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Antifungal Activity of Some Plant Extracts on *Alternaria alternata*, the Causal Agent of Alternaria Leaf Spot of Potato

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Abstract: Pure methanol (m) and methanol water (mw) extracts of 5 plants namely: peppermint, eucalyptus, lavandula, Russian knapweed and datura were screened for their antifungal ability against *Alternaria alternata,* the causal agent of Alternaria leaf spot of potato at 5, 10 and 15% concentrations *in vitro*. Fungicide mancozeb 0.2% was also used for better comparison. Poisoned food technique and spore germination assay method were used to evaluate the antifungal efficacy of plant extracts. Present findings showed that methanol extracts of eucalyptus, peppermint and lavandula had impressive antifungal effects in inhibiting the mycelial growth as well as spore germination of the pathogen. It was also found that methanol extracts were quite more effective than methanol water extracts in this regard. Methanol extracts of peppermint (15%), lavandula (15%), peppermint (10%) and eucalyptus (15%) demonstrated promising ability in inhibiting the mycelial growth of *A. alternata* by 0.13, 0.40, 0.43 and 0.50 cm, mycelial growth respectively, while majority of methanol water extracts had either less or no effects in this connection. Spore germination of *A. alternata* was prominently reduced by methanol extracts, while those of methanol water extracts had very less effects in this regard. Mancozeb (0.2%), methanol extracts of eucalyptus (15%) and peppermint (10%) by 2, 6 and 7% spore germination were best, while methanol water extracts of datura 10, 15 and 5%, lavandula 15 and 10% and also Russian knapweed 5% represented no effect and by 91, 89, 87, 87, 85 and 85% spore germination were at par with control. Findings from this study confirmed that plant extracts can be used as less hazardous natural fungicides in controlling plant pathogenic fungi, thus reducing the dependence on the synthetic fungicides. Methanol extracts of peppermint, eucalyptus and lavandula might be promising materials for natural formulations in controlling Alternaria leaf spot of potato in the field.

Key words: Plant extracts, *Alternaria alternata*, fungicidal effect, potato, mycelial growth, spore germination

INTRODUCTION

Alternaria species are important fungal agents mostly causing aerial diseases of many plants word wide. *Alternaria alternata* usually reported as a weak pathogen can sometimes attack vigorous plants and cause economic losses (Ellis and Ellis, 1985). Droby and Prosky (1984) reported that about 28% leaf infection of potato plants causes about 18% yield reduction due to this disease. This pathogen with more than 380 hosts has been recorded in USDA systematic botany and mycology fungus-host distribution database (Simmones, 1995). Early blight is an important disease of potato in Iran (Ershad, 1995) and Ommati and Karimi (2002) identified this disease from different provinces of this country indicating *A. alternata* as the dominant causal agent in majority of potato growing areas.

Although, with the application of several fungicides plant pathogens can be controlled but the hazardous effects of such products in human health and environmental aspects are known. A part from these problems, their excess applications may lead toward pest resistance. Natural plant products have the potential as safe alternatives for chemical fungicides in plant disease managements. During last decades several researches have been conducted on plant extracts and oils to find out such alternatives and valuable results have been achieved (Ayoub and Niazi, 2001; Bowers and Locke, 2000; Suprapta and Kalimi, 2009; Singh, 2004). Doltsinis *et al*. (2006) evaluated the efficacy of Milsana® VP 1999 and 2000 (a formulated plant extract of *Reynoutria sachalinensis*), known to induce resistance to powdery mildew on cucumbers, against *Leveillula taurica* on greenhouse tomato. In four out of five trials, Milsana® achieved a disease reduction ranging from 42.2 to 64.6%. Milsana® was equally effective to wettable sulphur indicating that its effect was rather preventive than curative. Laboratory tests showed that Milsana® (VP 1999)

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had a direct effect on conidial germination. Overall, results indicated that Milsana® could play an important role in disease management of powdery mildew in organic and low input tomato production. Rai *et al*. (2000) found that pure extract of *Adenocallima alliaceum* can completely inhibit the spore germination of *Alternaria alternata* and *Fusarium oxysporum*. Harish *et al*. (2004) working on rice brown spot (*Helminthosporium oryzae*) control with 15 seed extracts under laboratory condition found that 10% rhizome extract of turmeric (*Curcuma longa*), seed extracts of sundavathal (*Solanum indicum*) and vedpalai (*Wrightia tinctoria*) exerted maximum inhibition of the mycelial growth and spore germination of the pathogen. Khallil (2001) tested 20 plant extracts for their efficacy against two plant pathogens: *Alternaria solani* and *Saprolegnia parasitica* under laboratory condition and found extract of *Eugenia aromatica* to completely inhibit the spore germination of *A. solani* and exerted a highly significant depressive effect on the mycelial growth of the two tested species. He also reported that extracts of garlic and onion bulbs, eucalyptus leaves and pepper fruits could exhibit remarkable inhibitory effects against these two fungi. Feng and Zheng (2007) studied the antifungal activity of essential oils of five plants (thyme, sage, nutmeg, eucaptus and cassia) against *Alternaria alternata* at different concentrations (100-500 ppm) *in vitro* and *in vivo* and found that the cassia oil can completely inhibit the growth of *A. alternata* at 300-500 ppm. In their investigation the thyme oil exhibited a lower degree of inhibition (62% at 500 ppm). The cassia oil also reduced the percentage of decayed tomatoes at 500 ppm. Faria *et al*. (2006) from brazil reported that essential oil of *Ocimum gratissimum* aerial parts obtained by steam-distillation (1.1% w/w) inhibited the growth of several fungi including *Botryosphaeria rhodina*, *Rhizoctonia* sp. and two strains of *Alternaria* sp. In a study Singh (2004) evaluated five plant extracts including garlic and eucalyptus extracts at 10, 20 and 30% concentrations on leaves of Indian mustard artificially inoculated with early blight pathogen (*Alternaria brassicae*) and found the garlic extract as best biocide in controlling the disease. Abo El-Seoud *et al*. (2005) tested essential oils of fennel, peppermint, caraway, eucalyptus, geranium and lemongrass for their antimicrobial activities against some plant pathogens (*Fusarium oxysporum*, *Alternaria alternata*, *Penicillium italicum* and *Botrytis cinerea*) and on the basis of their effectiveness, selected essential oils of fennel, peppermint and caraway as active ingredients for formulating biocides. Hassanein *et al*. (2008) tested leaf extracts of neem (*Azadiracta indica*) and chinachery (*Melia azedarach*) extracted by ethanol, ethyl acetate and water against two tomato fungal pathogens at 5, 10, 15 and 20% concentrations and found that both ethanol and ethyl acetate extracts of neem leaves assayed at a concentration of 20%, completely suppressed the growth of *F. oxysporum* and inhibited *A. solani* by ratios between 52.44 and 62.77%. The corresponding values with chinaberry leaf extract were quite lower by 5.77% for *A. solani* and 6.55% for *F. oxysporum*. Shirzadian *et al*. (2009) evaluated extracts of 23 plants including 21 moss species and 2 leafy liverwort species obtained by ethanol, water and petroleum ether solvents against 7 pathogenic fungal pathogens including *Alternaria alternata* and found the broadest spectrum of antifungal activity by the ethanolic extracts of 6 moss species namely: *Philonotis marchica, Grimmia pulvinata, Plagiomnium rugicum, Haplocladium* sp. *Bryum pallens* and *Drepanocladus aduncus* followed by two liverworts: *Pellia epiphylla* and *Dumortiera hirsuta*. Hadizadeh *et al*. (2009) working on antifungal effect of essential oils from some medicinal plants of Iran: neetle (*Urtica dioica*), thyme (*Thymus vulgaris)*, eucalyptus (*Eucalyptus* sp.), rute (*Ruta graveolens*) and common yarrow (*Achillea millefolium*) on *A. alternata* of potato as a model pathosystem, found that both the neetle and the thyme oils exhibited proper antifungal activity against this pathogen. Spore germination and germ tube elongation of the pathogen in potato dextrose broth was strongly reduced in the presence of 1500 ppm of the nettle oil. Fawzi *et al*. (2009) used extracts of 5 plants (cinnamon, halfa barr, laurel, avocado and ginger) performed with either Cold Distilled Water (CDW) or Boiling Distilled Water (BDW) against *Alternaria alternata* and *Fusarium oxysporum*. And found that CDW extracts of halfa barr and ginger were the most effective to inhibit the growth of the tested fungi followed by avocado, cinnamon and laurel. Suprapta and Kalimi (2009) used extract formulations of four plant species (*Eugenia aromatica, Piper betle, Alpinia galangal* and *Sphaeranthus indicus*) for their antifungal activity. Preparing six formulations [f1, f2, f3, f4, f5 (mixture of 1st and 2nd) and f6 (mixture of 3rd and 4th)], they found that 5% solution of f5 formulation had the highest inhibitory effect against the radial growth of *F. oxysporum* f. sp. *Vanillae* on PDA. They also reported that the development of stem rot disease was obviously suppressed on vanilla seedlings grown in the soil treated with 5% solution of each plant extract. The lowest disease incidence was attained by f5, in which only 7% of the vanilla seedlings were infected.

The present investigation was conducted to select the most effective antifungal plant extract(s) among 5 tested plants for further research in formulating safe fungicides against plant diseases.

MATERIALS AND METHODS

Preparation of pathogenic isolate of the pathogen: A pathogenic isolate of *A. alternata* was obtained from the fungal collection of Shahrood Agricultural Research Center, Shahrood, Iran in 2008 and was recultured on PDA.

Preparation of plant extracts: Matured leaves of eucalyptus (*Eucalyptus camaldulensis*, family: Myrtaceae) and datura (*Datura stramanium*, family: Solanaceae), leaves and young flowering shoots of peppermint (*Mentha piperita*, family: Lamiaceae) and Russian knapweed (*Acroptilon repens*, family: Asteraceae) and young shoots of lavandula (*Lavandula officinalis*, family: Lamiaceae) were thoroughly washed in running water and kept in shade to dry. Dry materials were then ground finely by a blender. Pure methanol (96%) was used as solvent but in two separate experiments. In the first experiment, Pure methanol was used for extraction and in the second one methanol water was used as 1:1 proportions. Five hundred milliliter of solvents (pure methanol and methanol water) were added to 50 g of dry materials of each plant and homogenized for 20 min with the help of a homogenizer. Mixtures were then centrifuged at 447x g for 10 min to obtain clear extracts. The methanol was completely removed from the clear solutions using a rotary evaporator. Final extracts were passed through 0.2 μ seitz filters to remove any unwanted bacteria and were used as 100% pure extracts.

Antifungal evaluation of plant extracts on mycelial growth of *A. alternata in vitro*: Poisoned food technique (Schmitz, 1930) was used for evaluating the effect of different extracts on mycelial growth of *A. alternata*. Fifty milliliter of PDA were kept in 100 mL. Erlenmeyer flasks, sterilized for 20 min and kept under sterilized hood to cool up to 60°C. Exact amounts of pure extracts were then added to each flask and shaked gently to prepare PDA containing 5, 10 and 15% of extracts. Nine centimeter Petri dishes were poured with PDA containing known percentage of extracts. Five millilmeter discs of young culture of *A. alternata* were kept in the center of each Petri dish. Mancozeb 0.2% was used in the same manner for better comparison. Petri dishes were incubated at 25-27°C for 7 days and then the smallest and largest diameters of mycelial growth of each petri dish were measured and recorded. Three petri dishes were kept for each treatment and in the case of control PDA free of any extract was used.

Antifungal evaluation of plant extracts on spore germination of *A. alternata in vitro*: The spore germination assay (Suprapta and kalimi, 2009) was used for evaluating the effect of different extracts on spore germination of *A. alternata*. Exact extract concentrations (5, 10 and 15%) were prepared in sterilized water. One drop of extract solutions were poured in pits of pitted glasses and kept as such to dry. Then one drop of spore suspension of *A. alternata* (5×10^5 spores mL^{-1}) were poured in these pits and pitted glasses were kept inside desiccators at room temp. and 80% relative humidity for 48 h. Germinated spores were then counted under microscope with the help of a hemocytometer and recorded. Mancozeb 0.2% was used in the same manner for better comparison. Three pits were kept for each treatment and in the case of control only sterilized water was used.

Statistical analysis: The split plot design was used for analyzing the collected data. Main factors were two types of solvents (pure methanol and methanol + water) and sub factors were as:

1: Peppermint 5%, 2: Peppermint 10%, 3: Peppermint 15%, 4: Eucalyptus 5%, 5: Eucalyptus 10%, 6: Eucalyptus 15%, 7: Lavandula 5%, 8: Lavandula 10%, 9: Lavandula 15%, 10: Russian knapweed 5%, 11: Russian knapweed 10%, 12: Russian knapweed 15%, 13: Datura 5%, 14: Datura 10%, 15: Datura 15%, 16: Mancozeb 80WP (0.2%), 17: Control.

Results were analyzed with the help of MSTATC software and means compared by Duncan Multi Range Test.

RESULTS

Effect of solvents on antifungal properties of plant extracts: Significant differences were observed between effect of pure methanol extracts (m) and methanol water extracts (mw) regarding inhibition of mycelial growth as well as spore germination of the pathogen at 1% level of significance (Fig. 1a and b, Table 2). In this study methanol extracts showed quite more effectiveness than those of methanol water extracts against *A. alternata* The mean mycelial growth of *A. alternata* in case of methanol extracts was 2.86 cm. while the corresponding value was found to be 6.19 cm in the case of methanol water extracts. Also the mean spore germination of *A. alternata* in case of methanol extracts was 25%. in comparison with those of methanol water extracts which was 77%.

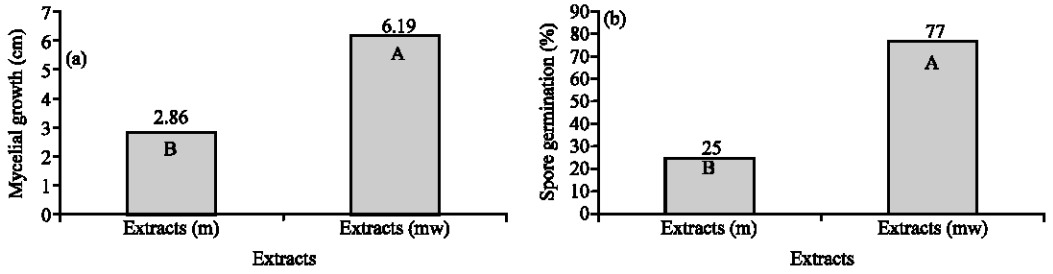

Fig. 1: Effect of solvents on plant extract constituents in relation to mycelial growth (a) and percentage of spore germination (b) of *A. alternata*

Table 1: Effects of treatments on mycelial growth and spore germination of *A. alternata*

	Mean mycelial growth (cm)			Mean spore germination (%)		
Extract applied	5%	10%	15%	5%	10%	15%
Control			7.26AB			93A
Datura (mw)	6.40BC	5.70CD	5.26CDE	87ABC	91A	89AB
Russian knapweed (mw)	8.16A	8.10A	7.93A	85ABCD	81CDEF	81CDEF
Lavandula (mw)	7.40AB	6.36C	5.53CD	79DEF	85ABCD	87ABC
Peppermint (mw)	7.98A	7.90A	7.83A	67GH	73FG	65H
Eucalyptus (mw)	3.23GHI	3.33GH	3.46GH	78DEF	81CDEF	74EFG
Datura (m)	4.94DEF	3.93FG	2.30HIJK	33J	28JK	48I
Russian knapweed (m)	6.96AB	3.33GH	2.10IJK	23K	27JK	29JK
Lavandula (m)	4.16EFG	3.60G	0.40L	27JK	24K	24K
Peppermint (m)	1.73K	0.43L	0.13L	10L	7LM	13L
Eucalyptus (m)	3.08GHIJ	1.96JK	0.50L	10L	13L	6LM
Mancozeb 0.2%			1.83K			2M

Values followed by same letters are not significantly different at 1% level of significance related to mycelial growth and spore germination of the pathogen separately

Table 2: ANOVA for effects of treatments on mycelial growth and spore germination of *A. alternata*

Source of variation	df	Mycelial growth (cm)	Spore germination (%)
Replication	2	0.97	0.33
Solvent	1	1370.9**	21072.68**
Error	2		
Treatments	16	62.25**	180.83**
Solvent×treatment	16	41.28**	59.37**
Error	64		
CV		9.91	6.54

*, **Significantly different at 5% and 1% level of significance, respectively

Antifungal effect of plant extracts on mycelial growth of the pathogen: Statistical analysis revealed that methanol extracts of peppermint (15%), lavandula (15%), peppermint (10%) and eucalyptus (15%) (Table 1, 2 and Fig. 2) had significant differences with other treatments in inhibiting the mycelial growth of the pathogen (p = 1%). These treatments by 0.13, 0.40, 0.43 and 0.50 cm mycelial growth had best efficacy in this way respectively. Extracts of peppermint 5% (m), mancozeb 0.2%, eucalyptus 10% (m). Russian knapweed 15% (m), datura 15% (m) and eucalyptus 5% (m) with 1.73, 1.83, 1.96, 2.10 and 2.30 cm mycelial growth had also good ability in this regard and were kept in the second group, While methanol water extracts of Russian knapweed (5 and 10%), peppermint (5%), Russian knapweed (15%), peppermint (10 and 15%) and lavandula (5%) by 8.10, 8.06, 7.96, 7.93, 7.90, 7.83 and 7.40 cm growth had no effect regarding inhibitory effect on colony growth of *A. alternata*, respectively (Fig. 2).

Antifungal effect of plant extracts on spore germination of the pathogen: As indicated in Table 1 and 2, significant differences could be observed between treatments in inhibiting the spore germination of *A. alternata* at 1% level of significance. Although, in this experiment Mancozeb 0.2% by 2% spore germination showed best inhibitory effect but had no significant difference with methanol extracts of eucalyptus (15%) and peppermint (10%). These two extracts by 6 and 7% spore germination were at par with the mancozeb. In the case of methanol extracts spore germinations of *A. alternata* varied between 6 to 48% , while in the case of methanol water extracts spore germinations were found to be 65 to 91%. In this study, methanol water extracts of datura (10 and 15%), lavandula (15%), datura (5%), lavandula (15%) and Russian knapweed (5%) by 91, 89, 87, 85 and 85% spore germination had least effect in inhibiting the spore germination of *A. alternata*, respectively.

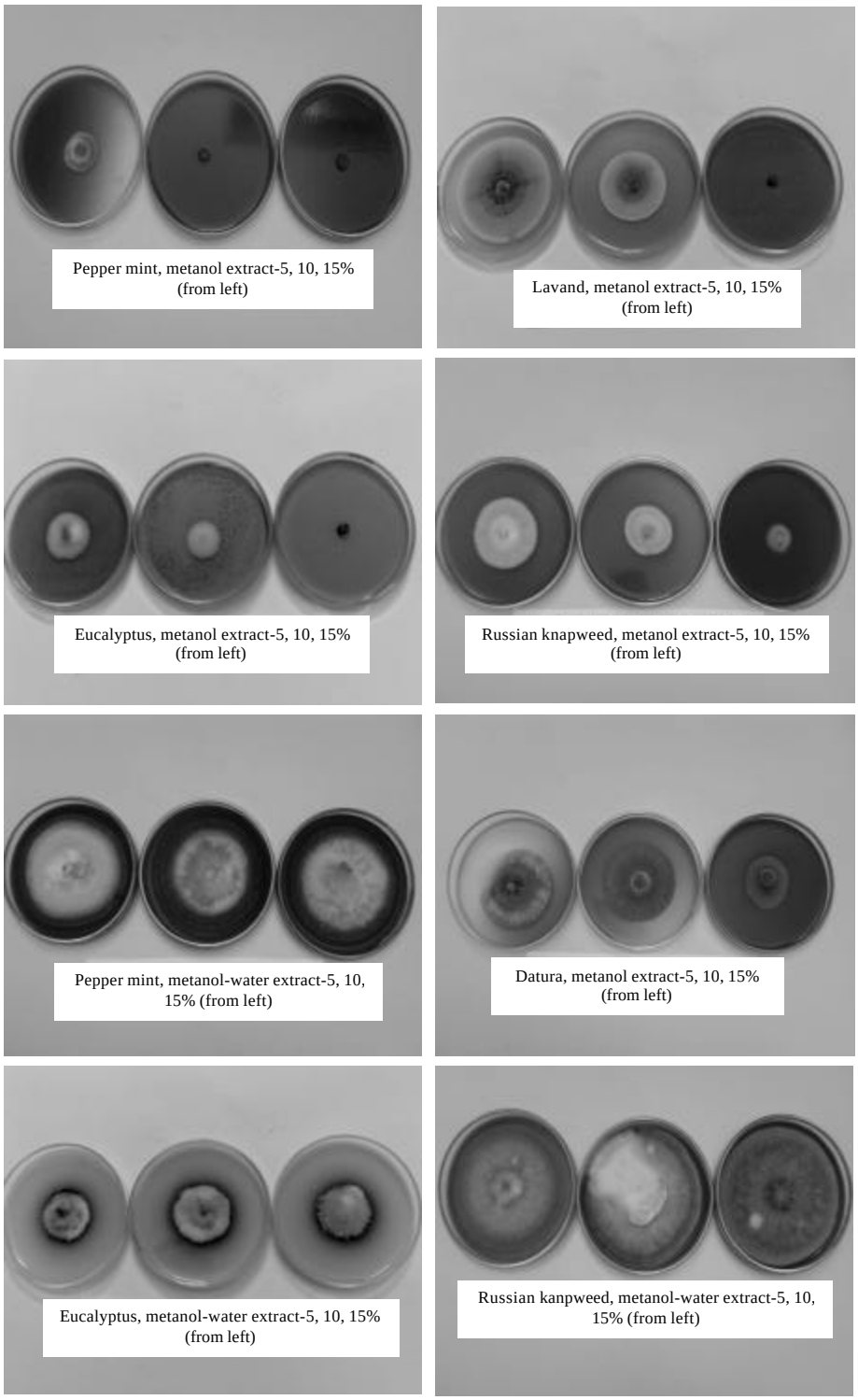

Fig. 2: Effect of methanol extracts of peppermint (first row, left), eucalyptus (second row, left), lavandula (first row, right), Russian knapweed (second row, right), datura (third row, right) and methanol water extracts of peppermint (third row, left), eucalyptus (forth row, left), Russian knapweed (forth row, right)

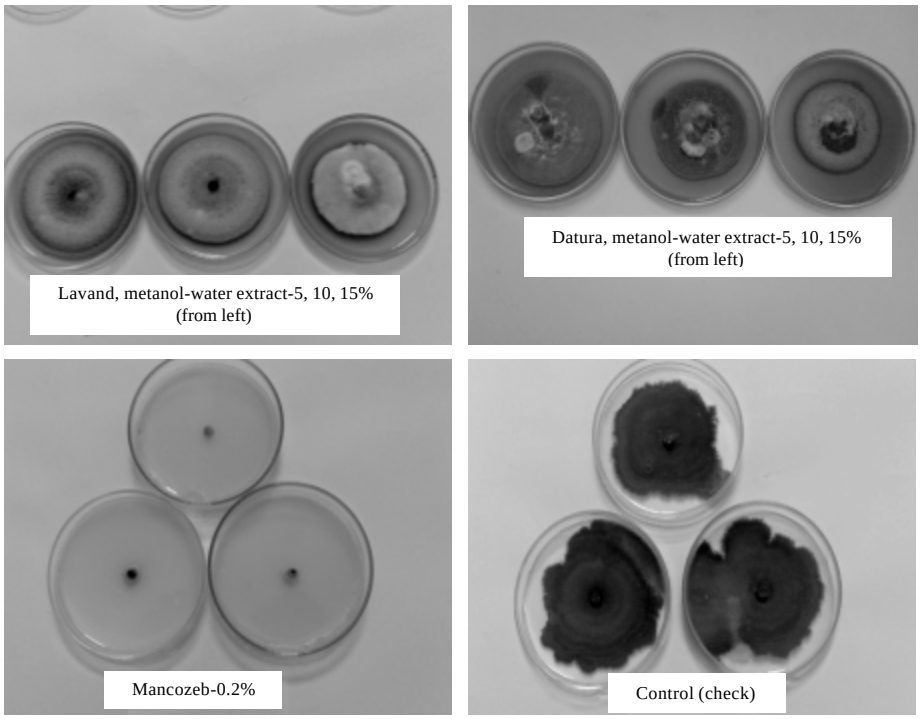

Fig. 2: Continued: lavandula (top left), datura (top right) in contrast with mancozeb 0.2% and control on mycelial growth of *A. alternata* after 7 days

DISCUSSION

Present findings showed that methanol extracts are quite more effective than methanol water extracts against *A. alternata in vitro*, There are some articles indicating more effectiveness of plant extracts performed with pure solvents such as ethanol in comparison with aqueous extracts, for instance Shirzadian *et al*. (2009) compared antifungal properties of ethanol, petroleum ether and water extracts of some plants against some pathogenic fungal pathogens including *Alternaria alternata*, found highest antifungal activity among ethanolic extracts. In this connection Hassanein *et al*. (2008) who screened ethanol, ethyl acetate and aqueous extracts of neem and chinachery against two tomato fungal pathogens found ethanol and ethyl acetate extracts of these plants to suppress growth of *F. oxysporum* and inhibited *A. solani* in comparison to aqueous extracts which were less effective. These findings are in agreement with our results stating that pure solvents might be more effective in extracting antifungal active ingredients. Among extracts of five tested plants of our study peppermint and eucalyptus extracts showed promising effects against *A. alternata*, these results have been confirmed by several researches, for examples Abo-El-Seoud *et al*. (2005) selected peppermint and eucalyptus essential oils as active ingredients for biocide formulations because of confirmation of their antimicrobial activity against some plant pathogens including *A. alternata*. In another study although, Singh (2004) who evaluated some plant extracts against early blight of Indian mustard at different concentrations found garlic extract as most effective in controlling the disease although he mentioned eucalyptus extract as less effective biocide in controlling the disease. In other side Hadizadeh *et al*. (2009) found eucalyptus essential oils not so effective in controlling *A. alternata* of potato which is against our results. Review of related articles showed that more articles are in support of eucalyptus having antifungal activity. In our study datura extracts did not demonstrate any antifungal property while Ayoub and Niazi (2001) found it effective in controlling wheat rust (*Puccinia recondita*) under controlled condition. Efficient anti-mycelial extracts of *A. alternata* had also prominent effects against spore germination of the pathogen. Present findings were in agreement with the results of Khallil (2001) who found eucalyptus extract as highly effective in preventing the spore germination of *Alternaria solani*. But Hadizadeh *et al*. (2009) in an experiment compared spore germination effect of neetle, thyme, eucalyptus, rute and common yarrow essential oils against *A. alternata* and found nettle and thyme oils to be more effective than

eucalyptus oil. On the basis of results of present study out of five plant extracts, methanol extracts of peppermint, eucalyptus and lavandula had high antifungal abilities against Alternaria leaf spot of potato *in vitro* which. may be incorporated for bioicide formulations in integrated management of this disease.

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