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Potential of *Inula racemosa* root extract and its fractions to suppress root-knot nematode *Meloidogyne incognita*

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Abstract: Nematicidal potential of chloroform root extract of *Inula racemosa* and its fractions was investigated on egg hatching and mortality of root knot nematode *Meloidogyne incognita*. Egg masses and second stage juveniles (J_2) of *M. incognita* were exposed to different concentrations (0.1-8.0 mg ml^{-1}) of *I. racemosa* root extract and its fractions. Observations on egg hatch were recorded on 1st, 3rd, 5th, 7th and 9th day and those of mortality studies were recorded on 2nd, 4th, 6th, 8th and 10th day, respectively. Significant mortality as well as egg hatch inhibition was observed for all the tested components at 5 %. The root extract was found to be most effective in controlling egg hatching as complete inhibition was observed at 8.0 mg ml^{-1} concentration on 1st day of treatment and nonpolar fraction was most effective in causing mortality of J_2 of *M. incognita* as 100 % inhibition was observed at 6.0 and 8.0 mg ml^{-1} concentration on 2nd day of treatment. Maximum inhibition of egg hatching was observed for root extract at 8.0 mg ml^{-1} concentration and 100 % mortality was observed for root extract as well as nonpolar fraction at the same concentration. The nonpolar fraction was most effective in causing mortality as maximum mortality was observed at 6.0 and 8.0 mg ml^{-1} concentration throughout the exposure time. Polar fraction was least effective among all the components both in egg hatch inhibition and J_2 mortality of *M. incognita*. Both the activities showed concentrations as well as time dependence. Results show different role of tested components on egg hatching and mortality of root knot nematode. The root extract of *I. racemosa* and its fractions showed a potential to develop new nematicide.

Keywords: Asteraceae, Egg hatching, Juvenile, Root knot nematode, Soxhlet extraction

INTRODUCTION

Natural products comprise large and diverse group of substances obtained from various sources such as plants, marine organisms, bacteria and fungi. Many plant species produce biochemicals that protect them by killing or repelling the pest that feed on them. Plant based natural products are gaining importance as they minimizes the effects caused due to residual pesticides (environmental hazards, secondary pest outbreaks, resistance etc.). Out of several pathogenic organisms, soil borne pathogens are of major concern as they significantly reduce the yield and quality of vegetables crops. Soil borne pathogens are categorized under different phyla-bacteria, fungi and nematodes. Out of these nematodes especially root-knot nematodes are causing severe damage to vegetables crops resulting in decrease in production as well as quality of the crop. Root knot nematodes, *Meloidogyne incognita* is responsible for causing damage to 1700 plant species and are among the most destructive nematodes in agriculture; causing an estimated yearly crop loss of $157 billion worldwide (Oka *et al*., 2000; Trudgill and Block, 2001, Singh *et al.,* 2015), out of this $40.3 is reported from India. It is the one of the major pest in vegetables and orchards. Although several synthetic pesticides and chemical fumigants are being used to control these soil borne parasites but most them are banned these days due to development of resistance in nematodes, which results into yield reductions of the crops (Noling and Becker, 1994). Moreover, their indiscriminate use causes serious damage to environment and human health. Agricultural production can be salvaged if these parasites are successfully combated. Therefore, scientists are developing alternative management techniques such as plant based chemicals, biological control agents, cropping systems soil amendments and judicious use of nematicides (Serfoji *et al*., 2010). Many scientists have carried out the research on plant extracts for the management of root knot nematodes. Chemicals produced by plants are potential source for the development of plant based pesticides. Several plant species are known to cause toxic affects against root knot nematodes (Onifade and Egunjobi, 1994; Nimbalkar and Rajurkar, 2009; Iruthaya and Devraj, 2011; Bharadwaj and Sharma, 2007) specifically members of family Asteracae (Chitwood, 2002; Moosavi, 2012). These plants or their parts (flowers, leaves, roots, seeds), either in crude form or as formulation, may be utilized as organic amendments

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or bio-pesticides (Perez *et al*., 2003).

Inula racemosa (Asteraceae) is an annual herbaceous plant found in North Western Himalayas (Khare, 2007), commonly known as pushkarmoola in Indian medicinal system. It is medicinally very important plant (Shabir *et al*., 2010) and used to treat many diseases in China since ancient times. Its seeds are aphrodisiac, roots are expectorant and are used to cure skin diseases and as adulterant for *Sassurea costus* roots (Sarin, 1996). This plant is also used by native Americans for treating tuberculosis (Moerman, 1986). Its roots are rich source of bioactive compounds. *I. racemosa* was found to show toxic effects against stored grain pests and many phytopathogenic fungi (Liu *et al*., 2006; Kataria and Chahal, 2013). Many investigators have reported the medicinal properties and biological activities of *I. racemosa* root extract, however little is known about the nematicidal effect of *I. racemosa* root extract and its fractions against *M. incognita*. However, toxicity of several other *Inula* species was reported against root knot nematodes as *Inula viscosa* powdered leaves and its extracts were found to have toxic effect against *M. javanica* (Oka *et al*., 2001).Therefore, in present research bioassay studies were undertaken to evaluate nematicidal properties of *I. racemosa* root extract and its bioactivity guided fractionation to assess its effect on second stage juveniles and eggs of *M. incognita* under laboratory conditions.

MATERIALS AND METHODS

Maintenance of pure culture of *Meloidogyne incognita*: The nematode *M. incognita* used in studies was identified by its characteristic perennial pattern. Pure culture was raised by single egg mass technique and multiplied on brinjal, a susceptible host for root knot nematode. For mass multiplication of *M. incognita* culture, the soil was autoclaved at 15 psi pressure and 121 ºC for at least 30 min. The autoclaved soil was then filled in the pot. Three weeks old seedlings were transferred in to earthen pots containing sterilized soil and inoculated with freshly hatched 2nd stage juveniles (J_2) collected from egg masses of pure culture. After sixty days of inoculation the egg masses were collected and were used for bioassay studies on egg hatching

Table 1. Effect of *I. racemosa* root extract, its non-polar and polar fraction on per cent egg hatch inhibition of *M. incognita* at different concentrations and durations.

Particulars	Duration (days)	Average % hatch inhibition at different concentrations (mg ml^{-1})						
		0.1	0.5	1.0	2.0	4.0	6.0	8.0
Root extract	1	11.24 (19.55)	28.7 (32.37)	47.04 (43.28)	60.65 (51.15)	74.85 (59.88)	100 (89.96)	100 (89.96)
	3	18.94 (25.79)	28.56 (32.29)	47.09 (43.32)	62.11 (51.99)	77.13 (61.41)	92.15 (73.73)	100 (89.96)
	5	21.24 (27.43)	39.73 (39.06)	49 (44.41)	65.25 (53.86)	79.00 (62.69)	94.83 (76.99)	100 (89.96)
	7	20.79 (27.12)	40.36 (39.43)	48.73 (44.26)	67.63 (55.29)	79.05 (62.73)	94.27 (76.12)	100 (89.96)
	9	24.84 (29.88)	40.89 (39.74)	50.67 (45.36)	67.85 (55.44)	80.26 (63.59)	99.71 (86.86)	100 (89.96)
Non-polar fraction	1	11 (19.35)	23.97 (29.29)	37.05 (37.48)	45.44 (42.36)	54.83 (47.752)	68.94 (56.11)	85.51 (67.59)
	3	18.67 (25.58)	34.76 (36.11)	41.06 (39.83)	48.99 (44.41)	61.86 (51.84)	73.69 (59.12)	84.09 (66.45)
	5	21.12 (27.35)	32.99 (35.04)	49.87 (42.05)	52.48 (46.40)	64.75 (53.56)	75.89 (60.57)	86.12 (68.11)
	7	22.61 (28.38)	41.77 (40.24)	53.22 (42.73)	55.07 (47.89)	65.61 (54.08)	74.46 (59.62)	89.87 (71.41)
	9	19.66 (26.23)	43.72 (41.37)	55.91 (43.30)	57.44 (49.26)	63.32 (52.70)	76.85 (61.22)	90.51 (72.02)
Polar fraction	1	5.92 (14.04)	23.37 (28.89)	31.86 (34.14)	39.75 (39.07)	46.68 (43.06)	62.16 (52.04)	71.31 (57.59)
	3	19.42 (26.13)	29.12 (32.69)	38.76 (38.48)	45 (42.11)	50.92 (45.51)	65.95 (54.29)	73.46 (58.97)
	5	15.21 (22.94)	31.07 (33.86)	40.85 (39.71)	47.34 (43.45)	54.61 (47.6)	73.45 (58.97)	77.01 (61.35)
	7	23.88 (29.24)	35.49 (36.55)	45.4 (42.33)	48.72 (44.25)	54.61 (47.63)	74.51 (59.65)	78.19 (62.14)
	9	25.76 (30.49)	35.71 (36.68)	48.58 (44.17)	46.74 (43.11)	54.64 (47.64)	74.46 (59.63)	79.14 (62.79)

Figures in parenthesis are arc sine transformed, CD 5% A (Compounds)=0.37,B (Days)=0.47, C (Concentrations)= 0.56, A× B (Interaction between compounds × days)=0.82, A × C (interaction between compounds × concentrations)=0.97, B × C (interaction between days × concentrations=1.25, A × B × C (interaction between compounds, days and concentrations) =2.18

and mortality of *M. incognita*.

Procurement of raw material: The powdered roots of *I. racemosa* were subjected to Soxhlet extraction for 6-8 hr using chloroform as the solvent and was concentrated with a rotary vacuum evaporator at 40 ºC to afford crude extract. The resulting chloroform extract was then partitioned into petroleum ether (60-80 ºC) and acetonitrile to have non-polar and polar fraction by liquid-liquid partitioning (Bahl and Bahl, 1992).

Preparation of test concentrations: Since, little work has been reported on the nematicidal activity of *I. racemosa* root extract and its fraction. Preliminary trials were conducted to standardize the range of concentrations to be used in trials and concentrations ranging from 0.1 to 8.0 mg ml^{-1} were found to be effective for carrying out nematicidal bioassay. The stock solution of concentration 8.0 mg ml^{-1} was prepared by dissolving 0.8 g of each component (root extract, non- polar and polar fraction) in 100 ml of water along with Tween 80 as emulsifier. The serial dilutions were made using distilled water as required. Three replications for each treatment were performed. As Tween 80 was not showing any difference in preliminary trails, therefore only water was used as negative control in further studies.

Bioefficacy: All bioassays were set up in laboratory conditions under controlled temperature and humidity (27±2 ^{0}C and 70±1 %). Egg hatch and mortality studies (second stage juveniles, J_2) were conducted against root knot nematode *M. incognita.*

Hatching test: For extraction of egg masses the infected plants of brinjal were uprooted, carefully washed under tap water for removal of soil. The egg masses were isolated from roots and collected in Petri dishes containing water. Ten egg masses, with an average of 200-250 eggs per egg mass, were placed in 5 ml of each concentration (0.1-8 mg ml^{-1}) and control (water only). The plates were covered with solid lid and wrapped with parafilm® and kept in an incubator. To avoid microbial contamination separate Petri dishes were used for each day. Number of hatched juveniles was counted on 1st, 3rd, 5th, 7th and 9th day after incubation under light microscope. Each treatment was repli-

Table 2. Effect of *I. racemosa* root extract and its non-polar and polar fractions on percentage mortality of *M. incognita* at different concentrations and durations.

Particulars	Duration (days)	Average % mortality at different concentration (mg ml^{-1})						
		0.1	**0.5**	**1.0**	**2.0**	**4.0**	**6.0**	**8.0**
Root extract	2	15.9 (23.49)	32.8 (34.92)	40.3 (39.39)	48.1 (43.89)	58.5 (49.87)	64.2 (53.25)	100 (89.96)
	4	27.8 (31.81)	35.8 (36.73)	49 (44.41)	52.5 (46.41)	59.6 (50.51)	68.7 (55.83)	100 (89.96)
	6	38.3 (38.22)	49.0 (44.41)	53.8 (47.160)	56.2 (48.54)	60.4 (50.86)	75.0 (58.70)	100 (89.96)
	8	47.2 (43.38)	59 (50.165)	66.7 (54.73)	75.0 (59.97)	81.0 (64.13)	86.7 (68.58)	100 (89.96)
	10	59.0 (50.16)	75.6 (60.37)	85 (67.19)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)
Non-polar fraction	2	25.9 (30.58)	30.6 (33.57)	42.7 (40.78)	45.3 (42.32)	55.8 (48.31)	100 (89.96)	100 (89.96)
	4	28.9 (32.51)	37.9 (37.98)	45.0 (42.11)	53.1 (46.76)	60.7 (51.16)	100 (89.96)	100 (89.96)
	6	35.5 (36.56)	44.2 (41.65)	58.2 (49.69)	66.2 (54.43)	72.1 (58.09)	100 (89.96)	100 (89.96)
	8	40.2 (39.02)	52.5 (46.41)	75.0 (59.97)	81.7 (64.65)	93.4 (75.08)	100 (89.96)	100 (89.96)
	10	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)
Polar fraction	2	0 (0.18)	0 (0.18)	0 (0.18)	25.0 (29.99)	30.0 (33.21)	50.0 (44.79)	90.0 (71.54)
	4	10.8 (19.18)	22.4 (28.24)	28.9 (32.51)	30.2 (33.19)	42.0 (40.38)	55.3 (48.02)	92.1 (73.61)
	6	15.4 (23.09)	25.6 (30.38)	39.2 (38.75)	43.5 (41.25)	50.2 (45.09)	61.5 (51.63)	92.9 (74.52)
	8	30.0 (33.61)	49.9 (44.93)	50.0 (44.98)	67.2 (55.04)	69.1 (56.21)	70.4 (57.01)	95.0 (77.05)
	10	45.0 (42.11)	50.2 (45.09)	70.2 (56.89)	80.3 (63.41)	85.7 (67.75)	90.0 (71.54)	100 (89.96)

Figures in parenthesis are arc sine transformed, CD 5% A (Compounds)=0.75, B (Days)=0.97, C (Concentrations)=0.11, A× B (Interaction between compounds × days)=0.167, A × C (interaction between compounds × concentrations)=0.19, B×C (interaction between days × concentrations)=0.25, A × B × C (interaction between compounds, days and concentrations) = 0.44

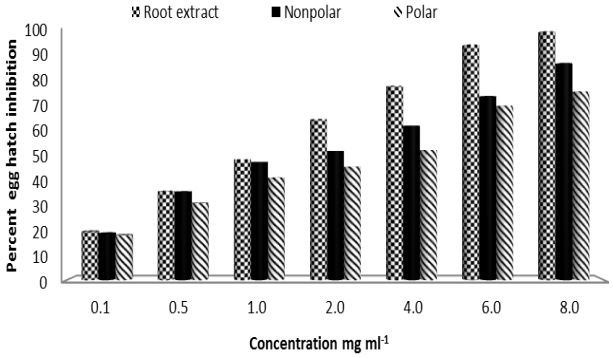

Fig. 1. *Comparative study of I. racemosa root extract and its fractions on average egg hatch inhibition of M. incognita exposed to different concentrations.*

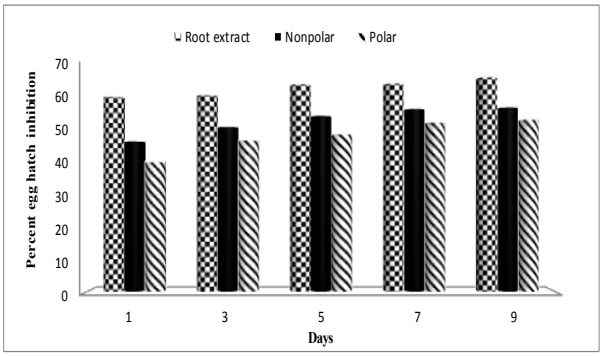

Fig. 2. *Comparative study of I. racemosa root extract and its fractions on average egg hatch inhibition of M. incognita w.r.t. duration.*

cated thrice. The percentage inhibition was calculated by the formula (Chahal *et al.,* 2016) and mean of three replications were presented (Table 1).

Mortality test: For mortality test, egg masses were handpicked using sterilized forceps from heavily infected roots. These egg masses were washed in distilled water, placed in 15 mesh sieve (8 cm in diameter) containing crossed layers of tissue paper to obtain freshly hatched juveniles. After complete hatching, nematodes were collected in a beaker and allowed to settle down. Excess water was decanted off. The number of nematodes juveniles was adjusted to 100 J_2/ml and number was counted under microscope. Counted number of juveniles were transferred to Petri dishes containing each concentration of test solution as well as control. Number of dead/alive nematodes was counted on 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} day of exposure. The plates were covered with solid lid and wrapped with parafilm® and kept in an incubator. The nematodes were considered dead if found motionless when probed with fine needle. After the last count, the inactive juveniles were maintained in distilled water for 24 hrs to observe their survival. The percent mortality was calculated (Chahal *et al*., 2016) using the formula and average mortality at a particular concentration was presented (Table 2).

Statistical analysis: Per cent egg hatch inhibition and per cent mortality data was subjected to statistical analysis using the factorial completely randomized design (CRD) statistical package. The critical differences in main effects *i.e.* compounds, concentrations and days as well as in their interactions were tested at P = 0.05.

RESULTS AND DISCUSSION

Egg hatching studies: The studies conducted on the *I. racemosa* root extract and its fractions inhibited egg hatching of *M. incognita* (Table 1). The maximum inhibition was observed at 0.8 mg ml^{-1} concentration for all the tested components. All the three components evaluated for their nematicidal efficacy were found to significantly (5%) reduce egg hatching of *M. incognita* as compared to control (water only). Maximum hatch inhibition was observed in root extract at 0.8 mg ml^{-1} where even at the lowest duration of 24 hrs egg hatching was completely inhibited. Minimum egg hatch inhibition was observed at lowest concentration of 0.1 mg ml^{-1}, where 11- 24 % decrease was observed in hatch inhibition of *M. incognita.* Egg hatching was inhibited in other concentration in the order of 0.1<0.5<1.0<2.0<4.0<6.0<8.0 mg ml^{-1}indicating that reduction in egg hatch was concentration dependent. The studies on the effect of duration (1, 3,5,7,9 days) revealed that hatching decreased from 1^{st} to 9^{th} day. Percent egg hatch inhibition was higher when the egg masses were exposed to longer duration (Fig. 1). Of the three components evaluated *I. racemosa* root extract and its fractions, root extract was found to be most effective at all the duration followed by non polar and polar fractions. Hence the order of activity was:Root extract > Non-polar > Polar fraction. Percent egg hatch inhibition by root extract ranged from 60-70 % at different durations while in non-polar fraction the inhibition was found to be between 40-60 % and in polar 40-50 % respectively (Fig. 2).

Mortality: The mortality of *I. racemosa* root extract and its fractions revealed that juvenile mortality increased with increase in exposure time and concentration and mortality differed significantly among different concentrations and exposure times (Table 2). Only 15.9 % mortality occurred at 0.1 mg ml^{-1} on 2^{nd} day and increased to 59 % on 10^{th} day, while at 0.5 mg ml^{-1} concentration 32.8 % J_2 mortality was observed on 2^{nd} day which increased upto 75.6 % on 10^{th} day of exposure. Maximum mortality was observed at 8.0 mg ml^{-1} of root extract and non-polar fraction where complete mortality was observed on 2^{nd} day of exposure. Significant difference (5%) in J_2 mortality was observed at different concentrations for root extract and the same effect was observed for non-polar and polar fractions. Nonpolar fraction and root extract were significantly at par at 0.5 mg ml^{-1} where J_2 mortality was observed between 30-50 %. In non polar fraction maximum mortality was observed for all the concentrations on 10^{th} day and for all the days at 6.0 and 8.0 mg ml^{-1}

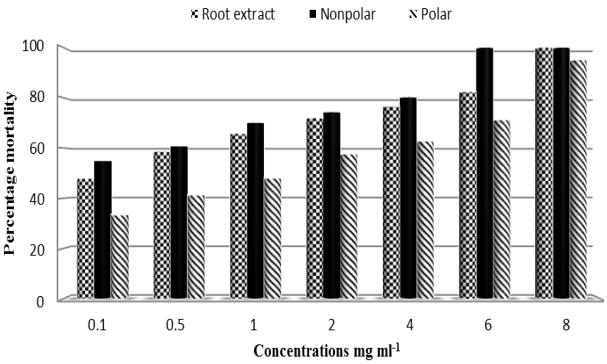

Fig. 3. *Comparative study of I. racemosa root extract and its fractions on percent mortality of juveniles of M. incognita exposed to different concentrations.*

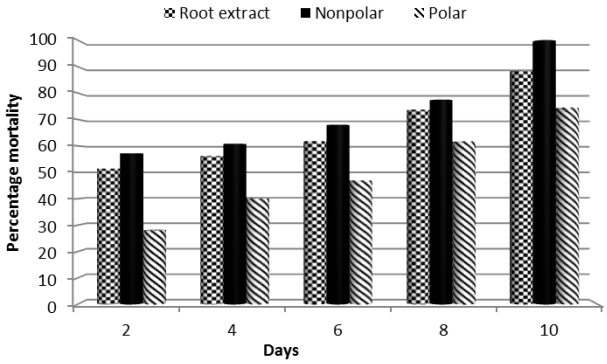

Fig. 4. *Comparative study of I. racemosa root extract and its fractions on percent mortality of juveniles of M. incognita w.r.t time.*

concentrations. Polar fraction was found to be least effective as no mortality was observed at lower concentrations (0.1-1.0 mg ml^{-1}) on 2nd day of exposure. On comparing the average mortality with duration (Fig. 3) and concentration (Fig. 4), all the three components show linear increase in number of days and concentrations. Therefore, the order of effectiveness against J_2 mortality is as follows: Non-polar > Root extract > Polar fraction.

In vitro evaluation of nematicidal properties of *I. racemosa* root extract and its fractions showed significant reduction in egg hatch count as well as enhanced mortality of second stage juveniles of *M. incognita.* Numbers of botanical plants and their constituents have been reported to possess nematicidal activity against root knot nematodes (Renco *et al*., 2014). Adegbite (2011) reported egg inhibitory effect of several indigenous plant extracts (*Azadirachta indica, Chromolaena odorata, Nicotiana tabucum,Carica papaya, Cannabis sativa, Cassia alata* and *Vernonia amygdalina*)against *M. incognita.* The nematicidal activity of essential oils extracted from several species(*Calendula maritima*, *Calendula officinalis*, *Calendula suffruticosa*, *Crysanthemum coronarium* and *Crysanthemum segetum*) of family Asteracae against *M. artiellia* has been investigated and the strongest nematicidal activity was exhibited by the essential oil obtained from the flower heads of *Chrysanthemum coronarium* (Perez *et al*., 2003). However very less reports are available on nematicidal properties of *I. racemosa* root extract and its fractions. Out of the three components tested, *I. racemosa* root extract was found to be most active as egg hatch inhibitor. The chemicals present in the plant extracts either affected the embryonic development or killed the eggs or even dissolved the egg masses. The egg hatch inhibitory effect of root extract might be due to permeability of egg shells of *M. incognita* to the compounds present in extract which are toxic to the developing juveniles. The plant extracts contained alkaloids, flavonoids, sesquiterpenoids, saponins, amides including benzamide and ketones that singly and in combination inhibited hatching (Adegbite, 2003; Goswamiand Vijayalakshmi, 1986; Chitwood, 2002). The nematicidal properties of *I. viscosa* leaf extract and its formulations have been reported against *M. javanica* (Oka *et al*., 2001; Oka *et al*., 2006) which was found to be due to the presence of some pharmacologically active compounds (Ulubelen *et al*., 1987; Wollenweber *et al*., 1991) including sesquiterpenes, sesquiterpene acids (Marongiu *et al*., 2003), azulenes, lactones, flavonoids and essential oils (Lauro and Rolih, 1990). Number of compounds with nematicidal activity had been isolated from species in family Asteracae (Chitwood, 2002; Gommers, 1981), out of these major compounds are sesquiterpenelactones which are very well documented for their biological activities (Hong *et al*., 2007). Sesquiterpenelactones isolated from different plant species have been known to show nematicidal, plant growth regulatory, antifungal and insecticidal properties (Datta and Saxena, 2001; Barrero *et al*., 2000; Liu *et al*., 2006; Ma *et al*., 2013, Wu *et al*., 2016). Chemical profiling of the root essential oil of *I. racemosa* showed the presence of mainly sesquiterpenes (60%),and other minor constituent were apiotaxene (22 %) and phenylacetonitrile (2 %) (Bokadia *et al*., 1986). Jamna *et al*. (2012) reported the presence of tannins, sterols and alkaloids and absence of triterpenoids and flavonoids in the roots of *I. racemosa*. The sesquiterpenelactones isolated from the roots of *I. racemosa* are mostly eudesmanolides with specific alantolide skeleton (Kalsi *et al*., 1989; Wang *et al*., 2000). The roots of *I. racemosa* are rich in sesquiterpenelactones (Seca *et al*., 2014; Goyal *et al*., 1990), specifically those containing α-methylene-γ-lactone moieties.

In present study, non-polar fraction of *I. racemosa* root extract was found to be most lethal against J_2 of *M. incognita,*whereas root extract was found to be more effective as egg hatching inhibitor, depicting larvicidal properties of non-polar fraction and ovicidal properties of root extract. The polar fraction was found to be least active among all the tested components, showing that less polar components were more active and the results are in accordance with the findings of Barrero *et al*., (2000) who reported low toxicity of polar as compared

to non-polar sesquiterpenelactones against fungus, *Cunningham ellaechinulata*. Alantolactone and isoalantolactone isolated from the roots of *I. racemosa* were found to be effective against juveniles of *M. incognita*. In the present study exposure of the nematode juveniles to 1100 µg ml^{-1} of alantolactone for 24 hrs caused a mortality of 97 % relative to the control. Sesquiterpenoid lactones having α-methylene-γ-lactone moiety were found to be more effective than other compounds devoid of this moiety. Alantolactone was found to be most effective among all the tested components (Mahajan*et al*., 1986). Alantolactone has also been known to possess anthelmintic activity (Bourrel *et al*., 1993).

I. racemosa root extract and its non-polar and polar fractions showed nematicidal properties against *M. incognita.*Root extract was found to be most active (100%) as egg hatch inhibitor at 8 mg ml^{-1}showing ovicidal properties and non-polar fraction at 6 and 8 mg ml^{-1}against J_2 of *M. incognita* showing larvicidal properties. Nematicidal properties of *I. racemosa* root extract and its fractions might be due to the presence of sesquiterpenelactones, (alantolactone and isoalantolactone) especially those containing α-methylene-γ-lactone moiety. The studies will be needed to test the efficacy of extract and its fractions under field conditions. Further studies are required to isolate bioactive compounds from this extract responsible for egg hatch inhibition as well as mortality of *M. incognita* so that commercial nematicide can be developed for its practical use throughout the world.

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