Journal of Medicinal Plant Research

Volume 9 Number 1, 3 January, 2015
ISSN 2009-9723

Academic Journals

ABOUT JMPR

The Journal of Medicinal Plant Research is published weekly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (weekly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JMPR are peerreviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: jmpr@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The Journal of Medicinal Plant Research will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editorial Board

Journal of Medicinal Plants Research

Table of Contents: Volume 9 Number 1, 3 Janruary, 2014

ARTICLES

Research Articles

academicJournals

Vol. 9(1), pp. 1-7, 3 January, 2015
DOI: 10.5897/JMPR2014.5512
Article Number: 8ECAA8950096
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Anti inflammatory properties of the root tubers of *Gloriosa superba* and its conservation through micropropagation

Nikhila, G. S., Sangeetha, G. and *T. S. Swapna

Department of Botany, University College, Thiruvananthapuram-34, Kerala, India.

Received 10 July, 2014; Accepted 11 November, 2014

Gloriosa superba **L. (Liliaceae) is an important medicinal herb of Asia and Africa. The plant is used to cure ulcers, piles, cancer, gout, scrofula and act as abortifacient, anthelmintic, antipyretic and anti-inflammatory drug. The main aim of the proposed work is to evaluate the anti-inflammatory activity of *G. superba* and also conserve the same plant through the *in vitro* propagation. The anti-inflammatory activity was evaluated by cycloxygenase inhibition assay and 5- lipoxygenase inhibition assay. In the cycloxygenase inhibition assay, the percentage inhibition were found to be 3.38%, 26.27%, 43.22% for sample concentration 100µg/ml, 500µg/ml, 1000µg/ml respectively and in the lipoxygenase inhibition assay percentage inhibition were found to be 49.23%, 76.92%, 84.61% for sample concentration 100µg/ml, 500µg/ml, 1000µg/ml respectively. In lipoxygenase inhibition assay the methanol extract of *G. superba* tuber showed close percentage inhibition with that of the standard aspirin. The present study thus confirmed that methanol extract of root tubers of *G. superba* possessed good anti-inflammatory activity. At present, the plant is on the way of extinction due to its misuse, over exploitation and unscientific collection. Micro propagation is an important method to conserve this highly anti-inflammatory medicinal plant. *In vitro* studies of *G. superba* includes induction of callusing and organogenesis, using various explants. The results indicated that medium supplemented (MS) with auxin (NAA) (0.15 mg/l) and Benzylamino purine (BAP) (0.25 mg/l) induced callusing, with 2,4-D (0.5 mg/l) and Kinetin (0.25 mg/l) induced somatic embryogenesis, NAA (0. 5mg/l) and BAP (0.25 mg/l) promoted the formation of the maximum number of shooting and with NAA (0.25 mg/l) and BAP (0.15 mg/l) rooting was induced. Micro propagation will be helpful for the conservation and maximum utilization of this plant with high antiinflamatory potential along with the identification and isolation of useful bioactive molecules.**

Key words: Colchicine, colchicoside, gloriosine, lipoxygenase, cycloxygenase.

INTRODUCTION

Inflammation is an inevitable part of the complex biological response of body to harmful stimuli and is also a localized, nonspecific response to infection. The infected cells release chemical signals such as interleukins, gamma-Interferon, and histamine which promote the dialation of blood vessels, increase flow of blood to the infection site and causes the area to become red and warm. They also increase the permeability of

capillaries in the area and also produce oedema. The symptoms of acute inflammation include heat, pain, swelling, redness, and loss of function (Ilango et al., 2013). By blocking each alarm signal molecule, or triggering stop signal, the inflammatory process can be inhibited or suppressed (Nathan, 2002).

Inflammation is mainly caused due to the presence of some enzymes such as Phospholipase A2. When the Phospholipase A2 is activated, a fatty acid called arachidonic acid is released from the phospholipid membrane, which is a substrate for two enzymes namely Cyclooxygenase (COX) and Lipoxygenase (LOX). COX is a key enzyme responsible for the formation of prostaglandins from arachidonic acid. It has two different isoforms, designated COX-1 and COX-2. COX-1 is also called "housekeeping" enzyme (Palomer et al., 2002) which is present in most tissues and also responsible for the kidney and platelet function. But COX-2 is primarily present at the site of inflammation and expressed only in brain and spinal cord tissue and can also be induced in a wide variety of normal tissues by the hormones of ovulation and pregnancy, growth factors, oncogenes, and tumour promoters (Abada et al., 2006). Cycloxygenase-2 is an inducible isoform of COX-1 that catalyses the rate limiting step in the formation of prostaglandin from arachidonic acid. Inductions of COX-2 accelerate the cell growth, enhance the cell mobility and inhibit apoptosis. Overexpression of COX-2 causes tumorigenesis and at the same time the inhibition of COX-2 results in the reduction of tumour. Therefore, the inhibition of COX-2 could be an effective remedy in the prevention and treatment of cancer (Cao et al., 2002). The consequence of overexpression of COX-2 is also reported by Funk (Funk, 2001). Lipoxygenases catalyse the deoxygenation of poly unsaturated fatty acids in lipids (Lewis et al., 1990) and helps in the conversion of arachidonic acid into proinflammatory mediators called leukotrienes, which are potent molecules having diverse biological actions (Iranshahi et al., 2009).

In the present study, anti- inflammatory activity of a medicinally potent plant *G. superba* was evaluated along with its *in vitro* propagation. *G. superba* is an important perennial climbing herb among the medicinal plants (Chi et al., 2001) with brilliant wavy edged yellow and red flowers. It is one of the exported medicinal plants of India, which cure many ailments but may prove fatal on misuse (Ashok et al., 2011). *G. superba* is used to cure various respiratory disorders. The leaf sap is used as smoothening agent for pimples and skin diseases. The medicinal property of *G. superba* is due to the presence of bioactive compounds in different parts of the plant. Several secondary metabolites have been isolated from tubers, leaves and seeds (Ravindra et al., 2009). The plant is seasonal and the seed dormancy is an important factor that interfere the cultivation of this highly potent plant. The plant is commercially propagated from its root tubers. *G. superba* produces a biforked tuber and each of these forks has only one growing bud. Root tubers are brittle and liable to break easily. If the growing bud is subjected to any kind of damage, the tuber will fail to sprout. The vigour of the vine, its flowering and fruiting depends on the size of the tuber.

The V- shaped tuber is used for the treatment of haemorrhoids, cancer, chronic ulcers, leprosy and also for inducing labour pains. The tuber is also used as abortifacient, tonic, stomachic, anti-inflammatory and anthelmintic. When the root tubers are boiled with sesamum oil, it's then applied to affected arthritis joints to reduce pain (Joshi, 1993). The cultivation using root tuber is not enough for the production of maximum numbers of *G. superba* for commercial purposes. So the tissue culture is one of the way to conserve this highly medicinal and ornamental endangered plant. Micro propagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. The present study is planned to develop an effective protocol for propagation of *G. superba* L. *in vitro* and transplant of the commercially useful plant from laboratory to field condition along with evaluation of anti-inflammatory action of the plant.

MATERIALS AND METHODS

Anti-inflammatory studies

The dried powdered tuber was defatted with petroleum ether (60 to 80°C) by hot extraction in a soxhlet apparatus for 48 to 72 h. The defatted powder was further extracted with methanol for 72 h. This methanol extract was used for the evaluation of anti-inflammatory potential of *G. superba* by cycloxygenase inhibition assay and 5-lipoxygenase inhibition assay.

Lymphocyte culture preparation

Roswell Park Memorial Institute (RPMI) 1640 [HIMEDIA] media was used for Human Platelet Lysate (HPL) culture and the medium was supplemented with 20% heat inactivated Foetal Bovine Serum (FBS) and 20% antibiotics (Penicillin). The culture was then filtered using 0.2 µm pore sized cellulose acetate filter in completely aseptic conditions followed by addition of fresh plasma at a concentration of $1x10^6$ cellsml^{-1}, and incubation for 72 h. After addition of 1µl LPS (Lipopolysaccharides), culture was incubated for 24 h. Standard drug such as aspirin was used in the concentration of 100 µgml^{-1} from a stock of 100 mg ml^{-1} and the sample was added in the concentration of 100 µgml^{-1}, 500µgml^{-1} and 1000 µg ml^{-1} from a stock of 100 mg ml^{-1}. Culture was incubated for 24 h and then centrifuged at 6000 rpm for 10 min.

*Corresponding author. E-mail: swapnats@yahoo.com.

Table 1. *In vitro* anti inflammatory activity of *Gloriosa superba* L.

Sample concentration *(µg/ml)	COX assay % of inhibition	LOX assay % of inhibition
Standard drug	95.34	96.38
100	3.38	49.23
500	26.27	76.92
1000	43.22	84.61

*µg/ml – Microgram / milliliter

Supernatant was discarded and 200 µl of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton) was added. The incubation was done for 30 min at 4°C and the assay was done in pellet suspended in a small amount of supernatant (Copeland et al., 1994; Ilango et al., 2013).

Cycloxygenase assay (COX assay)

Procedure: The reagents used were 100 mm Tris HCl (pH 8)**,** 5µm Hemoglobin**,** 200µm arachidonic acid**,** 10% Trichloroacetic acid (TCA) in HCl, and 1% Thiobarbituric acid. Arachidonic acid was added to the pellet and incubation was done at 37°C. Then added 0.2 ml of 10% TCA in 1N HCl, contents were heated in a boiling water bath for 20 min followed by adding 0.2 ml of TBA, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632nm for COX activity (Copeland et al., 1994; Ilango et al., 2013).

5-lipoxygenase assay (LOX assay)

Procedure: For LOX assay 70 mg of linoleic acid and equal weight of tween 20 was dissolved in 4 ml of oxygen free water and followed by sufficient amount of 0.5N. Sodium hydroxide (NaOH) was added to yield a clear solution and then made up to 25 ml using oxygen free water. This was divided into 0.5 ml portions and flushed with nitrogen gas before closing and kept frozen until needed. The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The increase in optical density (OD) was measured in 234 nm (Reddanna et al., 1990; Ilango et al., 2013).

% inhibition was calculated using the formula: (C-T/ C) ×100 (C =Optical density of control, T = Optical density of Test)

In vitro studies

Plant material: *G. superba* L. plant was collected from the garden of University College, Thiruvananthapuram. Leaves and intermodal portions were used as explants which were surface sterilized in mercuric chloride solution for ten minutes followed by rinsing with double distilled water.

Nutritional medium*:* The murashige and skoog's medium were used for *in vitro* culture of *G. superba* (Murashige et al., 1962). MS medium supplemented with combinations of plant growth regulators (PGRs) like IAA (Indole – 3- acetic acid) + BAP (6 – Benzyl aminopurine), IBA (Indole- 3- butric acid) + Kinetin, IBA + BAP, 2, 4 D (2, 4 Dichlorophenoxy acetic acid) + Kinetin, NAA (α-Naphthalene acetic acid) +BAP and 2, 4 D + BAP were used to induce callusing and organogenesis. The surface sterilized explants were placed on MS medium supplemented with different concentrations of NAA and BAP (0.1 – 0.5 mg/l) for callus induction. Different concentrations of Kinetin, 2, 4-D and IBA (0.1 - 0.5 mg/l) was used for inducing somatic embryogenesis. The leaves and internodes were inoculated in MS medium supplemented with different concentrations of BAP alone and BAP in combination with NAA, 2, 4-D and NAA (0.1 - 0.5 mg/l) for multiple shooting. Regenerated shoots were transferred to medium supplemented with different concentration of NAA+ BAP, IBA+ BAP and IAA+ BAP (0.1 - 0.5 mg/l) for root induction. Healthy shoots with well developed roots were transferred to plastic cups containing sand and soil in the ratio 1:2. Polyethylene covers were placed over the plantlets for the first 2 weeks for acclimatization and then transferred to green house and finally acclimatized in the field.

RESULTS

Anti-inflammatory studies

The results showed that the methanol extracts of root tubers of *G. superba* possessed good anti-inflammatory activity. In the cycloxygenase inhibition assay, the percentage inhibition were found to be 3.38%, 26.27%, 43.22% for sample concentration 100 µg/ml, 500 µg/ml, 1000 µg/ml respectively and in the lipoxygenase inhibition assay percentage inhibition were found to be 49.23%, 76.92%, 84.61% for sample concentration 100 µg/ml, 500 µg/ml, 1000 µg/ml respectively (Table1) (Figure1).

In vitro studies

Different explants showed different response in MS media with various hormonal combinations.

Callus induction*:* The results indicated that MS medium supplemented with NAA (0.15 mg/l) + BAP (0.25 mg/l) induced 96% callus proliferation from internode within 17 days of inoculation(Table 2) (Figure 2A).

Embryogenesis*:* Maximum somatic embryogenesis was observed (98%) on the medium supplemented with 2, 4-D (0.5 mg/l) + Kinetin (0.25 mg/l) after 17 days of inoculation from the internode (Table 3) (Figure 2B).

Shoot induction: The multiple shooting of *G. superba* L. were observed (100%) on the MS basal medium supplemented with NAA (0.5 mg/l) and BAP (0.25 mg/l) within 36 days from the leaf explants with mean shoot

Table 2. Effect of PGRs on callusing from internode (after 17 days).

PGRs	PGRconc (mg/l)	FCI (%)
NAA+BAP	0.1+0.25	75
	0.15+0.25	96
	0.25+0.25	70
IBA+BAP	0.1+0.25	50
	0.15+0.25	44
	0.25+0.25	42
2,4-D+KN	0.1+0.25	36
	0.15+0.25	32
	0.25+0.25	29

PGR- Plant growth regulator, FCI (%) - frequency of callus induction

Table 3. Effect of PGRs on somatic embryogenesis from internode (after 17 days).

PGRs	PGRconc (mg/l)	FSE (%)
2,4-D+KN	0.5+0.25	98
	0.15+0.25	90
	0.25+0.25	79
IBA+KN	0.5+0.25	42
	0.15+0.25	39
	0.25+0.25	44
NAA+BAP	0.5+0.25	28
	0.15+0.25	27
	0.25+0.25	31

FSE(%) - Frequency of somatic embryogenesis

number 9 and shoot length of 7 cm (Table 4) (Figure 2C, E, F).

Root induction: In the present study among the different hormone combinations tried, maximum frequency of root induction (100%) was observed in MS media supplemented with hormone combination of NAA (0.25 mg/l)+ BAP(0.15 mg/l) after 36 days of inoculation from internode (Table 5) (Figure 2D).

Acclimatization: The plantlets were taken out from the rooting medium and washed in running tap water to remove the remnants of agar. Then the plantlets were put into Low Minimal Salt Medium (LMSM) and then transferred to a cup that containing sterilized sand and soil in the ratio 1:2. Polyethylene covers were placed over the plantlets for the first 2 weeks for acclimatization and then transferred to green house and finally acclimatized in the field (Figure 2- G, H, I).

DISCUSSION

In lipoxygenase inhibition assay, the methanol extract of *G. superba* tuber showed close percentage inhibition with that of the standard aspirin and hence proved that the methanol extracts of *G. superba* exert a preferential effect on the 5-LOX pathway. The present study thus confirmed that *G. superba* could be used as potent anti-inflammatory agent. The results are found to be in correlation with the previous studies which were done in carrageenan induced animal models (Abhishek et al., 2011; Jomy et al., 2009; Joshi et al., 2010).

In vitro callus induction and regeneration of healthy plants of *G. superba* L. was reported by Anirudha (Anirudha, 2011) and maximum callus proliferation was induced in B5 medium supplemented with NAA (2 mg/l) and kinetin (0.5mg/l) after 5 weeks of inoculation. But in the present study, maximum callus proliferations were obtained within 17 days of inoculation in MS medium. In

Table 4. Effect of PGRs on regeneration responses from leaf (after 36 days).

PGRs	PGRconc (mg/l)	FSI (%)	MSN	MSL (*cm)
NAA+BAP	0.5+0.25	100	9	7
	0.15+0.25	80	5	2.5
	0.25+0.25	90	11	10.2
IBA+KN	0.5+0.25	40	3	3
	0.15+0.25	50	4	1.5
	0.25+0.25	42	7	2.1
NAA+BAP	0.5+0.25	70	1	2.9
	0.25+0.15	60	1	2.2
	0.25+0.25	76	2	3.3

FSI- Frequency of shoot Initiation, MSN- Mean Shoot Number, MSL- Mean Shoot Length, *cm - centimeter

Table 5. Effect of PGRs on rooting response from internode (after 36 days).

PGRs	PGRconc (mg/l)	FRI (%)	MRN	MRL (*cm)
NAA+BAP	0.2+0.15	84	19	2.1
	0.25+0.15	100	26	2
	0.25+0.2	72	14	5
IAA+BAP	0.2+0.15	-	-	-
	0.25+0.15	19	7	1.4
	0.25+0.2	24	1	2.5
IBA+BAP	0.2+0.15	32	4	1
	0.25+0.15	-	-	-
	0.25+0.2	-	-	-

FRI - Frequency of root Initiation, MRN- Mean Root Number, MRL – Mean Root Length, *cm - centimeter

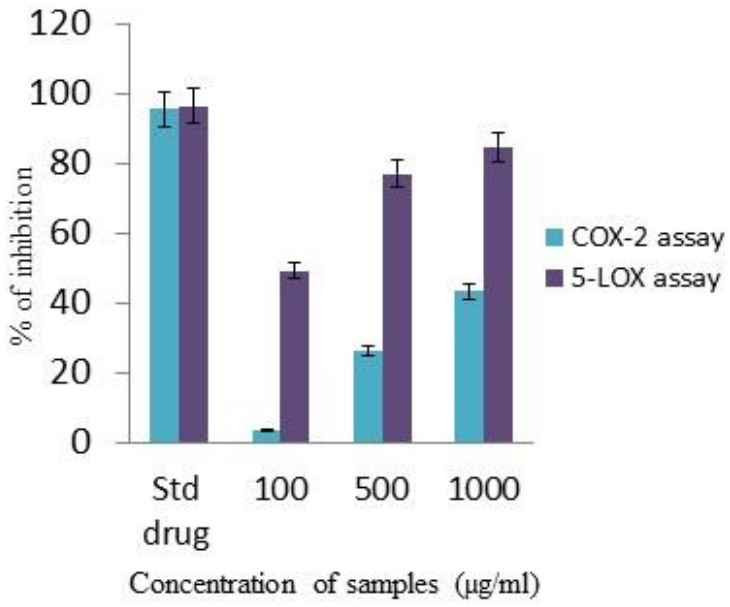

Figure 1. Percentage inhibition in COX and LOX assay.

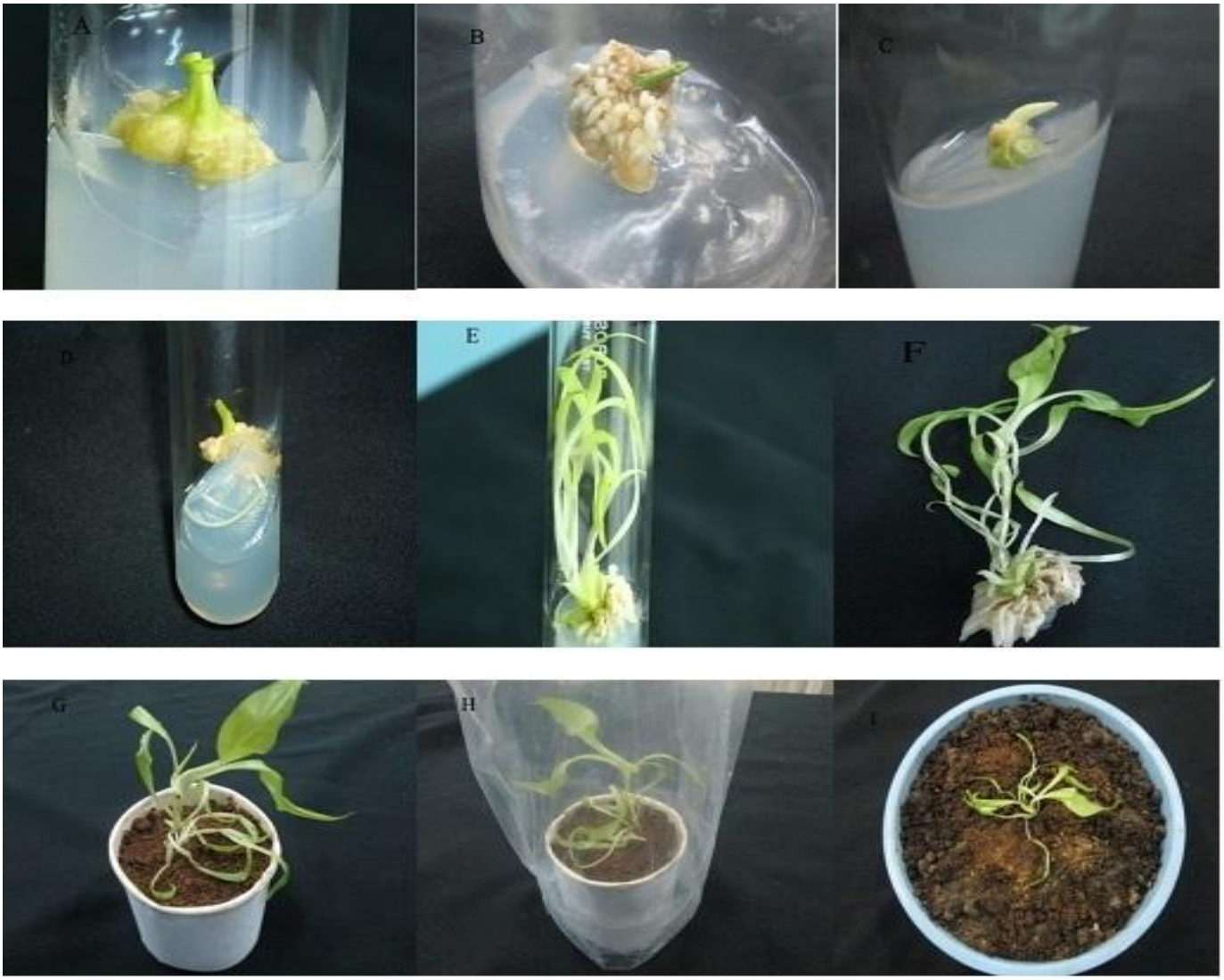

Figure 2. *In vitro* propagation of *Gloriosa superba* L. A- Callus induction, B- Somatic embryogenesis, C- Shoot induction, D- Root induction, E-F- Multiple shooting, G-I- Acclimatization.

comparison with the previous study, the hormone combinations used in the present study were better and faster for callus induction. Somatic embryogenesis and plant regeneration in *G. superba* L. was reported on MS medium supplemented with 2, 4-D (4 mg /l) + Kinetin (5 mg /l) + CH(10 mg /l) + CW(20%) (Jadhav and Hedge, 2001). But in the present study, maximum somatic embryogenesis was observed on the medium supplemented with 2, 4 -D (0.5 mg/l) + Kinetin (0.25 mg/l) along without CH and CW after 17 days of inoculation without subculture.

Multiple shoot formation in *G. superba* using tuber as explants were reported in MS and B5 medium (Ravindra et al., 2009). However, in the present study, NAA and BAP induced multiple shoots effectively from the explant. Indirect organogenesis with root induction of *G. superba* L. was reported in MS medium supplemented with 1.0mg/l IBA and 0.5 mg/l IAA (Sayeed et al., 2005) but direct root induction is not yet reported.

Conclusion

G. superba is found to be an important anti- inflammatory agent. This property may be due to the presence of bioactive compounds and the utilization of these potent compounds are helpful for the production of a new anti-inflammatory drug. Due to overexploitation and its unscientific collection *G. superba* has been endangered, therefore, there is an urgent need to conserve the plant by biotechnological approaches like tissue culture. In the present study, different hormonal combinations in MS

medium suitable for callusing, high frequency somatic embryogenesis and organogenesis of *G. superba* were standardized which will be helpful for conservation of the plant.

ACKNOWLEDGMENT

All authors are thankful to the Department of Botany, University College, Thiruvananthapuram, India for providing laboratory facilities to carry out this work.

Conflict of interests

The author(s) have not declared any conflict of interests.

REFERENCES

Abada MJ, Bessa AL, Ballarin B, Aragon O, Gonzales E, Bermejo P (2006). Anti-inflammatory activity of four Bolivian *Baccharis* species (Compositae). J. Ethnopharmacol. 103(3):338-344.

Abhishek M, Satish KV, Santosh KS, Deepika M, Prasad GBKS, Dua VK (2011). Investigation of anti-inflammatory properties of *Swertia chirayta* and *Gloriosa superba*. Recent Res. Sci. Technol. 3(3):40-43.

Anirudha R (2011). *In vitro* callus induction and regeneration of healthy plants of *Gloriosa superba* L. J. Fund. Appl. Life Sci. 1(1):64-65.

Ashok KK, Shagufta K, Sujata G, Amarjeet B (2011). *In vitro* shoot initiation from apical shoot buds and meristems of *Gloriosa superba* L. - an endangered medicinal herb of high commercial value. Res. 3(11):36- 45.

Cao Y, Prescott SM (2002). Many action of cyclooxygenase-2 in cellular dynamics and cancer. J. Cell. Physiol. 190(3):279-286.

Chi YS, Jong HG, Son KH, Chang HW, Kang SS, Kim HP (2001). Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: cyclooxygenases and lipoxygenases. Biochem. Pharmacol. 62(9):1185-1191.

Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S, Trazaskos JM (1994). Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. Proc. Natl. Acad. Sci. 91:11202-11206.

Funk CD (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294(5548):1871-1875.

Ilango K, Renjith AA (2013). Evaluation of *in vitro* anti-inflammatory activity of *Viburnum punctatum* Buch- Ham. Ex D. Don. Brit. Biomed. Bull. 1(1):18-22.

Iranshahi M, Askari M, Sahebkar A, Hadjipavlou-Litina D (2009). Evaluation of antioxidant, anti-inflammatory and lipoxygenase inhibitory activities of the prenylated coumarin umbelliprenin. DARU J. Pharm. Sci. 17(2):99-103.

Jadhav SY, Hedge BA (2001). Somatic embryogenesis and plant regeneration in *Gloriosa superba* L. Indian J. Exp. Biol. 39(9):943-946.

Jomy CJ, Jennifer F, Tanaji N, Samir RN, Alok S, Pradeep TD (2009). Analgesic and anti-inflammatory activities of the hydroalcoholic extract from *Gloriosa superba* L. Int. J. Green Pharm. 3(3):215-219.

Joshi CS, Priya ES, Mathela CS (2010). Isolation and anti- inflammatory activity of colchicinoids from *Gloriosa superba* seeds. Pharm. Biol. 48(2):206-209.

Joshi P (1993). Tribal remedies against snake bites and scorpion stings in Rajasthan. Glimpses Plant Res. 10:23-25.

Lewis RA, Austen KR, Soberman RJ (1990). Leukotrienes and other products of 5-lipoxygenase pathway, Biochemistry and relation to pathobiology of human disease. N. Engl. J. Med. 323(10):645-55.

Murashige TF, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 15(3):473-497.

Nathan C (2002). Points of control in inflammation. Nature 420:846-852.

Palomer A, Cabre F, Pascual J, Campos J, Trujillo MA, Enterna A, Gallo MA, Garcia A, Maulean D (2002). Identification of novel cyclooxygenase-2 selective inhibitors using pharmacophore models. J. Med. Chem. 45:1402-1411.

Ravindra A, Mahendra KR (2009). Review: Current advances in *Gloriosa superba* L. Biodiversitas 10(4):210-214.

Reddanna P, Whelan J, Maddipati KR, Reddy CC (1990). Purification of arachidonate 5-lipoxygenase from potato tubers. Methods Enzymol. 187:268-77.

Sayeed H, Shyamal KR (2005). Micropropagation of *Gloriosa superba* L. through high frequency shoot proliferation. Plant Tissue Cult. Biotech. 15(1):67-74.

academicJournals

Vol. 9(1), pp. 8-15, 3 January, 2015
DOI: 10.5897/JMPR2014.5622
Article Number: 8092BDA50098
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Compact callus cultures and evaluation of the antioxidant activity of *Hovenia dulcis* Thunb. (Rhamnaceae) under *in vivo* and *in vitro* culture conditions

Ivan Gonçalves Ribeiro[1], Carlos Roberto Machado Gayer[2], Tatiana Carvalho de Castro[1], Marsen Garcia Pinto Coelho[2] and Norma Albarello[1]*

[1]Laboratório de Biotecnologia de Plantas (Labplan), Departamento de Biologia Vegetal/IBRAG – Universidade do Estado do Rio de Janeiro, Brazil.
[2]Laboratório de Imunologia Aplicada e Bioquímica de Proteínas e Produtos Naturais (LIA-BPPN), Departamento de Bioquímica/IBRAG – Universidade do Estado do Rio de Janeiro, Brazil.

Received 12 September, 2014; Accepted 18 December, 2014

Plant biotechnology enables the production of biomass under controlled conditions, providing the synthesis of raw material in a continuous and homogeneous way. The aim of the present study was to establish *Hovenia dulcis* callus cultures and to evaluate their antioxidant potential in comparison with wild grown and *in vitro* plants. The best results for compact calli were obtained with the supplementation of 1-naphthaleneacetic acid (NAA) at 2.5 mg L^{-1} and the use of benzylaminopurine (BAP) enhanced callus growth. The medium supplemented with 2.5 mg L^{-1} NAA + 0.65 mg L^{-1} BAP produced 115.3±28.2 mg of dry weight. The auxin NAA was responsible for the production of light-green compact callus, while picloram and 2,4-D promoted mixed (friable and compact) calli. Total polyphenols and total flavonoids were found in higher concentrations in wild grown plants, whereas the reduction capacity and DPPH radical scavenging assays recorded higher antioxidant activity in calluses extracts. The protocols established here represent a viable and effective way for producing substances with medicinal interest.

Key words: Tissue culture, total phenolics, total flavonoids, 2,2-diphenyl-1-picrylhydrazyl (DPPH), medicinal plant.

INTRODUCTION

Plant-derived natural products have been widely investigated for the discovery and development of new pharmaceuticals. Plant tissue culture technology provides an attractive alternative for secondary metabolite production, offering the possibility of obtaining medicinal compounds and ensuring sustainable conservation and rational use of biodiversity (Coste et al., 2011; Coppede et al., 2014). Such techniques allow controlled cultivation, providing continuous and homogeneous synthesis of raw material, regardless of environmental and seasonal factors (Praveen and Murthy, 2011). Recently, plant tissue culture technology has been efficiently applied in

*Corresponding author. E-mail: albarellon@gmail.com. Tel: 55 21 2334-0293.

secondary metabolites production (Piekoszewska et al., 2010; Praveen et al., 2010; Pawar and Thengane, 2011). In addition, secondary metabolites are often presented in low amounts, which justify the search for alternative production methods. In this sense, callus cultures offer a useful system for *in vitro* production of secondary metabolites.

Hovenia dulcis Thunberg, known as Japanese raisin tree, is indigenous to East Asia. The natural occurrence ranges from Japan, Korea and East China to the Himalayas up to altitudes of 2000 m (Hyun et al., 2010). Extracts of *H. dulcis* act as detoxicant in alcohol poisoning and protect the liver against hepatotoxic substances (Kim, 2001; Xu et al., 2003). *H. dulcis* has medicinal properties, such as antigiardial activity (Gadelha et al., 2005) and others that were reviewed by Hyun et al. (2010).

The antioxidant activity of *H. dulcis* was reported in several extracts and related to different mechanisms. Antioxidant activity of pseudo fruit extracts was associated with the control of diabetes (Lee et al., 2005). So far there has been no research on the antioxidant activity of *H. dulcis* produced through tissue culture technologies.

Reactive oxygen species (ROS) and free radical mediated reactions have been implicated in degenerative or pathological processes (Tadhani et al., 2007). In living organisms, oxidative stress has been implicated in the formation of toxic compounds and cellular damage. Compounds capable of inhibiting or reducing injuries caused by ROS are known as antioxidants (Prior and Cao, 1999) and certain amounts of exogenous antioxidants are constantly required to maintain an adequate balance (Moo-Huchin et al., 2015). These molecules protect biological systems against damage caused by oxidation of macromolecules or cellular structures.

Taking into account the fact that *H. dulcis* is a tree and that its life cycle may be a step down for its exploitation, plant tissue culture techniques can supply the biomass necessary to its medicinal use. So far, callus and cell cultures of *H. dulcis* have not been studied in this context and may provide a reliable source of compounds of medicinal interest.

The purpose of this study was to evaluate the effects of plant growth regulators and explant source on the induction and establishment of *H. dulcis* callus cultures. This work also aimed to compare the antioxidant activity of cultured calluses, *in vitro* propagated plants and wild grown plants, in order to validate biotechnological strategies to produce bioactive compounds.

MATERIALS AND METHODS

Plant

Two-month-old seedlings obtained from *in vitro* germination and two-month-old *in vitro* propagated plantlets were used as explant sources. Seedlings were grown on MS medium (Murashige and Skoog, 1962) and the regenerated plantlets were obtained and maintained on MS medium containing 0.5 mg L^{-1} benzylaminopurine (BAP) and 0.5 mg L^{-1} 6-furfuryl-aminopurine (KIN) (Castro, 2001).

Leaves and seeds of *H. dulcis* from *in vivo* plants were collected after ripening in Teresópolis, Rio de Janeiro, Brazil. A voucher specimen (HRJ1426) is kept at the Herbarium of Rio de Janeiro State University.

Callus induction

Leaf (5 mm^2) and stem (5 mm) segments from both explant sources were inoculated in MS medium with 30 g L^{-1} sucrose and supplemented with the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), picloram (PIC) or 1-naphthaleneacetic acid (NAA) at different concentrations (0, 1.25, 2.5 or 5.0 mg L^{-1}). The medium pH was adjusted to 5.8 prior to autoclaving (121°C for 15 min) and solidified with 8 g L^{-1} agar (Merck). Five explants were inoculated in flasks (65 ×83 mm) containing 30 ml of culture medium in a total of five replicates, and the experiments were repeated three times. The flasks were kept in a growth chamber at 26±2°C under 16 h photoperiod provided by cool-white fluorescent tubes (45 μmol $m^{-2}s^{-1}$). Subcultures to media with the same composition were performed after 30 days. Callus morphological characteristics (consistency and color) and biomass growth based on dry weight (DW), were scored after 60 days. Dry weight of the callus was obtained after drying to constant weight at 45°C for 24 h.

Establishment of callus lines

Based on the prior experiment, stem explants from *in vitro* propagated plants and epicotyls from seedlings obtained through *in vitro* germination were inoculated in MS medium supplemented with the cytokinin BAP (0.65 or 1.25 mg L^{-1}) associated with 2,4-D (1.25 mg L^{-1}), PIC (1.25 mg L^{-1}) or NAA (2.5 mg L^{-1}). Cultures were maintained and analyzed under the previously mentioned conditions.

The experiments followed a sequential and completely randomized experimental design (Compton, 1994; Compton and Mize, 1999). The results were submitted to D'Agostino & Pearson omnibus normality test, analysis of variance (ANOVA) and means were compared by Tukey's test. The tests were performed using GraphPad Prism 5.0 software.

Antioxidant activity

Compact calli obtained from two-month-old *in vitro* germinated seedlings, grown on MS medium supplemented with 2.5 mg L^{-1} NAA + 0.65 mg L^{-1} BAP, two-month-old *in vitro* regenerated plantlets cultivated on MS medium supplemented with 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} of KIN and leaves of *in situ* tree were used as extraction sources. The material was dried at 45°C for 24 h. The samples (5 g) were immersed in 50 ml ethyl alcohol PA (Merck) for two weeks at 25±2°C and kept under agitation. Extracts were filtered on Whatman paper (No.1) and dried on rotary evaporator at 40°C, until constant weight and kept in the dark, at 10°C.

Evaluation of total phenolic compounds

Total phenolics were estimated according to the colorimetric method of Folin-Ciocalteu (Singleton and Rossi, 1965). The extracts were diluted in ethanol (90%), and a 100 µl sample was mixed with 100 µl of the Folin-Ciocalteu reagent (50%). After 5 min,

Table 1. Effect of explant source and auxins on the establishment of *H. dulcis* callus cultures grown on MS medium after 8 weeks.

Plant growth regulator (mg L^{-1})	*In vitro* plantlet stem segments			Seedlings epicotyls			Seedlings hypocotyls		
	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (g)
MS0	-	0	-	-	0	-	-	0	-
1.25 2,4-D	F/M	88	27.5±13.0[b]	F/M	64	17.29±5.9[b]	F/M	40	17.5±6.5[b]
2.50 2,4-D	F/M	68.18	16.7±3.7[b]	-	0	-	-	0	-
5.00 2,4-D	F/M	64	21.7±10.4[b]	-	0	-	-	0	-
1.25 PIC	F/M	80	24.2±9.2[b]	F/M	56	16.9±5.7[b]	F	20	-
2.50 PIC	F/M	76	26.2±11.3[b]	F/M	48	16.2±2.9[b]	F/M	0	-
5.00 PIC	F/M	52	13.4±2.2[b]	F/M	16	-	F/M	5	-
1.25 NAA	C/M	33.33	-	C	92	27.2±14.5[a]	C	45.83	27.9±12.2[b]
2.50 NAA	C	100	57.2±31.7[a]	C	60	34.3±14.0[a]	C	55	56.9±27.0[a]
5.00 NAA	C	100	45.5±13.6[a]	C	24	-	C	35	-

C: Compact callus; F: Friable callus; M: Mixed callus; -: No callus formation. Values represent mean ±SD of three replicates. Different small letters within each column indicate significant ($P < 0.05$) differences among treatments. MS0 = Growth-regulator free MS medium.

2 ml of Na_2CO_3 (2%) were added and incubated at room temperature for 1 h. The absorbance was measured at 750 nm. Total phenolic compounds content was determined using a standard curve prepared with gallic acid and results were expressed as microgram of gallic acid equivalent (GAE) per milligram of dry weight. The samples were analyzed twice in duplicate.

Total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Costa, 1982). The extracts were diluted in ethanol (90%), and 1 ml of sample was mixed with 1 ml of $AlCl_3$ (2%) and 2 ml of ethanol. Absorbance was measured at 425 nm after 5 min of incubation at room temperature. Total flavonoids were determined using a quercetin standard curve and results expressed as microgram of quercetin equivalent (QE) per milligram of dry weight. The samples were analyzed twice in duplicate.

Reducing power assay

The reducing power was determined based on the method of Yen and Chen (1995). A 400 µl sample (diluted in ethanol 20%) was added to 400 µl of phosphate buffer pH 6.6 (50 mM) and 400 µl of potassium ferrocyanide [$K_2Fe(CN)_6$] (1%). After 20 min of incubation 400 µl of trichloroacetic acid (TCA) (10%) and 4.4 ml of deionized water were added. After that 3 ml of the solution were withdrawn and mixed with 400 ml of 0.1% ferric chloride ($FeCl_3$). Absorbance was measured at 700 nm. The results were obtained using a gallic acid standard curve and samples were analyzed twice in duplicate.

DPPH radical scavenging assay

Free radical scavenging activity of extracts was quantified spectrophotometrically using DPPH assay (Brand-Williams et al., 1995). A 1 ml extract sample was dissolved in 50% dimethyl sulfoxide (DMSO)/50% ethanol, and mixed with 1 ml of DPPH. After 30 min of incubation, absorbance was measured at 517 nm, twice, in duplicate. The results were expressed as percentage of capture of DPPH radical calculated by the equation:

$$\frac{[1-(A_1-A_2)]}{A_0} \times 100$$

where A_0 = Absorbance of blank; A_1 = Absorbance of sample with DPPH; A_2 = Absorbance of sample without DPPH

RESULTS AND DISCUSSION

Auxins and callogenesis

Callus formation was not observed in leaf explants. The addition of 2,4-D to cultures of regenerated plants resulted in the induction of friable calli at all tested concentrations, while only the lowest concentration (1.25 mg L^{-1}) promoted calli formation from *in vitro* seedlings. Despite being predominantly friable, these calli presented compact areas and therefore were classified as mixed callus. The addition of PIC induced the formation of the same types of calli on explants obtained from *in vitro* regenerated plants and on cultures initiated from *in vitro* germinated seedlings (Figure 1). The addition of NAA promoted the establishment of compact light green calli (Figure 1A and 1B) on all types of stem explants at all concentrations (Table 1).

Majority of callus induction processes described to date employ transcriptional or post-transcriptional regulators that lead to changes in gene expression or protein translation (Ikeuchi et al., 2013). Auxins are supposed to promote a physiological change in previously differentiated plant tissue, leading cells to differentiate and start division (Machakova et al., 2008). Lo Schiavo et al. (1989) suggested that an increase in DNA methylation in the presence of auxins could cause cellular reprogramming and such changes in DNA methylation were observed

Table 2. Effect of explant source and BAP on *H. dulcis* callus cultures grown on MS medium after 8 weeks.

Growth regulator (mg L^{-1})	*In vitro* plantlet stem segments			Seedlings epicotyls		
	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (mg)
1.25 2,4-D+0.65 BAP	M/F	100	73.3±54.3[a]	F/M	100	32.2±15.5[b]
1.25 2,4-D+1.25 BAP	M/F	93.33	100.6±48.19[a]	F//M	40	43.5±31.3[b]
1.25 PIC+0.65 BAP	-	0	-	-	0	-
1.25 PIC+1.25 BAP	M	100	80.6±35.9[a]	F/M	76	55.63±36.5[b]
2.50 NAA+0.65 BAP	C	91.66	110.8±40.92[a]	C	100	115.3±28.2[a]
2.50 NAA+1.25 BAP	C	80	75.0±25.3[a]	C	100	95.6±19.04[a]

C: Compact callus; F: Friable callus; M: Mixed callus; -: No callus formation. Values represent mean ±SD of three replicates. Different small letters within each column indicate significant ($P < 0.05$) differences among treatments.

in callus cultures (Lambe et al., 1997). Recently, genome-wide transcriptional analysis showed that genes involved in auxin signaling and meristem development were methylated within the callus (Li et al., 2011). The addition of exogenous auxin seems to be re-quired for *H. dulcis* callus induction since in the absence of such compounds callus formation was not observed.

In the present study, friable calli were observed when 2,4-D and PIC were added to the culture medium, while only NAA induced compact callus which is in contrast with Jeong et al. (2009) findings, where 2,4-D promoted the formation of compact callus in leaf explants. Similar results were reported for other species such as *Gossypium hirsutum*, where 2,4-D promoted only friable callus while NAA was responsible for compact callus formation (Baksha et al., 2006). This response may be related to 2,4-D strength, since other studies suggested this growth regulator as more suitable to promote callus friability (Cardoso and Oliveira, 1996; Salman, 2002).The degree of activity of individual auxins in different processes is very variable. It may differ not only from species to species, but also from organ to organ, tissue to tissue, cell to cell and, with the age and physiological state (Davies, 2004). Often, after uptake into plant tissues, 2,4-D and NAA are converted to conjugates, and this process may regulate levels of free active substances (Machakova et al., 2008).

NAA has been mentioned as weaker auxin when compared with 2,4-D and PIC. Thus, it may be inferred that endogenous cytokinin action would be responsible for cell aggregation, which could explain compact callus formation when NAA was added to the culture medium. This consideration could also explain the green color of the compact callus, since cytokinins, in culture, tend to induce chlorophyll formation in callus, while auxins may be inhibitory (Machakova et al., 2008). Abdellatef and Khalafallah (2008), working with *G. hirsutum*, found that 2,4-D induced callus ranging from yellow to brown while the NAA produced callus ranging from yellow to green, and IAA, which is considered weaker than the NAA, promoted green callus. The increase of IAA concentration led to the reduction in chloroplast development in callus (Wolzny et al., 1973). These data corroborate our findings and could explain the formation of compact, green callus on media supplemented with NAA.

Dry weight analysis (Table 1) showed that tested concentrations of PIC and 2,4-D had no significant difference regarding biomass accumulation. In the present paper, the best results for compact callus were obtained with the supplementation of NAA at 2.5 or 5.0 mg L^{-1} (Table 1). Explant source also had no significant effect on callus growth. Considering the collected data, the culture media supplemented with 1.25 mg L^{-1} 2,4-D, 1.25 mg L^{-1} PIC and 2.5 mg L^{-1} NAA were selected to continue the study, and as explant source, stem segments from *in vitro* regenerated plants and epicotyls of *in vitro* seedlings.

Cytokinin and callogenesis

The addition of BAP had no effect on callus morphology (Table 2). None of the media containing 2,4-D or PIC were able to establish homogeneous friable callus cultures. The cultures inoculated in MS medium supplemented with 1.25 mg L^{-1} PIC + 1.25 mg L^{-1} BAP showed a high degree of oxidation. Dry weight measurements showed that BAP concentration had no significant effect on biomass production (Table 2). Therefore, the medium supplemented with 2.5 mg L^{-1} NAA + 0.65 mg L^{-1} BAP was considered the most suitable to produced *H. dulcis* compact callus. This medium was considered preferable since the same results were obtained using less growth regulators, which may induce somaclonal variation. This genetic modification was observed in *Amorphophallus albus* plants obtained from callus induced by combinations of NAA and BAP (Hu et al., 2008).

The addition of cytokinin to the media in order to increase culture growth proved to be effective. In many species, the association of cytokinins and auxins has been reported to promote the best growth and maintenance of callus (Park et al., 2002; Salman, 2002; Santos et al., 2011).

Compact callus cultures obtained on the present study were subcultivated for at least 6 months and no morphological

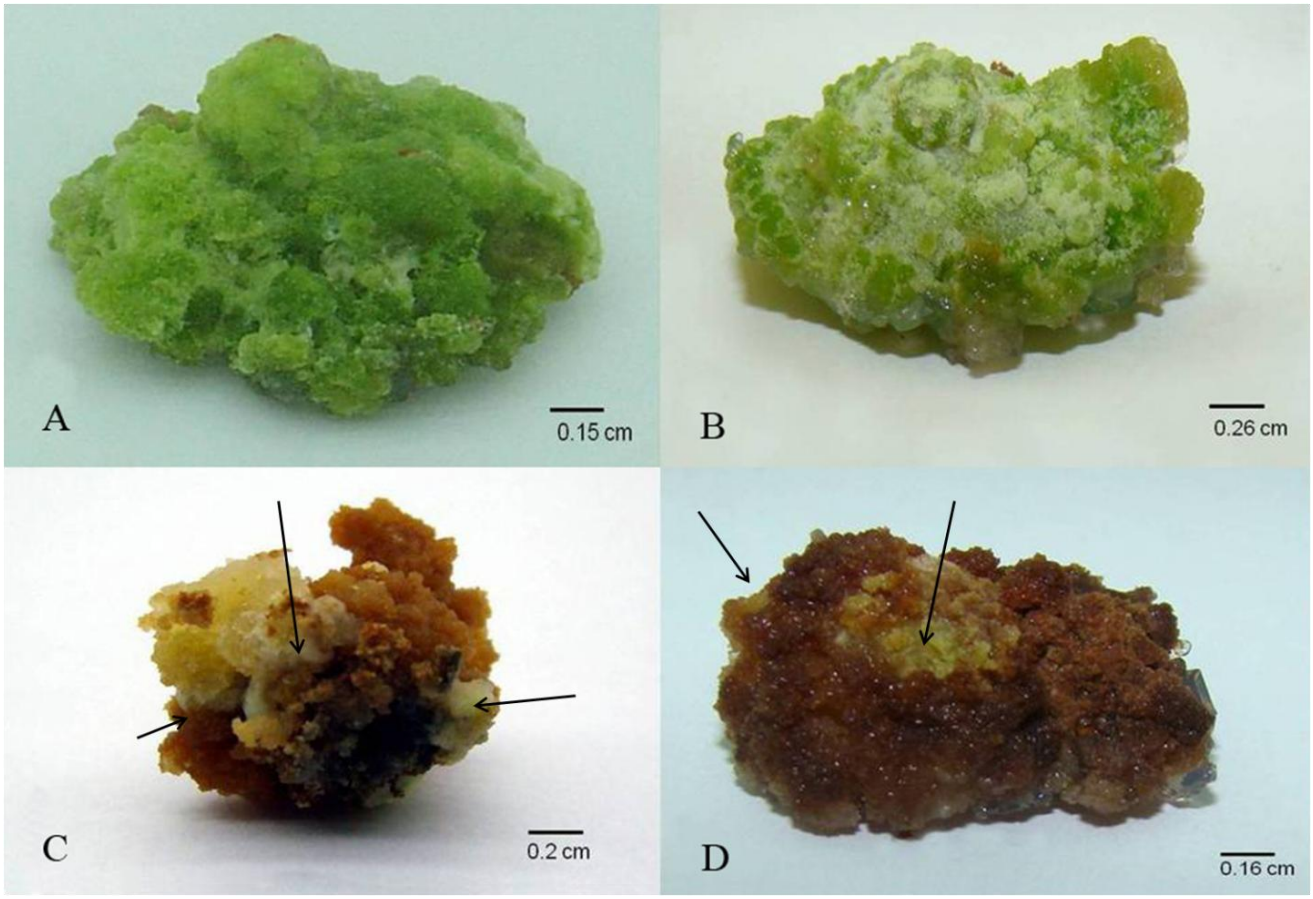

Figure 1. *H. dulcis* callus cultures established on MS medium supplemented with different auxins and BAP, after 8 weeks. Light-green compact callus grown on MS + 2.5 mg L^{-1} NAA (A) and 2.5 mg L^{-1} NAA + 1.25 mg L^{-1} BAP (B). Mixed (friable and compact) callus grown on MS + 1.25 mg L^{-1} PIC (C); and 1.25 mg L^{-1} BAP 1.25 mg L^{-1} PIC (D). Arrows indicate compact areas.

changes were observed. These results are highly desirable, since Jeong et al. (2009) observed oxidation in *H. dulcis* non-organogenic calli after 20 days.

Antioxidant activity

The Folin-Ciocalteu method, used to analyze polyphenol content, revealed that wild grown plants had the highest concentration of these compounds with the equivalent of 59.73±1.37 mg of gallic acid/mg extract (Figure 2A). Phenolic compounds are commonly found in plants and are important in defense against infections and injuries. They are recognized as antioxidants (Kahkonen et al., 1999) and their presence is considered an indication of antioxidant activity of plant extracts. The Folin-Ciocalteau has become a routine method and is considered sensitive and accurate (Huang et al., 2005). As observed for phenolic compounds, *in situ* plants also presented the highest flavonoid concentration with the equivalent to 12.20 ± 0.87 mg of querciting/mg extract (Figure 2B).

In some cases, the antioxidant activity can be significant and not correlated with the presence of total polyphenols. Wanasundara et al. (1995), working with canola, concluded that polyphenol content was not the crucial factor in determining the antioxidant capacity. The authors found a fraction with low polyphenol content with higher antioxidant activity in comparison with fractions of higher polyphenol content. These data are consistent with what was observed in *H. dulcis* callus extracts. The reducing power assay showed that callus extracts had the highest capacity of reducing iron, yielding 0.420 + 0.012 mg of gallic acid equivalent/mg extract (Figure 2C). The DPPH radical scavenging assay showed that callus extracts had the highest percentage of radical scavenging. The compounds present in callus extract were able to scavenge 57.31% of available radicals (Figure 2D).

The data obtained from these assays suggest that the highest antioxidant activity observed in the callus extracts is due to compounds different from polyphenols or flavonoids that were found in lower amounts in callus. Saponins are one of the major groups of secondary metabolites present in *H. dulcis*. Among several

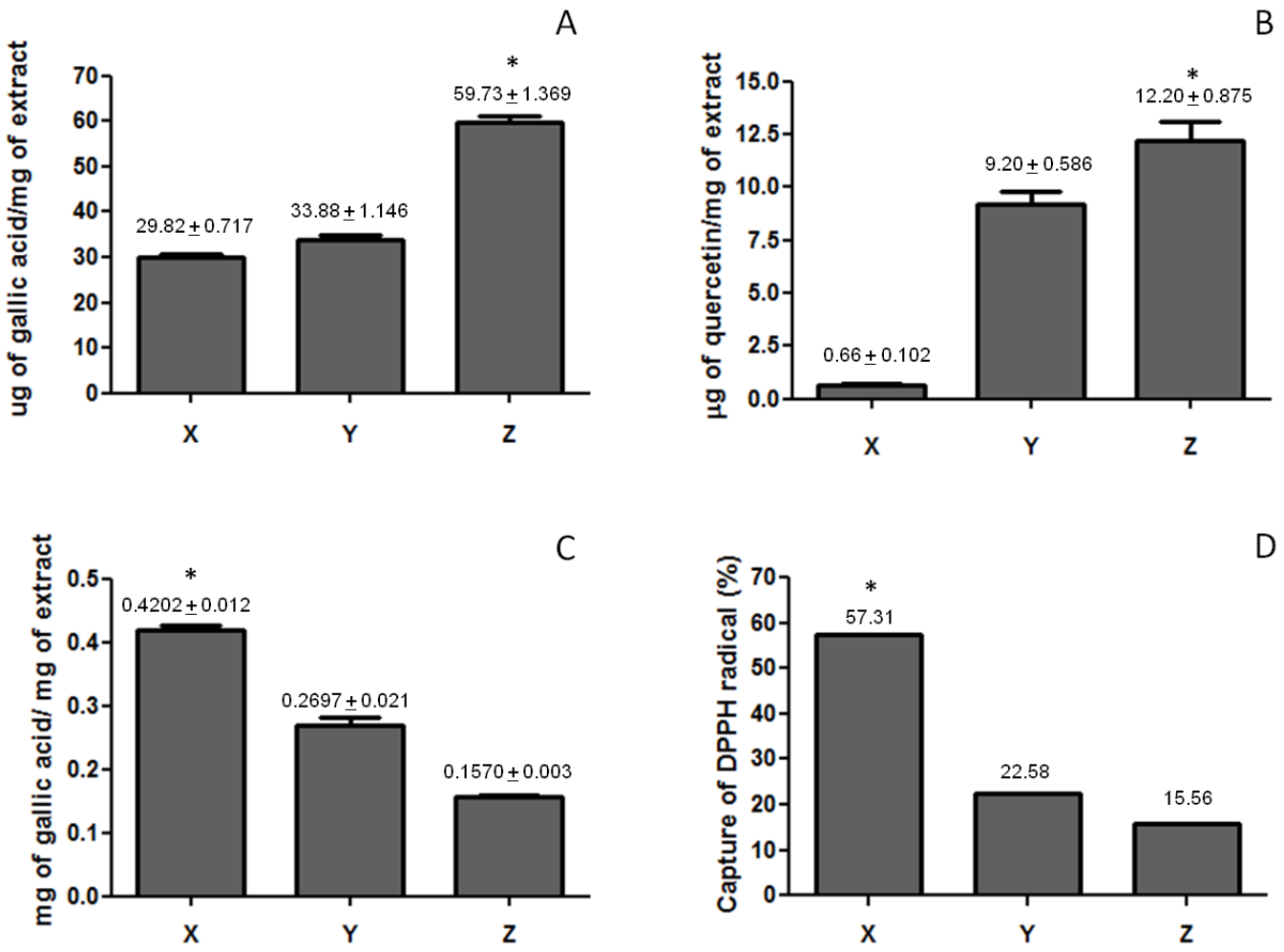

Figure 2. Antioxidant activity of *H. dulcis* extracts from compact callus (X), *in vitro* propagated plants (Y) and field grown plants (Z) evaluated by the total polyphenols assay (A), total flavonoids assay (B), reducing power assay (C) and DPPH radical scavenging assay (D). Values represent mean±SD of three replicates (A, B and C) and mean (D). * indicate significant ($P < 0.001$) differences between samples by the Tukey test.

antioxidant properties (Sparg et al., 2004; Ayaz et al., 2014) and therefore, might be responsible for the observed results. The highest antioxidant activity of callus in comparison to other plant materials is in accordance to what was observed in *Sylibum marianum* (Abbasi et al., 2010).

Conclusions

In conclusion, the auxins 2,4-D and PIC produced mixed friable beige and light green compact callus, while NAA induced compact green callus formation. The addition of BAP to the culture medium increased biomass growth. Callus extracts presented the highest antioxidant potential, which shows that *in vitro* production of compounds of medical interest by callus culture is a viable alternative in comparison to traditional methods, being able to exceed the productivity of *in situ* plant. Further investigation on the phytochemistry of these calli is of interest in order to elucidate which molecules are responsible for the higher antioxidant activity.

ACKNOWLEDGEMENTS

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are grateful to Maria Francisca Santoro Assunção for the valuable technical assistance. The authors wish to thank Dr. Timothy P. Moulton for english corrections.

Conflict of interest

All authors declare that they have no conflict of interest.

Abbreviations:

ROS, Reactive oxygen species; **BAP,** benzylaminopurine; **KIN,** 6-furfuryl-aminopurine; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **PIC,** 4-amino-3,5,6-trichloro picolinic acid; **NAA,** 1-naphthaleneacetic acid; **DW,** dry weight; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl.

REFERENCES

Abbasi BH, Kahn MA, Mahmood T, Ahmad M, Chaudhary MF, Kahn MA (2010). Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. Plant Cell Tiss. Org. Cult. 101:371-376.

Abdellatef E, Khalafallah MM (2008). Influence of growth regulators on callus induction from hypocotyls of medium staple cotton (*Gossypium hirsutum* l). BARAC B-67. J. Soil Nature 2:17-22.

Ayaz M, Junaid M, Ahmed J, Ullah F, Sadiq A, Ahmad S, Imran M (2014). Phenolic contents, antioxidant and anticholinesterase potentials of crude extract, subsequent fractions and crude saponins from *Polygonum hydropiper* L. BMC Complement. Altern. Med. 14:145.

Baksha R, Mavlanov GT, Nasirova GB, Djataev SA (2006). Gossypol accumulation and morphogenesis in cotton (*G. hirsutum* L.) callus cultures. J. Biol. Sci. 6:1126-1129.

Brand-Williams W, Cuvelier ME, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol. 28:25-30.

Cardoso MA, De Oliveira DE (1996). Tissue culture of *Hypericum brasiliense* Choisy: Shoot multiplication and callus induction. Plant Cell Tiss. Org. Cult. 44:91-94.

Castro TC (2001). Germinação, micropropagação e perfil fitoquímico por CLAE da espécie medicinal Hovenia dulcis Thunb. (Rhamnaceae). Master's Thesis, Universidade do Estado do Rio de Janeiro, Rio de Janeiro.

Compton ME (1994). Statistical methods for the analysis of plant tissue culture data. Plant Cell Tiss. Org. Cult. 37:217-242.

Compton ME, Mize CW (1999). Statistical considerations for in vitro research: I – Birth of an idea to collecting data. *In Vitro* Cell. Dev. Biol. Plant 35:115-121.

Coppede JS, Pina ES, Paz TA, Fachin AL, Marins MA, Bertoni BW, França SC, Pereira AMS (2014). Cell cultures of *Maytenus ilicifolia* Mart. are richer sources of quinone-methide triterpenoids than plant roots in natura. Plant Cell Tiss. Org. Cult. 118:33-43.

Costa AF (1982). Farmacognosia – Farmacognosia Experimental. Lisboa: Calouste Gulbenkian.

Coste A, Vlase L, Halmagyi A, Deliu C, Coldea G (2011). Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss. Org. Cult. 106:279-288.

Gadelha AP, Vidal F, Castro TC, Lopes CS, Albarello N, Coelho MGP, Figueiredo SFL, Monteiro-leal LH (2005). Susceptibility of *Giardia lamblia* to *Hovenia dulcis* extracts. Parasitol. Res. 97:399-407.

Hu J, Gao X, Liu J, Xie C, Li J (2008). Plant regeneration from petiole callus of *Amorphophallus albus* and analysis of somaclonal variation of regenerated plants by RAPD and ISSR markers. Bot. Stud. 49:189-197.

Huang D, Ou B. Prior RL (2005). The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53:1841-1856.

Hyun TK, Eom SH, Yu CY, Roitsch T (2010). *Hovenia dulcis* – An Asian Traditional Herb. Planta Med. 76:943-949.

Ikeuchi N, Sugimoto K, Iwase A (2013). Plant Callus: Mechanisms of Induction and Repression. Plant Cell 25:3159-3173.

Jeong MJ, Song HJ, Park DJ, Min JY, Jo JS, Kim BM, Kim HG, Kim YD, Kim RM, Karigar CS, Choi MS (2009). High frequency plant regeneration following abnormal shoot organogenesis in the medicinal tree *Hovenia dulcis*. Plant Cell Tiss. Org. Cult. 98:59-65.

Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem. 47:3954-3962.

Kim OK (2001). Protective effects of *Hovenia dulcis* Thunb. on hepatotoxicity in carbon tetrachloride intoxicated rats. J. Korean Soc. Food Sci. Nutr. 30:1260-1265.

Lambe P, Mutambel HSN, Fouche JG, Deltour R, Foidart JM, Gaspar TH (1997). DNA methylation as a key process in regulation of organogenic totipotency and plant neoplastic progression? In Vitro Cell. Dev. Biol. Plant 33:155-162.

Lee YA, Chae HJ, Moon HY (2005). Effect of *Hovenia dulcis* THUMBERG var. *koreana* Nakai fruits extracts on glucose, lipid metabolism and antioxidant activities in streptozotocin induced diabetic rat. J. Exp. Biomed. Sci. 11:533-538.

Li W, Liu H, Cheng ZJ, Su YH, Han HN, Zhang Y, Zhang XS (2011). DNA Methylation and Histone Modifications Regulate De Novo Shoot Regeneration in Arabidopsis by Modulating WUSCHEL Expression and Auxin Signaling. PLoS Genet. 7(8):e1002243.

Lo Schiavo F, Pitto L, Giuliano G, Torti G, Nutironchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor. Appl. Genet. 77:325-331.

Machakova I, Zazimalova E, George EF (2008). Plant Growth Regulators I: Introduction; Auxins, their Analogues and Inhibitors. In: George EF, Hall MA, De Clerk GJ (Eds.), Plant propagation by tissue culture 3rd edition. Vol. 1. The background. Springer, The Netherlands. pp.175-204.

Moo-Huchin VM, Moo-Huchin MI, Estrada-Leon RJ, Cuevas-Glory L, Estrada-Mota IA Ortiz-Vazquez E, Betancur-Ancona D, Sauri-Duch E (2015). Antioxidant compounds, antioxidant activity and phenolic content in peel from three tropical fruits from Yucatan, Mexico. Food Chem. 166:17-22.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Phyisiol. Plant 15:473-498.

Park JB, Lee KB, Lee S (2002). Histological study of callus formation and root regeneration from mung bean (*Vigna radiata* W.). J. Plant Biol. 45: 170-176.

Pawar KD, Thengane SR (2011). Influence of abiotic elicitation on production of dipyranocoumarins in suspension cultures of *Calophyllum inophyllum* L. Acta Physiol. Plant 33:2149-2158.

Piekoszewska A, Ekier, H, Zubek S (2010). Arbutin production in *Ruta graveolens* L. and *Hypericum perforatum* L. *in vitro* cultures. Acta Physiol. Plant 32:223-229.

Praveen N, Murthy HN (2011). Effects of macroelements and nitrogen source on biomass accumulation and withanolide-A production from cell suspension cultures of *Withania somnifera* (L.) Dunal. Plant Cell Tiss. Org. Cult. 104:119-124.

Praveen N, Naik PM, Manohar SH, Nayeem A, Murthy HN (2010). *In vitro* regeneration of brahmi shoots using semisolid and liquid cultures and quantitative analysis of bacoside A. Acta Physiol. Plant 31:723-728.

Prior RL, Cao G (1999). *In vivo* total antioxidants capacity: Comparison of different analytical methods. Free Rad. Biol. Med. 27:1173-1181.

Salman MN (2002). Establishment of callus and cell suspension cultures from *Gypsophila paniculata* leaf segments and study of the attachment of host cells by *Erwinia herbicola* pv. gypsophilae. Plant Cell Tiss. Org. Cult. 69:189-196.

Santos AV, Arrigoni-Blank MF, Blank AF, Diniz LEC, Fernandes RMP (2011). Biochemical profile of callus cultures of *Pogostemon cablin* (Blanco) Benth. Plant Cell Tiss. Org. Cult. 107:35-43.

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

Sparg SG, Light ME, Van Staden J (2004). Biological activities and distribution of plant saponins. J. Ethnopharmacol. 94:219-243.

Tadhani MB, Patel VH, Subhash R (2007). *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. J. Food Compos. Anal. 20:323-329.

Wanasundara UN, Amarowicz R, Shahidi F (1995). Partial characterization of natural antioxidants in canola meal. Food Res. Int. 28:525-530.

Wolzny A, Gwodz E, Sweykowska A (1973). The effect of 3-indoleacetic acid on the differentiation of plastids in callus cultures of *Cichorium intybus* L. Protoplasma 76:109-114.

Xu BJ, Deng YQ, Jia XQ, Lee JH, Mo EK, Kang HJ, Sung CK (2003). A rapid screening for alcohol detoxification constituents of *Hovenia dulcis* by microplate reader. Agric. Chem. Biotechnol. 46:105-109.

Yen GH, Chen HY (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 43:27-32.

Related Journals Published by Academic Journals

- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals