RESEARCH PAPER

Accepted 22 February 2002
Revised 29 January 2002
Received 22 August 2000
Indian J. Pharm. Sci., 2002, 64(3): 217-221

Rotenoids and Rutin in Callus Cultures of *Tephrosia purpurea* (L) Pers

R. ZAFAR* AND M. MUJEEB
Plant Tissue Culture Laboratory
Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy,
Jamia Hamdard, Hamdard Nagar, New Delhi-110 062.

The root, stem and leaf calli of *Tephrosia purpurea* L. pers were successfully developed and maintained on Murashige and Skoog's medium supplemented with various plant growth regulators. The content of rotenoids and rutin in the callus cultures were estimated by spectrophotometric method.

Tephrosia purpurea (L). Pers (Fabaceae) is an important indigenous medicinal plant used in Indian system of medicine and is commonly known as Saraphunkha[1]. It is an important component of some marketed herbal preparations such as Tephroli and Yakrifit used for liver disorders[2,3]. Various parts of this plant are used as remedy for impotency, asthma, diarrhoea, gonorrhoea, rheumatism, and urinary disorders[1]. The seed extract has shown insecticidal and insect repellant properties[4]. The ethanolic extract of entire plant was found to exhibit cytotoxic activity against KB cells in culture[5]. The aqueous extract of seeds showed significant *in vivo* hypoglycaemic activity in diabetic rabbits[6]. The plant is also considered useful in bilious fibrile attacks, obstruction of liver and spleen[7-11]. Previous phytochemical investigation of this plant has resulted in the isolation of coumarin[12], flavonoids, rotenoids[13-14], flavones and isoflavones[15-16].

In the present investigation, an attempt has been made to develop the static culture of root, stem and leaf of *T. purpurea* on Murashige and Skoog's (MS) medium. Rotenoids were found to be present in leaf, stem and root calli, while rutin was found to be present only in the leaf callus, which was extracted, separated by preparative TLC and quantitatively estimated by a spectrophotometric method.

*For correspondence:
E-mail: root@hamduni.ren.nic.in

MATERIALS AND METHODS

Tephrosia purpurea viable seeds and herbal parts (leaves, stems and roots) were collected from mature plants grown in the herbal garden of Jamia Hamdard Campus during September/October. The plant and seeds were identified by the National Institute of Science Communication (NISCOM) Pusa, New Delhi. A herbarium specimen was kept in the department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi.

Surface sterilization of seeds:

The seeds were subjected to usual physical and chemical treatments to improve the rate of germination. They were first kept in dilute detergent solution (cedepol) for a minute and then washed under running tap water followed by double distilled sterile water. The seeds were then surface sterilized by treating with 80% ethanol for a min and then by mercuric chloride (0.4%) for 5 min followed by washing with double distilled sterile water. The surface sterilized seeds were kept in seed germinator for germination in dark at 25°.

Initiation of callus:

Pieces of leaf, stem and root (each about 12-15 mm) were excised from 7 d old seedlings and inoculated for initiation of callus on 100 ml of MS medium supplemented with 2,4 dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA) kinetin (kin), 6-benzyladenine (6-BA) and naphthalene acetic acid (NAA) either alone or in combination as given

TABLE 1: EFFECTS OF THE PLANT GROWTH REGULATORS ON CALLUS INITIATION IN MS MEDIUM.

Growth regulators (μM)	Days required for callus initiation	Callus initiation
2,4-D (4.52)	10-12	+Stem
		+++Root
Kin (4.64)	12-15	++Stem
IAA (5.70)	12-15	+++Root
		++Stem
6BA(4.43)	15-17	++Stem
2,4-D + Kin (4.52)+(4.64)	12-15	++Root
		++Stem
2,4-D + IAA (4.52)+(5.70)	12-15	+++Root
		++Stem
2,4-D+ 6-BA (4.52)+(4.43)	18-20	++Stem
NAA +IAA (5.37)+ (5.70)	15-18	++Root
NAA + IAA (5.37)+(22.8)	20-22	++Root
NAA + 6-BA (5.37)+(4.43)	12-15	++Stem
		+Root
IAA+6BA (5.70)+(4.43)	12-15	++Stem
NAA+2,4-D (5.37)+(4.52)	18-20	++Root
NAA + Kin (5.37)+(4.64)	18-20	+Stem
		+Root
Kin+6-BA (4.64)+(4.43)	20-25	++Stem
2,4-D+IAA+NAA+Kin	12-15	++Stem
(4.52)+(5.70)+(5.37)+(4.64)		+++Root
2,4-D+IAA+Kin(4.52)+(5.70)+(9.28)	7-8	++Stem
		+++Root ++Leaf

Kinetin (Kin); 6-benzyladenine (6-BA); 2,4-dichlorophenoxy acetic acid (2,4-D); Indole acetic acid (IAA) and Naphthalene acetic acid (NAA) were used as plant growth regulators to initiate prominent (+++); moderate (++) and feeble (+) callus.

in Table 1. The cultures were maintained at 25±2° under 16-h diffused light (1600 lux)/ 8 h darkness cycle.

Maintenance of independent calli:

The initiated calli of root and stem were successfully maintained in MS medium supplemented with 2,4-D (4.52 μM) + IAA (28.5 μM) + kin (9.29 μM). While MS medium supplemented with 2,4-D (4.52 μM) + IAA (28.5 μM) + 6-BA (8.86 μM) was used for the maintenance of leaf callus. The cultures were aseptically transferred to 100 ml of fresh medium contained in a 250 ml Erlenmeyer flask after an interval of 3-4 w and kept under the conditions mentioned above.

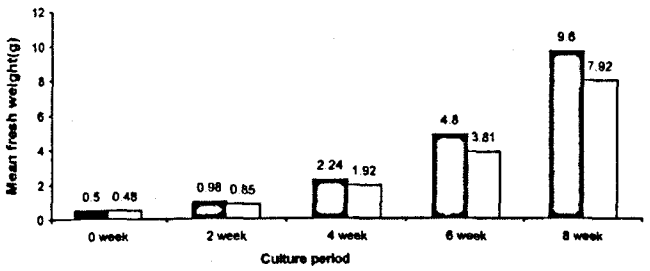

Fig. 1: Rate of growth of root and stem calli.

The mean fresh weight of root callus (■) and stem callus (□) were recorded at 0,2,4,6 and 8 w.

Growth pattern of root and stem calli:

The pieces of 30 d old root and stem calli were transferred aseptically to the previously weighed four flasks each containing 100 ml of MS medium. The initial fresh weight of callus was recorded. Subculturing was carried out at 2, 4, 6 and 8 w and the increase in fresh weight of callus was recorded at the interval of 2 w. The rate of growth was determined on the basis of increase in fresh weight of calli after every two weeks and the data obtained is presented in fig. 1.

Quantitative estimation; rutin:

For separation and quantitative estimation of rutin, 6 g of each of leaf, stem and root calli (6 m old) were soxhlet extracted with alcohol for 8 h. The extracts were filtered and concentrated. The extracts obtained from different calli were subjected to thin layer chromatography (silica gel G) using ethyl acetate: formic acid: water (10:2:3) as solvent system. Only the leaf extract showed the presence of rutin which was separated by PTLC and quantitatively estimated at 420 nm by UV spectrophotometer[17]. The percentage of rutin (w/w of callus) was calculated by taking the average of three readings.

Estimation of rotenoids:

Seven grams of each of root, stem and leaf calli (6 m old) were soxhlet extracted separately with acetone for 8-h for rotenoids estimation. The extracts were filtered and concentrated. The extracts so obtained from different calli were quantitatively estimated for rotenoids content by UV spectrophotometric method at 540 nm as described by Jones[18]. The results are given in Table 2. The percentage of rotenoids was calculated by taking the average of three readings.

Qualitative phytochemical analysis:

The extracts of the calli of leaf, root and stem along with the natural parts of the plant were also subjected to general chemical tests for different types of constituent such as alkaloid, flavonoids, anthraqunone, saponin, steroids, phenols, protein amino acids and reducing sugar.The tests are as follows:

TABLE 2: CONTENT OF ROTENOIDS IN DIFFERENT CALLUS SAMPLES.

Callus Samples	Content of rotenoids (w/w)*
Root	0.83%
Stem	0.50%
Leaf	0.72%

* Stand for percentage yield of rotenoids.

Alkaloids:

Five milliliters of alcoholic extracts of leaf, stem, root and their calli were evaporated to dryness. The alcoholic residues were taken in 5 ml of 2 % hydrochloric acid, and filtered. Filtrates were tested with alkaloid reagents such as Mayor's reagent (KI+HgCl2 solution), Dragendorffs reagent (solution of potassium bismuth iodide) and Hager's reagent (saturated solution of picric acid in water) which on addition of 2 ml of filtrates of the extracts gave creamy, reddish brown and yellow precipitates respectively. Besides these reagents, solution of ammonium phosphomolybdate or tannic acid solution also gave precipitate when added to different filtrates indicating the presence of alkaloids in all the samples except in stem, leaf and their calli.

Anthraquinones:

The plant materials stem, root, leaf and their calli were extracted with water and it was heated in a boiling water bath for 15 min with dilute sulphuric acid. Then the aqueous extract was extracted with chloroform. Equal volume of diluted ammonia solution was added to the chloroform layer. No purple colour was observed in ammonia layer in any sample there by indicating the absence of anthraquinone.

Amino acids:

The plant materials (stem, root leaf and their calli) were extracted with 50% methanol. These extracts were then concentrated to 1/4th of their volume. Few drops of ninhydrin reagent were added to these extracts and heated on a water bath. Appearance of pinkish red to violet colouration indicated the presence of amino acids in all samples.

Phenolics:

To the aqueous or 30% methanolic extract of different

samples of callus and plant parts a few drops of acidified ferric chloride solution were added. The appearance of blue, green or brown colouration indicated presence of phenolic compounds, in all the samples.

Flavonoids:

The plant materials and callus were extracted with 30% methanol and then heated on a water bath after acidification for 15 min. Then it was extracted with chloroform. To the separated chloroform layer a few pieces of zinc granules were added followed by a drop of concentrated HCl. Appearance of pink colouration indicated the presence of flavonoids in all samples.

Steroids:

The plant material and callus were extracted with chloroform and the extract was concentrated to 1/4th of its volumes. To these, few drops of acetic anhydride and a drop of cone sulphuric acid were added. Appearance of green or brown colour in all samples indicated the presence of steroids.

Reducing sugar:

Aqueous or 30% methanolic extracts and callus extracts were heated with 2 ml of each Fehling's A and B solutions. Appearance of red colouration in all samples except in leaf and its callus showed the presence of reducing sugars.

Saponins:

The dried powdered extract of plant material and callus on shaking with water produced soap like froth, which sets for sometime indicated the presence of saponins in the sample. Also on addition of a small portion of methanolic extract in a drop of blood on a microscopic slide, a haemolytic zone surroundings the extract were formed. Froth and haemolytic zone appeared, saponins were found to be present in all samples.

Protein:

To the methanolic extracts of plant material and callus, few drops of Million's reagent were added. A white precipitate is obtained in the presence of protein which turned red on heating. It indicated the presence of protein.

RESULTS AND DISCUSSION

The callus was successfully initiated on the different explants (excised individual parts of the germinated seedlings) of *T. purpurea*. MS medium supplemented with 2,4-D (4.52 µM) or IAA (5.70 µM) or 2,4-D + IAA (4.52 µM + 5.70 µM) or 2,4-D + IAA + NAA + kin (4.52 µM + 5.70 µM + 5.37 µM + 4.64 µM) and 2,4-D + IAA + kin (4.52 µM + 5.70 µM + 4.64 µM) was found to be better medium for initiation of callus of root . The initiation of callus on. stem was found in almost 75% of the combinations tried with MS medium as given in Table 1. It was also found that out of all the combinations tried only MS medium supplemented with 2,4-D (4.52 µM) + IAA (5.7 µM) + kin (9.29 µM) showed initiation of callus on leaf. As compared to other parts, the mass of callus initiated on root was more prominent and vigorous.

The calli initiated on root and stem were subsequently maintained independently in MS medium supplemented with 2,4-D (4.52 µM) + IAA (28.5 µM) + kin (9.29 µM). It was obvious that increase in the concentration of IAA (28.5 µM) caused vigorous growth of the calli. In the initiation of callus the quantity of IAA used was only 5.7 µM. It was also observed that the leaf callus did not grow properly in the above medium but it could be maintained in MS medium supplemented with 2,4-D (4.52 µM) + IAA (28.5 µM)+ 6-BA (8.86 µM), while in this combination root and stem calli showed very slow growth.

Calli obtained from the different explants (root and stem) were capable of growing on MS medium supplemented with 2,4-D (4.52 µM) + IAA (28.5 µM) + kin (9.29 µM) for 5 m through successive subculturing. The rate of growth of callus was found to be gradual and uniform. The growth pattern of leaf callus could not be studied as the growth of callus was slow. However, the calli were soft and brown or dark brown in colour. The maximum increase in fresh weight of the calli of root and stem at the end of eight weeks were found to be 9.6 g and 7.92 g respectively fig. 1. The results of qualitative analysis for phytochemical constituents are presented in Table 3.

Rutin was found to be present only in leaf callus (0.98% w/w with respect to callus). Rotenoids were present in leaf, stem and root calli. In an earlier report by Sharma *et al.*[19]; it has been reported that 1.25% w/w of rotenoids were present in the static culture, while in the present investigation the total content of rotenoids in the calli developed from different parts of *T. purpurea* was found to be 2.05% w/w (Table 2).

Thus, the results obtained in the present investigation are significant from the point of view that the biomass obtained from different parts of the plant is capable of producing higher amount of rotenoids. Rutin, which is an important bioactive constituent is also being produced in the leaf cal-

TABLE 3: PHYTOCHEMICAL SCREENING.

Sample	Alkaloid	Flavonoid	Anthraquinone	Saponin	Steroids	Phenol	Protein	Amino acids	Reducing sugar
RE	+	+	-	+	+	+	+	+	+
SE	-	+	-	+	+	+	+	+	+
LE	-	+	-	+	+	+	+	+	-
RCE	+	+	-	+	+	+	+	+	+
SCE	-	+	-	+	+	+	+	+	+
LCE	-	+	-	+	+	+	+	+	-

Root extract (RE), Stem extract (SE), Leaf extract (LE), Root callus extract (RCE), Stem callus extract (SCE) and Leaf callus extract (LCE) were tested to check the presence (+) and absence (-) of different phytoconstituents.

lus and its presence is reported for the first time in the callus culture of *T. purpurea*. However, further studies are required to be carried out for rapid growth of the leaf callus in order to obtain the sufficient amount of the biomass for getting better yield of rutin.

ACKNOWLEDGEMENTS

We thank Mr Qaisar Naeem Khan, Department of Reprography, Jamia Hamdard for his help. We are grateful to University Grant Commission (UGC) and Jamia Hamdard (Hamdard University) for their financial assistance.

REFERENCES

1. Jain, S.K. and Filipps, R.A., In; Medicinal Plant of India, Orient Longman Limited, Chennai, 1991, 340.
2. Sankaran, J.R., **J. Natl. Integ. Med. Asson.**, 1984, 26, 255.
3. Kumar, A., Dutta, M., Bhatt, T.K. and Dalal, D.S., **Indian Vet. J.**, 1997, 74, 422.
4. Saxena, B.N., Dubey, D.N. and Nair, A.L., **Def. Sci. J.**, 1974, 24, 43, Through, **Chem. Abs.**, 1975, 82, 691782.
5. Aswal, B.S., Bhakuni, P.S., Goel, A.K., Kar, K. and Mehrata, B.N., **Indian J. Exp. Biol.**, 1984, 22, 487.
6. Rahman, H., Kashifudduja, M., Tyed, M. and Saleemuddin, M., **Indian J. Med. Res.**, 1985, 81, 418.
7. Dymock, W., Warden, C.J.H. and Hooper, D., In; Pharmacographia India., A History of the Principle Drugs of Vegetable Origin, Delhi Periodical Experts, Delhi, 1976, 415.
8. Kirtikar, K.R. and Basu, B.D. In; Indian Medicinal Plants, Delhi Periodical Experts, Delhi, 1975,724.
9. Nadkarni, K.M., In; Indian Materia Medica. Popular Prakashan Pvt. Ltd., Bombay, 1989, 561.
10. Wealth of India. A Dictionary of Indian Raw Materials and Industrial Products Rev. Edn., CSIR, New Delhi, 1976, 153.
11. Plea, G.I. and Hewitt, W.R. Eds., In; Toxicology of Liver, Raven Press, New York, 1982, 103.
12. Rajani, P. and Sharma, P.N., **Phytochemistry**, 1979, 18, 1581.
13. Venkata Rao, E. and Ranga Rao, N., **Phytochemistry**,1984, 23, 2339.
14. Venkata Rao, E. and Ranga Rao, N., **Phytochemistry**, 1979, 18, 1581.
15. Husseini, F.A. and Shoeb, A., **Planta Med**, 1986, 52, 220.
16. Gupta, R.K., Krishnamurty, M. and Parthasarathy, J., **Phytochemistry**, 1980, 19, 1680.
17. Sethi, P.D., In; Quantitative Analysis of Drugs in Pharmaceutical Formulations, 3rd Edn., CBS Publishers and Distributors, Delhi, 1997, 525.
18. Jones, M.A., **J. Assn. Off. Agr. Chemist**, 1945, 28, 352.
19. Sharma, R. and Khanna, P., **Indian J. Exp. Biol**, 1975, 13, 84.