Indian J. Plant Physiol., Vol. 14, No. 2, (N.S.) pp. 124-129 (April-June, 2009)

ANTIOXIDANT ACTIVITY OF MIMONOSIDES ISOLATED FROM *IN VITRO* REGENERATED PLANTS OF *MIMOSA HAMATA* WILLD.

SATISH C. JAIN[1]*, RENUKA JAIN[2] AND RENU SINGH[1]

[1]Medicinal Plants and Biotechnology Laboratory, Department of Botany, [2]Department of Chemistry, University of Rajasthan, Jaipur-302 004, India

Received on 8 April, 2009, Revised on 26 June, 2009

SUMMARY

Plants are major source for the discovery of new products of medicinal value for drug development. Different strategies including use of *in vitro* system, have been extensively studied to improve the production of plant chemicals. The present study is focused on the application of tissue culture technology for the production of some important plant pharmaceuticals. Under this, biosynthetic potentialities of *in vitro* regenerated plants of *Mimosa hamata* Willd. on Murashige and Skoog's (MS) medium have been evaluated for their phytochemicals and antioxidant activity. Three mimonosides A, B and C were isolated and identified on the basis of physical and spectral data from the n-butanol fraction of *in vitro* regenerated roots, using column chromatography. Similarly, in both *in vivo* and *in vitro* plant parts (root, stem and leaves) free radical scavenging activity was also studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and compared with standard antioxidants (quercetin and ascorbic acid). The extract of *in vivo* roots was found to be a good scavenger of DPPH radicals (RC_{50} 5 µg/ml) as compared to *in vitro* root extract (RC_{50} 9 µg/ml) comparable to the standards. It is evident from the results that *M. hamata* synthesizes useful bioactive metabolites and a potential source of natural antioxidant(s).

Key words: **Antioxidant activity, DPPH, *in vitro* regeneration, mimonosides, *Mimosa hamata***

INTRODUCTION

Mimosa hamata Willd., a much-branched straggling shrub occurs in tropical areas and widely distributed in India and Pakistan (Hussain *et al.* 1979). Traditionally, the rural people use this plant to cure urinary complaints and as a tonic against weakness. A paste of leaves is applied over glandular swellings and is used in dressing for sinus, sores and piles. Its roots possess contraceptive efficacy while seeds are used as a blood purifier (Nadkarni and Nadkarni 1954, Chopra *et al.* 1956). Various bioefficacies, viz. antifungal activity of deproteinized leaf extract (Mukadam *et al*. 1976, Umalkar *et al.* 1977), antibacterial activity of alcoholic extract of aerial parts (Hussain *et al*. 1979) and antiviral activity of the methanolic extract of roots (Jain *et al.* 1997a) have been studied. Various compounds, viz. 4-ethylgallic acid from flowers (Mehta *et al*. 1988), a triterpenic saponin B (3-O-L-arabinosyl-D-glucosyl morolic acid) from roots (Jain and Arora 1997), ethyl gallate and gallic acid from leaves (Hussain *et al.* 1979) and mimonoside A, B, C, and saponin A (3-O-D-glucosyl-L-rhamnosyl morolic acid) from its roots (Jain *et al*. 1997b) have been reported. Callus tissue is of particular use in micropropagation where it can be used to grow genetically identical copies of plants with desirable characteristics. However, no study on the cell cultures of *M. hamata* has been carried out so far. Therefore, in the present study we evaluated the phytochemical and antioxidant activity and biosynthetic potentialities of *in*

*Corresponding author, E-mail: jainnatpro3@rediffmail.com

vitro regenerated plants from *M. hamata* and compared with the *in vivo* system.

MATERIALS AND METHODS

Plant materials: Mature seeds of *M. hamata* Willd. were collected from the fields of Ajmer, Rajasthan, India, during July-August 2006 and identified by one of the authors, Prof. S. C. Jain. A voucher specimen (RUBL 9565) has been deposited in the Herbarium in the Department of Botany, University of Rajasthan, Jaipur, India. Seeds were washed under running tap water for 5 min, treated with a laboratory detergent (Labolene, Qualigens, India; 55% v/v) for 1 min followed by 3-4 washings with sterile distilled water. Later, the seeds were surface-sterilized by dipping in 70% ethanol for 30 s, later immersed in 0.1% (w/v) mercuric chloride by intermittent shaking for 2 min and washed thoroughly (3x) with sterile distilled water. These seeds were grown on growth hormone free basal Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) for 25 days in a growth cabinet with a 16 h photoperiod under normal light.

In vitro plant regeneration: Hypocotyl explants (~ 0.5×0.5 cm) were sectioned from the germinated seedlings (~ 5 cm in height) and incubated on MS medium supplemented with varying levels of growth regulators i.e. 1-naphthaleneacetic acid (NAA) and kinetin (Kn), either singly or in combination. Developed callus was transferred on MS medium (0.8% agar, pH 5.8-6.0) supplemented with different concentrations of NAA (0.5 -2.0 mg/l), Kn (0.5-3.0 mg/l), indole-3-acetic acid (IAA; 0.01-0.3 mg/l) and 6–benzylaminopurine (BAP; 0.5-2.0 mg/l) for shoot formation. The number of regenerated shoots was recorded after 4 weeks and excised from mother tissue, and cultured on MS medium supplemented with different concentrations of IAA (0.5-1.5 mg/l) + indole-3-butyric acid (IBA; 0.5-1.5 mg/l) for root induction. All the culture flasks were placed in growth chamber at 25±2ºC under 24 h photoperiod provided by white fluorescent light with 2000 Lux intensity and 60-65% relative humidity. Rooted shoots were hardened in thermocol cups (10 cm diameter) filled with autoclaved soil and manure (1:1). These were covered with clear polyethylene bags to ensure high humidity and maintained at 25±2ºC under 16/8 h light/ dark cycle in growth chamber. After 15 days, the micropropagated whole plants were collected, different plant parts (root, stem and leaf) were separated, dried in an oven at 60°C for 24 h and used for further studies. All the experiments were repeated thrice and the data was statistically analyzed.

Phytochemical analysis: 50 g of *in vitro* regenerated roots collected after 4 subcultures were dried, powdered and Soxhlet extracted with methanol for 24 h. The resultant extract was filtered and concentrated *in vacuo* (3.30%). Later, it was suspended in water and fractioned with petroleum ether, ether and n-butanol (cold). The resultant n-butanol fraction (reddish-brown) was slowly poured in acetone with continuous stirring until a light pink precipitate was obtained. The precipitate on filtration and drying under reduced pressure afforded an amorphous powder which gave a positive response for saponin test with vanillin-sulphuric acid (blue-violet colour). The dried precipitate was subjected to column chromatography over silica gel using chloroform and methanol (in order of increasing polarity) as solvents (Jain *et al.* 1997b; Table 2). Various fractions (25 ml each) were collected, concentrated and screened by thin layer chromatography (TLC; Si gel G). Fractions 1-9 (eluent: $CHCl_3$, $CHCl_3$-MeOH, 9:1 and 8:2) did not give any solid on drying, and were discarded. Fractions 10-19 (eluent: $CHCl_3$-MeOH, 7:3 and 6:4) resulted in three compounds which were purified after repeated crystallization. The melting points of the isolated compounds were determined in capillary tubes (Toshniwal Melting Point Apparatus) and each compound was subjected to infra-red spectral studies (Perkin Elmer 337, Grating Infra Red Spectrophotometer).

Determination of antioxidant activity: 10 g each of *in vivo* and *in vitro* regenerated plant parts (root, stem and leaf) were separately extracted with 100 ml methanol on a steam bath for 24 h. Each extract was filtered through Whatman No. 1 paper, freeze-dried (Heto Power Dry PL 3000) and stored in a refrigerator until used. For antioxidant assay, method of Fogliano *et al.* (1999) was adopted with minor modifications. For qualitative assay, extracts and quercetin as standard (20 mg) were dissolved in 1 ml methanol, out of which 1 µl

was applied on TLC plates (Si gel G $F_{254;}$ 20x20 cm; E. Merck). Later, these plates were sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH; 20 mg/10 ml) and exposed to daylight until discoloring of the background (6 h). The resulting yellow colour on the plates was determined as active antioxidant constituent(s). This method was also used for standards (ascorbic acid and quercetin). For quantitative assay**,** 8 mg of each of the extract was dissolved separately in 10 ml methanol and various concentrations (80, 60, 40, 20 and 10 µg) were prepared. 2.5 ml of the extract each was mixed with DPPH (2.5 ml; 20 mg/ 10 ml) and allowed to stand (30 min) for any reaction to occur. The absorbance of the colour developed was measured at 517 nm by UV spectrophotometer (Varian type Cary PCB 150 Water Peltier System with standard cuvettes). The standard ascorbic acid and quercetin as controls were also processed similarly. Three replicates were taken and the average mean was noted for each concentration. Data was processed using EXCEL and concentration, that cause 50% reduction in absorbance (RC_{50}), was calculated. Percent inhibition of DPPH was also calculated following Lee *et al.* (1998): % Inhibition = 1–(A_1/A_2) x100, where, A_1 is the absorbance of the test sample and A_2 is the absorbance of control reaction.

RESULTS AND DISCUSSION

In vitro plant regeneration: In *M. hamata*, swelling of the hypocotyl explants of seedlings was observed after 10-12 days of inoculation for induction of callus. Maximum response (80%) was observed on MS medium containing 20.0 mg/l NAA and 0.8 mg/l Kn. A maximum of 29.0±0.57 shoots were observed in the combination of Kn+IAA (3.0+0.1 mg/l) followed by at 3.0+0.2 mg/l (27.6±0.66) and 2.0+0.1 mg/l (22.0±0.57) shoots, respectively (Fig. 1). The regenerated shoots attained a length of 3-5 cm within 20 days. However, growth hormone free basal MS medium was least effective (2.0±0.3) in shoot proliferation. The results of rooting were observed (3.5±0.2 in number) on the MS media supplemented with 0.5 mg/l IBA having the frequency of 70% (Table 1). The well rooted plantlets were transferred to plastic cups containing a mixture of soil and manure (1:1). *In vitro* regeneration holds tremendous potential for the production of high quality plant-based medicines and successful secondary metabolites from various medicinal plants in cell cultures have been reported (Murch *et al.* 2000). In *Mimosa* species, only *in vitro* micropropagation of *M. tenuiflora* and *M. pudica* has been reported so far (Villarreal and Gabrielata 1999, Munshi *et al.* 2001). Therefore, this is the first report of whole plant regeneration in *M. hamata* on MS medium.

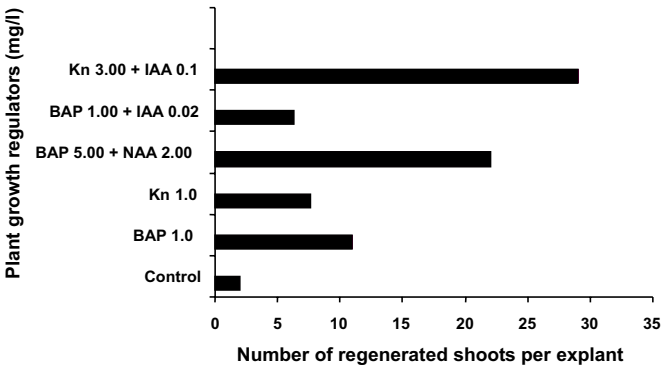

Fig. 1. Effect of plant growth regulators on shoot regeneration of *M. hamata*

Table 1. Rooting of *in vitro* derived shoots of *M. hamata* on MS medium fortified with different concentrations of IAA and IBA.

Hormone	Concentration (mg/l)	% of rooting	No. of roots/ shoots (Mean ±S.E.)
IAA	0.5	30	2.20 ± 0.10
	1.0	30	2.00 ± 0.30
	1.5	40	2.50 ± 0.20
IBA	0.5	70	3.50 ± 0.20
	1.0	50	2.80 ± 0.20
	1.5	40	2.50 ± 0.40

S.E. = Standard Error.

Phytochemical analysis: On column chromatographic separation, three mimonosides A, B and C were isolated from the n-butanol fraction of *in vitro* regenerated roots (Fig. 2) and identified on the basis of melting point (m.p.) and co-infra red (IR) with the authentic samples (Yamaguchi, 1970, Dev *et al.* 1989, Jain *et al.* 1997b).

Mimonoside A: White powder (3.48 mg); m.p. 248-50°C, gave positive test for saponins (blue-violet colour

Table 2. Mimonosides isolated from *in vitro* regenerated roots of *M. hamata.*

Fraction No.	Eluent	Crystallization solvents	Colour	Yield (mg)	m.p. (°C)	Identified compounds
1-4	$CHCl_3$	-	-	-	-	-
5-9	$CHCl_3$-MeOH (9:1, 8:2)	-	-	-	-	-
10-16	$CHCl_3$-MeOH (7:3)	MeOH+ $CHCl_3$	White	3.48	248-50	Mimonoside A
		MeOH+ $CHCl_3$	Light buff	2.97	240-42	Mimonoside B
17-19	$CHCl_3$-MeOH (6:4)	MeOH+ $CHCl_3$	White	3.12	252-54	Mimonoside C

$CHCL_3$, Chloroform; MeOH, Methanol.

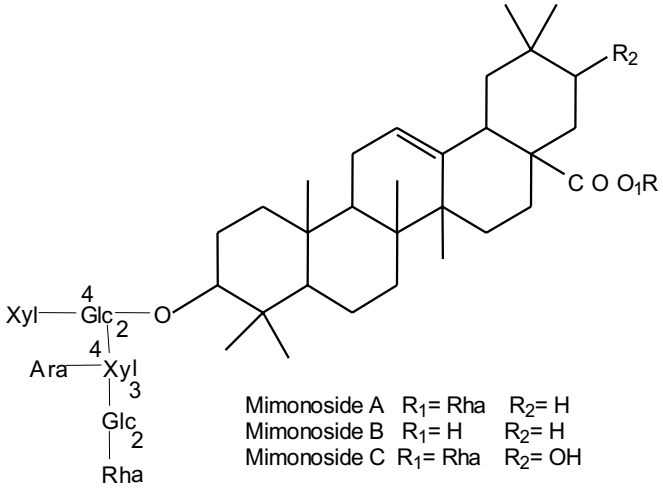

Fig. 2. Structures of isolated Mimonosides A-C

with vanillin-sulphuric acid), triterpenes (Liebermann-Burchard test), IR(υ_{max}) cm^{-1}: (KBr) 3350 (broad-OH groups), 1740, 1650, 1260, 1070, 1040.

Mimonoside B: Light buff amorphous powder (2.97 mg), m.p. 240-42°C, gave positive test for unsaturated triterpenoid saponins, IR (υ_{max}) cm^{-1}: (KBr) 3400-2750 (broad –COOH and –OH groups), 1735, 1640, 1250, 1085, 1030.

Mimonoside C: White amorphous powder (3.12 mg), m.p. 252-54°C, gave positive test for saponins (blue-violet colour with vanillin-sulphuric acid), triterpenes (Liebermann-Burchard test) and unsaturation (TNM test), IR(υ_{max}) cm^{-1}: (KBr) 3400 (broad –OH groups), 2920, 2880, 1745, 1650, 1480, 1385, 1365, 1250, 1085, 1030. Earlier these three mimonoside A, B, C were reported from roots of *M. hamata* and from *M. tenuiflora in vivo* conditions (Anton *et al.* 1993, Jain *et al.* 1997b) and no report on isolation of these mimonosides from *in vitro* conditions are available to date. It is evident from the results that this plant showed the similar biosynthetic potentialities of *in vitro* system in comparison to *in vivo* system.

Antioxidant activity: Highest antioxidant activity *in vivo* extracts, was demonstrated in roots, that is similar to standard ascorbic acid (RC_{50} 5 µg/ml), and in *in vitro* extracts, stem also possessed very high activity (RC_{50} 8.5 µg/ml) which is also comparable to standard quercetin (RC_{50} 8 µg/ml; Fig. 3). Similarly, *in vivo* roots had significant potential in % inhibition of DPPH (97.07%) which is similar to standard ascorbic acid and *in vitro* system, stem part showed highest inhibition (68.95 %) of DPPH free radicals (Table 3).

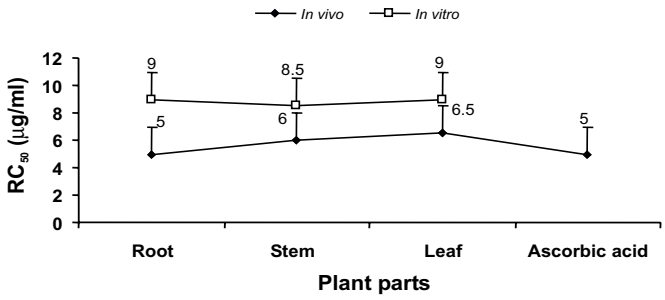

Fig. 3. *In vivo* and *in vitro* antioxidant activity of *M. hamata*

The DPPH antioxidant assay is based on the principle that this is a stable free radical, is able to decolourise in the presence of free radical scavengers

Table 3. Antioxidant activity of methanolic extracts from *M. hamata.*

Plant parts	RC_{50} (µg/ml)		Percent inhibition of DPPH (µg/ml)									
			In vivo					*In vitro*				
	In vivo	*In vitro*	10	20	40	60	80	10	20	40	60	80
Leaf	6.5	9.0	81.50	82.20	84.52	85.42	88.32	59.60	60.81	62.32	62.91	63.01
Stem	6.0	8.5	83.25	89.72	92.37	93.45	96.12	64.91	65.08	66.92	67.31	68.95
Root	5.0	9.0	95.10	96.00	96.55	96.62	97.07	54.10	55.69	66.01	67.80	68.60
Ascorbic acid	5.0	-	97.60	97.60	97.60	97.70	97.70	-	-	-	-	-
Quercetin	8.0	-	62.42	80.58	93.38	93.82	94.71	-	-	-	-	-

(antioxidants). In the quantitative DPPH assay, both *M. pudica* and *M. rubicaulis* were the most active and showed a strong antioxidant activity with RC_{50} values of 2.10×10^{-2} and 1.90×10^{-1} mg/ml respectively (Genest *et al*. 2008). Similar results were obtained in this study that certifies that *M. hamata* can be used as a potential antioxidant agent.

In vitro propagation of medicinal plants with enriched bioactive principles is found to be highly useful for commercial production of medicinally important compounds. An improved understanding of the secondary metabolite pathway in economically important plants has resulted in their increased use (Vanisree *et al.* 2004). In the present study, a protocol has been established for whole plant regeneration from the cell cultures of *M. hamata* and three mimonosides A-C, which are potential bioactives, have been isolated from *in vitro* regenerated roots. The biological effect of these mimonosides has shown significant cytotrophic properties on murine (LMTK) and human cultured fibroblasts (Jiang *et al.* 1991a, b, Anton *et al.* 1993, Rivera-Arce *et al.* 2007). The significant mitogenic effect and cytotrophic properties on human fibroblast cells of these mimonosides can contribute to explain the traditional use of *M. hamata*. This study also suggests that the *in vivo* and *in vitro* extracts of this plant are a potential source of natural antioxidant agents.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the partial financial support from UGC and ICMR, New Delhi.

REFERENCES

Anton, R., Jiang, Y., Weniger, B. and Beck, J.P. (1993). Pharmacognosy of *Mimosa tenuiflora* (Willd.) Poiret. *J. Ethnopharmacol.* **28:** 153-157.

Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1956). Glossary of Indian Medicinal Plants. CSIR, New Delhi.

Dev, S., Gupta, A.S. and Patwardhan, S.A. (1989). CRC Handbook of Triterpenoids. Triterpenoids. Vol. II: Pentacyclic and Hexacyclic Triterpenoids, CRC Press, Inc., Florida.

Fogliano, V., Verde, V., Randazzo, G. and Retieni, X. (1999). A method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *J. Agric. Food Chem.* **47:** 1035-1040.

Genest, S., Kerr, C., Shah, A., Rahman, M.M., Saif-E-Naser, M.M., Nigam, P., Nahar, L. and Sarker, S.D. (2008). Comparative bioactivity studies on two *Mimosa* species. *BLACPMA* **7:** 38-43.

Hussain, N., Modan, M.H., Ghulan, S.S. and Zaidi, S.A.H. (1979). Antimicrobial principles in *Mimosa hamata*. *J. Nat. Prod.* **42:** 525-527.

Jain, R. and Arora, R. (1997). 3-O-L -Arabinosyl-D-glucosyl morolic acid from *Mimosa hamata*. *J. Med. Arom. Plant Sci.* **19:** 22-23.

Jain, R., Arora, R., Alam, S. and Jain, S.C. (1997a). Pharmacological evaluation of *Mimosa hamata* roots. *Fitoterapia* **68:** 377-388.

Jain, R., Arora, R. and Jain, S.C. (1997b). Saponins from the roots of *Mimosa hamata* Willd. *Indian J. Chem.* **36:** 61-64.

Jiang, Y., Anton, R., Massiot, G., Lavand, C.L., Teulon, J.M. and Girechot, C. (1991a). Structure of a new saponin from the bark of *Mimosa tenuiflora*. *J. Nat. Prod.* **5:** 1247-1253.

Jiang, Y., Massiot, G., Lavaud, C.L., Teulon, J.M., Guechot, C., Hagg-Berrurier, M. and Anton, R. (1991b). Triterpenoid glycosides from the bark of *Mimosa tenuiflora*. *Phytochem.* **30:** 2357-2360.

Lee, S.K., Mbwambo, Z.H., Chung, H.S., Luyengi, L., Games, E.J.C. and Mehta, R.G. (1998). Evaluation of the antioxidant potential of natural products. *Com. Chem. High T. Scr.* **1:** 35-46.

Mehta, B.K., Sharma, S. and Dubey, A. (1988). 4-Ethylgallic acid from two *Mimosa* species. *Phytochem.* **27:** 3004-3005.

Mukadam, D.S., Balkhande, L.D. and Umalkar, G.V. (1976). Antifungal activities in deproteined leaf extracts of weeds and non-weeds. *Indian J. Microbiol.* **16:** 78.

Munshi, M.K., Hossian, S.N., Islam, R., Hakim, L. and Hossain, M. (2001). Micropropagation of *Mimosa pudica* L. through *in vitro* culture and using gamma irradiation. *Plant Tissue Cult.* **11:** 151-157.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15:** 473-497.

Murch, S.J., Krishna, R.S. and Saxena, P.K. (2000). Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. *Plant Cell Rep.* **19:** 698-704.

Nadkarani, A.K. and Nadkarni, K.M. (1954). Indian Materia Medica. Popular Book Depot, Bombay.

Rivera-Arce, E., Chávez-Soto, M.A., Herrera-Arellano, A., Arzate, S., Agüero, J., Feria-Romero, I.A., Cruz-Guzmán, A. and Lozoya, X. (2007). Therapeutic effectiveness of a *Mimosa tenuiflora* cortex extract in venous leg ulceration treatment. *J. Ethnopharmacol.* **109:** 523-528.

Umalkar, G.V., Mukadam, D.S. and Nehemiah, A.K.M. (1977). Fungistatic properties of some deproteinised leaf extracts. *Sci. Cult.* **43:** 437.

Villarreal, M.L. and Gabrielata, R. (1999). *In vitro* propagation of *Mimosa tenuiflora* (Willd.) Poiret., a Mexican medicinal tree. *Plant Cell Rep.* **16:** 80-82.

Vanisree, M., Lee, C.Y., Lo, S.F., Nalawade, S.M. and Lin, C.Y. and Tsay, H.S. (2004). Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot. Bull Acad. Sin.* **45:** 1-22.

Yamaguchi, K. (1970). Spectral Data of Natural Products Vol. I. American Elsevier, New York.