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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, dyspepsia, cancer, gout, scrofula and act as abortifacient, anthelmintic, antipyretic and anti-inflammatory. The medicinal properties of this plant is due to the presence of many secondary metabolites such as colchicine, colchicoside, chelidonic acid, luteolin and gloriosine. The main aim of the proposed work is to evaluate the anti-inflammatory activity of *Gloriosa 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 of the plant extract 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 *Gloriosa superba* tuber showed closest percentage inhibition with that of the standard aspirin. The present study thus confirmed that methanol extract of root tubers of *Gloriosa 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 *Gloriosa superba* includes induction of callusing and organogenesis, using various explants such as leaf, internodes, axillary buds and tuber. The results indicated that MS medium supplemented with NAA (0.15mg/l) and BAP (0.25mg/l) induced callusing, with 2,4-D(0.5mg/l) and Kinetin (0.25mg/l) induced somatic embryogenesis, NAA (0. 5mg/l) and BAP (0.25mg/l) promoted the formation of the maximum number of shooting and with NAA (0.25mg/l) and BAP (0.15mg/l) rooting was induced. Micro propagation will be helpful for the conservation and maximum utilization of the plant along with the identification and isolation of useful bioactive molecules.

Keywords : Colchicine; Colchicoside; Cycloxygenase; Gloriosine; Lipoxygenase.

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 alarm signals, which promote the dilation 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 produce oedema. The symptoms of acute inflammation includes heat, pain, swelling, redness, and loss of function.

Inflammation is mainly caused due to the presence of some enzymes such as Phospholipase A2. When the Phospholipase A2 activated, a fatty acid called arachidonic acid is released from the phospholipid membrane, which is a substrate for two enzyme 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[1] 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[2].

Cycloxygenase-2 is an inducible isoform of

COX-1 that catalyses the rate limiting step in the formation of prostaglandin from arachidonic acid. Induction 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 to be an effective remedy in the prevention and treatment of cancer[3]. The consequence of overexpression of COX-2 is also reported by Funk[4]. Lipoxygenases catalyse the deoxygenation of poly unsaturated fatty acids in lipids[5] and helps in the conversion of arachidonic acid into proinflammatory mediators called leukotrienes, which are potent molecules having diverse biological actions[6].

In the present study anti- inflammatory activity of a medicinally potent plant *Gloriosa superba* was evaluated and plants were propagated *in vitro*. *Gloriosa superba* is an important perennial climbing herb among the medicinal plants[7] 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[8]. *Gloriosa 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 *Gloriosa 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. 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. *Gloriosa 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 were 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 and applied on arthritis affected joints, pain could be reduced[9]. The cultivation using root tuber is not enough for the production of maximum number of *Gloriosa 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 *Gloriosa superba* L. *in vitro* and transplant the commercially useful plant from laboratory to field condition along with evaluation of anti-inflammatory action of the plant.

Material and Methods

Evaluation of Anti-inflammatory activity - The dried powdered tuber (500g) was defatted with petroleum ether (60 to 80°C) by hot extraction in a soxhlet apparatus for 48-72 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 *Gloriosa superba* by cycloxygenase inhibition assay and 5-lipoxygenase inhibition assay.

Lymphocyte culture preparation: 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 0.2µm pore sized cellulose acetate filter in completely aseptic conditions followed by addition of fresh plasma at a concentration of 1×10^6 cellsml^{-1}, and incubation for 72 hours. After addition of 1µl LPS (Lipopolysaccharides), culture was incubated for 24 hours. Standard drug such as aspirin was used in the concentration of 100µgml^{-1} from a stock of 100mg ml^{-1} and the sample was added in the concentration of 100µgml^{-1}, 500µgml^{-1} and 1000 µg ml^{-1} from a stock of 100 mg ml^{-1}. Culture was incubated for 24 hours followed by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 200µl of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton) was added .The incubation was done for 30 minutes at 4°C and the assay was done in pellet suspended in a small amount of supernatant [10].

Cycloxygenase Assay (COX assay):

Procedure: Arachidonic acid was added to the pellet and

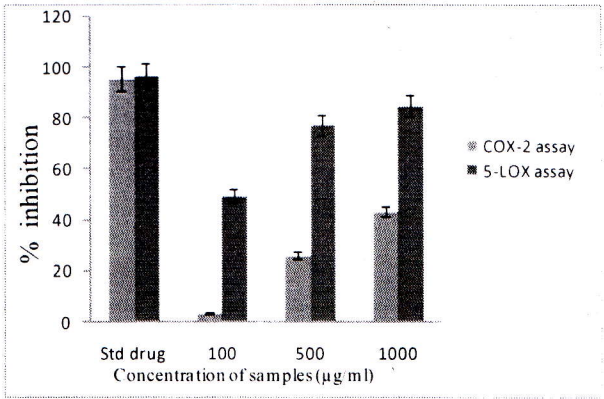

Fig.1. Percentage inhibition in COX and LOX assay.

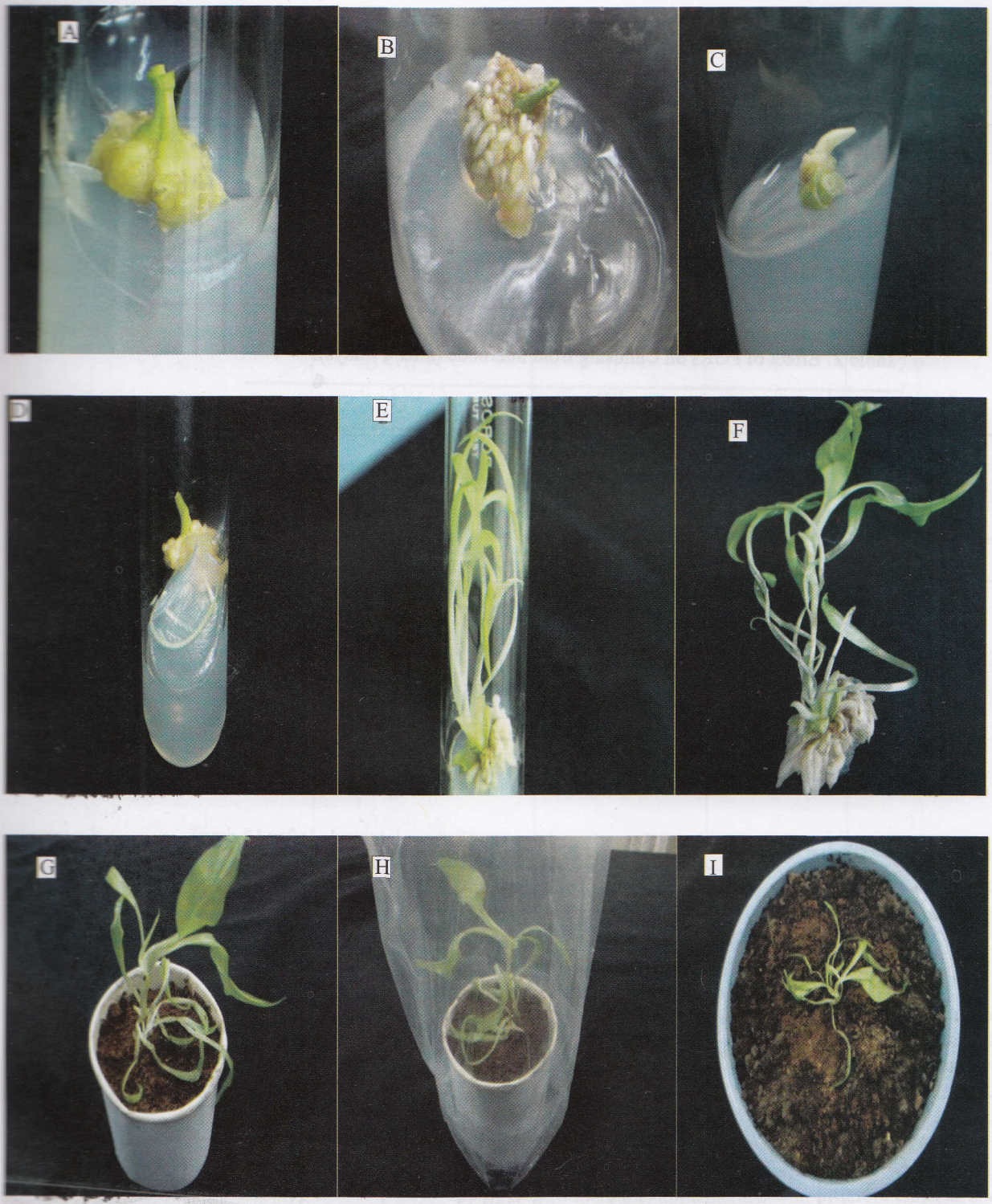

Fig.2. *In vitro* propagation of *Gloriosa superba* L. A-Callus induction, B- Somatic embryogenesis; C- Shoot induction, D- Root induction; E-F- Multiple shooting, G-I- Acclimatization

incubation was done at 37°C. Then added 0.2ml of 10% TCA in 1N HCl, mixed and contents were heated in a boiling water bath for 20 min followed by adding 0.2ml of thiobarbituric acid (TBA), cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632nm for COX activity".

5-lipoxygenase Assay (LOX assay):

*Procedure:*For LOX assay 70mg of linoleic acid and equal weight of tween 20 was dissolved in 4ml of oxygen free water and followed by addition of sufficient amount

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 (100µg/ml)	95.34	96.38
100 µg/ml	3.38	49.23
500 µg/ml	26.27	76.92
1000 µg/ml	43.22	84.61

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

S.No.	PGRs	PGRconc.	FCI(%)
1	NAA+BAP	0.1+0.25	75
		0.15+0.25	96
		0.25+0.25	70
2	IBA+BAP	0.1+0.25	50
		0.15+0.25	44
		0.25+0.25	42
3	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).

S.No.	PGRs	PGRconc.	FCI(%)
1	2,4-D+KN	0.5+0.25	98
		0.15+0.25	90
		0.25+0.25	79
2	IBA+KN	0.5+0.25	42
		0.15+0.25	39
		0.25+0.25	44
3	NAA+BAP	0.5+0.25	28
		0.15+0.25	27
		0.25+0.25	31

FSE(%)- Frequency of Somatic Embryogenesis

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

S.No.	PGRs	PGR conc.	FCI(%)	MSN	MSL(cm)
1	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
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
3	NAA+BAP	0.5+0.25	70	1	2.9
		0.15+0.25	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

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

S.No.	PGRs	PGR conc.	FCI(%)	MSN	MSL(cm)
1	NAA+BAP	0.2+0.15	84	19	2.1
		0.25+0.15	**100**	26	2
		0.25+0.2	72	14	5
2	IAA+BAP	0.2+0.15	-	-	-
		0.25+0.15	19	7	1.4
		0.25+02	24	1	2.5
3	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

of 0.5N NaOH to yield a clear solution and then made up to 25ml using oxygen free water. This was divided into 0.5ml 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 OD was measured in 234nm[11].

% 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: Gloriosa superba L. plant was collected from the Botanical 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 was used for *in vitro* culture of *Gloriosa superba*[12]. MS medium supplemented with combinations of plant growth regulators (PGRs) like IAA + BAP, IBA + Kinetin, IBA + BAP, 2, 4 D + Kinetin, NAA +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.

Results and Discussion

Evaluation of Anti-inflammatory potential- The results showed that the methanol extracts of root tubers of *Gloriosa 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 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 (Table1) (Fig.1). In lipoxygenase inhibition assay the methanol extract of *Gloriosa superba* tuber showed closest percentage inhibition with that of the standard aspirin and hence proved that the methanol extracts of *Gloriosa superba* exert a preferential effect on the 5-LOX pathway. The present study thus confirmed that *Gloriosa superba* can 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[13-15].

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 (0.15mg/l) + BAP (0.25mg/l) induced 96% of frequency of callus induction (FCI) from internode within 17 days of inoculation. *In vitro* callus induction and regeneration of healthy plants of *Gloriosa superba* L. was reported by Anirudha[16]. Where maximum callus proliferation was induced in B5 medium supplemented with NAA (2mg/l) and kinetin (0.5mg/l) after 5 weeks of inoculation. But in the present study maximum callus proliferations were obtained within 12 days of inoculation in MS medium in NAA (0.15mg/l) + BAP (0.25mg/l) (Table 2) (Fig. 2- A). So comparison with the previous study, the hormone combination used in the present study was better and faster for callus induction.

Embryogenesis: Maximum frequency of somatic embryogenesis (FSE) was observed (98%) on the medium supplemented with 2, 4-D (0.5mg/l) + Kinetin (0.25mg/l) after 17 days of inoculation from the internode (Table 3) (Fig. 2- B). Somatic embryogenesis and plant regeneration in *Gloriosa superba* L. was reported on MS medium supplemented with 2, 4-D (4mg /l) + Kinetin (5 mg /l) + CH(10 mg /l) + CW(20%)[17]. 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) alone without CH and CW after 17 days of inoculation without subculture.

Shoot induction: The multiple shooting of *Gloriosa superba* L. were observed (100%) on the MS basal medium supplemented with NAA (0.5 mg/I) and BAP (0.25 mg/l) within 36 days from the leaf explants with mean shoot number (MSN) 9 and mean shoot length (MSL) of 7 cm (Table 4) (Fig. 2- C, E, F). Multiple shoot formation in *Gloriosa superba* using tuber as explants were reported in MS and B^5 medium by Jadhav *et al.*[18]. But in the present study NAA and BAP induced multiple shoots effectively from the leaf explant.

Root induction: In the present study among the different hormone combinations tried, maximum frequency of root induction (FRI) 100% was observed in MS media supplemented with hormone combination of NAA (0.25mg/l) + BAP(0.15 mg/l) after 36 days of inoculation from internode (Table 5) (Fig. 2- D) with maximum mean root number (MRN)[26]. Indirect organogenesis with root induction of *Gloriosa superba* L. was reported in MS medium supplemented with 1.0 mg/l IBA and 0.5 mg/l IAA[19]. But effective direct root induction is not yet reported.

Acclimatization: The plantlet is taken out from the rooting medium and washed in running tap water to remove the remnants of agar. Then the plantlet is 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 (Fig. 2- G, H, I).

The present study concluded that *Gloriosa superba* is an important medicinal plant with high anti-inflammatory potential. This property may due to the presence of bioactive molecules and the utilization of these potent compounds are helpful for the development of a new anti- inflammatory drug. Due to overexploitation and its unscientific collection *Gloriosa superba* has been endangered, therefore, there is urgent need to conserve the plant by biotechnological approaches like tissue culture. In the present study an efficient protocol for fast induction of callusing, high frequency somatic embryogenesis and organogenesis of *Gloriosa superba* was identified which will be helpful for conservation of the plant.

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