**Steroids**
+	++	_	+	_	_	**Triterpenoids**
_	++	+	+	_	++	**Reducing Sugars**
_	+	+	_	+	+	**Alkaloids**
_	+	+	_	_	+	**Phenolic compounds**
_	_	_	_	_	_	**Catachins**
_	_	_	_	_	_	**Amino acids**
_	_	_	_	_	_	**Anthraquinones**
_	_	_	_	_	+	**Tannins**
+	_	_	+	+	+	**Saponins**
+	+	_	_	_	+	**Flavonoids**

Table3. Preliminary phytochemical screening of leaf extracts of *Basil*

Various Extracts						
Hexane	Ethanol	Methanol	Chloroform	Ethyl acetate	Petroleum ether	**Chemical Compounds**
+	+	_	+	+	_	**Steroids**
_	+	_	+	_	+	**Terpenoids**
_	_	_	+	_	+	**Reducing Sugars**
+	+	+	_	+	++	**Alkaloids**
_	++	_	_	_	_	**Phenolic compounds**
_	_	+	_	_	+	**Cardiac Glycosides**
_	_	+	_	_	_	**Amino acids**
_	_	_	_	_	+	**Anthraquinones**
_	+	_	_	_	_	**Tannins**
+	_	_	+	+	+	**Saponins**
+	+	+	_	_	+	**Flavonoids**

Table 4. Antimicrobial activity of *N.oleander* extracts against yeast and fungi isolates tested based on disk diffusion method (mm)

Different solvent extracts								
Hexane	**GSF[1]**	**CIP[2]**	Ethanol	Methanol	Chloroform	Ethyl acetate	Petroleum ether	**Microorganisms**
12	22	–	15	13	12	11	14	***R.solani***
15	25	–	13	16	14	16	13	***Fusarium sp.***
12	32	–	15	16	13	11	13	***Pythium sp.***
12	34	–	16	15	14	12	11	***Verticillium sp.***
20	–	33	24	21	17	14	17	***P.syringae pvs.***
16	–	26	17	14	15	13	15	***X.campestris pvc.***
14	–	27	21	19	16	15	16	***R.solanacearum***
14	–	31	19	17	15	16	15	***E.carotova***

1-Griseofulvin 2- Ciprofloxacin

Table 5. Antimicrobial activity of *O.basilicum* extracts against fungi isolates tested based on disk diffusion method (mm)

Different solvent extracts								
Hexane	**GSF[1]**	**CIP[2]**	Ethanol	Methanol	Chloroform	Ethyl acetate	Petroleum ether	**Microorganisms**
14	22	–	20	16	15	15	11	***R.solani***
12	25	–	19	17	12	11	13	***Fusarium sp.***
14	32	–	21	20	13	12	14	***Pythium sp.***
16	34	–	22	18	16	14	12	***Verticillium sp.***
11	–	33	16	14	13	11	10	***P.syringae pvs.***
13	–	26	17	14	11	10	13	***X.campestris pvs.***
10	–	27	14	12	9	11	12	***R.solanacearum***
15	–	31	17	15	13	13	14	***E.carotova***

1-Griseofulvin 2- Ciprofloxacin

Table 6. MICs and MBCs of N.oleander ethanol extract against selected bacterial phytopathogens (µg/ml)

Ciprofloxacin		***N.oleander***		**Antimicrobial activity**
MBC	MIC	MBC	MIC	
0.085	0.065	0. 73	0.33	***P.syringae pvs.***
0,024	0.024	0.224	0. 17	***X.campesteris PVS.***
0,095	0.095	0. 53	0. 26	***R.solanacearum***
0.024	0.024	0.31	0.026	***E.carotova***

Table 7. MICs and MFCs of *O.basilicum* ethanol extract against selected fungal phytopathogens (µg/ml)

Griseofulvin		***O.basilicum***		**Antimicrobial activity**
MBC	MIC	MBC	MIC	
0.024	0.024	1.25	0.12	***R.solani***
0.098	0.098	0.486	0.12	***Fusarium sp.***
0.091	0.091	2.5	1.25	***Pythium sp.***
0.095	0.095	2.5	0.12	***Verticillium sp.***

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