International Journal of Biology
www.ccsenet.org/journal.html
Vol. 1, No. 1
January 2009

Study of Bioassay the Allelopathical Effect of Neem (*Azadirachta indica*) n-hexane, Acetone and Water-soluble Extracts on Six Weeds

Zoheir.Y.Ashrafi (Corresponding author)
M. Sc. of Weed Science Faculty of Agronomy and Animal Science
University college of Agricultural and Natural Resource, Karaj Paradaise
University of Tehran and Lecture at Payame Noor University (PNU), Tehran, Iran
Tel: 98-261-280-1011 E-mail: zoheir1980@gmail.com

Sedigheh Sadeghi
M. Sc. of Weed Science Faculty of Agronomy and Animal Science
University college of Agricultural and Natural Resource, Karaj Paradaise
University of Tehran and Lecture at Payame Noor University(PNU), Tehran, Iran
Tel: 98-229-236-1357 E-mail: sadeghi58@gmail.com

Hassan.M.Alizade
Professor of Department Weed Science, Faculty of Agronomy and Animal Science
University college of Agricultural and Natural Resource, Karaj Paradaise, University of Tehran

Hamid.R.Mashhadi
Professor of Department Weed Science, Faculty of Agronomy and Animal Science
University college of Agricultural and Natural Resource, Karaj Paradaise, University of Tehran.

Ebrahim.R.Mohamadi
M. Sc. of Weed Science Faculty of Agronomy and Animal Science
University college of Agricultural and Natural Resource, Karaj Paradaise, University of Tehran

The research is financed by University of Tehran (Sponsoring information)

Abstract

Azadirachta indica, or Neem Tree, is an evergreen tree native to Southeast Asia. All parts of the tree have been used medicinally for centuries. The allelopathic potential of extracts of *Azadirachta indica* L., which is one of the most dominant weeds in tropical regions of South-west Asia, was investigated under laboratory conditions. The n-hexane-soluble, acetone-soluble and water-soluble fractions obtained from the acetone extract of *A. indica* shoots inhibited the germination and the growth of roots and shoots of six test plant species. The inhibitory activity of the water-soluble fraction was greatest, followed by that of the n-hexane-soluble and acetone-soluble fractions in all bioassays. Significant reductions in the germination and growth of the roots and hypocotyls were observed as the extract concentration increased. The concentration-dependent responses of the test plants to the fractions suggested that all three fractions might contain allelochemicals, but that the greatest potential was in the water-soluble fraction. These

results indicate that *A. indica* may produce potent allelochemicals, which should be investigated further in the laboratory and the field.

Keywords: Neem, *Azadirachta indica*, Bioassay, Allelopathy, Germination, Tropical weed

1. Introduction

A number of weed and crop species have been reported to possess allelopathic activity on the growth of other plant species (Rice, 1984). Chemicals with allelopathic activity are present in many plants and in many organs, including leaves, flowers, fruits and buds (Ashrafi et al, 2007; Putnam & Tang, 1986; May & Ash, 1990; Mahall & Callaway, 1991; Inderjit, 1996).

Azadirachta indica, or Neem Tree, is an evergreen tree native to Southeast Asia. All parts of the tree have been used medicinally for centuries. It is widely used in toothpastes, soaps and lotion today, as well as being a biological insecticide. *Azadirachta* is a genus of two species of trees in the flowering plant family Meliaceae. Numerous species have been described in the genus but only two are currently recognized, *A. excelsa* (Jack) Jacobs, and the economically important Neem tree, *A. indica* A. Juss. (Ashrafi et al, 2008; Mabberley et al. 1995, Pennington & Styles 1975). The need to reduce harmful environmental effects from the overuse of herbicide has encouraged the development of weed management systems, which are dependent on ecological manipulations rather than agrochemicals (Liebman and Ohno, 1997). Allelopathy has been defined as an adverse influence of one plant or micro-organism on another (Rice, 1984). In agricultural practice, allelopathy is exploited for weed control (Kohli et al., 1998). Neem (*Azadirachta indica*. A. Juss) is a versatile tree native to South and South-East Asia, Japan, tropical USA, South America, Australia and Africa. Its various plant parts have been traditionally used to control domestic insects, pests in stored grains, crop pests and in human and livestock medicine. Recently, these properties have been attributed to hundreds of chemicals present in the tree. Neem trees have many unique compounds that have been identified (Sankaram, 1987). The more common and the most analyzed compounds include nimbin (anti-inflammatory), nimbidin (anti-bacterial, anti-ulcer, analgesic, anti-arrhythmic, anti-fungal), nimbidol (anti-tubercular, anti-protozoan, anti-pyretic), gedunin (vasodilator, anti-malaria, anti-fungal), sodium nimbinate (diuretic, spermicide, anti-arthritic), quecetin (anti-protozoal), salannin (repellent), and azadirachtin (repellent, anti-feedant, anti-hormonal) (Sankaram, 1987). Because neem may contain a number of useful chemicals, with multiple uses and adaptability to diverse habitats and climatic conditions, interest in the tree has increased. However, very few reports of neem's allelopathy have been published. Under certain conditions, these compounds are released into the environment, either as exudates from living tissues or by decomposition of plant residues in suficient quantities to affect neighbouring or successional plants (Ashrafi et al, 2007; Bhowmik & Doll, 1982; Putnam, 1988; Inderjit & Dakshini, 1992; Einhellig, 1996). Evidence for allelopathy has accumulated in the literature over many years and many kinds of allelochemicals have been isolated and characterized from various plants (Bell, 1981; Duke, 1986; Putnam, 1988; Gross & Parthier, 1994; Seigler, 1996). However, little information is available concerning the allelopathic potential of tropical and subtropical plants. *Azadirachta indica* L., a perennial and prostrate-growing member of the *Meliaceae*, is one of the most dominant weeds of fields in tropical regions of Iran, South-east and South-west Asia. It was therefore of interest to test the allelopathic potential of this species, using extracts obtained under laboratory conditions.

2. Materials and methods

2.1 Plant material and extraction

Shoots of *Azadirachta indica* var, hirsute were harvested from an experimental field at Zabol University (Sistan State), Iran, washed thoroughly with tap water and rinsed with distilled water. After blotting dry with filter paper (No. 1; Whatman), the shoots (1 kg fresh weight) were homogenized in 5 L of 70% (V/V) cold aqueous acetone and the homogenate was filtered through filter paper (No. 1; Whatman). The residue was homogenized again with 5 L of 50% (V/V) cold aqueous acetone and filtered. The concentrate was divided into n-hexane-soluble, acetone-soluble and water-soluble fractions, and the fractions were evaporated to dryness as described by Kato-Noguchi et al. (1994).

2.2 Bioassay for germination studies

Six species, *Amaranthus rotundus* L. (cockscomb), Canada thistle (*Cirsium arvense*) (L.), *Digitaria sanguinalis* L. (crabgrass), Wild mustard (*Sinapis arvensis* L.), lettuce (*Lactuca sativa* L.) and ryegrass (*Lolium ultiforum* Lam.) were chosen for bioassay as test plants because of their known germination behaviors.

The residues of n-hexane-soluble (3.5 g), acetone-soluble (2.3 g) and water-soluble (8.2 g) fractions were dissolved in a small volume of n-hexane, acetone and distilled water respectively. Each of the solutions was added to a sheet of filter paper (No. 2; Whatman) in a 9-cm Petri dish and dried. The filter paper in the Petri dish was moistened with 10 mL of 3 mM phosphate buffer (pH 7.0) containing 0.05% (V/V) Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma). The concentrations of the residues of each fraction in the bioassay were 0, 0.01, 0.03, 0.1, 0.3 and 1 mg mL^{-1}.

Seeds of the test species were sterilized in a 2% (wt/V) solution of sodium hypochlorite for 15 min and rinsed in distilled water four times. Fifty seeds of each species were sown on filter paper in Petri dishes and allowed to germinate in the dark at 25 °C for 2 days (cress, lettuce, ryegrass and timothy) or 3 days (*A. caudatus* and *D. sanguinalis*). Then the germinated seeds were counted and the percentage germination was calculated by reference to that of control seeds which had been treated with plain solution without residue (0 mg mL^{-1}).

2.3 Bioassay for growth studies

The residues of n-hexane-soluble, acetone-soluble and water-soluble fractions were dissolved and added to a sheet of filter paper in a Petri dish and the filter paper was moistened with 10 mL of 3 mM phosphate buffer (pH 7.0) containing 0.05% (V/V) Tween 20, as described above. After sterilization and germination in the dark at 25 °C for 2 or 3 days, 50 germinated seeds of each of the six species were arranged on filter papers in Petri dishes and allowed to grow in the dark at 25 °C for 2 days (cress, lettuce and ryegrass) or 3 days (*A. caudatus*, *D. sanguinalis* and timothy). The shoot and root lengths of the seedlings were then measured with a ruler and the percentage length of seedlings was calculated by reference to the length of control plants treated with plain solution without residue (0 mg mL^{-1}).

2.4 Measurement of osmotic potential

The residues of n-hexane-soluble, acetone-soluble and water-soluble fractions were dissolved and added to a sheet of filter paper in a Petri dish. The filter paper was moistened with 10 mL of 3 mM phosphate buffer (pH 7.0) containing 0.05% (V/V) Tween 20 as described above and stored in the dark at 25 °C for 2 or 3 days. After filtration of solution in each Petri dish, the osmotic potential of the solution was determined by a Vapor Pressure Osmometer (5500; Wescor). Standard solutions of mannitol were prepared at different concentrations, as described by Hu & Jones (1997), and seeds or germinated seeds of test plants were incubated in the solutions in the dark at 25 °C. After 2 or 3 days, the germinated seeds were counted and the lengths of roots and shoots of the plants were measured as described above.

2.5 Statistical analysis

All experimental treatments were replicated five times in completely randomized block designs. The percentages of seed germination and seedling length were scaled so that control was 100% as described above, and means and SEs from five replicate experiments with 50 plants each were calculated.

3. Results and discussion

3.1 *Effect* of osmotic potential on bioassay

As extreme osmotic potential in test solutions for bioassay inhibits germination and growth of several plant species (Haugland & Brandsaeter, 1996; Hu & Jones, 1997), the effects of the osmotic potential of test solutions on the bioassay in these experiments were analyzed. The osmotic potential of all test solutions was less than 70 mmol kg^{-1}. Test plants for the bioassays were also incubated in a range of solutions with known osmotic potential. No effect of osmotic potential on germination, root growth and shoot growth of the test plants was detected up to 150, 100 and 300 mmol kg^{-1}, respectively, in agreement with the results of Hu & Jones (1997). Thus, the osmotic potential of the test solutions did not significantly affect germination, root growth and shoot growth of the test plants for the bioassay.

3.2 Activity on germination

The allelopathic potential of n-hexane-soluble, acetone-soluble and water-soluble fractions obtained from extracts of shoots of *A. indica* was tested with seed germination of lettuce (Fig. 1). All three fractions suppressed the germination of the seeds, but by far the greatest inhibition was observed in the bioassay of the water-soluble fraction. When the percentage germination rate was plotted against the logarithm of the concentrations, the response curves of the n-hexane-, acetone- and water-soluble fractions were linear between 10 and 40%, 10 and 30% and 10 and 90% inhibition respectively. The activities of the n-hexane- and acetone-soluble fractions were weak and complete response curves were obtained only with the water-soluble fraction. The concentrations required for 25% inhibition in the assay (defined as I_{25}) were 0.11, 0.61 and 0.026 mg mL) 1 for the n-hexane-, acetone- and water-soluble fractions, respectively, as interpolated from the response curves. Comparing I_{25} values, the inhibitory activity of the water-soluble fraction was 4.2- and 23-fold greater than that of the n-hexane- and acetone-soluble fractions respectively. The effects of the three fractions on seed germination of all six test species are summarized in Table 1. They were measured 2 or 3 days after the onset of the bioassay, once more than 70% of control plants had germinated. As described above, I_{25} values were determined after drawing the concentration-response curves. In all bioassays, the I_{25} value of the water-soluble fraction was smallest, followed in order by the n-hexane- and acetone-soluble fractions, confirming that the water-soluble fraction caused the greatest inhibition of seed germination. Additionally, all three fractions were more effective on the germination of dicotyledonous species (*A. caudatus*, cress and lettuce) than on the germination of monocotyledonous test species (*D. sanguinalis*, timothy and ryegrass).

3.3 Activity on seedling growth

Figure 2 shows the effects of the n-hexane-, acetone- and water-soluble fractions on the root growth of lettuce. All three fractions inhibited the growth of the roots, with the most marked inhibition being achieved by the water-soluble fraction. When the percentage length was plotted against the logarithm of the concentrations, although complete response curves were obtained only with the water-soluble fraction, the response curves of the n-hexane-, acetone- and water- soluble fractions were linear between 0 and 60%, 0 and 30%, and 0 and 90% inhibition respectively. As interpolated from the response curves, the I_{25} values in the assay were 0.032, 0.14 and 0.01 mg mL^{-1} for the n-hexane-, acetone- and water-soluble fractions respectively. Figure 3 shows the effects of the n-hexane-, acetone- and water-soluble fractions on the shoot growth of lettuce. These fractions inhibited shoot growth to a considerably less extent than root growth, the I_{25} values in the assay being 0.24, 0.78 and 0.049 mg mL^{-1} for the n-hexane-, acetone- and water-soluble fractions respectively. Increasing the concentrations of all fractions increased the inhibition of both root and shoot growth. The effects of these fractions on the root and shoot growth of all six test species were measured 2 or 3 days after onset of the bioassay and the I_{25} values were determined as described above (Tables 2 and 3). In all bioassays, the I_{25} value of the water-soluble fraction was smallest, followed in order by the n-hexane- and acetone-soluble fractions. The effectiveness of all three fractions on the roots of the test species was greater than that on the shoots of the same species. This observation agrees with that of Stachon & Zimdahl (1980) who found the ethanol extracts of *Cirsium arvense* L. (Canada thistle) more inhibitory to cucumber (*Cucumis sativis* L.) roots than to hypocotyls. The distinction between dicotyledonous and monocotyledonous species was less clear in shoot and root tests than in germination tests. Plant-to-plant interference is a complex combination of competition for resources such as light, nutrients and water, and allelopathic reaction (Fuerst & Putnam, 1983; Qasem & Hill, 1989), and distinguishing allelopathic effects from the competitive interference is diffcult (Leather & Einhelling, 1988; Inderjit & Dakshini, 1994). However, the seedlings of each test species used in these experiments were grown in a single Petri dish without intraspecies competition for resources, as young seedlings withdraw nutrients from the seeds and light is unnecessary in the developmental stage (Fuerst & Putnam, 1983). Thus, germination and growth inhibition of the test species are likely to have been caused by the allelopathic reaction rather than by competitive interference.

Significant reductions in the germination and growth of the roots and shoots were observed as the extract concentration increased. The results are in agreement with previous investigations in that the activity of either water-extracts or weed residues was directly related to the concentration of the residue rates (Caussanel, 1979; Chung & Miller, 1995; Babu & Kandasamy, 1997). Such concentration-dependent responses of the test plants to the fractions suggest that each fraction separated from the extract of *A. indica* might contain allelochemicals, that the allelopathic potential of the water-soluble fraction was greatest and that this fraction may contain the most active allelochemicals. This preliminary research suggests that *A. indica* contains potent allelochemicals that may enhance its efficiency as a weed. On the other hand, residues or aqueous extracts of the plant may be useful for weed management. It has been shown that some plant residues and extracts can work as weed inhibiting agents (Bhowmik & Doll, 1982; Putnam & Tang, 1986; Einhellig, 1996).

References

Ashrafi,Z. Y., H. R.Mashhadi, S. Sadeghi. (2007). Allelopathic Effects of Barley (*Hordeum vulgare*) on Germination and Growth of Wild Barley (*Hordeum spontaneum*). Pak. J.weed Sci.Res.13(1-2): 99-112.

Ashrafi,Z. Y., H. R.Mashhadi, S. Sadeghi, H. M. Alizade. (2008). Effects Allelopathical of sunflower (*Helianthus annuus*) on Germination and Growth of Wild Barley (*Hordeum spontaneum*). J. Agric. Tech. 4(1): 219-229.

Babu, R.C & O.S Kandasmy. (1997). Allelopathic effect of Eucalyptus globulus Labill. on Cyperus rotundus L. and Cynodon dactylon L. Pers. Journal of Agronomy and Crop Sciences 179, 123-126.

Bell, A. B. (1981). Biochemical mechanisms of disease resistance. Annual Review of Plant Physiology 32, 21-81.

Bhowmik, P. C & J.D Doll. (1982). Corn and soybean response to allelopathic effects of weed and crop residues. Agronomy Journal 74, 601-606.

Caussanel, J.P. (1979). Non-competitive effects between lamb's quarters (Chenopodium album L.) and maize (INRA 258). Weed Research 19, 123-135.

Chung, I. M & D. A Miller. (1995). Natural herbicide potential and alfalfa residue on selected weed species. Agronomy Journal 87, 920-925.

Duke,S. O. (1986). Naturally occurring chemical compounds as herbicides. Reviews of Weed Science 2, 17-65.

Einhelling, F.A. (1996). Interactions involving allelopathy in cropping systems. Agronomy Journal 88, 886- 893.

Furest,E.P. & A. R Putnam. (1983). Separating the competitive and allelopathic components of interference: theoretical principles. Journal of Chemical Ecology 8, 937-944.

Gross, D. & B. Paritheir. (1994). Novel natural substances acting in plant growth regulation. Journal of Plant Growth Regulation 13, 93-114.

Haugland, E. & L. O. Brnand. (1996). Experiments on bioassay sensitivity in the study of allelopathy. Journal of Chemical Ecology 22, 1845-1859.

Inderjit. (1996). Plant phenolics in allelopathy. Botanical Review 62, 186-202.

Inderjit & KM. Dakshini. (1992). Formation 7-O-glucoside (ononin), an additional growth inhibitor in soils associated with the weed, Pluchea lanceolata (DC) C.B. Clarke (Asteraceae). Journal of Chemical Ecology 18, 713-718.

Inderjit & KM. Dakshini. (1994). Allelopathic effect of Pluchea lanceolata (Asteraceae) on characteristics of four soils and tomato and mustard growth. American Journal of Botany 81, 799-804.

Kato,N.H, et al. (1994). Allelopathy of oats. I. Assessment of allelopathic potential of extract of oat shoots and identification of an allelochemical. Journal of Chem. Ecol. 20, 309-314.

Leather,G.R & F.A Einhelling. (1988). Bioassay of naturally occurring allelochemicals for phytotoxicity. Journal of Chemical Ecology 14, 1821-1828.

Mahall, B. E & R.M.Callaway. (1991). Root communication among desert shrubs. Proceedings of the National Academy of Science of the United State of America 88, 874-876.

May, F.E & J. E Ash. (1990). An assessment of the allelopathic potential of Eucalyptus. Australian Journal of Botany 38, 245-254.

Putnam, A. R. (1988). Allelochemicals from plants as herbicides. Weed Technology 2, 510-518.

Putnam, A. R & C. Tang. (1986). The Science of Allelopathy. John Wiley & Sons, New York, USA.

Qasem, J.R & T.A. HILL. (1989). On difficulties with allelopathy methodology. Weed Research 29, 345-347.

Seligler, D.S. (1996). Chemistry and mechanisms of allelopathic interactions. Agro. J. 88, 876-885.

Zimdahl, R.L. & W.J. Stachon. (1980). Allelopathic activity of Canada thistle (Cirsium arvense) in Colorado. Weed Science 28, 83-86.

Mabberley, D. J. et al. (1995). *Azadirachta*, pp. 337-343. In: *Flora Malesiana* ser. 1 Spermatophyta 12(1): 1-407.

Pennington, T. D. & B. T. Styles. (1975). A generic monograph of the Meliaceae. *Blumea* 22: 419-540.

Kohli, R.K., (1993). Allelopathic impact of sunflower on weeds and crops of Punjab. PSCST Project Report, India.

Kohli, R.K., Batish, D. & Singh, H.P., (1998). Allelopathy and its implications in agroecosystems. *J. Crop Prod.* **1**, pp. 169–202.

Liebman, M. & Ohno, T. (1997). Crop rotation and legume residue effects on weed emergence and growth: applications for weed management. In: Hatfield, J.L. *et al.*, 1997. *Integrated Weed and Soil Management*, Ann Arbor Press, Chelsea, MI, pp. 181–221.

Rice, E.L. (1984). Allelopathy, 2nd Edition. Academic Press, London, pp. 309–316.

SAS Institute. (1997). SAS User's Guide: Statistic. SAS Institute, Cary, NC.

Sankaram, A.V.B. (1987). Chemistry, biological activity and utilization aspects of some promising neem extractives. In: Proceedings of the Third International Neem Conference, Nairobi, Kenya, 10 July 1986, pp. 127–148.

Notes

Note 1. A laboratory experiment was conducted at Agricultural Campus, Tehran of University, in Karaj city May-2008.

Table 1. I_{25} of n-hexane-, acetone- and water-soluble fractions obtained from shoot extracts of Azadirachta indicafor seed germination. Means ±SE from five replicate experiments with 50 plants each are shown

		L_{25}(mg ml^{-1})		Control plants germination %
Test species	n-hexane soluble	Acetone soluble	Water soluble	
A. rotundus	0.11	0.71	0.021	76
Canada thistle	0.22	0.76	0.018	89
lettuce	0.09	0.64	0.031	84
D. sanguinalis	0.24	0.79	0.044	71
Wild mustard	0.29	0.89	0.047	85
Ryegrass	0.34	0.94	0.053	69

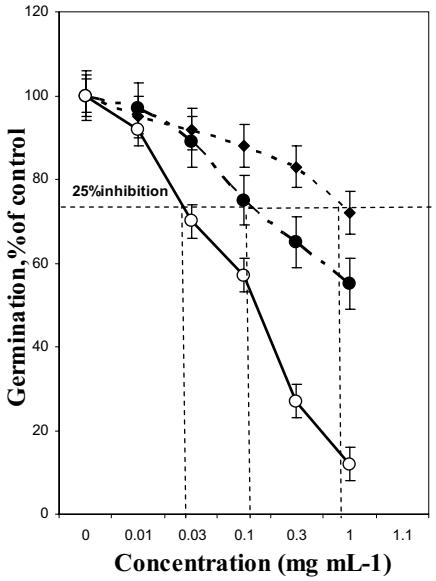

Figure 1. Effects of n-hexane- (●), acetone- (♦) and water-soluble (○) fractions obtained from shoot extracts of *Azadirachta indica* on germination of lettuce seeds. Means ± SE from 50 seeds are shown. Germination rate of control plants was 88 ± 7.7%.

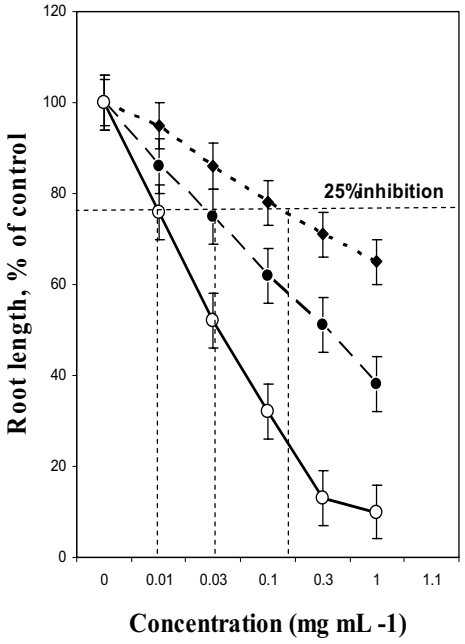

Figure 2. Effects of n-hexane- (●), acetone- (♦) and water-soluble (○) fractions obtained from shoot extracts of *Azadirachta indica* on root growth of lettuce. Means ± SE from 50 plants are shown. Length of control seedlings was 19.1 ± 1.4 mm.

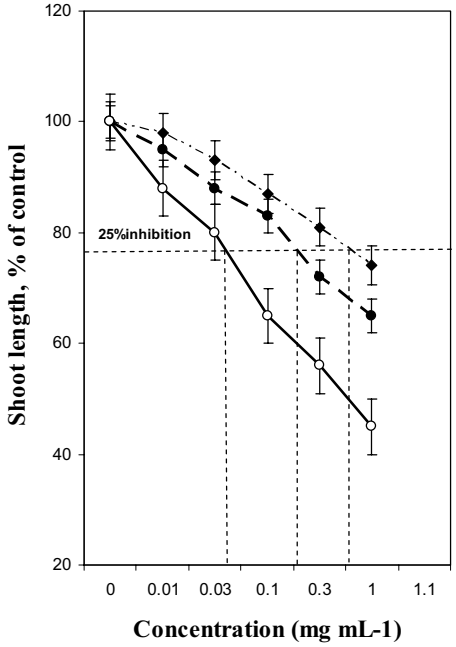

Figure 3. Effects of n-hexane- (●), acetone- (♦) and water-soluble (○) fractions obtained from shoot extracts of *Azadirachta indica* on shoot growth of lettuce. Means ± SE from 50 plants are shown. Length of control seedlings was 13.3 ± 0.7 mm.