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In vivo and *In vitro* Investigations on Rotenoids from *Cyperus Rotundus* (Linn.)

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ABSTRACT

Cyperusrotundus is an important medicinal plant widely used in traditional medicine around the world. An *in vitro* tissue culture of this plant was raised for investigating its naturally occurring insecticidal compound- the rotenoids. Callus of *C. rotundus*was initiated from young rhizomes on MS basal medium supplemented with different concentrations of growth hormones. Five rotenoids viz., elliptone, tephrosin, rotenone, sumatrol and deguelin were identified from plant parts (rhizome and leaves) as well as callus cultures on the basis of thin layer-chromatography, Gas liquid-chromatography and high performance liquid -chromatography. Rotenoid content was higherin rhizome (6.7 mg/g.dw) in comparison to leaf and calli (4.6 mg/g.dw and 6.2 mg/g.dw respectively).

Keywords: Cyperus rotundus, Callus culture, rotenoids

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INTRODUCTION

Cyperus rotundus Linn. (family Cyperaceae), also known as purple nutsedge or nutgrass, is common perennial weed with slender, scaly creeping. It is commonly known as "Nagarmotha" and is originated in India as a weed and it is now widely distributed in the tropics and subtropics[1].The medical uses of *Cyperus* have been used in medicine for thousands of years. The phytochemical investigation of *Cyperus rotundus* rhizome have revealed the presence of polyphenol, flavonol glycoside, alkaloid,saponins,sesquiterpenoids and essentialoil[2]. Although *C. rotundus* has been acknowledged as the most troublesome agricultural weed much about its basic growth and physiology is not well understood. Neither this species nor any other member of the sedge family (Cyperaceae) has been maintained as a callus *in vitro*, although many grasses and other monocotyledons have been grown successfully in tissue culture[3].Smith[4](1968) and Mohan Ram and Batra[5] (1970) grew young inflorescence meristems of *Carex* and *Cyperus* in tissue culture, respectively but did not subculture or induce callus. The growth of nutsedge*in vitro* should provide a powerful tool in fundamental studies of growth and development of the species. *C. rotundus*is a multipurpose plant, widely used in traditional medicine around the world to treat stomach aliments, wounds, boils and blisters[6]. Rotenoids are second largest group of naturally occurring insecticides and are a group of ketonic compounds consisting of four fused ring system called chromano-chromanone, biosynthesized from an iso-flavonone nucleus and have been mainly confined to the family fabaceae and are considered as naturally occurring poison to fishes and show insecticidal property[7]. They are of special value for the control of leaf chewing beetles, caterpillars and for pesticide activity. These natural insecticides score over synthetic insecticides because of their extremely low residual toxicity to mammals, non persistence and their wide spectrum of activity on many insects. They can be used with safety in many situations of public health demand and score over synthetic insecticides due to their extremely low residual toxicity to mammals, non-persistence and wide spectrum of activity on many insects[8]. Due to their insecticidal and pesticidal activity, they have agricultural and horticultural importance[9]. With low toxicity and relatively long residual action to warm blooded animals, rotenoids are used as a fish poison[10,11]. An important prerequisite for a sustainable ecosystem is the prevention of environmental pollution. It arise from the improper and indiscriminate use of hazardous toxicants. Therefore rotenoids appear ecologically sound and fulfill the criterion of international code of FAO for using them in integrated pest management schedules..Various methods for the chemical analysis of rotenoids have been described with the

help of association of official agricultural chemist for the separation of various fraction by using improved procedure, such as Thin layer densitometry[12], Gas liquid chromatography[13]and HPLC[14].Larvicidal properties of rotenoids from different plant species have also been worked out[15,8]. Rotenoids have also been reported from various plant species of the family cyperaceae.[16,17,18,19,20,21,22,23] However, there is no report on the production of rotenoids from tissue culture of *Cyperusrotundus* hence this study was undertaken.

MATERIAL AND METHODS

Callus induction and establishment

Surface sterilized rhizome tips of the plants were collected from mature plants and were used as explants for callus formation, establishment and development. The least contamination occurred when no adventitious roots or root primordial were visible on the surface of the young rhizome. All of surfaces blackened within a few days. callus proliferation were noticeable within 2-3 week on Murashige and Skoog's (1962) media(MS) supplemented with 2.0-6.0 mg/lt 2,4-D. Callus of each plant raised on MS-medium *in vitro* was maintained for one year by frequent subculturings on fresh medium after every 4-5 weeks, for its biomass production. Callus was also subcultured on fresh medium in order to evaluate whether the callus showed any morphogenic response or continued to proliferate as such. Fresh weight of callus was recorded by weighing the callus tissue after separating it from the plant. Over 25 clones of calluses were established and subcultured. Cultures were divided into quarters and transferred to new MS media every 6-8 week. Some clones have been subcultured for over one year. Callus was pale white and almost slimy surface. The best growth of undifferentiated callus occurred on 2.5 and 6.0 mg/litre 2, 4-D in MS medium.

Rotenoids Extraction procedure

Each of the powdered material (root and leaves samples) of *C. rotundus* was extracted separately with acetonitrile saturated with n-hexane for 72 h at room temperature.[24] Each sample mixture was filtered and concentrated to dryness *in vacuo,* separately. The concentrated extract was dissolved in acetone and filtered with Whatmann No. 1 filter paper. The filtrate was run on column of inert alumina to eliminate impurities. The various fractions were pooled together, dried and weighed for crude rotenoid content in different plant parts. The dried extracts were stored at 5ºC in the refrigerator until used for further studies.

Thin layer chromatography

To examine rotenoids, thin layer chromatography was performed using silica gel G (E Merck)

coated plates (20x20 cm; wet thickness 0.2-0.4 mm) which were dried at room temperature, activated at ~100ºC for 30 min and brought to room temperature before use.

Qualitative analysis

Extracts of the shade dried plant material were analysed by CO-TLC using rotenoids viz., rotenone, elliptone, sumatrol, tephrosin and deguelin as standard reference markers. Each of the fractions was applied 1 cm above the edge of the activated TLC plates and developed separately two dimensionally in two solvent system; solvent system (I) chloroform: ether; 95:5 in first direction and solvent system (II) chloroform: acetone: acetic acid; 196:3:1 in the second[25] (Delfel and Tallent, 1969). The spots were visualized under UV lamp and sprayed with hydroiodic reagent (HI) and heated at 120ºC for 20 min to develop the chromatograms.

Preparative thin layer chromatography

Preparative thin layer chromatography of various test extracts was performed on silica gel G (0.3-0.4) coated plates along with standard markers in a solvent system. The spots were visualized under UV lamp and sprayed with hydroiodic reagent (HI) and heated at 120ºC for 20 min to develop the chromatograms. The fluorescent spots corresponding with those of standard reference compounds were marked separately in each case and scrapped along with silica gel from about 150-200 plates and eluted with methanol. The eluted fractions were filtered, dried in *vacuo* and weighed.

Identification

Each of the isolated compound was subjected for its melting point and spectral studies along with respective standard markers. The compounds were identified on the basis on TLC behavior and spectral studies.

Gas Liquid Chromatography (GLC)

The isolated rotenoides were subjected to gas chromatography adopting following operating conditions (Gas chromatogram; Nucon 5700 model, equipped with column: OV 17. (30mx0.25 mm id x 0.25 □m film thickness) with flame ionization detector; Carrier gas helium at a flow rate 1.5 ml/min length; oven temperature programming: 60ºC-250ºC injector temperature – 230ºC; transfer line – 260ºC; chart speed was 4-10 cm/minutes). GLC curves were obtained after reduction of rotenoids with sodium borohydride. About 100 □m of 100% concentrated extract was injected. Retention time was calculated separately and compared with those of reference compounds.

High Performance Liquid Chromatography (HPLC)

The high performance liquid chromatography for the determination of rotenoids was performed

on a Millipore Waters Model 501 fitted with pump solvent delivery system, injector (Model; 6UK) by using □□ Bondapak C_{18} stainless steel column (30cmx3.9 mm ; temperature 24ºC±2ºC). The mobile phase used for the separation was isocratic solvent (3000 ml of acetonitrile diluted with 2000 ml of deionized water; 60:40) and the flow rate was adjusted to 0.7 ml/min (eluent program: 40 min 100% methanol). The effluent was monitored by UV absorption at 294 nm with a detector adjusted at attenuation of 0.5 AUFS (Lambda Max Model 481 LC spectrophotometer detectors; Waters). The extract was pretreated, filtered, dissolve in 0.5□l of the mobile phase and a 10□l volume of the sample was injected the calibration graphs were constructed by plotting the ratio of the peak area for determination.

RESULTS AND DISCUSSIONS

In the present investigation, five rotenoids*viz.*, elliptone, tephrosin, rotenone, sumatrol and deguelin were isolated and identified from plant parts (rhizome and leaves) as well as callus tissue (2, 4, 6, and 8 weeks) of *C. rotundus.* They were confirmed on the basis of GLC which showed the peaks of isolated rotenoids were similar to that of the standard reference compounds (Figure. 1), TLC behavior, color, melting point, UV and IR spectral studies *viz.*, elliptone (Rf 0.76; purplish blue after spraying with HI; mp 178- 180°C; UVnm□$_{max}$ 221, 223), tephrosin, (Rf0.28;pink after spraying with HI, mp 190- 198°C; UVnm□$_{max}$ 222, 225), rotenone (Rf 0.53 after spraying with HI; mp 160-164°C; UVnm□$_{max}$ 221, 223), sumatrol (Rf 0.32; bluish grey after spraying with HI; mp 198-200°C, UVnm□$_{max}$ 223, 225) and deguelin (Rf 0.69; pink after spraying with HI; mp 194-196°C, UVnm□$_{max}$ 222, 224) (Table 1). Quantitative analysis reveals that maximum amount of rotenoid was found in rhizomeas comparison to leaf and calli in *C. rotundus.*In all therotenoids (rotenone, elliptone, degulin, sumatrol and tephrosin) the content varied in amount present in plant parts and callus cultures. Among the plant parts, total amount of rotenoids was found to be higher in rhizome (6.7mg/g.dw) as compared to leaves (4.6 mg/g.dw; Table 2). In callus culture the rotenoids content (6.2mg/g.dw; Table 3) was found to be maximum in 6 weeks old callus culture as the maximum growth index was observed in 6 week old callus (4.78;Table 3). A progressive increase in rotenoids content was found from 2 to 6 weeks old callus culture which decreased in 8 weeks old callus.

Table-1:Chromatographicbehavior of isolated rotenoids of *C. rotundus*

Isolated Rotenoids	Chemical formula	Colour after spray Hydroiodic acid	Rf	UV nm λ_{max}	Melting point
Rotenone	$C_{23}H_{22}O_6$	Blue	0.53	221, 223	160-164°C
Elliptone	$C_{20}H_{16}O_6$	Purplish blue	0.76	221, 223	178-180°C
Deguelin	$C_{23}H_{22}O_6$	Pink	0.69	222, 224	194-196°C
Sumatrol	$C_{23}H_{22}O_7$	Bluish grey	0.32	223, 225	198-200°C
Tephrosin	$C_{23}H_{22}O_6$	Pink	0.28	222, 225	190-198°C

Table 2: Yield of isolated rotenoids (mg/g.dw) from plant parts of *C. rotundus*

Plant parts	Rotenoids content (mg/g.dw)					Total Rotenoids content
	Rotenone	Elliptone	Degulin	Sumatrol	Tephrosin	
Rhizome	1.3	0.6	1.9	0.7	2.2	6.7
Leaves	0.7	1.1	1.5	0.5	0.8	4.6

Table 3. Yield of isolated rotenoids (mg/g.dw) from *in vitro* cultures of *C.rotundus*

Age of tissue	G.I.	Rotenoids content (mg/g.dw)					Total Rotenoids content
		Rotenone	Elliptone	Degulin	Sumatrol	Tephrosin	
2	1.26	0.2	0.4	0.1	0.5	0.6	1.8
4	2.60	0.8	0.2	0.6	1.0	0.8	3.4
6	4.78	1.0	0.9	1.5	0.8	2.0	**6.2**
8	3.97	0.5	1.8	0.9	1.2	1.0	5.4

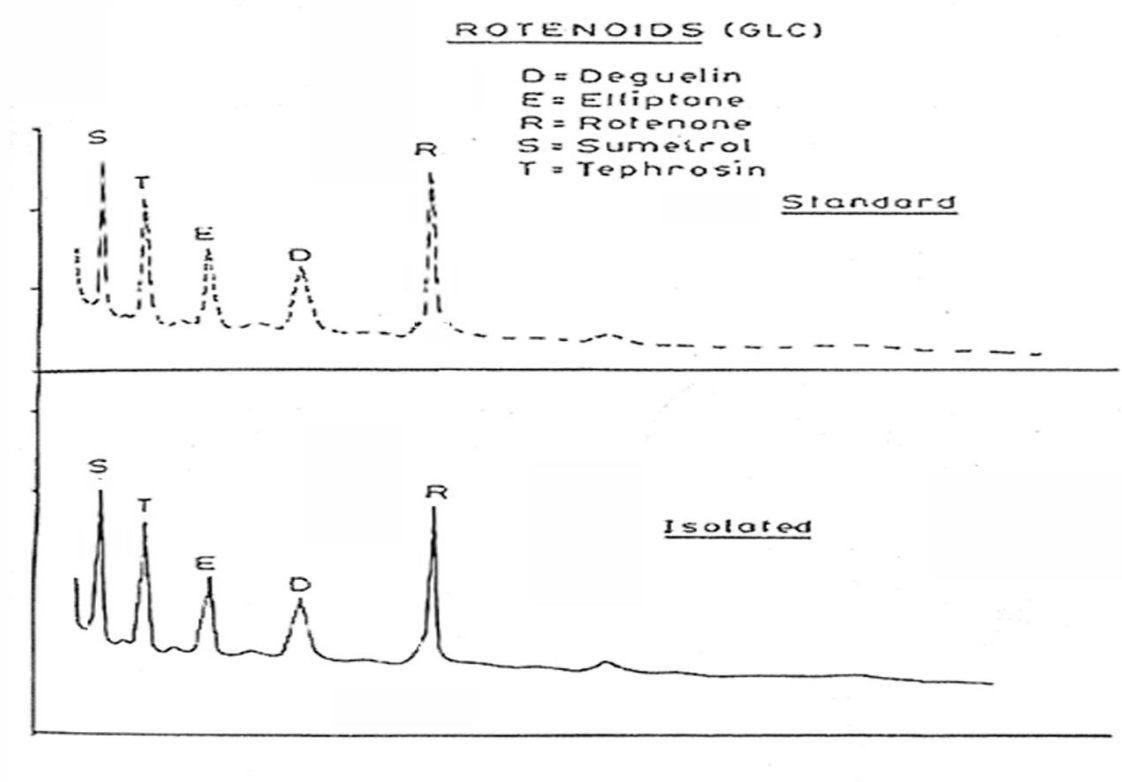

Figure 1: GLC chromatogram of isolated rotenoids and standards.

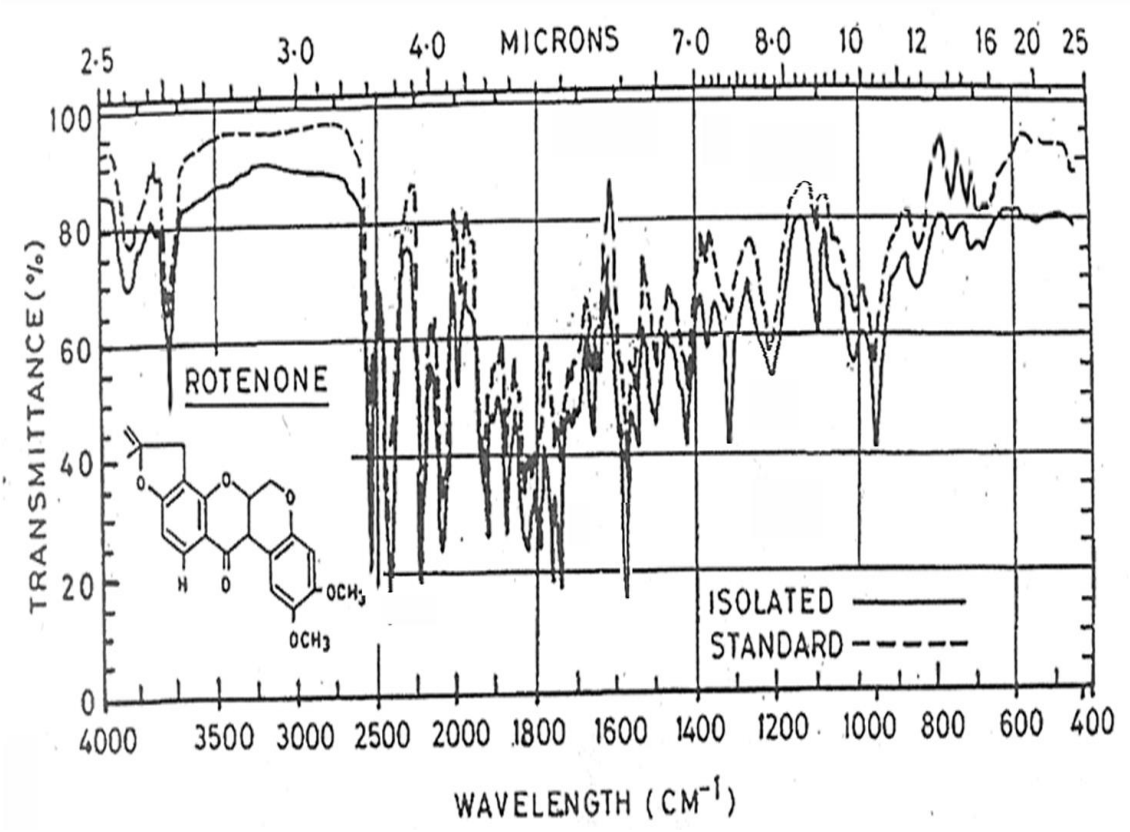

Figure 2: Infrared spectra of standard and isolated Rotenone

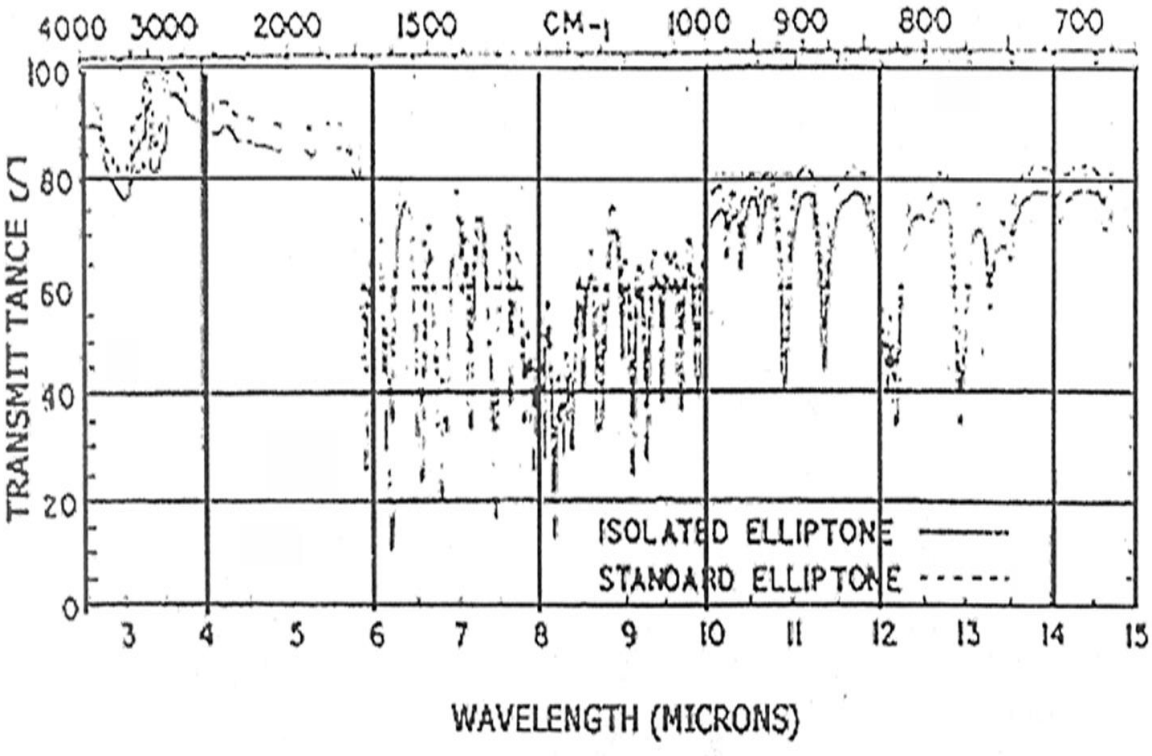

Figure 3: Infrared spectra of standard and isolated Elliptone

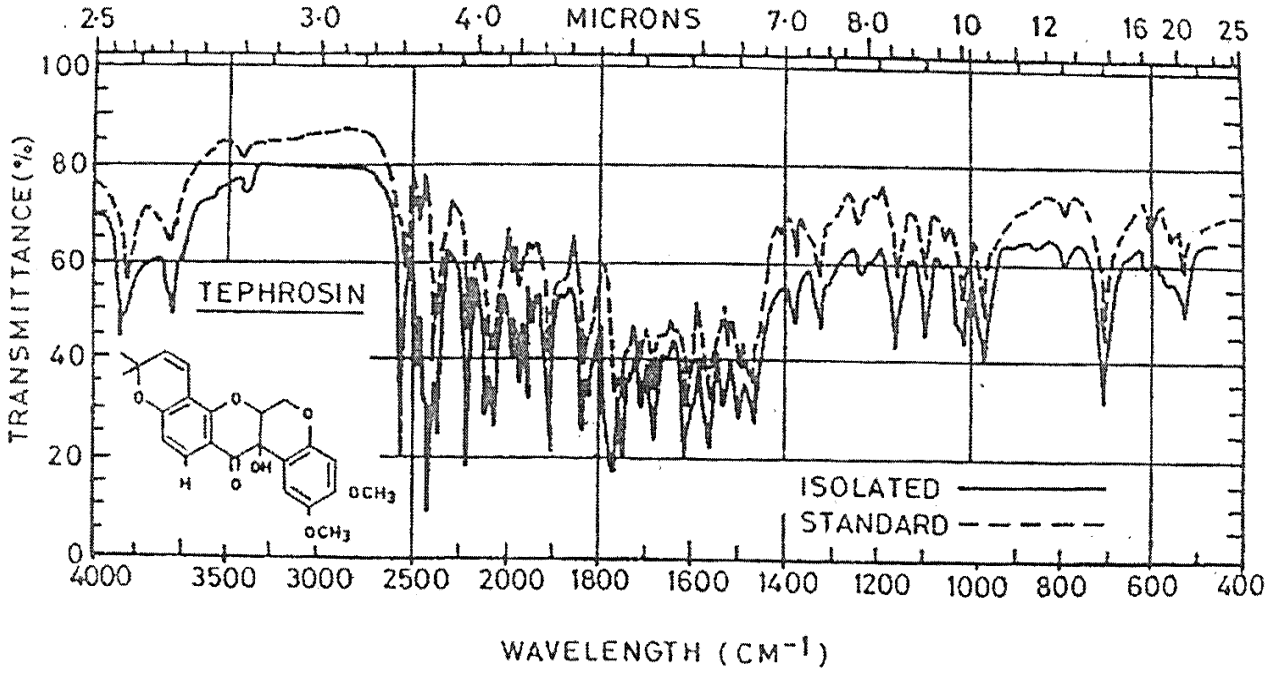

Figure 4: Infrared spectra of standard and isolated Tephrosin

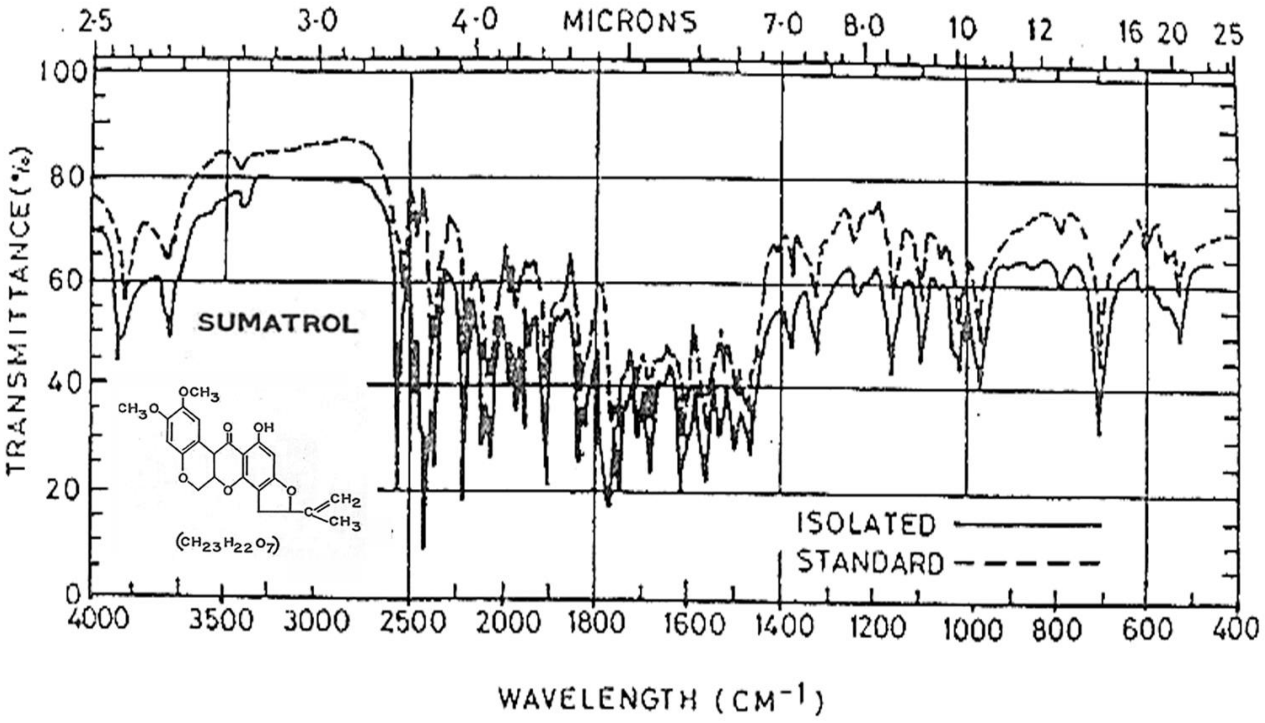

Figure 5: Infrared spectra of standard and isolated Sumatrol

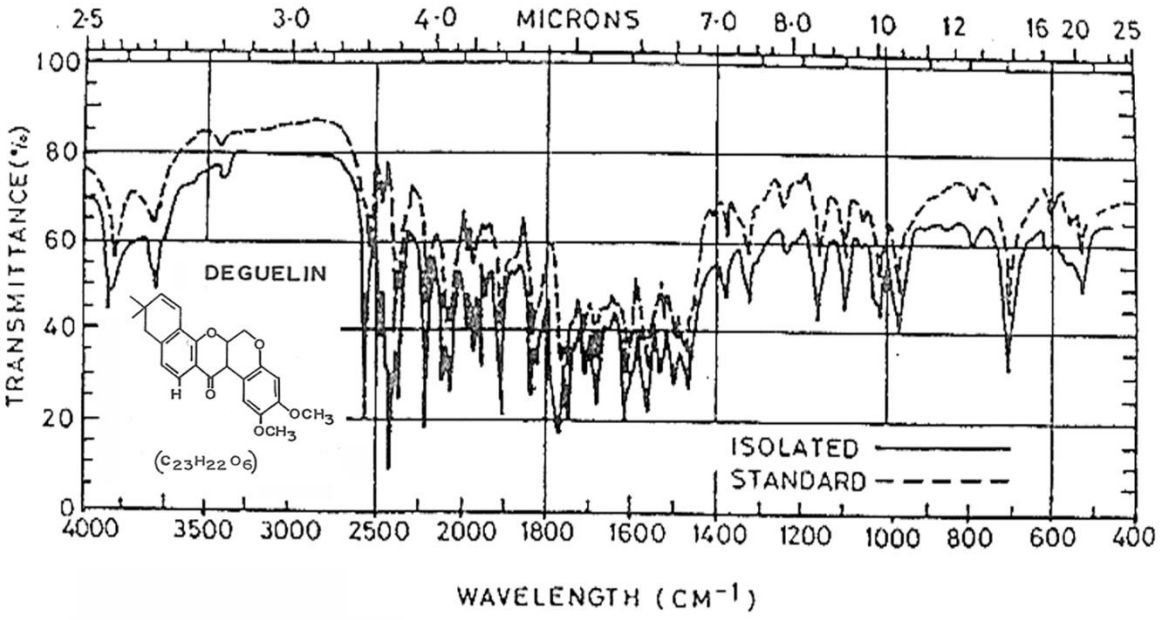

Figure 6: Infrared spectra of standard and isolated Deguelin

CONCLUSION

The present investigation revealed that callus culture of the plant species of *C. Rotundus* possess rotenoids biosynthetic potential which can be exploited commercially for the production of rotenoids in the interest of human welfare.

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