Phytochemical constituents of cultured cells of *Eucalyptus tereticornis* SM.

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ABSTRACT

Callus induced from shoot explants of *Eucalyptus tereticornis* was maintained for eight months on a defined MS medium. The lipid composition of the callus of *E. tereticornis* were β -sitosterol, stigmasterol and cholesterol. In addition, we report the presence of a flavanoid glycoside, aglycon identified as Kaempferol. Further, the presence of 2,3-dihydroxybenzaldehyde and 3,4-dihydroxyphenyl acetic acid was established from the methanol fraction.

List of Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid, Kn = Kinetin, NAA = 1-Naphthaleneacetic acid, IAA = Indole-3-acetic acid, BA = 6-Benzylaminopurine.

INTRODUCTION

Eucalyptus is a large genus of subtropical and warmtemperate evergreen aromatic trees indigenous to Australia, Tasmania and New Guinea. About 100 species have been tried in India for valuable timber, pulp and paper production.

Most of the species are popularly known as Gum trees although the exudation from them is not a gum but an astringent, tanniferous polyphenol called Kino. Bark of *Eucalyptus* species contains varying proportions of polyphenols. They constitute an important source of tanning materials (Linchan, 1981).

Investigations of the phenolics of *Eucalyptus* have revealed the presence of eucalyptin, betulinic acid and urosolic acid from *E. citriodora* Wild. (Dayal Rameshwar, 1982), euglobul III from *E. globulus* (Sawad, 1980), robus-teol-A (Qin Cuo-We, 1981) and later 7-O-methyl aroma-dendrin, aromadendrin and fustin from *E. citriodora* (Dayal Rameshwar, 1981). Morolic acid was characterised from *E. grossa* by Cannon (1981). Earlier Wollenweber (1981) reported flavanoids from the cuticular layer of the leaf of *E. ovata*. Abd-Alla (1980) identified flavanoid glyco-sides in two chemical races of *E. camaldulensis* corres-ponding to north and south populations of Australia.

Attempts to propagate *Eucalyptus* through tissue culture have been successful (De Fossard 1978, Gupta et al 1978, 1982, 1983, Hartney and Barker 1980, Mehra-palta 1982, Bennet and McComb 1982, Durand-Cresswel 1982, Sankara Rao and Venkateswara 1985). The phyto-chemical investigations of the callus have not been car-ried out so far and we wish to report the generation of callus and subsequent phytochemical investigations of

E. tereticornis.

MATERIAL AND METHODS

Nodal and internodal segments from tender portions of actively growing branches of *E. tereticornis* plants raised in the Indian Institute of Science nursery were collected. They were surface sterilized with 0.1% mercuric chloride solution for 15 minutes and rinsed thoroughly with sterile distilled water. The material was further cut into pieces of suitable size (10mm) and implanted onto culture media.

Culture media composed of mineral salts and vita-mins of Murashige and Skoog (1962) with 2% sucrose and 800 mg/L Polyvinylpyrrolidone (PVP). Only agar solidified media (0.7%) were used.

All chemicals used were of analytical grade (British Drug House, E. Merck, Sigma and Difco). The following combinations of growth supplements were used for callus initiation.

1. MS Basal medium + 2,4-D (5 mg/L) + Kn (0.5 mg/L)
2. MS Basal medium + 2,4-D (2 mg/L)
3. MS Basal medium + NAA (1 mg/L) + IAA (0.2 mg/L) + BA (1 mg/L).

Callus was successfully maintained for 8 months with a passage period of 3 weeks on MS(2) medium.

Culture media in tubes were autoclaved at 15 lbs/cm² for 15 minutes. Cultures were incubated at 25 $\pm$ 2°C, exposed to an artificial neon light of 200 μ m/m²/sec with dark light cycles of 8/16 hours.

Phytochemical Examination

Fresh callus (100 gm) at the end of six week period was separated from the media and washed with double distilled water and lyophilised. The lyophilised callus powder was extracted exhaustively with n-hexane in soxhlet apparatus. The hexane fraction was separated and the marc was dried and extracted with ethyl acetate followed by another extraction with methanol.

The hexane fraction was dried and to the residue chloroform was added. The chloroform soluble fraction was separated to which hexane was added and kept at room temperature. A white residue (A) was obtained posi-tive to sterols. The chloroform insoluble hexane fraction was a greasy yellow semisolid (60 mg). The white residue (A), (25 mg) was chromatographed on a silicagel column (1.5x20 cm). The column when eluted with hex:CHCl₃

(50:50), CHCl_3 (100), CHCl_3 :EtOAc (70:30) gave spots corresponding to 2 phytosterols. A silicagel TLC of the fraction with hex: CHCl_3 :EtOAc (70:20:10) indicated homogeneity of the samples.

Ethyl acetate fraction when partitioned with ether and chromatographed on silicagel column (1.5 x 20 cm) gave fractions positive to phenolics. The ether:EtOAc (50:50) fraction upon repeated crystallization in acetone-ether mixture gave pale yellow crystals (8 mg) which was identified as a flavanoidal glycoside by chemical degradation and spectral analysis.

Methanol fraction when chromatographed on silicagel column gave fractions positive to phenols. A TLC of the fractions with authentic phenols was carried out using the following systems. CHCl_3 :methanol (80:20), EtOAc:methanol:HAC (80:18:2) and 2% HCOOH .

Phytosterols were characterised by chromatography and spectroscopy. Silicagel TLC of the samples with authentic samples of cholesterol, cholesterol acetate, stigmasterol, β -sitosterol was carried out. Cochromatography of a mixture of authentic sterols and sample was carried out in solvent systems hex: CHCl_3 :EtOAc (70:20:10), CHCl_3 :hex (50:50), ButOH:HAC: H_2O (50:40:10). The IR spectra were recorded on Perkin-Elmer IR spectrophotometer by the nujol method.

The flavanoidal glycoside (5 mg) was dissolved in 20% HCL (6 ml) and boiled for 10 minutes to hydrolyse it. The solution was cooled after addition of water (100 ml), then it was washed thrice with benzene and dried in vacuum. The yield of aglycon was 3.2 mg. The aglycon when treated with Shinoda's reagent gave a bright red colour which was identical with that of authentic kaempferol. Chromatographic analysis was done on Whatman No.10 paper. Solvent systems used were isoprop: NH_3 : H_2O (80:10:10), 2% HCOOH , benzene:HAC: H_2O (40:40:20).

Analysis of phenols in methanol fractions: Chromatographic analysis was done with paper on Whatman No.10 paper and TLC on silicagel. Authentic samples tried include, 2,3-dihydroxybenzaldehyde, 3,4-dihydroxyphenylacetic acid, DL-4-hydroxy 3-methoxy mandelic acid, 3,5-dihydroxyphenylacetic acid, protocatechuic acid, t-cinnamic acid, p-coumaric acid. Spots were identified by fluorescence and R_f values. Freshly diazotised p-nitroaniline and sodium hydroxide solutions were used as spray reagents.

RESULTS AND DISCUSSION

Callus from the shoot segments of *E.tereticornis* were induced under light at $25 \pm 2^\circ\text{C}$. The callus was obtained with all the three MS (1),(2) and (3) media tried. Callus was white and friable in medium supplemented with 2,4-D with or without Kn whereas the medium containing BA, NAA and IAA produced coloured compact slightly nodulated callus. The callus grew vigorously in MS(2) media and required subculturing every three weeks. During routine passage, portions of the callus were seen to develop red pigment. The chemical constituents of the callus were repeatedly extracted in n-hexane, EtOAc and methanol. The hexane fraction upon partition with CHCl_3 and the subsequent silicagel column chromatography of CHCl_3 soluble fraction afforded pure fractions of a major and two minor sterols identified by chromatographic behaviour on TLC. IR spectra of major sterol - 3200 cm^{-1} (-OH), 1800 cm^{-1} (C=C), 800 cm^{-1} (- CH_2 -) was superimposable with authentic β -sitosterol - 3200 cm^{-1} , 1750 cm^{-1} , 900 cm^{-1} . Quantitatively the components of phytosterols in the callus were different with β -sitosterol as the main component (70%), stigmasterol and cholesterol present only in trace amounts.

The ultra violet absorption spectra of the aglycon measured in methanol containing 0.01% HCL had an absorption maxima of carbonyl group at 315 nm (kaempferol

Fig.1. IR spectra of the flavanoidal aglycon from cultured cells of *E.tereticornis*
Continuous line: Kaempferol, broken line: sample.

320nm) and aromatic peak at 280nm (kaempferol 280nm). The IR spectra of the sample - 3420 cm^{-1} , 3380 cm^{-1} , 3220 cm^{-1} , 3180 cm^{-1} (multiple OH peaks), strong peak at 1700 cm^{-1} (carbonyl), 1600 cm^{-1} (Aromatic) and kaempferol - 3430 cm^{-1} , 3350 cm^{-1} , 3300 cm^{-1} , 3150 cm^{-1} , 1700 cm^{-1} , 1600 cm^{-1} was superimposable. We identified the aglycon moiety by hydrolysing the glycoside. Qualitative analysis, chromatographic and spectral data proved that aglycon was kaempferol. The Fehlings reaction was positive indicating the glycosidic linkage (Fig.1, Table 1).

The phenolics of the methanol fraction upon silicagel chromatography afforded fractions positive to phenolics identified by spot tests with freshly diazotised p-nitroaniline and NaOH solutions. We could identify only two phenolics P_1 and P_2 by chromatography with authentic samples. The IR spectra of P_1 - 3350 cm^{-1} (-OH), 1650 cm^{-1} (carbonyl), 1660 cm^{-1} (Aromatic) and 2,3-dihydroxybenzaldehyde - 3300 cm^{-1} , 1650 cm^{-1} , 1600 cm^{-1} and P_2 - split peak at 3400 cm^{-1} , 3350 cm^{-1} (-OH groups), 1700 cm^{-1} (carbonyl), 1590 cm^{-1} (aromatic) and 3,4-dihydroxyphenylacetic acid - 3440 cm^{-1} , 3320 cm^{-1} , 1700 cm^{-1} , 1600 cm^{-1} , 900 cm^{-1} were superimposable.

Table 1

Properties of the aglycon from the cultured cells of E. tereticornis

	R_f values (X 100)			Spectral Data	
	Isoprop : $\text{NH}_3:\text{H}_2\text{O}$ 80:10:10	Benzene : $\text{HAC}:\text{H}_2\text{O}$	2% HCOOH	λ max (nm)	
Sample from cultured cells	45	30	70	315	280
Authentic kaempferol	45	28	65	320	280

During the tissue culture of E. tereticornis the accumulation of phenolics in the absence of PVP in the culture media was a routine problem which lead us to investigate the chemical composition of the aromatic metabolites, in cultured cells. To our knowledge, this is the first report on the phytochemistry of the callus of Eucalyptus although report of flavanoid glycosides from the leaves of E. citriodora, E. ovata, E. camaldulensis are available (Dayal Rameshwar 1980, 1981, Wollenweber 1981, Abd-Alla 1980). Abd-Alla (1980) identified kaempferol-3-glycoside from the leaf of E. camaldulensis. We report for the first time isolation of kaempferol glycoside from cultured cells of E. tereticornis. It appears, that a major hydroxylation system

is active as seen from the nature of aromatic products in the callus of E. tereticornis. We believe it is possible to alter the biosynthetic profile of phenolics in the callus by changing cultural conditions like light and hormonal manipulation.

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