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Research Article

Phytochemical evaluation and nematicide effect of a leaf aqueous extract of *Eucalyptus globulus* against *Pratylenchus vulnus* infecting apple

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ABSTRACT

Nematicidal activity of an aqueous extract from dried leaves of *Eucalyptus globulus* was evaluated towards the root lesion nematode *Pratylenchus vulnus in vitro* and pot experiment. In both experiments, the application of four concentrations of the leaf aqueous extract (100%, 60%, 30%, 15% w/v) significantly reduced the number of females and males on apple rootstock MM106 roots. *In vitro* test showed that the highest net mortality was recorded with the concentration 100% (96%) after 72hrs of exposure time. Pot experiment studies indicated that the concentration 100 increased significantly ($P < 0.05$) the female and male reduction rates (84.43% and 91.40% respectively) compared to others leaf extracts concentrations. The chemical treatment with Oxamyl G (Oxamyl granule) reduced significantly female and male rates by 98.30% and 100%, respectively. The chemical analysis of dried leaves of *Eucalyptus globulus* showed high levels of total phenols and total flavonoids contents and exhibited high antioxidant capacity. The results suggest that the leaf aqueous extracts of *Eucalyptus globulus* had a promising nematicide potential against root lesion nematodes.

Keywords: Apple rootstock, Pratylenchus vulnus, *Eucalyptus globulus*, Biological control, Bioactive metabolites

INTRODUCTION

Plant parasitic nematodes are dangerous enemies of several agricultural and horticultural crops. The estimated annual yield loss due to plant parasitic nematodes on major crops of the world is 12.3% [1]. The *Pratylenchus* species are economically important pests of many crops [2]. The root lesion nematode, *Pratylenchus vulnus* is recognized as a pathogen of apples and has been shown to be a serious nematode pest causing yield losses in apple orchards in warm mediterranean environments [3]. This species is also pathogenic to almond [4], peach [5] and plum [6].

The plant parasitic nematodes management is commonly based upon chemical treatment. Due to the harmful toxicity in the environment and risks to human health, the use of this type of materials is discussed. Plant derived nematicides could be promoting alternative to pesticides use and fit well to the principles of integrated pest management. Natural products obtained from plants have been used as pest management agents. They served also in some commercial bio pesticides [7,8]. *Eucalyptus globulus* (Myrtaceae) is an indigenous tree of Australia, widely applied in medicine [9]. The leaves of several species of *Eucalyptus* have shown biological activities including anti-microbial, fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematicidal effects [10,11]. The chemical profile of *E. globulus* leaves, in particular the essential oil, has widely studied [12]. The leaves of this medicinal tree contain 70% of eucalyptol (1,8-cinéole). A large number of monoterpenoids have been identified, mainly alpha-pinene, s-pinene, δ-limonene, para-cymene, camphene, alphaphellandrene, alphafenchene, γ-terpinene. The aromadendrene and alloaromadendrene are the most found

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sesquiterpenoids. The monoterpens glycosides (globulisin, cypellocarpin, euglobulin), flavonoid (quercetin, rutin), polyohenols (catechol, cafeic acid, gallol, etc.) are other compounds most found in E. globulus leaves [13-15]. The nematicidal activity of several plants are attributed to these chemical compounds [16-20].

The present study aimed at evaluating the nematicidal effect of dried leaf aqueous extracts of Eucalyptus globules against *Pratylenchus vulnus in vitro* and *in vivo* experiments. Additionally, the chemical screening of total phenol and total flavonoïd contents and antioxidant activity of the aqueous extracts were analyzed.

MATERIALS AND METHODS

Plant collection and extracts preparation

Eucalyptus leaves were collected from wild trees in the region Oueslatia (center of Tunisia) and dried in the shade at 30 to 40°C for fifteen days. A leaf powder was prepared by crusher the leaves using commercial mortar. To obtain the aqueous extract, 30 g of powdered leaves were placed in glass flasks containing 100 ml of sterilized distilled water. The flasks were placed under an orbital shaker for 48 hours at room temperature (about 25°C). After two days; the water suspension in the flasks was filtered through a what man filter paper N°1 and the filtrate was used directly for *in vitro* and pot essays. The obtained filtrate was considered as a concentrated solution (100%) and other concentrations (60%, 30%, 15% w/v) were prepared by adding the required amount of sterilized distilled water at the time the bio test and pot experiments were performed.

Phytochemical analyses

The preparation of sample and determination of total flavonoids, total phenol, antioxidant activity and HPLC-DAD for phenolic profile were determined as described by Hajji-Hedfi, et al.

In vitro nematicide assay

A suspension of *Pratylenchus vulnus* specimens provided from roots of MM106 rootstock, after root extraction by double centrifugation method [21] was used. Fifty individuals of *P. vulnus* were placed in a plate containing 1 ml of leaf aqueous extract (four concentrations were used; 100%, 60%, 30% and 15%) with ten replicates for each treatment. Plates with distilled water were used as a negative control (0%). The plates were maintained at 25°C in darkness. Then, the effect of the aqueous extracts on *P. vulnus* viability was checked at 24, 48 and 72 hrs. The response of individuals was observed and the dead nematodes were counted under a stereoscopic microscope. The moved nematodes during 24hrs were considered alive, whereas those not responding were considered dead. The experiment was repeated twice.

In vivo experiment

A pot experiment was conducted under greenhouse conditions at higher agronomic institute of Chatt-Mariem, Sousse university, Tunisia. Total of 42 trees of the rootstock 'MM106' (one year old each tree) were transplanted each one into 10 kg soil pots filled with a sterilized sandy loam soil (1:1:1v/v). The initial population is determined from all rootstock tree naturally infected with the root lesion nematode (1390 females and 123 males per 1 g of roots), and originated from the region of Oued Melliz of the governorate of Jendouba (north of Tunisia). Four concentrations of leaf aqueous extract of *E. globulus* were used (100%, 60%, 30% and 15%). Each pot was treated by pipetting 10 ml of each concentration into two holes at 2–4 cm deep around root system. Additional pots were treated with 3 g of Oxamyl G and were used as positive control for comparison. Plants naturally infected and not treated are considered as negative control. All treatments were arranged in a completely randomized design with seven replicates.

After 2, 4 and 6 months of the treatments; a root sampling from each treatment was realized. Taking 10 g of each replication and mixed in order to have a representative sample of each treatment. The *P. vulnus* root population was determined via root extraction and observation under a stereoscopic microscope. At the end of experiment (after 6 months of the treatments), the growth parameters were measured including: The foliage tree diameter, plant height, trunk diameter, branch number, and number of leaves per branch.

Statistical analysis

The data were subjected to *analysis of variance* (ANOVA) using SPSS 20.0 for windows. The bioassays data were analyzed by probit regression to obtain the LD values. Laboratory and pot experiments were repeated twice and the treatment means were compared by the Tukey's multiple range tests when the F-tests were statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical analyses of dried leaves composition

The results for flavonoid and total phenolic contents and antioxidant activities measured by DPPH method from aqueous extracts from dried leaves of E. globules showed that the flavonoid and total phenols contents were 14183,28 µg QE/ g D.W and 185,45 µg GAE/g D.W, respectively. It was observed that aqueous extracts contained a notable antioxidant activity reached 62,19%.

The overall chemical composition of *E. globulus* aqueous extract permitted the identification of a number of phenolic acids such as quinic acid and gallic acid, in addition the quercetin, main flavonoids constituent. Nevertheless, other phenolic acids occurred in leaves of *E. globulus* as Catechin (+), caffeic acid, 4-0-caffeoylquinic 3,4-di-O-caffeoyquinic acidand 1,3-di-O-caffeoyquinic acid but at low rates. The phytochemical analysis of dried leaves showed the high levels of flavonoid and phenol contents and an interesting antioxidant capacity. *E. globulus* has been reported previously containing biologically active terpenoids, flavonoids, polyphenols and high antioxidant activity [22,23]. Several recent studies have shown that the biocidal activity was attributed to several bioactive molecules revealed the capacity of plant extracts in the prevention and control of plant diseases is due to presence of some natural compounds such sterols, saponins, tannins, flavonoids and alcohols [24-27]. The toxicity of root knot nematodes (*M. javanica*) treated by *F. vulgare* and *M. spicata* were achieved essentially by tanins, saponsides, estragol, phenol [28]. The phenols were reported involved in plant defense against pathogens. The tomato roots immersed in five solutions of phenols showed the decrease of radopholus reniformis infection. The quercetin (flavonoid) inhibited the development of *Meloidogyne javanica*. The rotenone as flavonoid demonstrated the nematicide activity against *Caenorhabditis elegans* (Table 1) [29].

Table 1. Phytochemical screening of phenolic constituents in dried leaves of *E. globulus.*

Name	Concentration (ppm)
quinic acid	5726.766
Rutin	973.7393333
quercetin	309.378
Gallic acid	249.4513333
Quercetrin (quercetin-3-o-rhamonosid)	113.5566667
Chlorogenic acid	141.2693333
syringic acid	96.02866667
Hyperoside (quercetin-3-o-galactoside)	63.349
4-O-caffeoylquinic acid	55.346
trans frulic acid	32.50233333
Cirsiliol	22.312
p-coumaric acid	16.01066667
Luteolin	5.978666667
caffeic acid	4.796
Cirsilineol	4.193
Apegenin	2.853666667
Catechin (+)	2.81
1,3-di-O-caffeoyquinic acid	2.665333333
kampherol	0.649333333
3,4-di-O-caffeoyquinic acid	0.395

In vitro Effect of *E. globulus* on root lesion nematode

The efficacy of *E. globulus* leaf aqueous extracts on *P.vulnus* mortality, under laboratory conditions, is presented in (Table 2). The results showed that the four concentrations enhanced significantly the *P. vulnus* mortality as compared with control. After 24 and 48 hours, the undiluted extract (100%) exhibited the highest mortality as compared with the diluted extracts at 60%, 30%and 15%. After 72 hours exposure, both treatment with C1 and C2 exhibited the greatest net mortality (P<0.05). The lethal doses LD_{50} and LD_{90} of *E. globulus* aqueous extracts were 83, 19 µg/ml and 189, 12 µg/ml respectively. The *in vitro* tests confirmed previous observations on the strong biocidal effect of *E. globulus* (Figure 1). A crude extract from fruits of *E. globulus* showed strong antimicrobial activities [30,31]. The results of biocide activity are in line also with the finding of when they reported a high antimicrobial potential for the essential oil of *E. globulus* when compared with the antibiotics used (ceftriaxone, amykacine and tetracycline). Additionally, a number of investigations have demonstrated acaricide effect against *Boophilus microplus* [32] and insecticide activity against several insects genera [33-35].

Table 2. Effect *in vitro* of different concentrations of aqueous extract of *Eucalyptus globulus* on mortality of *P.vulnus*.

Treatment	Mortality% 24 h	Mortality% 48 h	Mortality% 72 h	Mortality% Net
***Eucalyptus globulus* (30 g/100 ml)**	62.00 ± 10.32^{c}	85.00 ± 5.27^{d}	96.00 ± 5.16^{c}	95.78 ± 5.46^{b}
***Eucalyptus globulus* (20 g/100ml)**	45.00 ± 17.15^{b}	73.00 ± 6.74^{c}	92.00 ± 6.32^{c}	91.39 ± 7.50^{b}
***Eucalyptus globulus* (10 g/100ml)**	41.00 ± 9.94^{b}	58.00 ± 4.21^{b}	78.00 ± 7.88^{b}	77.17 ± 7.66^{a}
***Eucalyptus globulus* (5 g/100ml)**	$43.00 \pm 8{,}2\ ^{b}$	59 ± 3.16^{b}	78.00 ± 4.21^{b}	77.05 ± 4.05^{a}
Control	49.33 ± 0^{a}	72.00 ± 0^{a}	88.67 ± 6.99^{a}	-
Values are means ± standard deviation of five replicates. Values in columns followed by the same letter do not differ at P < 0.05 according to Tukey's multiple-range test.				

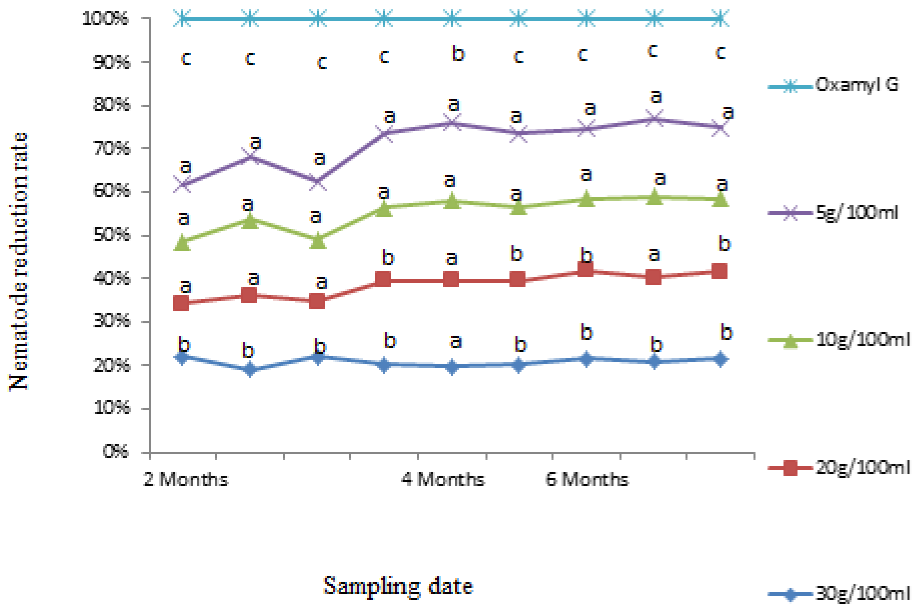

Figure 1. Mean effect of different concentrations of *Eucalyptus globulus* aqueous extract on of *Pratylenchus vulnus* development on apple roots (Values followed by the same letter do not differ at P <0.05 according to Tukey's multiple range test).

Effectiveness of aqueous leaves extract of *E. globulus* against *P. vulnus in vivo*

Nematode development

After 6 months' post treatment, the concentration 100% reduced significantly (P<0.05) root lesion nematode development and showed the lowest female and male reduction rate by 84.43% and 91.40% respectively. However, the chemical treatment with Oxamyl G registered important lowest for female and male reduction rates with 98.30% and 100%, respectively. The *P. vulnus* females were reduced significantly at concentration 100% and concentration 60% but males were only reduced at concentration 100%. In general, the invasion of apple roots by *P. vulnus* decreased significantly by Oxamyl G followed by concentrations 100%, 60%, 30% and 15% (Table 3).

Table 3. Effect *in vivo* of different concentrations of aqueous leaves extracts of *Eucalyptus globulus* on apple root stock MM106 growth.

Treatments	**Foliage tree diameter (cm)**	**Branch number**	**Trunk diameter (cm)**	**Plant height (cm)**	**Leaves Number/ branch**
***Eucalyptus globulus* (30 g/100ml)**	10.29^{a}	6.86^{b}	2.71^{ab}	97.14^{ab}	10.43^{bc}
***Eucalyptus globulus* (20 g/100ml)**	9.57^{a}	6.00^{ab}	2.57^{ab}	89.29^{ab}	8.57^{ab}
***Eucalyptus globulus* (10 g/100ml)**	8.43^{a}	5.86^{ab}	2.36^{ab}	90.00^{ab}	7.86^{ab}
***Eucalyptus globulus* (5 g/100ml)**	8.21^{a}	5.71^{ab}	2.36^{ab}	90.29^{ab}	7.85^{ab}
Oxamyl G (3 g)	17.71^{b}	9.43^{c}	2.93^{b}	98.57^{b}	11.71^{c}
Control	7.57^{a}	4.86^{a}	2.21^{a}	80.00^{a}	5.57^{a}

Values are means ± standard deviation of four replicates. Values in columns followed by the same letter do not differ at P<0.05 according to Tukey's multiple range tests.

After 2 months, the undiluted aqueous extract, reduced (P<0.05) female and male rate by 56.23% and 60.13%, respectively, compared to others extract concentrations. Oxamyl G enhanced female and male reduction rate respectively by 96.61% and 100%. After 4 months, the concentration 100% showed significantly (P<0.05) female and male reduction rate (74.50% and 82.52% respectively) compared to the chemical treatment female and male reduction rate by 97.51% and 100%, respectively. The potential uses of plant extract to control the root lesion nematode, *Pratylenchus spp*. have been reported by several researchers [36]. Reported the nematicidal effect of Chenopodium ambrosioides towards *Pratylenchus brachyurus*. The aqueous extract of *Melia azedarah* displayed nematicide activity against *P. thornei* [37]. Furthermore, 75% mortality of *P. vulnus* juveniles recorded after 96 hours of exposure to 15 µg ml-1 of essential oil from Rosmarinus officinallis [38].

Plant growth

Oxamyl G enhanced significantly apple growth comparing with control trees and those treated with leaf aqueous extract solutions [39]. The apple trees treated with the highest concentration (undiluted concentration) of leaf aqueous extract showed maximum growth parameters as compared with both concentrations 60% and 30% (P<0.05) (Table 4). Both, chemical treatment and leaf extracts at all concentrations improved significantly the number of leaves per branch compared with control.

Table 4. Effect *in vivo* of different concentrations of *Eucalyptus globules* aqueous extract on *Pratylenchus vulnus* development in apple roots.

Treatment	Female reduction (%)	Male reduction (%)	Total reduction (%)	Sex ratio
***Eucalyptus globulus* (30 g/100ml)**	84.43 ± 4.45[b]	91.40 ± 3.76[b]	84.84 ± 4.24[b]	1,08 b ± 0,06
***Eucalyptus globulus* (20 g/100ml)**	77.97 ± 4.32[b]	82.84 ± 5.87[a]	78.30 ± 4.15[b]	0,97 b ± 0,22
***Eucalyptus globulus* (10 g/100ml)**	64.95 ± 9.66[a]	81.05 ± 5.71[a]	65.84 ± 9.38[a]	1,26 a ± 0,14
***Eucalyptus globulus* (5 g/100ml)**	63.76 ± 7.66[a]	78.53 ± 3.10[a]	64.59 ± 7.26[a]	1,24 a ± 0,12
Oxamyl G (3 g)	98.30 ± 0.34[c]	100 ± 0[c]	98.40 ± 0.31[c]	1,01 c ± 0,03
Values are means ± standard deviation of four replicates. Values in columns followed by the same letter (a, b, c) by colomn do not differ at P <0.05 according to Tukey's multiple range tests.				

CONCLUSION

Considering the results of total flavonoid and phenol, contents and the antioxidant capacity, the dried leaves of the medicinal plant *Eucalyptus globulus* contained high levels of phenols and flavonoids and showed a high level of the (DPPH) scavenging. Furthermore, the aqueous extracts of dried leaves showed a significant potential on reducing development of the root lesion nematode *Pratylenchus vulnus in vitro* and *in vivo*. These findings provide scientific evidence to support the value of medicinal plants for pest management and indicate that *E. globulus* could be a promising source for potential biological control agent. Further work should be realized on the identification; isolation of the specific compounds from the leaf extracts which are responsible for the higher nematicidal action.

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