ORIGINAL ARTICLE

Isolation, quantification and anti-microbial screening of flavonoids from callus of *Vitex negundo* Linn

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Context: Natural habitats for medicinal plant are disappearing fast and together with environmental instabilities, it is increasingly difficult to acquire plant-derived compounds. This has prompted scientists to consider the possibilities of using cell cultures as an alternative supply for the production of plant natural products. **Aim:** The present investigation deals with the establishment of callus cultures from leaf explants of *Vitex negundo*, to extract flavonoids from the raised callus and to assess their anti-microbial potential. **Materials and Methods:** Callus culture of *Vitex negundo* has been established on Murashige and Skoog's medium supplemented with 2, 4-D using leaf explants. Growth index (GI) was calculated for the tissues obtained after 2, 4, 6 and 8 weeks, respectively. Calli of 2, 4, 6 and 8 weeks old were used for extraction of total flavonoids (free and bound) using standard method. Flavonoid extract of 6 weeks old callus with maximum GI and maximum flavonoid content was then tested against *Staphylococcus aureus, Agrobacterium tumefaciens* (bacteria), *Candida albicans* (yeast) for their anti-microbial potential. Minimum inhibitory concentration, minimum bactericidal/fungicidal concentrations and total activity were also evaluated. **Results:** GI was found maximum in 6 weeks old tissue (0.76). Total flavonoids from 6 week old tissue showed maximum zone of inhibition against *A. tumefaciens.* Kaempferol was identified from total flavonoids of 6 week old callus through, thin layer chromatography (TLC), preparative TLC, Melting point and Infra red spectral studies. Content of kaempferol in 6-week-old calli was also estimated using standard Spectrophotometric method, which came out to be 54.25 µg/mg d.wt (dry weight) of calli. **Conclusion:** The protocol of callus induction of *V. negundo* opens new vistas that could facilitate phytochemical production, extraction of pharmaceuticals and their screening against different human pathogens from the callus without harvesting the plant itself.

Key words: Anti-microbial potential, callus, flavonoids, *Vitex negundo*

INTRODUCTION

Plant-derived compounds have become a topic of great interest, owing to their versatile applications.[1] It has been observed that natural habitats for medicinal plants are disappearing fast along with environmental and geopolitical instabilities, which have made difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals.[2] Under present situation, biotechnological approaches, especially plant tissue cultures, are found to be a good alternative of the traditional agriculture in the industrial production of plant metabolites.[3,4]

In the present investigation, an effort has been made to establish callus cultures of *Vitex negundo*, to extract flavonoids and to assess their anti-microbial potential. *Vitex negundo* Linn., locally termed as Nirgundi (in Hindi) is a deciduous, woody aromatic and multi-purpose medicinal shrub belonging to the Verbenaceae family. It is usually found in South-Asian countries**.** The major form of healthcare in the rural parts of developing countries is traditional medicine, which is mostly plant-based. Along with the utilisation in traditional medicine by local practitioners and healers, this plant also reportedly showed diverse pharmacological properties including analgesic,[5] anti-nocieceptive,[6] anti-inflammatory,[7-10] anti-fertility,[11] anti-feedant,[12,13] anti-histamine,[14] anti-oxidant,[15,16] anti-hyperglycemic effect,[17] cytotoxicity for human cancer cell line,[18] hepatoprotective activity against liver damage induced by d-galactosamine,[19]commonly used tubercular drugs,[20] and carbon tetrachloride,[21,22] laxative activity,[23] immunomodulatory effect[24] and mosquito repellent effect.[25,26]

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MATERIALS AND METHODS

Plant Collection and Authentication

Vitex negundo L was collected in the month of September and October 2009 from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at department of Botany, University

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of Rajasthan and (voucher specimen no: RUBL20838) was submitted to the herbarium, Botany department, University of Rajasthan. The plants were grown in pots and were kept in natural environment and maintained for fresh explants.

Preparation of Media

Murashige and Skoog Media (1962) was used to develop cultures. For callus induction the medium was supplemented with 2,4-D in three different concentrations in three different sets (0.5, 1 and 1.5 mg/l). Media containing flasks were sterilised by autoclaving at 15 lbs pressure for 15 min.

Inoculation of Explants

For callus initiation, young leaves of 1.5-2 cm were excised from the *ex vitro* plant and washed in the liquid detergent and rinsed thoroughly in tap water followed by distilled water. The cleaned explants were kept in sterile distilled water and were surface sterilised with aqueous 0.1% mercuric chloride solution for 1-2 min followed by five rinses in sterile distilled water before inoculating them on the culture media (Murashige and Skoog's media supplemented by 2,4-D). Explants were incubated and kept at 25 ± 2°C, light intensity of 1200 lux using fluorescent tubes (40 W). Two explants per flask and twenty flasks per treatment were used. The cultures were regularly observed and examined twice a week.

Callus Induction and Growth

Among all the tested concentrations for callus induction 1 mg/l was observed to be the best in comparison to other tested concentrations. Therefore this concentration (1 mg/l 2, 4-D) was used repeatedly for callus induction.

Calli so produced from leaf explants were transferred periodically after every 4-6 weeks to the freshly prepared supplemented culture medium till a good amount of calli was obtained.

Determination of Growth Characteristics

The morphological data regarding colour and texture were recorded in the callus initiation stage and thereafter at 2, 4, 6 and 8 weeks.

Calli of all ages of leaf explants were harvested regularly and their growth index (GI) was calculated on the fresh weight basis by the well established formula. Five such replicates were examined in each case and average values were calculated [Table 1].

$$GI = \frac{\text{Final fresh weight of the tissue - Initial fresh weight of the tissue}}{\text{Initial fresh weight of the tissue}}$$

Extraction and Quantification of Flavonoids

Calli of 2, 4, 6 and 8 weeks old were harvested from 10 flasks each, separately dried, finely powdered, weighed and subjected to extraction, following the method of Subramanian and Nagarajan.[27] The dried callus tissue was Soxhlet extracted with 80% methanol on a water bath for 24 h and filtered. Filtrate was re-extracted successively with petroleum ether, ethyl ether and ethyl acetate using separating funnel. Petroleum ether fractions were discarded due to being rich in fatty substances, whereas ethyl ether and ethyl acetate fractions were analysed for free and bound flavonoids, respectively. Ethyl acetate fraction of each sample was hydrolysed by refluxing with 7% H_2SO_4 for 2 h. Thereafter, it was filtered and re-extracted with ethyl acetate. The extract thus obtained was washed with distilled water to neutrality, dried and weighed as bound flavonoids. Similarly, ethyl ether fractions were also dried and weighed as free flavonoid [Table 2, Figure 1].

Determination of Anti-microbial Activity

Anti-microbial Susceptibility Testing

Bacterial strain *Staphylococcus aureus* (MTCC 87), *Agrobacterium tumefacians* (MTCC 431) and yeast *Candida albicans* (MTCC 183) were procured from IMTECH, Chandigarh, India. Bacterial strain was grown and maintained on Muller–Hinton Agar (MHA) medium, while yeast was maintained on Sabouraud Dextrose Agar (SDA) medium.

To check the anti-microbial activity of the extracts, 'Disc diffusion assay',[28-30] was carried out. MHA and SDA base plates were prepared and seeded with the bacterial and fungal inocula, respectively (inoculum size 1×10^8 CFU/ml for bacteria and 1×10^7 CFU/ml for yeast). Sterile filter paper discs (Whatman no. 1, 6 mm in diameter) were impregnated with 100 µl of each of the extract (10 mg/ml

Table 1: Growth characteristics of calli obtained from leaf explant of *Vitex negundo*

Callus age (weeks)	Colour	Texture	Growth index
2	Brownish yellow	Loose	1.32
4	Greenish brown	Compact	0.40
6	Greenish brown	Compact	0.76
8	Blackish brown	Tightly compact	0.55

Table 2: Flavonoid containt of callus of different age group of *Vitex negundo* Linn

Callus age (week)	Flavonoids mg/g d.wt		
	Free	Bound	Total
2	1.5	0.5	2
4	4	1.5	5.5
6	7.5	4.4	11.9
8	3	1.2	4.2

concentration) to give a final concentration of 1 mg per disc and were left to dry *in vacuo* so as to remove residual solvent, which might interfere with the determination. Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with streptomycin and terbinafine (1 mg per disc) as standard drugs for bacteria and fungi, respectively. The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were incubated at 37°C for bacteria (24 h) and 27°C for fungi (48 h). AI for each extract was calculated by the following well-established formula [Table 3, Figure 2].

$$GI = \frac{\text{Final fresh weight of the tissue - Initial fresh weight of the tissue}}{\text{Initial fresh weight of the tissue}}$$

Determination of Minimum Inhibitory Concentration, Minimum Bactericidal/Fungicidal Concentrations and Total Activity

Minimum inhibitory concentration (MIC) of the extracts for test organisms was determined by the broth micro dilution method.[31] Extracts were re-suspended in acetone to make 10 mg/ml final concentration and were then twofold serially diluted; added to broth media of 96-wells of micro titre plates. Thereafter, 100 ml inoculum (1×10^8 CFU/ml for bacteria and 1×10^7 CFU/ml for yeast) was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. The micro plates were incubated at 37°C for 24 h for bacteria and 27°C for 48 h for yeast. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtitre plate that showed no turbidity after incubation. The turbidity of the wells in the microtitre plate was interpreted as visible growth of microorganisms. The minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC) was determined by sub culturing 50 ml from each well showing no apparent growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC/MFC [Table 4].

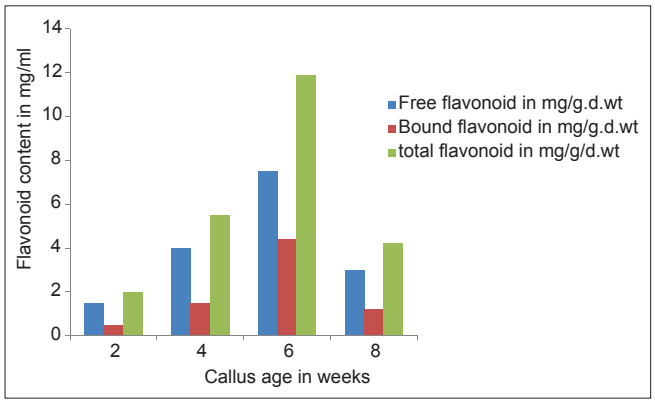

Figure 1: Flavonoid content of calli of different age group of *Vitex negundo*

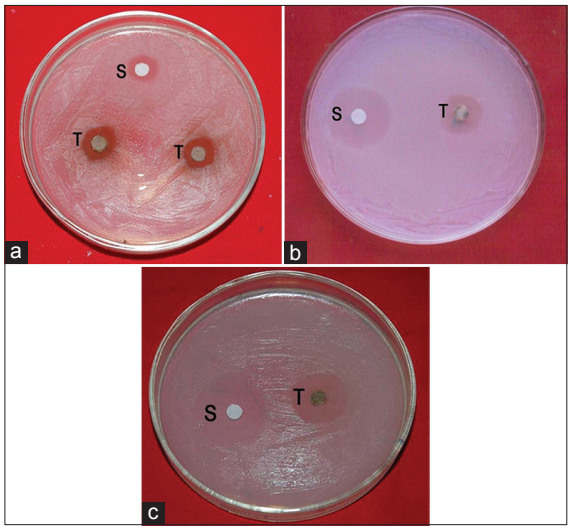

Figure 2: Anti-microbial activity of total flavonoids of 6 weeks old calli of *Vitex negundo*. Activity against (a) *C. albicans*; (b) *S. aureus*; (c) *A. tumefaciens*

Total activity (TA) is the volume at which test extract can be diluted retaining the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.[32] [Table 4].

Identification of Flavonoids

Total flavonoid extract from 6 weeks old callus was selected for the compound identification. Thin layer chromatography (TLC) was performed on silica gel coated (0.2-0.3 mm thick) and activated glass plates using an

Table 3: Anti-microbial activity of total flavonoids of 6 week old callus of *V. negundo*

Extract	Pathogens					
	S. aureus		*A. tumefacians*		*C. albicans*	
	IZ	AI	IZ	AI	IZ	AI
6-week-old callus	11.2± 0.882	0.911± 0.033	18.4± 0.773	0.877± 0.667	10.12± 0.033	1.012± 0.033

IZ – Inhibition zone in mm (mean value: Including 6 mm diameter of disc); AI – Activity index (IZ developed by extract/IZ developed by standard); ± – SEM; (-) – No activity; E1 – Free flavonoids; E2 – Bound flavonoids. IZ of standard drug candid v6 against *C. albicans* (14 mm). IZ of standard drug streptomycin against *S. aureus* (21 mm), *A. tumefacians* (28 mm)

Table 4: MIC, MBC/MFC and and TA of total flavonoids of 6 week old callus of *V. negundo*

	Pathogens								
	S. aureus			*A. tumefacians*			*C. albicans*		
	MIC	MBC	TA	MIC	MBC	TA	MIC	MBC	TA
6-week-old callus	0.156	0.312	76.28	0.078	0.156	152.56	0.625	0.625	21.12

MIC – Minimum inhibitory concentration,(in mg/ml); MBC/MFC – Minimum bactericidal/fungicidal concentration,(in mg/ml); Total activity – Extract per gram dried plant part in ml/g MIC of extract

organic solvent system (benzene, acetic acid and water in the ratio 125:72:3). Extract was dissolved in ethanol and applied on TLC plates along with standard kaempferol. Spots coinciding with the standard was marked and visualised under ultraviolet (UV) light. Identification of compound was further confirmed by exposing the plates to iodine vapours and spraying the plates with 5% ethanolic ferric chloride solution. Rf values of test extracts and standards were calculated and compared.

Preparative TLC was carried out for the flavonoids of 6-week-old callus showing the best activity. About 300 silica gel coated (0.4-0.5 mm thick) and activated plates were run, and the spots of standard Rf values were eluted. Elute was co-chromatographed with standard to test the purity of compound. The isolated compound was then crystallised.

Further confirmation of isolated compound was done by IR spectral study along with authentic sample of standard compound [Figure 3].

Spectrophotometric Quantification of Isolated Kaempferol

Quantitative estimation of kaempferol was carried out colorimetrically with the help of a spectrophotometer [SHIMADZU, UV-visible spectrophotometer, UV-1700 (pharrmaspec)] following the methods of references.[33-35] Stock solution (1 mg/ml) of kaempferol was prepared by dissolving the authentic substance in methanol. Different concentrations (25-150 µg) of the substance were spotted separately on silica gel coated and activated glass plates. Separate glass plated for each of the concentrations of kaempferol were used and these chromatograms were developed in the same solvent system as was used for qualitative purpose (benzene: Acetic acid: Water in the ratio of 125:72:3). Such developed chromatograms were dried at room temperature and visualised under UV light. The fluorescent spots were marked and collected along with the adsorbent in separate test tubes. To each of the test tubes 5 ml of spectroscopic methanol was added, shaken vigorously, centrifuged and the supernatants were collected separately. The volume of the elutes was made up to 10 ml by adding spectroscopic methanol. To each of these samples, 3 ml of 0.1 M Aluminum chloride was added, the tubes Stoppard tightly and the mixtures were shaken vigorously. Such tubes were kept at room temperature for 20 min. Ten such replicates were prepared in each case and their optical densities measured using the spectrophotometer set at the 423 nm for kaempferol against a blank (10 ml spectroscopic methanol + 3 ml of 0.1 M aluminium chloride) and their average values calculated. Various concentrations of kaempferol were then plotted against their respective optical densities which followed the Beer's Law.

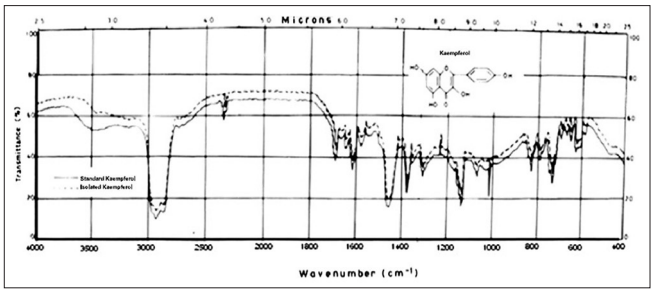

Figure 3: IR spectra of isolated Kaempferol

Each of the test extract was dissolved in 1 ml of spectroscopic methanol and applied on silica gel coated and activated glass plates along with the authentic kaempferol as marker and developed as above. Fluorescent spots coinciding with that of the reference compound were marked, scrapped and eluted with spectroscopic methanol. Samples were prepared and the optical density in each case was recorded by spectrophotometer as above. Concentration of kaempferol in test sample was studied in the regression curve plotted earlier on the basis of their optical densities.

RESULTS AND DISCUSSION

MS media, supplemented with 2, 4-D (1 mg/l), successfully induced the callus from leaf explants of *V. negundo* and the callus was established within 15 days. Callus from *V. negundo* has been induced earlier. The un-organised callus so produced was greenish white, healthy and fast growing, which gradually became brownish yellow after 2 weeks. It was greenish brown after 4-6 weeks and turned to blackish brown after 8 weeks. Regarding texture, initial callus was loose and soft which gradually became slightly compact after 2 weeks. However after 4, 6 and 8 weeks, it gradually became compact. GI of tissues obtained showed gradual increase in value up to 6 weeks, thereafter decline was observed in 8 weeks. GI was maximum in 6-week-old tissue (0.76), followed by 4 weeks (0.40), 8 weeks (0.55) and 2 weeks (0.18) and initial callus being 1.10 [Table 1].

Total flavonoid (free + bound) content was found to be maximum in 6 weeks old callus (11.9 mg/g d.wt) and minimum was in 2 weeks old callus (2 mg/g d.wt). Similarly, maximum free and bound flavonoid content was observed in 6 week old callus (7.5 and 4.4 mg/g d.wt, respectively), whereas minimum free and bound flavonoids were observed in 2 weeks old callus (1.5 and 0.5 mg/g d.wt, respectively) [Table 2, Figure 1].

Total flavonoids from 6-week-old callus was tested for anti-bacterial and anti-fungal activity against *S. aureus*, *A. tumefaciens* and *C. albicans*, respectively. All the tested pathogens were found to be sensitive to the tested extract at 1 mg per disc concentration. Maximum activity was

seen against *A. tumefaciens* with significant inhibition zone (IZ = 18.4 ± 0.773 mm, AI = 0.877 ± 0.667) and MIC and MBC was found to be 0.078 and 0.156 mg/ml, respectively. Tested extract had shown positive response against *S. aureus* (IZ = 11.2 ± 0.882, AI = 0.911 ± 0.033). Total flavonoids showed significant zone of inhibition against *C. albicans* (IZ = 13.2 ± 0.033 mm, AI = 1.012 ± 0.003) and similar MIC and MFC values (0.625 mg/ml). Same values of MIC and MBC/MFC indicate bactericidal/fungicidal nature, whereas different values indicate bacteriostatic/fungistatic nature of tested extracts. Total flavonoids extract of 6 weeks old callus has shown maximum TA values against *A. tumefaciens* and *S. aureus* (152.56 and 76.28 ml/g, respectively) [Tables 3 and 4, Figure 2].

Active compound in the extract was also identified and confirmed as Kaempferol (Rf -0.86) using solvent system of benzene, acetic acid and water (125:72:3) [Figure 1]. Colour of flavonoid in UV light was fluorescent yellowish blue. Colour observed after spraying with 5% Ethanolic $FeCl_3$ was brown. Melting point of identified kaempferol was also similar to their respective standard compounds (271-273°C). IR spectrum along with authentic sample further confirmed the presence of the compound [Figure 3]. Content of kaempferol was quantified through standard spectrophotometric method. It came out to be 54.25 µg/mg d.wt of calli, which was also compared with the kaempferol content obtained from flowers of *V. negundo* (108 µg/mg.d.wt). Although the content obtained from flowers is more but in the light of the fact, that flowers from the plant cannot be obtained regularly in all seasons, besides the land available for growing plant is gradually shrinking, the importance of tissue culture enhance as continuous supply of the compound can be manipulated through bioreactors, of course the need of the hour is to reduce the cost of raising and maintaining the potential of tissue culture for producing the compound with same or increased rate.

An efficient protocol was established for *in vitro* shoot multiplication from shoot tip of *V. negundo*.[36] Leaf explants of *V. negundo* had been used to induce indirect organogenesis.[37] Effect of different phytohormones had been seen to induce callus from leaf and intermodal explants of *V. negundo*.[38] Anti-microbial effects of *V. negundo* had also been demonstrated[39,40] previously but so far very little work has been carried out to establish culture of *V. negundo* and no effort has been done to extract, screen and identify the pharmaceutical compounds from cultures of *V. negundo*.

CONCLUSION

The results of this study advocate that large scale production of callus of *V. negundo* can be a good source for commercial production of secondary metabolite and to prepare future anti-microbial drugs. It would also release the pressure on the plant and would help in protecting biodiversity.

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