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Enhancement of antioxidant compound in *Tylophora indica* (Asclepeadaceae) callus

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ABSTRACT

The accumulation of kaempferol was evaluated in undifferentiated callus of Tylophora indica through TLC, HPTLC analysis with standard reference compound. Kaempferol is a strong antioxidant and help to prevent oxidative damage to our cells, lipids and DNA. In the present investigation, we have enhanced the kaempferol content in Tylophora indica tissue culture by using precursors like sallicyclic acid, ornithine, cinnamic acid, tyrosin and phenylalanine in different concentration(10 and 20 mg/100 ml).Here we have used static as well as suspension culture to enhanced the kaempferol concentration . The callus of Tylophora indica was initiated and maintained on MS (Murashige and Skoog's) medium supplemented with 3% of sucrose, while Zenk production media was used as production media. A remarkable enhancement in kaempferol content was obtained by using 20 mg /100 ml of tyrosin (1.49% dw; control -0.096%dw) in suspension culture ,which is more than tenfold increase. The enhancement of kaempferol in callus of Tylophora indica by using these amino acids was reported for the first time.

Keywords: Callus; NAA; BAP; Phenylalanine; Ornithine; Tyrosin; Cinnamic acid; Zenk media

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INTRODUCTION

Medicinal plant is defined as a plant which has been proven or thought to have medicinal remedies [1].Plant tissue cultures have now emerged as an alternative to plants which have acquired world-wide dimensions in improving genetic potential of plants and their preparative use in methodologies. Cultures have been proved to retain biosynthetic capacities, as various compounds and enzyme systems of plants are found to be present in cultures too, Review of literature reveals that lot of work has been done in the field of flavonoids tissue culture [2,3,4,5]. Flavonoids are metabolites produced as part of plant defense, especially against the effects of ultraviolet radiation., Flavonoids ,one of secondary metabolite are potent antioxidants, free radical scavengers, and metal chelators and lipid peroxidation inhibitor. They have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, vasodilatory actions [6],.Kaempferol seems to prevent arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood. Studies have also confirmed that kaempferol acts as a chemopreventive agent, which means that it inhibits the formation of cancer cells. It is a flavonoid classified as flavonol that widely distributed group of polyphenolic compounds, characterized by a common benzo-pyronestructure .Biotransformation is a process through which functional groups of organic compounds are modified by living cells. Biotransformation done by plant cell culture system can be desirable when a given reaction is unique to a plant cell and the product of reaction has a high market value. The useful natural products are synthesized through secondary metabolism. Growth inhibition is often associated with cytodifferentiation and the induction of enzymes for secondary metabolism, so dual culture system is preferred. Dual culture system involves biomass production in a medium optimum for cell proliferation followed by transfer

of healthy cells to a different medium which is favorable for product yield. This strategy was used by Zenk et al., 1975[7] for the production of indole alkaloids by *Catharanthusroseus* cells. In the present study, this strategy was used to enhance the production ofkaempferol in *Tylophora indica*callus. Cytokinin concentration in the media also supports to enhance the secondary metabolite. Khanna et al., 1988 [4]have reported the enhancement of secondary metabolite by feeding the callus with precursor in the tissue culture of various medicinal plant sps. So,keeping all this background we have selected sallicyclicacid,cinnamicacid,ornithine ,tyrosin and, phenylalanine as precursor for enhancement of kaempferol in callus of *Tylophora indica* . Zenk media was used as production media.

Tylophora indica(Burm f.)Merill.(Family: Asclepidaceae) commonly knownasAntmul is a twining perennial plantdistributed throughout southern andeastern part of India in plains, forests, andhilly places .The use of leaves for tea and pharmaceutical preparations is very common and agreement with the high levels of secondary metabolites in this organ *Tylophora indica*(Burm f.) Merill.(Family: Asclepidaceae) commonly known as Antmul is a twining perennial plant distributed throughout southern andeastern part of India in plains, forests, andhilly places The plant is found growingnormally in Uttar Pradesh, Bengal, Assam,Orissa, Himalayas and sub Himalayas inIndia It is a branching climber or shrubthat grows up to 1.5 meters, leaves areobvate-oblong to elliptic-oblong, -10cm long and 1.5-7cm wide.The plant has been reported to contain 0.2-0.46% alkaloids .It contain Tylophorine, , kaempferol, wax, resins, and tannins.*Tylophora indica* has been exploited by phytochemically as well as biotechnologically[8-15] Many scientists have documented the medicinal values and tissue culture studies on *T.indica*[16-19].

The biosynthsis of kaempferol in callus of *Tylophora indica* is not reported so far . In the present investigation *Tylophora indica* callus was initiated , maintained and used to examine the kaempferol content as experimental material . A novel method is described we have enhanced the kaempferol content in *Tylophora indica* tissue culture by using elicitor like salicyclic acid and various amino acids like ornithine, cinnamicacid,tyrosin ,phenylalanine in different concentrations. The enhancement of kaempferol in callus of *Tylophora indica* by using these precursor was reported for the first time.

MATERIALS AND METHODS

The plant material collected from the Kelkar farmhouse, Mulund, was used for the initiation of callus. The young leaves of the plant were surface sterilized with 0.1% of mercuric chloride and washed with sterile double distilled water. The surface sterilized leaves were cut in to pieces (1 cm) and aseptically inoculated in to the sterilized MS (Murasinge and skoog's) basal media supplemented with 3 % of sucrose, and various growth hormone in different combination separately. The media was solidified with 0.7 % of agar .It was adjusted to 5.8 pH before autoclaving (121^0c for 15 min.) .Various hormones like 2,4D,Kinetin ,IAA ,IBA in different combination were used but 2ppm of NAA and 0.2 ppm of BAP gave the best results. The cultures were incubated at 25^0c at 8 hours of dark and 16 hour of photo period (1000 Lux). The growth index in all the samples were calculated (Tab.1).After twenty days of incubation proliferation of leaves skin can be marked and finally bursting of the epidermis can be seen .The callus initiation started from midrib portion of the leaves. Sub culturing in to fresh media was carried out at the time interval of 4 weeks. The developed callus was then transferred to media i.ezenk media supplemented with precursor like salicyclic acid ,ornithine, cinnamicacid,tyrosin and phenylalanine in different concentration(10 and 20 mg/100 ml). All the samples were harvested at the age of six weeks, dried and subjected for methanolic cold extraction.These cold extracts were dried in vacuo and subjected for qualitative as well as quantitative analysis of kaempferol with that of the standard compound. The qualitative analysis was carried out by Thin Layer Chromatography (TLC),while quantitative estimation was carried out by HPTLC.

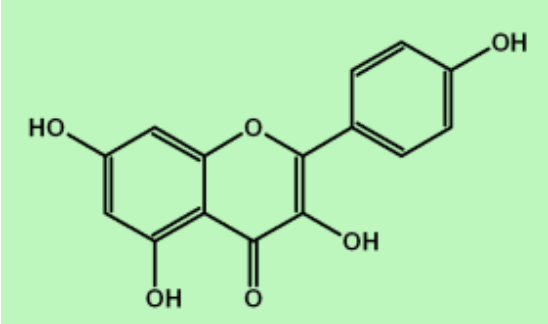
HPTLC(High Performance Liquid Chromatography) analysis

HPTLC analysis was carried out in Anchrom Test Lab Pvt. Ltd using silica gel plates (60F254 Manufacturer E. MERCK KGaA),Sample application was carried out on CAMAG Linomat 5 Instrument **(**CAMAG Linomat 5 "Linomat5_080222" S/N 080222).Inert gas was used as spray gas. Sample solvent type was methanol. Dosage speed was 150nl/s and syringe size was 100µl and the analysis wave length was 430 nm. Toluene: Ethyl acetate (14:2) was used as mobile phase.

RESULTS AND DISCUSSION

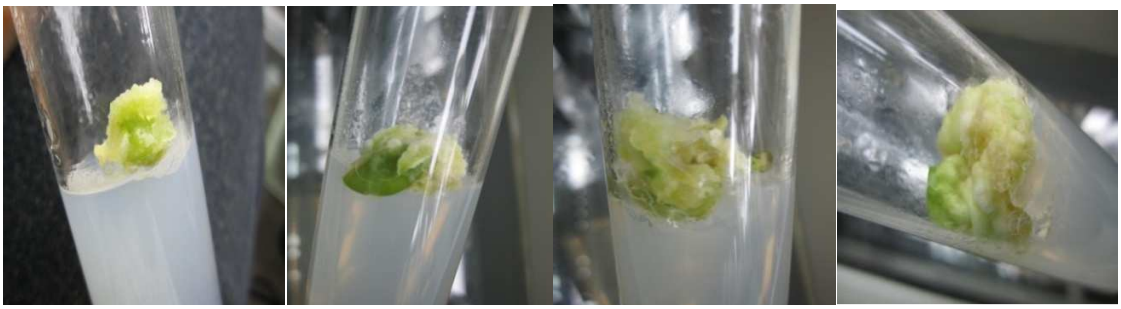
The callus of *Tylophora indica* was successfully initiate and established in the laboratory by using 0.2 ppm of BAP and 2ppm of NAA in the MS medium supplemented with3% of sucrose and0.8% of agar. The callus then transferred

to the Zenk production media to enhance the content of kaempferol.Zenk media is helps to produce the secondary metabolite in tissue culture.



Kaempferol (Rf 0.56; Uv fluorescent - bright yellowish blue, ammonia - deep yellow, Fecl3-brown; m.p. 270-273^{0}C

Figure1.: Showing the chemical structure of kaempfero



A **B** **C** **D**

Figure 2.: The Developmental stages of Callus (A-D) initiated from leaf explants in *Tylophora indica*

Table 1: Effect of different growth hormones on growth of callus (6 weeks old) initiated from the leaf explant.± represent the mean value s.e. of three replecates
GI=Final weight-initial weight/initial weight

Sr. No	IBA (ppm)	NAA (ppm)	BAP (ppm)	Kinetin (ppm)	Growth Index (GI)
A	-	2	0.2	-	5.69±0.011
B	-	1	0.1	0.1	3.11±0.052
C	-	1	0.1	0.2	3.98±0.041
D	-	2	-	0.1	5.12±0.032
E	0.5	-	0.2	-	2.11± 0.011
F	1.0	-	0.1	-	2.18 ±0.019

Table 2.: The enhancement of kaempferol in callus of *Tylophora indica*
± represent the mean value s.e. of three replicate

s. no	compound	Kaempferol content in Static culture(%)		Kaempferol content in Suspension(%)	
		10 mg/100ml	20 mg/100ml	10mg/100ml	20 mg/100 ml
1.	Salicyclicacid	0.21±0.076	0.18±0.096	-	-
2.	Cinnamic acid	0.47 ±0.056	0.12±0.054	-	-
3.	Ornithine	0.15±0.064	0.93±0.058	-	-
4.	Tyrosin	-	-	0.54±0.047	1.49±0.048
5.	Phenylalanine	-	-	0.153±0.078	0.239±0.038
6. 7.	Callus Leaves	0.096±0.076 0.1609±0.054	-	-	

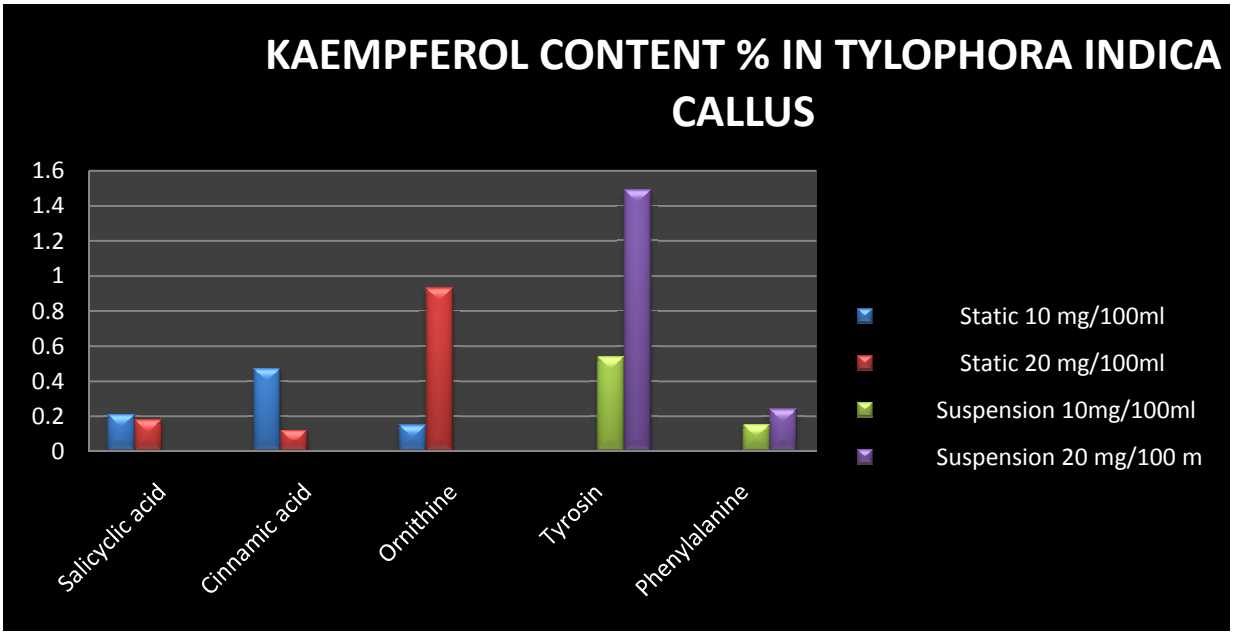


Figure 3 : The enhancement of kaempferol in callus of *Tylophora indica*

The present study describes the enhancement of kaempferol content in callus The different precursor like cinnamicacid,ornithine,tyrosin,phenylalanine, and salicyclic acid were used in 10 and 20 mg/100ml each separately.salicyclic acid and cinnamic acid and ornithine were used in static culture where as tyrosin and phenylalanine were used in suspension culture.Zenk media was used as a basal media in all the instances.the production of kaempferol in callus of *Tylophora indica* was increased considerably.The results showed that the tyrosin proved as a best precursor of kaempferol as dicussed in literature.maximum enhancement in kaempferol was found in the tyrosin treated callus which is around 10 fold increase than control.

.In addition to many essential nutritional components, plants contain phenolic substances, a large and heterogeneous group of biologically active non-nutrients .Flavonoids are divided into many categories, including flavonols, flavones, catechins, proanthocyanidins, anthocyanidins and isoflavonoids[6] Phenolic acids present in plants are hydroxylated derivatives of benzoic and cinnamic acid . Phenylalanine, produced in plants via the shikimate pathway, is a common precursor for most phenolic compounds in higher plants [20,21] Similarly, hydroxyl cinnamic acids, and particularly their coenzyme A esters, are common structural elements of phenolic compounds, The enzymes catalyzing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonialyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and hydroxycinnamate: coenzyme A ligase (C4L). These three steps are necessary for the biosynthesis of phenolic compounds) [20-21]. Tyrosine is really an essential amino acid, as it is synthesized by the hydroxylation of phenylalanine, an essential amino acid.In the present study,tyrosin in suspension culture gave the best results than others because suspension culture enhanced the production of secondary metabolite *in vitro* due to the reasons that it gives the better aeration in agitation, more contact of cells to nutrients and also more tyrosindecarboxilase activity which leads to maximize the kaempferol production. Further experiments related to the feeding of the other precursor in suspension will be carried out in future . The present study will also helpful in industries to commercial production of kaempferol,a valuable compound .It has been described that cinnamic acid is the better precursor of flavonoid than phenyl alanine [22],our results also support these findings.This type of study was carried out for the first time in *Tylophora indica* callus *in vitro.*

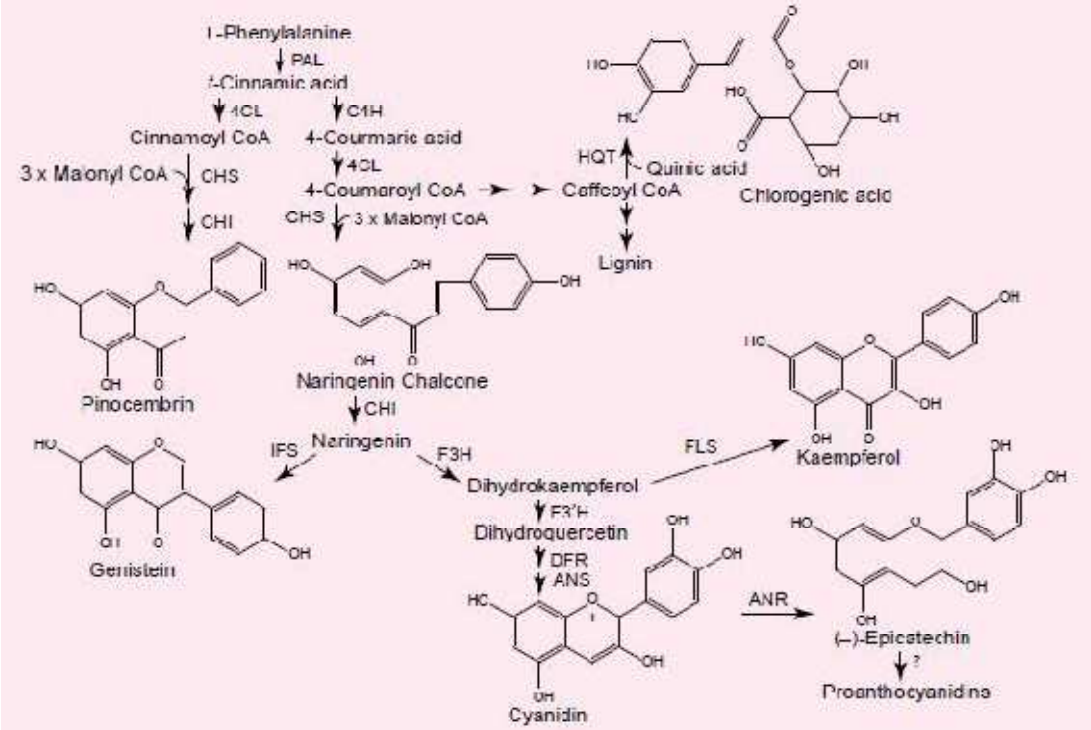


Figure 4. The biosynthetic pathway of kaempferol

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