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

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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.

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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)
	0.5+0.25	100	9	7
NAA+BAP	0.15+0.25	80	5	2.5
	0.25+0.25	90	11	10.2
	0.5+0.25	40	3	3
IBA+KN	0.15+0.25	50	4	1.5
	0.25+0.25	42	7	2.1
	0.5+0.25	70	1	2.9
NAA+BAP	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)
	0.2+0.15	84	19	2.1
NAA+BAP	0.25+0.15	100	26	2
	0.25+0.2	72	14	5
	0.2+0.15	-	-	-
IAA+BAP	0.25+0.15	19	7	1.4
	0.25+0.2	24	1	2.5
	0.2+0.15	32	4	1
IBA+BAP	0.25+0.15	-	-	-
	0.25+0.2	-	-	-

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

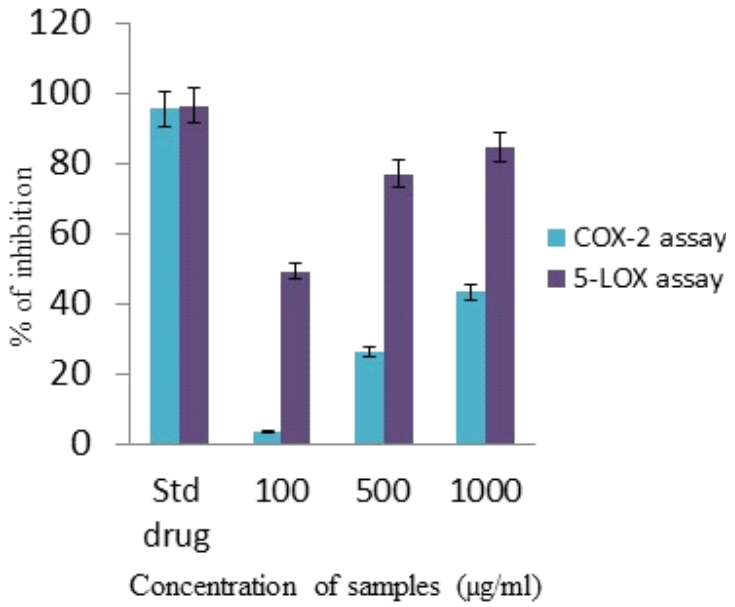

Figure 1. Percentage inhibition in COX and LOX assay.

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.

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Conflict of interests

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

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