



APPLICATION OF VOLATILE FRACTIONS FROM *AGERATUM HOUSTONIANUM* AND *TAGETES ERECTA* AS SAFE MANAGEMENT OF SOME ROOT PHYTOPATOGENIC FUNGI

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ABSTRACT

Ageratum houstonianum Mill and *Tagetes erecta* L. (Asteraceae) were subjected to hydrodistillation as well as the stepwise extraction with organic solvents. Crude extracts and the hydrodistilled essential oils (E.O) were bioevaluated against two phytopathogenic fungi *Rhizoctonia solani* and *Phytophthora megasperma* *in vitro*. The volatile fraction from both plants showed a good antifungal activity towards the tested fungi, EC_{50s} were 91 and 1369 ppm for *R. solani* and 84 and 1571 ppm for *P. megasperma* with *Ageratum* essential oil (E.O) and *Tagetes* E.O, respectively. But generally; *Ageratum* E.O was found to be more effective in reducing mycelium growth of *R. solani* and *P. megasperma* (EC₅₀ = 91 and 84 ppm respectively) than *Tagetes* E.O. Chemical composition of *Tagetes* E.O and *Ageratum* E.O; fractions F1 (solid) and F2 (liquid) fractions were investigated by GC-MS analysis. Interestingly, heterocyclic benzopyrane compound (Precocene II) was only component which has been detected in *Ageratum* E.O fraction F1. Precocene II seems to be the fungitoxic active components in *Ageratum* E.O and its fractions. However, monoterpene hydrocarbons were correlated with the fungitoxic effect of *Tagetes* E.O. In the green house experiments, *Tagetes* E.O and *Ageratum* E.O fraction F1 showed a clear selectivity towards tested patho-

gens; *Rhizoctonia solanii* and *Phytophthora megasperma*. *Ageratum* E.O; fractions F1 was found to be much more fungitoxic activity than *Tagetes* E.O. *Tagetes* E.O and *Ageratum* E.O fractions F1 were controlled the 90.91% of Root-Rot disease in bean caused by *Phytophthora megasperma*, while the percentage of disease control was only 36.36% for *Rhizoctonia solani*.

INTRODUCTION

Natural products are organic chemical compounds that produced from the secondary metabolic pathways in organs of higher plants, fungi, bacteria and marine organisms (González *et al* 1991 and Kim *et al* 2004). Since most of the synthetic chemicals used in plant protection have side effects and cause pesticidal pollution to the ecosystem by their persistence, there is a demand for the development of new safe, biodegradable alternatives which have a maximum efficacy with minimal environmental impact and danger for the consumer (Mishra and Duby 1990).

Ageratum spp is an annual herbaceous plant and it has a history of use in traditional medicine (Ming 1999), bioactivity of plant extract has been reported against bacteria and Fungi (Okunada 2002). Also, *Tagetes* spp essential oil has been found to effective, nonphytotoxic and easily biodegradable, the essential oil has exhibited very promising antifungal efficacy against many tested organisms (Gary and Dengre 1988; and Bruce *et al* 2002). Antifungal activity of *Tagetes* extracts and essential oil have been investigated by several

authors (Miah *et al* 1990; Kishore and Dwivedi 1991; Montes and Garcia 1997; Srivastava *et al* 1998 and Bii *et al* 2000).

Successful control of several phytopathogenic fungi using various plant extracts and essential oils including citrus essential oils; Lavander and Rosemary leave extracts; Garlic, Camphor and Neem extracts; and Lemon grass and clove essential oils were applied worldwide against *Penicillium digetatum*; *P. italicum*; *Alternaria citri*; *Phytophthora spp.*; *Fusarium spp.*; *Macrophomina phaseolina*; *Rhizoctonia solani* and *Scorotium rolfosii* (Tohamy *et al* 2002; Abd El-Naeem *et al* 2004; Widmer and Laurent 2006 and Mohamed *et al* 2006).

This paper describes the impact of *Ageratum houstonianum* and *Tagetes erecta* volatile oil on some phytopathogenic fungi in laboratory tests. It also discusses a strategy on how this application can be used to reduce root rot disease of beans as model. Joining the observed activity with the chemical composition of the fungitoxic fractions was considered in the present investigation.

MATERIALS AND METHODS

Plant materials

Two plant species from family *Asteraceae* have been chosen for this study. Mist flower (Borgoman) *Ageratum houstonianum* Mill, and Marigold *Tagetes erecta* L. the aerial parts of the flowering plants were collected in the period between 2002 and 2003 from the Botanical Research Farm, Faculty of Agriculture, Shobra El-Khema. Fresh plant samples were cleaned and washed by tap water then dried in attempt room temp in the shade. After dryness, the samples were grinded and kept into tightly sealed plastic pages and stored in closed brown glass bottles.

Preliminary chemical analysis and Volatile oil hydrodistillation

Moisture content and total ash content were determined in collected plant samples according to AOAC (1990). Volatile content was steam distilled according to British Pharmacopoeia (1980). Essential oils of both *A. houstonianum* and *T. erecta* were hydrodistilled in Clevenger's apparatus on a productive scale from the fresh aerial parts according to Clevenger (1928).

Plant extracts preparation

The air dried powder plant samples (20g) were exhaustively extracted in a continuous extraction apparatus with n-hexane, chloroform and then methyl alcohol (99%) The powder after each extraction was freed from the solvent before the next trip. The extract was filtered and the solvent was distilled off. The percentage of the crude extract has been calculated according to Khalil (1982). The preparative extracts were obtained by soaking the sample onto a cold solvent for excessive period of time as described by Iqbal *et al* (2004).

Fractionation of *A. houstonianum* essential oil

A dehydrated essential oil from *A. houstonianum* was noticed to solidify at low temp Dixit *et al* (1995). This observation was noticed also in our present research, this phenomenon has been developed to standardize the fractionation of the *Ageratum* E.O. The dehydrated E.O was dissolved in dry diethyl ether with the ratio of 2:1(w/v) and cooled at -5 °C for 7 days. The white amorphous crystals were identified as F1 and the liquid phase was identified as F2 fraction.

GC-MS analysis

Sample of 1 µl solution (1mg/ml) in n-hexane was injected in HP-5970 GC-MSD instrument, MS detector unit with EI and CI modes. Mass range is 50-800 m/z, D 3-5 capillary column, 30m length x 0.25mm I.D, 0.15µm film. The temp program was set to get the best separation.

Fungal isolates

Pathogenic isolates of *Phytophthora megasperma* and, *Rhizoctonia solani* originally isolated from diseased snap bean plants were used for all experiments. *Phytophthora megasperma* isolate was maintained on clarified V8 media while *Rhizoctonia solani* isolate was maintained on PDA media.

Solid agar bioassay

To test fungitoxic activity in vitro of *T. erecta* crude extracts and E.Os as well as the *A. houstonianum* E.O fractions F1 and F2 against *Rhizoctonia solani*, and *Phytophthora megasperma*, each E.Os and fractions were diluted w/v in agar diluted methods according to Hammer *et al* (1999). V8 agar plates were used for *P. megasperma* and

PDA plates were used for *R. solani*. Culture media in different final concentrations of each E.Os or fractions were inoculated by each pathogenic fungus separately. Each experiment was replicated fourth, fungal growth diameters were measured daily up to day 8 after inoculations, incubation temperature was 22°C. IC₅₀ was calculated by using regression equation between log concentrations and probit of percentage growth inhibition of fungi according to Abd El Naeem *et al* (2004).
Seedling bioassay

The *in-vivo* practical applicability of the *T. erecta* E.O and *A. houstonianum* E.O fraction F1 was tested in pot experiment by the modification the technique used by Kishore and Dwivedi (1991). In one set, the uninoculated control, 6 pots were filled with normal sterilized soil. In another set, which served as the inoculated control, each of twelve pots contained equal amount of soil infected with mycelia of either *R. solani* or *P. megasperma* isolates. In inoculated and uninoculated controls snap bean seeds (Pronco vr.) were soaked only in natural gum solution. The treatment sets, snap bean seeds (Pronco vr.) soaked separately in 3000ppm and 400ppm for *T. erecta* E.O and *A. houstonianum* E.O fraction F1 respectively prepared in natural gum solution. Snap bean seeds (Pronco vr.) were sown after one hour soaking time at rate of 3seed/ pots, three replicates were considered for each treatment. Records were made on pre-emergence after 7 days of planting as well as post-emergence damping-off after 15 days. The percentage of disease control were determined after 15 days of sowing, also height, dry weight and fresh weight of survival seedling were recorded.

RESULTS

1. Preliminary chemical analysis and Volatile oil hydrodistillation

The data in Table (1) presents the percent of moisture content, total ash and volatile compounds in the air dried *T. erecta* and *A. houstonianum* samples. Percentage of moisture content was almost the same for the two samples as well as the percentage of total ash, while the percentage of volatile content was higher in *A. houstonianum* plant (0.61%) than the *T. erecta* (0.12%). The percentage of chloroform extract was the same in both samples (1.29%) while the percentage of n-hexane and methanol extract were higher in *A. houstonianum* compared with *T. erecta*.

Table 1. Preliminary chemical analysis and percentage of the crude extracts with organic solvents of the air dried *A. houstonianum* and *T. erecta* (Asteraceae) plants

Chemical & crude extracts properties	<i>T. erecta</i> <i>A. houstonianum</i>	
	<i>erecta</i>	<i>houstonianum</i>
Moisture content (%)	11.56	11.50
Total Ash (%)	20.19	17.71
Volatile compounds(%)	00.12	00.61
Hexane Extr. (%)	02.85	05.07
Chloroform Extr. (%)	01.29	01.29
Methanol Extr. (%)	06.10	10.25

2. GC-MS analysis

Data in Table (2) presented the major constituents of both *T. erecta* E.O and *A. houstonianum* E.O fractions F1 and F2. In *T. erecta* E.O there were a five major components had been identified against the authentic samples. Monoterpenoid α -pinene was found to be the major component (42.86%) followed by monoterpenoides piperitone (32.22%) and D- Limonene (12.16%) while sesquiterpene β - Caryophyllene was encountered as 3.34% of total constituents of *T. erecta* E.O.

Table 2. GC-MS analysis of *T. erecta* E.O and *A. houstonianum* E.O fractions (F1 and F2)

Fraction	% Volatile concentration in dry samples		
	<i>T. erecta</i>		<i>A. houstonianum</i>
	E.Os	F1	F2
α -Pinene*	42.86	-	-
D-Limonene*	12.16	-	-
Piperitone*	32.22	-	-
β -Caryophyllene**	03.34	-	20.80
Caryophyllene Oxide**	-	-	11.78
γ -Cadenine**	-	-	09.43
Precocene II***	-	100	48.82
Unknown	03.04	-	-

* Monoterpenoides compound

** Sesquiterpene compound

*** Heterocyclic benzopyrane compound

Interestingly, precocene II was the only major component which has been detected in fraction F1 (Table 2) by GC-MS. The heterocyclic benzopyrane compound precocene II was also detected in F2 fraction; it was formed 48.82% of total components. Sesquiterpenoids β - Caryophyllene, Caryophyllene oxide and γ - Cadinene were also detected in F2 fraction. The percent of the corresponding compounds were 20.80, 11.78 and 9.43% respectively.

3. Solid agar bioassay

The fungitoxic activity of *A. houstonianum* E.O fractions F1, F2 and *T. erecta* E.O were tested against, *Phytophthora megasperma* and *Rhizoctonia solani*. Data in Table (3) presented the EC₅₀ of all treatments. It was noticed that EC₅₀ of *A. houstonianum* E.O F2 was much higher than the other two E.Os fractions. However the EC₅₀ for *T. erecta* E.O against *R. solani* and *P. megasperma* were 1571 and 1369 p.p.m respectively. EC₅₀ of fraction F1 was found to be much lower than the EC₅₀ of *T. erecta* E.O for two tested phytopathogens. The EC₅₀ was 84 and 91 p.p.m for both *P. megasperma* and *R. solanii* respectively.

4. Seedling bioassay

Data in Table (4) and Figure (1) showed the effect of treatment with *T. erecta* E.O or *A. houstonianum* E.O fraction F1 on the controlling the root rot disease in snap bean seeds (Pronco vr.) caused by either *P. megasperma* or *R. solani* *In vivo*. Better control results was recorded with *P. megasperma*.

Seed inoculation 90.9% percentage of disease control (% of plant survival) was for both treatments while it was 36.36% for *R. solani*. Percentage of germination, seedling height and fresh weight were in corresponding with the percentage of disease control, they were lower in seedlings infected with *R. solani* than those infected with *P. megasperma* compared with uninoculated control.

DISCUSSION

The recovery of the volatile fraction from leaves of *A. houstonianum* was ranged from 0.11-0.6 % Dixit *et al* (1995), and Wandji *et al* (1996). The steam distillation of *T. erecta* leaves yielded 0.2% of essential oil Machado *et al* (1994), meanwhile the percent of volatile fraction was found to

be a species dependent in *Tagetes* spp plant Héthelyi *et al* (1986).

Stepwise extraction has been carried out to catch the different compounds depending on the gradient differences in their polarity, Aquino *et al* (2002). Héthelyi *et al* (1987) were identified Limonene, β - Caryophyllene, Piperitone and γ -Terpinolene in *T. erecta* E.O by using GC-MS analysis. That was supported the obtained results. In another investigation Krishna *et al* (2002) were found that the hydrodistilled E.O from the shoots of *Tagetes* spp contain Limonene, (cis)- β -Ocimene, Terpinoline Piperitone and β - Caryophyllene. Also Singh *et al* (2003) reported that the β - Ocimene was the major constituents in Indian *Tagetes erecta* E.O. In *A. houstonianum* E.O fractions F1 and F2, the heterocyclic benzopyrane compound precocene I was detected as a major constituents, those findings were in agreement with several authors; Kasali *et al* (2002); Sundufu and Shoushan (2004). Chromene compounds, Sesquiterpenoids and monoterpenoids were contained in *Ageratum spp* E.O at 71.05, 13.95 and 5.17 % respectively. The main constituents of the hydrodistilled *A. houstonianum* E.O were precocene I, precocene II and beta- caryophyllene at levels 23.3, 43.99 and 9.18 respectively Suresh *et al* (1996).

Success of essential oils as biodegradable and environmentally safe fungi toxicants have shown the possibilities for their exploitation as natural fungicides Dikshit *et al* (1983); Dubey *et al* (1983); Asthana *et al* (1986). It was found that the *A. houstonianum* E.O fraction F1 was much higher than fraction F2 as fungitoxicant against the tested pathogens concluding that the active fungitoxicant (s) may contain in fraction F1. The essential oil of *A. coryzoides* exhibited a broad range of activity inhibiting 22 out of 35 fungi tested Dixit *et al* (1995). Similarly, *T. erecta* E.O showed a strong anti-fungal activity against 20 tested phytopathogens at 2000 p.p.m Kishore and Dwivedi (1991).

A. houstonianum E.O fraction F1 and *T. erecta* E.O have shown a strong fungitoxicity towards the tested phytopathogens *in-vitro*. It was found that the *Phytophthora spp* were much more susceptible than *Rhizoctonia spp* for the treatment with fraction F2. From the obtained results it could be noticed also that the fungitoxic activity of F1 fraction was higher than *T. erecta* E.O. the obtained results were found to be in agreement with several reports; Héthelyi *et al* (1986), Gary and Denger (1988), Lapis and Durancas (1978).

Table 3. Antifungal activity of *T. erecta* E.O and *A. houstonianum* E.O fraction F1 and F2 against two phytopathogenic fungi (*Rhizoctonia solani* and *Phytophthora megasperma*) in-vitro

Plant EOs extracts	<i>R. solani</i>			<i>P. megasperma</i>		
	EC ₅₀ ppm	Reg. Equation	(R ²)	EC ₅₀ ppm	Reg. Equation	(R ²)
<i>T. erecta</i> E.O	1369	Log Y=0.521X+3.365	0.999	1571	Log Y=2.621X-3.378	0.998
<i>A. houstonianum</i> E.O						
Fraction F1	0091	Log Y=2.129X+0.832	0.979	0084	Log Y=2.621X-0.375	0.949
Fraction F2	7949	Log Y=0.584X+2.723	0.961	0393	Log Y=1.173X+1.957	0.972

Reg. Equation = Regression equation between log concentration (log y) and percent of % fungus growth inhibition (x)
 (R²) = Correlation coefficient of Y and X
 EC₅₀ = the effective concentration at 50 % growth inhibition in ppm.

Figure 1. Effect of treatment with *T. erecta* E.O or *A. houstonianum* E.O fraction F1 on the Root-Rot disease in snap bean infected with *P. megasperma* (A) or *R. solani* (B)Table 4. Effect of treatment with *Tagetes* E.O and *Ageratum* E.O fraction F1 on root-rot diseases caused by *P. megasperma* or *R. solani* on snap beans

Pathogen inoculated	EOs Treatment	Pre-emergence %	Efficacy %*	Height /seedling (g)	Fresh weight /seedling (g)	Dry weight /seedling (g)
<i>R. solani</i>	<i>Tagetes</i> E.O	22.2	36.36	6.6±5.4	1.75	0.25
	<i>Ageratum</i> F1	22.2	36.36	3.7± 0.8	1.50	0.38
	Untreated	27.78	45.45	8.8±2.3	1.40	0.33
<i>P. megasperma</i>	<i>Tagetes</i> E.O	55.56	90.91	18.7±2.3	2.90	0.36
	<i>Ageratum</i> F1	55.56	90.91	14.8±5.81	2.80	0.33
	Untreated	44.4	72.72	16.7± 5.95	2.63	0.33
Non -infected	Untreated	61.11	-	19.2± 6.17	2.18	0.26

* Efficacy of treatment = (control - treatment) / control %

In-vivo experiment showed that the treatment with either *T. erecta* E.O or *A. houstonianum* E.O fraction F1 was effective in management of Root-Rot disease caused by *Phytophthora megasperma* much higher than *Rhizoctonia solani*. Even though the treatments were affected both *Phytophthora spp* and *Rhizoctonia spp* in *-vitro*, the obtained results cleared a high selectivity towards the *Phytophthora spp*. The *A. houstonianum* E.O fraction F1 was achieved the same effect of *T. erecta* E.O at Conc Level of 400 p.p.m compared with 3000 p.p.m of *T. erecta* E.O. it could be concluded that the *A. houstonianum* E. O fraction F1 was higher fungitoxic than *T. erecta* E.O . The potential of *T. erecta* E.O and *A. houstonianum* E.O fraction F1 as a promising fungitoxicant indicate the possibility of their exploitation as a novel fungitoxicants for the management of Root-Rot disease in snap bean. Kishore and Dwivedi(1991) reported that the possibility of control the Damping -off disease in tomato by using 2000-3000 p.p.m of *Tagetes* E.O . Also Dixit et al (1995) reported that the whole *Ageratum* E.O was successful in control blue mold -rot in mandarins.

Structure - fungitoxic relationship of F1 fraction and *T. erecta* E.O have been investigated. Monoterpenic hydrocarbons were 87.24% of total components in *T. erecta* E.O. There was a positive correlation between the monoterpenic content and the fungitoxic activity. Several reports have been pointed out to such correlation between the monoterpenoids and the antifungal activity [Kim et al (1995), Caccioni et al (1998), Arras and Usai (2001)]. The mechanism of action was suggested that the monoterpenoids may interact with the fungal cell membrane and disrupting it Thompson (1996). On the other hand, monoterpenoids were detected as minor constituents in *A. houstonianum* E.O and its fractions; instead, the heterocyclic benzopyrane compound precocene II was detected as a major component. More over, fraction F1 was found to contain the precoceneII mainly and this resulted in increasing the fungitoxicity of F1 fraction. The antifungal effect was decreased dramatically in F2 fraction as a result of decreasing the chromene content. From those observations it could be concluded that the precocene II, a member of chromene compounds, could be correlated with the antifungal activity of *A. houstonianum* E.O. Chromene compounds showed an antifungal activity against *Colletotrichum gloeosporioids* (Bandara et al 1992) *Phytophthora spp* (Widmer and Laurent 2006).

Further separation and isolation guided by antifungal evaluation are required to prove whether or not the chromene compound and / or any other constituents in F1 fraction are responsible for the observed antifungal activity. Despite the work that still needs to be done, this study provides the basis for an alternative method to synthetic chemical application that can be developed quickly for farmer use.

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الاستخدام التطبيقي للزيوت الطيارة المستخلصة من البرجمان والقطفية لإدارة المكافحة الآمنة لبعض الفطريات الممرضة للجذور

[١٧]

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نباتات الفاصوليا المنزرعة في اصص بالفطريات
المسببة لغفن الجذور *R. solani* , *P. megasperma*
اوضحت النتائج ان كلا من F1 والزيوت العطرية
للقطفية يقاومان الاصابة بغفن الجذور الناتج عن
الاصابة بفطر *P. megasperma* بنسبة ٩٠% في حين
كانت نسبة مقاومة غفن الجذور الناتج عن *R. solani*
هي ٣٦,٣% . مما يشير الى التأثير الاختياري على
المسببات المرضية محل لدراسة.

خلال دراسة التركيب الكيميائي لزيت القطفية
العطرية و الجزء الصلب المتبلور F1 لنبات البرجمان
اتضح ان المركبات التربينية *monoterpenoids* ترتبط
ارتباطا موجبا مع النشاط البيولوجي لزيت القطفية في
حين وجد ان المركب الحلقى الغير متجانس
precocene II قد يكون هو المسؤول عن التأثير
المضاد للفطريات في F1 .

وتوصى الدراسة بإمكانية استخدام الزيوت
العطرية الطبيعية ومستخلصاتها المجزأة منها والتي
اجرى العمل عليها في مكافحة مرض تعفن الجذور
في الفاصوليا والناتجة عن فطر *P. megasperma*
بالرغم من الحاجة الى اجراء المزيد من الدراسات
على المركبات الطبيعية المسؤولة عن النشاط
البيولوجي لمزيد من الاضاح عن دور التي تقوم به
لمكافحة هذه الفطريات الممرضة لجذور النباتات .

استخدم في هذه الدراسة نوعان نباتيان من العائلة
المركبة وهما البرجمان *Ageratum houstonianum*
ونبات القطفية *Tagetes erecta* حيث استخلصت هذه
النباتات بواسطة المذيبات العضوية بطريقة
الاستخلاص المتتابع كما تم استخلاص الزيوت
العطرية منها عن طريق التقطير المائي . تم تجزئة
الزيت العطري المستخلص من نبات البرجمان
بطريقة البلورة على البارد حيث تم الحصول على
بللورات بيضاء (F1) تم فصلها عن الجزء السائل
(F2) عند اختبار الزيت العطري المستخلص من
القطفية و الجزء الصلب المتبلور من زيت
البرجمان F1 على نوعين مختلفين من الفطريات
الممرضة للنبات وهما *Rhizoctonia solani* ,
Phytophthora megasperma اظهرت الزيوت
المختبرة اختياريية في التأثير على فطريات *R. solani*
P. megasperma كما اظهر F1 تأثيرا اقوى من
الزيت العطري للقطفية بصورة كبيرة كما توصلنا
قيم EC_{50} التركيز النصفى الفعال حيث كانت ٨٤ و ٩١
جزء في المليون لكلا من فطريات *P. megasperma* ,
R. solani على الترتيب في حالة F1 مقارنة ب
١٥٧١ ، ١٣٦٩ جزء في المليون في حالة الزيت
العطري للقطفية. عند اختبار الجزء الصلب المفصول
من زيت البرجمان F1 والزيت العطري للقطفية على