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CHARACTERIZATION OF UV-ABSORBING BENZOPHENONES FROM NATURAL AS WELL AS *IN-VITRO* ROOTS OF *GARCINIA INDICA* (THOUARS) CHOISY USING AN EFFICIENT HYPHENATED TECHNIQUE HPTLC-MS

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Keywords:

Garcinia indica, Ultraviolet radiation (UV), Benzophenones, Garcinol, HPLTC-MS, Column Chromatography

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ABSTRACT: Nowadays increase in UV induced skin ailments and adverse effects of synthetic UV filters, prompted researchers to focus on natural compounds capable of absorbing UV rays. *Garcinia indica* is a rich source of benzophenones, which are present in all plant parts. As per earlier reports, ethyl acetate root extract of it exhibits the highest SPF (7) than other plant parts. The present study aims to characterise anti-UV benzophenones and screening of *in-vitro* roots for it. Natural root extract subjected to silica gel column chromatography. The column fractions were analysed using a spectrophotometer and thin-layer chromatography. Initial fractions eluted in hexane to hexane: ethyl acetate 90:10 or 80:20 displayed a fluorescent blue band on the TLC plate and showed absorbance in the UV B region. As the concentration of ethyl acetate increased (Hexane: Ethyl acetate 70: 30) the eluted fractions exhibited an additional fluorescent green band. These fractions had a broad-spectrum UV absorption activity contributing to SPF and Boot star ratings. HPTLC-MS analysis of these two bands characterized garcinol (*m/z* 603.53) and 7 epi-isogarcinol (*m/z* 625.28) compounds from the green band and 14-deoxy-7-epi-isogarcinol (*m/z* 609.36) and 7 epi-isogarcinol (*m/z* 587.45) compounds from the blue band. Comparative HPTLC analysis of fruit and root extracts revealed that garcinol content is comparatively higher in fruit (9.3 %) than root (4.1%) which constitutes a higher boot star rating (5) in fruit extract. Root extract displayed an intensified blue band region which conferred a higher SPF (6.25) to root extract.

INTRODUCTION: In recent years, the frequency of conditions related to ultraviolet (UV) radiation has increased [1]. UV rays can harm the skin, causing changes in collagen and elastic fibres. These harmful effects can lead to DNA damage, which is linked to accelerated skin ageing and an increased risk of skin cancer [2, 3, 4].

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An effective way to mitigate the negative effects of UV radiation is through photoprotection, which can be achieved by using sunscreen. However, commercially available sunscreens that contain chemical UV filters are generally not stable when exposed to sunlight and can cause adverse effects on the skin.

Phytochemicals or natural active ingredients have antioxidant properties and therefore have the potential to prevent various cellular and molecular degenerative processes caused by UV radiation. These compounds are receiving significant attention for their role in protecting against UV-induced photodamage [5, 6].

Synthetic forms of benzophenones such as benzophenone-3 or benzophenone-4 are widely used in cosmetic formulations as a strong UV filter but are known to be susceptible to light degradation. Isoprenylated benzophenones, exclusively found in the Clusiaceae family, are natural complex compounds with powerful antioxidant properties [7]. In *Garcinia indica* (Thouars) Choisy, these isoprenylated benzophenones are present throughout the plant, but the highest sun protection ability was observed in root extracts, with a sun protection factor (SPF) of 7.19, compared to leaf extracts (SPF 1.27), seed extracts (SPF 2.70), and fruit extracts (SPF 3.67) [8]. Therefore, our study focused on the roots of *G. indica* for their ability to protect against the sun. However, due to the plant being an indigenous slow-growing tree species, it was not recommended to collect root material from mature trees. Thus, we conducted a study to establish *in-vitro* root cultures and utilize them for sun protection activities. This study aimed to examine *in-vitro* roots of *G. indica* at different stages of development for the production of isoprenylated benzophenones responsible for their sun protection properties, as well as to characterize and identify these benzophenones.

MATERIALS AND METHODS:

Plant Material Collection:

Matured Root: Matured roots of *Garcinia indica* trees were collected from about 15 years old fully grown plant at Dapoli, Maharashtra, India. The matured roots were initially stripped with a chisel and subsequently pulverized using a Philips mechanical blender to obtain finely cut pieces.

In-vitro and Hardened Roots:

Establishment of Shoot Cultures: The shoot cultures of *G. indica* were initiated as per the protocol described by Kulkarni and Deodhar [9]. Surface sterilized seeds of *G. indica* are cut into 2-4 segments and inoculated aseptically for seed germination on MS medium with 3% sucrose supplemented with NAA (2.69 μM/L), BAP (8.9 μM/L) and KN (0.93 μM/L). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ^{O}C for 15 min. The medium was solidified with 0.8% agar-agar (Himedia Pvt. Ltd., India). After 4 weeks of culture initiation, the seed segments with the induced adventitious shoot buds were subculture and maintained on the same freshly prepared MS medium incorporated with NAA (2.69 μM/L), BAP (8.9 μM/L), KN (0.93 μM/L) and 3% sucrose till shoot buds developed into shoots of approximately 8 to 10 mm in height. Seed segments were cut into small pieces with at least 3 – 5 shoots per piece and were transferred on MS medium supplemented with 3.0 % sucrose and 8.87 μM BAP for shoot multiplication. For shoot elongation, individual shoots from a bunch of shoots were removed and inoculated on half-strength MS medium supplemented with NAA (0.54 μM), BAP (0.44 μM), KN (0.93 μM) and 3% sucrose.

***In-vitro* Root Initiation:** Elongated shoots (height 15 to 25 mm) were cultured on a root induction medium under different culture conditions. Different strengths (full, ½ or ¼) and types (Solid or liquid) of WPM medium supplemented with different concentrations of (2.0, 3.0 %) sucrose and auxins (IBA) (0.0, 9.8, 14.8 μM) were used for root induction. The bridge method has been used in liquid media to support plantlets and bridges have been formed using Whatman filter paper 1 as a support matrix.

Hardening of the *In-vitro* Plantlets: Rooted shoots were successfully hardened to natural conditions using the protocol described by Chabukswar and Deodhar [10]. The roots of the 5 to 6-month-old hardened plant were trimmed and the plant was repotted into the plastic bags. The roots brought to the laboratory were washed under running water to remove the soil. The roots are dried in a hot air oven at 60 ºC, crushed, weighed and stored for benzophenone extraction.

Establishment of Adventitious Root Cultures:

Effect of Different Culture Conditions: The roots developed from elongated shoots of 4 to 5 cm lengths were cut into small segments each of approximately 1 cm in length. These root segments are exposed to different strengths (full, ½ or ¼) and types (Solid or liquid) of WPM medium supplemented with different concentrations of (2.0, 3.0 %) sucrose and auxins (IBA) (0.0, 9.8, 14.8 μM) for adventitious root branching.

Effect of Tryptophan: Root segments were also exposed to different (50, 100, 150, or 200 μM)

concentrations of tryptophan to see its effect on adventitious root branching.

The *in-vitro* roots from suspension cultures and from *in-vitro* hardened plants were harvested, dried in a hot air oven, and finely crushed with a Philips mechanical blender.

Extraction of Benzophenones: The crushed natural roots (NR) of *G. indica* were extracted using the maceration method with ethyl acetate solvent. The process involved taking 200g of crushed natural roots in conical flasks and adding 1L of ethyl acetate solvent. The flask was intermittently shaken for 2-3 days at room temperature, after which the roots were separated and the extract was collected. The extract was then concentrated using a rotary evaporator at 60ºC to recover the solvent. The resulting extract was dried, weighed, and stored at room temperature for further use. The same extraction process was used for *in-vitro* roots (IVR) and *in-vitro* hardened (IVHR) roots of *G. indica*. Crushed 5gm of IVR and 5 gm of IVHR were extracted using 100 ml of ethyl acetate solvent.

UV-VIS Spectrum Screening of NRE, IVRE and IVHRE and *In-vitro* SPF Determination of Three Root Extracts (RE): The UV absorbance of NRE, IVRE, and IVHRE from *G. indica* was examined using a UV-VIS Spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific). To perform the UV-VIS analysis, a concentration of 10 mg/ml of dried NRE, IVRE and IVHRE was reconstituted separately in ethyl acetate solvent and screened under UV light at wavelengths between 200-400 nm. The mean UVB (200-280 nm), and UVA (280-320 nm) absorbance were calculated for NRE, IVRE and IVIVHRE. The theoretical SPF and UVA/ UAB ratio of three samples were also determined using the method outlined by Mansur *et al.*[11].

The UVB absorbance readings from 290-320 nm were used to SPF using the formula,

SPF spectrophotometric = CF x ∑_290^320〖EE (λ) x I (λ) x Abs (λ)

Where EE (λ) is the erythemal spectrum, I (λ) is the solar intensity spectrum, Abs (λ) is the absorbance of the extract, and CF is the correction factor (=10).

Using formula 1, the SPF was calculated by measuring the absorbance from 290-320 nm at an interval of 5 nm.

Preparative Thin Layer Chromatographic Analysis of NRE, IVRE and IVHRE for Determination of Benzophenone Derivates: A TLC plate measuring 8 ×10 cm and coated with silica gel 60 F254 of 0.2 mm thickness was utilized to fractionate the NRE, IVRE, and IVHRE. The root extracts were dissolved in ethyl acetate solvent at a concentration of 10mg/ml, while standard garcinol (Cayman Chemicals, Michigan United States) was used at a concentration of 0.5mg /ml methanol. To load the root extracts (10 µl) and garcinol (5 µl), a micropipette was used, placing the samples 10 mm from the lower edge on an 8 ×10 cm TLC plate.

Various solvent systems were employed as mobile phases for separating benzophenones from all three root extracts, including Toluene: acetone: formic acid (6:6:1), Chloroform: methanol (9:1), Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26), Ethyl acetate: methanol: water (10:1:1), Hexane: ethyl acetate (30:70), and Hexane: ethyl acetate (70:30). After loading, the plate was developed in a pre-saturated developing chamber containing the aforementioned solvent systems up to a distance of 80 mm. The developed plate was then dried at room temperature. Chromatograms on the plate were visualized, and images were subsequently captured under white and ultraviolet light (254 and 365 nm) using CAMAG's TLC visualizer 2.

Fractionation of NRE using Column Chromatography: Root extract was fractioned using a 10 ml glass syringe packed with 6 g silica gel (100-200 mesh) mixed with 25 mL hexane. Silica-coated 250 mg of NRE was loaded onto the packed bed column. The solvent system selected was a combination of non-polar to mid-polar to highly polar solvents. The column was first eluted with 100% hexane followed by a gradient of hexane: ethyl acetate (90:10, 80:20, 70:30, 50:50), ethyl acetate: methanol (90:10) and 100% methanol respectively. The selected solvent systems were slowly added one by one and flowed through the packed column at a uniform rate. Every 2 mL solvent that flowed out from the packed column

was collected as plant fraction 1 until 27 and used for further analysis.

UV-Spectrophotometric Analysis and TLC: The UV light absorption capacity of column fractions numbered 1 to 27 was evaluated using a UV-Vis Spectrophotometer. The relative absorbance of all the fractions was measured between the wavelengths 200-400nm. A graph was plotted to illustrate the absorption spectrum, with the x-axis indicating the wavelength (nm) and the y-axis showing the relative absorbance. Additionally, the average UV B (absorbance between 200-280 nm) and UV A(280-320 nm) absorbance levels were calculated for each fraction. The Mansur *et al.*[11] method was used to measure the theoretical SPF and UVA/B of the fractions.

Fractions having significant absorbance in UV A and UV B regions were selected for TLC analysis. Selected fractions were examined using a TLC plate (20 ×10 cm) precoated with silica gel 60 F_{254} of 0.2 mm thickness. The developing solvent was Hexane: Ethyl Acetate (70:30). Fraction (20 µl) and Crude NRE (10 µl) were loaded with capillary. The loaded plate was developed in a pre-saturated TLC chamber containing the above-mentioned solvent system up to a distance of 8.0 cm. The developed plate was dried at RT. Chromatograms on the plate were visualized and images were subsequently captured under white and ultraviolet light (254 and 365 nm) using CAMAG's TLC visualizer 2.

HPTLC-MS Analysis of NRE: HPTLC analysis was performed at Anchrome Enterprises India Pvt. Ltd. Mulund (E), Mumbai. A CAMAG (Switzerland) semi-automated HPTLC system was used for the separation of UV-protective benzophenones from NRE. The sample was applied to silica gel pre-coated aluminium plates (DC-Fertigfolien ALU-GRAM1Xtra SIL G/UV254, Germany) by Linomat 5 semiautomatic applicator (CAMAG, Switzerland) fitted with 100 µl Hamilton microsyringe and connected to a nitrogen supply. For analysis, 10 mg of fruit extract and 0.5 mg of garcinol standard were separately reconstituted in 1 ml of HPLC-grade methanol. Aliquots (2 µl) of the extract and standard (2 µl) were applied as 8 mm bands, 5 mm from the lower edge on a 10 ×10 cm TLC plate precoated with silica gel 60 F_{254} of 0.2 mm thickness. Thereafter, each plate was transferred to the ADC2 development chamber, consisting of a glass twin-trough chamber (20 cm - 10 cm) with metal lids. Plates were developed with Toluene: Ethyl acetate: Formic acid (4:1:0.5) to a migration distance of 80 mm in the pre-saturated chamber. The developed plates were dried at RT. The photo documentation of the plate was carried out using CAMAG TLC visualizer 2 under white and ultraviolet light (254 and 365 nm) and stored using vision CATS version 3.2 planar chromatography manager software.

MS analysis was performed at a Sophisticated analytical instrument facility (SAIF) at IIT Bombay, Powai, Mumbai, Maharashtra. After visualization under UV light, the interested bands on the TLC plate were identified, marked and lifted using a CAMAG TLC-MS-Interface 2. The HPTLC-MS interface is equipped with a flow pump that has an inlet connected to a UPLC pump and an outlet attached to a Micromass-LCT Premier time-of-flight (ToF) mass spectrometer. Acetonitrile (HPLC grade) was utilized as the eluent at a flow rate of 0.3 mL/min to eliminate the target bands from the silica gel plate.

Incorporation of NRE in the Sun-protective Formulation and Determination of *In-vitro* SPF using Transpore Plates: To determine the lowest concentration of NRE with strong SPF activity, we added lower concentrations of NRE (2, 2.5, and 5%) to the sun screening cream base. Cream based used for preparing SPF formulation was as described by Dike et al.[8]. The creams were formulated as follows using the formulation mentioned in **Table 1**. The table includes base components (10 g) 1. Base cream (without any actives), 2. 2 % NRE, 3. 2.5 % NRE 4. 5 % NRE. The SPF was measured with the help of a UV-2000S Ultraviolet Transmittance Analyzer (Labsphere Inc., USA) at Kelkar Trust's Scientific Research Centre, V.G. Vaze College, Mumbai, and Mulund, Mumbai.

TABLE 1: COMPONENTS OF SUN PROTECTION FORMULATION (10 G) WITH OR WITHOUT NRE

Components	Base	2%NRE	2.5 % NRE	5 % NRE
Oil Phase (g)				

CCTG	0.4	0.4	0.4	0.4
IPM	0.4	0.4	0.4	0.4
Cetostearyl Alcohol	0.2	0.2	0.2	0.2
Stearic acid	0.3	0.3	0.3	0.3
Propyl Paraben	0.002	0.002	0.002	0.002
NRE	-	0.2	0.25	0.5
Water Phase (g)				
EDTA	0.02	0.02	0.02	0.02
Glycerine	0.4	0.4	0.4	0.4
TEA	0.042	0.042	0.042	0.042
Methyl Paraben	0.025	0.025	0.025	0.025
Add water to make the final volume 10 ml				

RESULT AND DISCUSSION: The genus *Garcinia* comprises a diverse array of biologically potent compounds, which have gained significant interest in recent years. These extracts are abundant in poly isoprenylated benzophenone derivatives, polyphenols, bioflavonoids, and xanthones. Extracts of the pericarp, epicarp and seeds of *Garcinia* have demonstrated antioxidant, anti-inflammatory, leishmanicial and antiprotozoal activities [12]. Recently, various research teams have published analytical techniques that include different chromatographic conditions and spectrophotometric technologies used for the identification, characterization and determination of these chemical components of *Garcinia* species.

The characterization and quantification of benzophenones are frequently carried out using LC/MS and HPLC methods. Kumar and Chattopadhyay [13] used HPLC and LC-ESI-MS methods for the identification and quantification of two polyisoprenylated benzophenones xanthochymol (Garcinol) and isoxanthochymol (isogarcinol) in different parts of *Garcinia indica*. Garcinol and isogarcinol function as anti-cancer agents against different types of malignancies including lung cancer, breast cancer, colorectal cancer, prostate cancer, and cervical cancer [14].

Jackson and co-workers [15] isolated xanthochymol from fruits of *G. xanthochymus* by reverse-phase column chromatography. The structure was elucidated by one-dimensional and two-dimensional NMR and MS. The authors reported xanthochymol induced apoptosis in *Candida albicans* hyphae and inhibited biofilm production by this fungus. Upon conducting an analysis with RP-HPLC coupled with the NMR/MS method, the fruits of *G. combogia* produced benzophenones guttiferone-I, -J, -K, -N, and -M in the diethyl ether extract [16]. According to studies by Di-Micco *et al.* [17], guttiferone-K and guttiferone-M act as inhibitors of topoisomerase II [18]. Earlier, Bharte and co-workers [19] carried out quantification and validation of two isomeric anticancer compounds, garcinol and isogarcinol, in ultrasound-assisted extracts of *Garcinia indica* fruits using HPTLC. A similar methodology was used by Patel and colleagues [20] for the quantification of garcinol content in herbal formulations. In the present publication, our objective is to characterize and quantify the benzophenones present in ethyl acetate extracts of *G. indica*, which are most potent in providing UV protection.

***In-vitro* Studies:**

Establishment of Shoot Cultures using Seed as an Explant: The initiation of shoot cultures from seeds, *in-vitro,* shoot multiplication, and subsequent shoot elongation was carried out in the present study following the protocol outlined by Kulkarni and Deodhar [9]. The results are depicted in **Fig. 1**.

In the present study, a maximum of 80% of the immature seed segments inoculated on MS medium with 3% sucrose and supplemented with NAA (2.69 µM/L), BAP (8.9 µM/L) and KN (0.93 µM/L) responded after 4 weeks of culture initiation to the development of multiple shoot primordia with an average of 12–15 shoots per seed segment **(Fig. 1A)**. For shoot multiplication, segmented seed pieces (3-5 shoots per piece) inoculated on MS medium supplemented with 8.87 µM BAP developed multiple shoots (16-18 shoots) within 3-4 weeks after culture **(Fig. 1B)**. The multiple shoots obtained were repeatedly subcultured on the same shoot multiplication medium at an interval of 30 days to obtain a maximum number of shoots.

FIG. 1: INITIATION OF SHOOT CULTURES FROM SEED EXPLANT, *IN-VITRO* ROOTING AND HARDENING OF *IN-VITRO* ROOTED SHOOTS A: INDUCTION OF SHOOT BUDS, B: SHOOT BUDS AFTER 3 WEEKS, C: ROOTING ON AGAR, D: ROOTING ON WHATMAN FILTER PAPER NO. 1, E: HARDENING OF *IN-VITRO* GROWN PLANTLETS IN SMALL PLASTIC POTS, F: MAINTENANCE OF HARDENED PLANTS IN PLASTIC BAGS, G: HARDENED PLANTLET

Root Induction: Kulkarni and Deodhar [9] in their *in-vitro* studies with *G. indica* found that IAA did not stimulate root growth in the cultured shoots. Among the NAA and IBA hormones tested for *in vitro* root formation, they achieved the maximum rooting with IBA treatment. Similarly, Chabukswar and Deodhar [10] employed a pulse treatment of IBA to stimulate root development in *G. indica*. Malik *et al.* [21] and Deodhar *et al.* [22] also utilized IBA to induce root formation in selected female *G. indica* plants and identified it as the most effective hormone for this purpose. Therefore, in the current study, only the auxin hormone IBA was used to stimulate root growth.

Initially, half-strength MS with different (0.0, 9.8 or 14.8 µM) concentrations of IBA was tried but no root initiation was observed. So we shifted from MS media to WPM media for rooting. Shoots inoculated in half-strength WPM fortified with different (0.0, 9.8 or 14.8 µM) concentrations of IBA and 3 % sucrose showed rooting response only at 14.8 µM IBA. At this concentration, 72% of shoot cultures responded to rooting and the mean number of roots obtained was 1.3±0.6 after 51 days of inoculation **Table 2**. When sucrose concentration was reduced from 3 % to 2% the rooting response was observed in 45±5 days and a little improvement in % shoots was seen from 72% to 75% **Table 2**. The number of roots remained the same (1.3 ± 0.6). Sha Valli Khan *et al.* [23] studied the effect of varied concentrations of agar, sucrose, MS basal medium strength, and polyamines on *in vitro* rooting in *Syzygiumalternifolium*(Wight.) Walp. They notice maximum rooting at low (2%) sucrose concentration incorporated in half-strength MS medium enriched with 1. 0 mg dm^{-3} IBA, 10 µM spermine and 0.8 % agar. The full-strength WPM media with or without supplementation of IBA did not induce any rooting.

Therefore, we have reduced the strength of WPM basal media to half (½) or quarter (¼). Shoots inoculated in half-strength WPM supplemented with 14.8 µM IBA, and 2% sucrose showed 75 % rooting response after 48 ± 6 days of inoculation with 1.3 ± 0.6 minimum number of roots. Whereas when ¼WPM media was used, 83% of shoots responded to rooting within 45 ± 5 days with no improvement in mean number of roots seen. Manzanera and Parados [24] and Purohit et al. [25] reported the beneficiary effect of reducing MS basal concentration on *in vitro* rooting in *Quercus sobur* and *Wrighatia tomentosa* respectively. Liquid media as compared to solid media provides high availability of nutrients and water due to low

resistance to diffusion and closer contact between the explant and culture medium which in turn promote better growth and subsequent rooting *in vitro* shoots [26, 27, 28]. Thus, we have also used liquid WPM basal medium with reduced strength (½ or ¼) along with the solid media. In liquid media bridge method was implemented and Whatman filter paper 1 was used as a support matrix. Shoot cultures when inoculated on ½ strength WPM solid media supplemented with 3% sucrose and 14.8 µM /L of IBA, the rooting response was observed after 51±8 days of inoculation and % rooting was 72%. After culturing shoots in a liquid medium keeping other parameters the same, the rooting response was observed in 35±2 days. The % response was also improved from 72% to 88% and the mean number of roots per explant improved from 1.3 ±0.6 to 2. Shoot cultures grown in liquid medium in ¼ strength WPM and 2% sucrose and 14.8um IBA still reduced the number of days of root initiation to 30 days rooting % was improved to 94% and there was a little improvement in the number of roots per explant (1.6±0.6). Roots produced on liquid media containing filter paper bridge were seen to be thin and white and resembling the natural roots as compared to those thick and greenish roots produced on solid media **Fig. 1 C-D**. Puchooa *et al.* [29] reported that filter paper support provided in liquid media gave better anchorage owing to its porosity that facilitated absorption through its surface area.

TABLE 2: EFFECT OF DIFFERENT STRENGTHS OF WPM MEDIUM SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF AUXIN IBA AND SUCROSE ON ROOTING IN SHOOT EXPLANT OF *GARCINIA INDICA*

WPM Media strength	IBA (µM)	Sucrose (%)	Type of medium	Rooting response in days (after inoculation)	Shoot response to rooting (%)	No. of roots per shoot
½ or ¼ basal	0.0	2 or 3	Solid/liquid	-	-	-
½	9.8	2 or 3	Solid/liquid	-	-	-
¼	9.8	2 or 3	Solid/liquid	-	-	-
½	14.8	3	Solid	51±8	72	1.3±0.6
½	14.8	3	Liquid	35±2	88	2
½	14.8	2	Solid	48 ± 6	75	1.3±0.7
½	14.8	2	Liquid	40± 3	80	1.3±0.6
¼	14.8	3	Solid/liquid	-	-	-
¼	14.8	2	Solid	45±5	83	1.3± 0.6
¼	14.8	2	Liquid	30	94	1.6±0.6

Hardening of Rooted Shoots to Acquire Hardened Roots for Benzophenone Extraction: Rooted shoots were successfully hardened to natural conditions using the protocol described by Chabukswar and Deodhar [10] and are depicted in **Fig. 1(E-G)**. After 5 to 6 months of hardening, the roots of the hardened plants rise to 18 -20 cm in length with the initiation of secondary branches. At this stage roots were harvested from the plants and plants were repotted in the pot. These harvested roots were used for the extraction of benzophenones.

Establishment of Adventitious Root Cultures:

Effect of Different Culture Conditions: For initiation of adventitious root culture, root fragments from *in vitro* rooted plants were used as an explant. The effect of different (½ or ¼) strengths and types (solid or liquid) of WPM media fortified with various concentrations (0.0, 9.8 or 14.8 µM) of auxins IBA and (2 or 3 %) sucrose were studied on adventitious root formation in root fragments of *G. indica*. Among the different (0.0, 9.8 or 14.8 µM) concentrations of IBA used, the adventitious root formation in the root segment was noticed in the presence of a 9.8 µM/L concentration of IBA after 58 ± 3 days of inoculation. 50 % of root segments responded to lateral rooting and the mean number of roots formed was 0.6±0.6. Higher concentrations (14.8 µM/L) of IBA inhibited adventitious root formation **Table 3**.

In the case of adventitious rooting from the root segment a drop in sucrose concentrations from 3% to 2% resulted in a delayed root segment response to lateral root production, along with a reduction in rooting percentage and a mean number of roots per root segment **Table 3**. Cui *et al.* [30] varied sucrose concentration from 1 to 10 % (w/v) to find out the optimal sucrose level for the growth of the

adventitious root cultures of *Hypericum perforatum*. They reported 3 % (w/v) sucrose as the optimal concentration for root biomass development. The full-strength WPM with or without IBA supplemented with 2 or 3 % sucrose does not induce adventitious roots on root fragments. While the intensity of WPM is reduced from ½ to ¼ does not produce a discernible positive or negative impact on the average number of roots generated per root segment. Instead, the rooting response of root segments is seen to have fallen from 50% to 20%. In *Withania somnifera*, ½ strength MS medium was observed to favour maximum adventitious root formation. Whereas full strength MS medium reduced the lateral root growth [31]. Reducing sucrose concentration or reducing the strength of WPM does not make any noticeable change in the number of days required by roots to initiate lateral rooting, per cent rooting response or the average number of lateral roots formed per segment. Thus, to enhance the potential of root segments to form more adventitious roots, we have used a liquid culture system. As mentioned in **Table 3** root segments inoculated on ½ strength of WPM solid medium enriched with 9.8 µM IBA and 3 % sucrose concentration responded after 58±3 days. The 50 % of root segments responded to lateral root formation with the mean number of roots per root segment being 0.6±0.6. After culturing in a liquid medium keeping other parameters the same, the rooting response was seen in 36 days. The % response was also improved from 50 % to 78% and the mean number of lateral roots per root explant improved from 0.6±0.6 to 1.3 ±0.6. The ¼ strength WPM liquid medium with the same concentrations of IBA or sucrose as that of the ½ strength liquid WPM does not produce any significant change in rooting parameters and the mean number of lateral roots per root segment. Whatman filter paper 1 used as a support matrix in liquid culture system was found to be superior in terms of root morphology **Fig. 2 A-B**. Therefore, the present study revealed that half-strength WPM liquid media with a lower (9.8 µM) concentration of IBA and 3 % sucrose concentration was found to be effective for the induction of adventitious roots in root explants. However, the number of lateral roots formed in above mentions media composition was very low in terms of mass production and extraction of benzophenones.

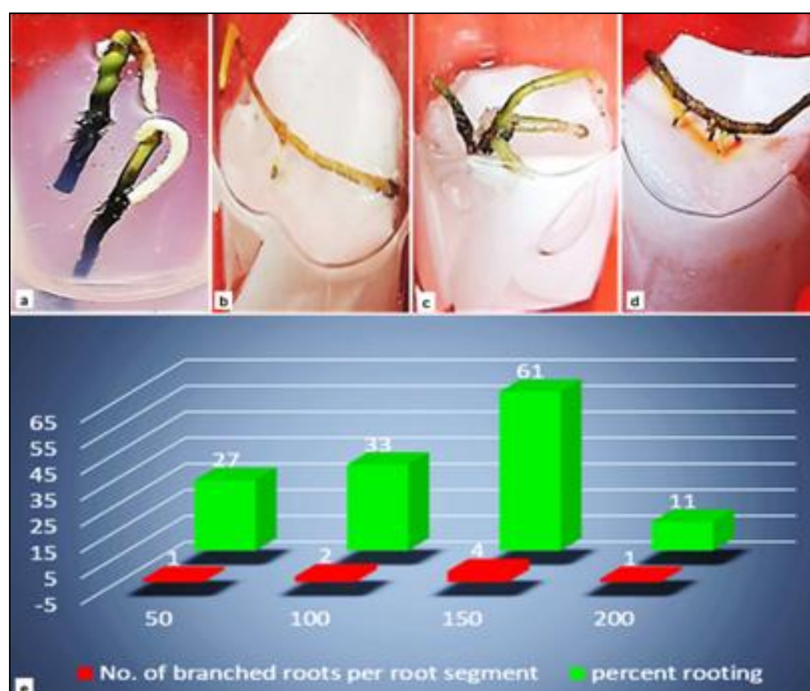


FIG. 2: EFFECT OF SUPPORT MEDIUM AND TRYPTOPHAN ON ADVENTITIOUS ROOTING INDUCED FROM ROOT EXPLANTS CULTURED ON ½ WPM SUPPLEMENTED WITH 9.8 µM IBA AND 3 % SUCROSE A ADVENTITIOUS ROOTING ON AGAR B ADVENTITIOUS ROOTING ON WHATMAN FILTER PAPER NO. 1, C & D ADVENTITIOUS ROOT FORMATION AT 150 µM TRYPTOPHAN CONCENTRATION, E GRAPHICAL REPRESENTATION OF EFFECT OF DIFFERENT (50, 100, 150, OR 200 µM) CONCENTRATIONS OF TRYPTOPHAN ON NUMBER OF BRANCHED ROOTING AND PERCENT ROOTING IN *G. INDICA*

TABLE 3: EFFECT OF DIFFERENT STRENGTHS OF WPM MEDIUM SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF AUXIN IBA AND SUCROSE ON ADVENTITIOUS ROOT FORMATION IN *GARCINIA INDICA*

WPM Media strength	IBA (µM)	Sucrose (%)	Type of medium	Rooting response in days (after inoculation)	Shoot response to rooting (%)	Avg. roots per explant
½ / ¼	0.0	2 or 3	Solid/liquid	-	-	-
½	9.8	3	Solid	58±3	50	0.6±0.6
½	9.8	3	liquid	36	78	1.3±0.6
½	9.8	2	Solid	68±1.4	15	0.4±0.6
½	9.8	2	Liquid	63±1.2	22	0.6±0.6
½	14.8	2 or 3	Solid/liquid	-	-	-
¼	9.8	2	solid	53±5	25	0.6± 0.6
¼	9.8	2	liquid	35±2	33	1.3±0.6
¼	9.8	3	Solid	60±1.1	20	0.6±0.6
¼	9.8	3	liquid	45±4	30	0.6±0.6
¼	14.8	2 or 3	Solid/Liquid	-	-	-

Effect of Tryptophan: Tryptophan is known to be a physiological precursor of auxins in higher plants. Tryptophan is one of the essential amino acids that can increase root growth and increase the level of endogenous auxin (e.g., IAA), which is helpful in the rooting of plants. In the current investigation, the most suitable media composition for the initiation of adventitious roots was determined to be ½ concentration WPM liquid medium enriched with 9.8 µM IBA and 3 % sucrose. However, the formation of adventitious roots in this medium was insufficient for the extraction of benzophenones.

To enhance this process we examined the impact of different concentrations of tryptophan (50, 100, 150, or 200 µM) in combination with ½ concentration liquid WPM fortified with 9.8 µM IBA and 3 % sucrose, results are shown in **Fig. 2 (C-E)**. Lower concentrations (50 or 100 µM) of tryptophan did not have a significant effect on lateral root formation in root explants. In contrast, a concentration of 150 µM tryptophan was found to produce the highest number of lateral roots (4) along **(Fig. 3 A-B)** with a good % root initiation response (61) in root explants **(Fig. 3 E)**. A higher concentration (200 µM) of tryptophan was found to inhibit lateral root growth. Therefore, a concentration of 150 µM tryptophan was determined to be effective in increasing adventitious rooting in *G. indica*. The present result thus showed that the addition of tryptophan in rooting media increases endogenous auxin (IAA) levels which synergistically with IBA showed enhancement in lateral root branching. Gohlot *et al.*[32] while working with *Azadiracta indica* A. Juss (Neem)also reported the positive effects of the inclusion of tryptophan in rooting media on its rooting potential. They observed early rooting with the increase in the number of roots and root length in *A. indica*. The adventitious roots developed after tryptophan treatment were further used for the extraction of benzophenones.

Three Types of Roots used for Screening UV Protective Ability:

Natural Roots (NR): Natural roots used in the present study were collected from about 15 years old fully grown plant at Dapoli.

Hardened Roots (HR): Approximately 6-month-old hardened roots were utilized for the extraction of benzophenones.

***In-vitro* Roots Generated from Suspension Cultures (IVR):** The well-grown *in-vitro* roots with secondary branches were utilized for Benzophenone extraction.

Extraction of Benzophenones: Previously, many organic solvents (methanol, ethanol, n-butanol, ethyl acetate and hexane) have been used in the processing of *G. indica* fruit rinds to extract benzophenones. However, among these various organic solvents, only the ethyl acetate extract showed significant UV absorption in UV A as well as UV B region [33]. Hence, in the present study ethyl acetate was used to extract benzophenones from natural, *in-vitro* and hardened roots. Biomass to the solvent ratio for NRE, IVRE and IVHRE was 20, 5 and 5 % (w/v) respectively. All three extracts (NRE, IVRE and IVHRE) were concentrated in a rotary evaporator at 60ºC and dried at RT.

The yield of NRE was noted to be 15 % w/w, IVRE was 10 % w/w and IVHRE was 12 % w/w.

Comparative UV Absorbance Analysis of NRE, IVRE, and IVHRE using UV-VIS Spectrophotometer: The comparative UV absorbing ability of all three root extracts (NRE, IVRE, IVHRE) were determined by using a Genesis 10S UV- VIS spectrophotometer. Absorbance was measured in the UV visible region from 200nm to 400nm. All three extracts NRE, IVRE and IVHRE were reconstituted at a concentration of 0.1 mg/ml in ethyl acetate solvents and they were used for spectrophotometric analysis. The findings demonstrated that NRE and IVRE both exhibit excellent UV absorbance activity that is identical to that of NRE **(Fig. 3)**. The average UV A absorbance of NRE, IVRE, and IVHRE was observed to be 3.51, 3.62, and 3.49 respectively. NRE was observed to have an average UV B absorbance of 4.07, IVRE of 4.24, and IVHRE of 4.25. Results demonstrate that all three root extracts had comparable UV absorption activity in both the UV A and UV B regions. This indicates that IVRE and IVHRE have the potential to absorb UV radiation and may thus be used in sunscreen formulations in place of NRE. Based on this result the study is taken further to perform *in-vitro* SPF analysis.

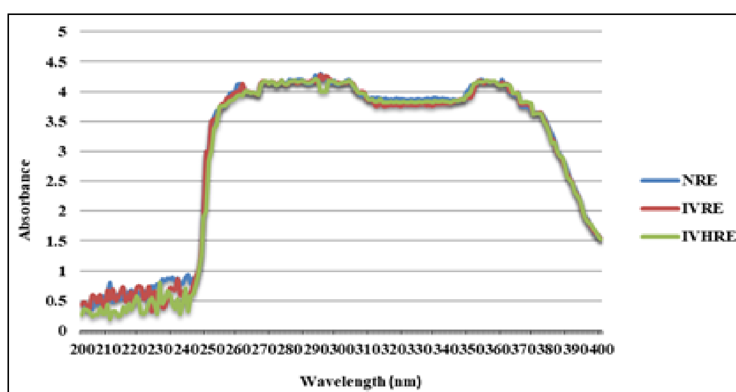


FIG. 3: COMPARATIVE UV ABSORBANCE POTENTIAL OF NRE, IVRE AND IVHRE

***In-vitro* SPF Determination of the Three Root Extracts NRE, IVRE and IVHRE by Mansur's Equation:** Recent progress in sunscreen technology has prioritized the development of efficient broad-spectrum (UVB and UVA absorbing) sunscreen products [12]. The term broad spectrum defines protection from harmful UV A and UV B rays. UV B rays are the stronger of the two and can cause sunburns and most skin cancers. UVA rays are typically responsible for premature ageing but may also contribute to some skin cancers. SPF defines protection against UV B rays and not about effectiveness against UV A rays.

Therefore, in the present investigation in addition to SPF, UV A/UV B ratios were taken into consideration which defines broad spectrum UV protection ability. If the UVA/UVB ratio is greater than 0.800 the formulation is regarded to have broad-spectrum UV protection. The ratio of UVA to UVB offers valuable insight into which UV region is effectively shielded by the substances. Moreover, the UVA/UVB ratio can be utilized to determine the Boots Star Rating, which categorizes products into groups ranging from 0 to 5 stars. The Boots Star Rating holds significance in assessing the consistency of the produced photoprotection levels, as the constituents of the sunscreens may deteriorate [34].

In the present study, the UV B absorbance readings from **Fig. 3** of NRE, IVRE and HRE from 290-320 nm at an interval of 5 nm were used to determine SPF using the Mansur equation as described in material and methods. The results of the *in-vitro* SPF analysis are shown in the **Table 4**. The SPF of NRE, IVRE and IVHRE was observed to be 40.45, 40.52 and 40.49 respectively. The boot star rating of NRE, IVRE and IVHRE was observed to be 4. This data demonstrates that the SPF values for IVHRE and IVRE were precisely the same and matched the SPF value for NRE. The SPF and boots star ratings of IVRE and IVHRE have once again demonstrated broad-spectrum ability to block UV rays. As a result, IVRE and IVHRE can be used effectively in a sunscreen formulation.

TABLE 4: *IN-VITRO* SPF DETERMINATION OF NRE, IVRE AND IVHRE USING THE MANSUR EQUATION

Wavelength	EE X I	Absorbance			EE X I X Absorbance		
		NRE	IVRE	IVHRE	NRE	IVRE	IVHRE
290	0.015	4.277	4.156	4.216	0.064	0.062	0.063
295	0.0817	4.151	4.232	4.192	0.339	0.346	0.3542
300	0.2874	4.148	4.132	4.14	1.192	1.188	1.190
305	0.3278	4.055	4.101	4.078	1.329	1.344	1.337
310	0.1864	3.89	3.863	3.877	0.725	0.720	0.723
315	0.0839	3.887	3.86	3.874	0.326	0.324	0.325
320	0.018	3.856	3.77	3.813	0.069	0.068	0.069
∑ (EE X I X A)					4.045	4.052	4.049
SPF (∑ (EE X I X A) X Correction Factor 10)					40.45	40.52	40.49
Average UV B (280 - 320 nm)					4.07	4.24	4.25
Average UV A (320 - 400 nm)					3.51	3.62	3.49
Average UV A / Average UV B					0.862	0.854	0.821
Boot star rating					****	****	*****

Preparative Thin Layer Chromatographic Separation of NRE, IVRE and IVHRE for Determination of Benzophenone Derivates: In *G. indica*, polyisoprenylated benzophenone derivatives and bioflavonoids are present as a major class of secondary metabolites. Polyisoprenylated benzophenone derivatives, such as garcinol, isogarcinol, xanthochymol and isoxanthochymol are isolated from *G. indica* fruits, dry rinds and leaves [35, 36, 37, 38, 31, 40, 41].

Benzophenone is a class of organic aromatic compounds. Plant metabolites with aromatic rings often exhibit a wider absorption spectrum, that encompasses the 200–400 nm region. Earlier Dhaval and Deodhar [42] screened various fruit rind extracts by HPLC. These extracts showed prominent peaks which coincided with standard garcinol (Cayman Chemicals, Michigan United States). They noticed as the amount of garcinol in the extract increased the UV-protecting ability of the extract also increased.

In the present study, the HPTLC method was followed to screen three root extracts for the presence of isoprenylated benzophenones and their UV protective ability was studied. Initially, a simple TLC method was standardised to visualize isoprenylayed benzophenones from NRE, IVRE, and IVHRE.

A TLC plate (8 ×10 cm) precoated with silica gel 60 F_{254} of 0.2 mm thickness was used. All three root extracts NRE, IVRE and HRE, were reconstituted in ethyl acetate at the concentration of 10mg/ml. Standard garcinol (Cayman Chemicals, Michigan United States) was reconstituted in methanol at the concentration of 0.5mg /ml. Root extracts (10 µl) and garcinol (5 µl) were loaded on the plate with the aid of a micropipette.

Among the various solvent systems tried (Toluene: acetone: formic acid (6:6:1), Chloroform: methanol (9:1), Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26), Ethyl acetate: methanol: water (10:1:1), Hexane: ethyl acetate (30:70) and Hexane: ethyl acetate (70:30)) the best resolution was obtained in hexane and ethyl acetate (70:30). Hence this system was shortlisted for the fractionation of root extracts in *G. indica*.

The results of thin layer chromatographic analysis of various root extracts of *G. indica* are depicted in **Fig. 4**. Standard garcinol exhibited a fluorescent green band that was visible at 366 nm at Rf value 0.42 **(Fig. 4 Lane D)**. The natural root extract exhibits a similar fluorescent greenish band at Rf value similar to garcinol **(Fig. 4 Lane A)**. A similar band of garcinol is also seen prominently with *in-vitro* hardened roots **(Fig. 4 Lane C)**.

But at Rf value 0.42 similar band was not observed in IVRE **(Fig. 4 Lane b)**. Along with garcinol the *in-vitro* hardened plants exhibited many other prominent fluorescent bands (like a yellow band at Rf value 0.48, two blue bands at Rf values 0.63 and 0.68 and a red band at Rf value 0.78) which might contribute to UV absorption. IVRE exhibited a completely different array of compounds. Though *in-vitro* root extracts do not exhibit a fluorescent green band coinciding with garcinol but exhibit many other fluorescent bands which are red.

These might be the precursors or intermediate compounds of garcinol or other anti-UV compound synthesis pathways and hence contribute to UV absorption in both UV A and UV B regions.

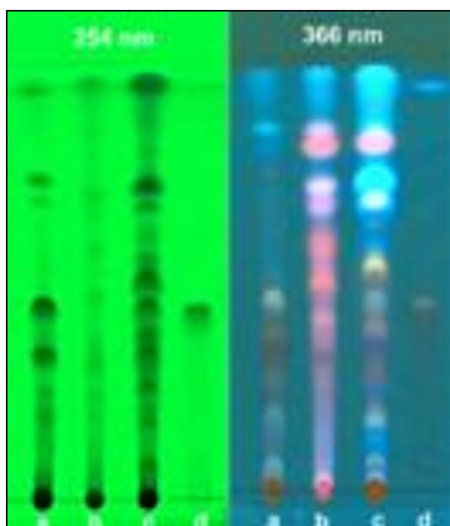


FIG. 4: TLC OF NRE, IVRE, IVHRE AND GARCINOL STANDARD. (A: NRE, B: IVRE, C: IVHRE AND D: GARCINOL STANDARD)

Fractionation and Isolation of Anti-UV Compounds from NRE through Column Chromatography followed by Spectrophotometric Analysis of Fractions and their TLC:

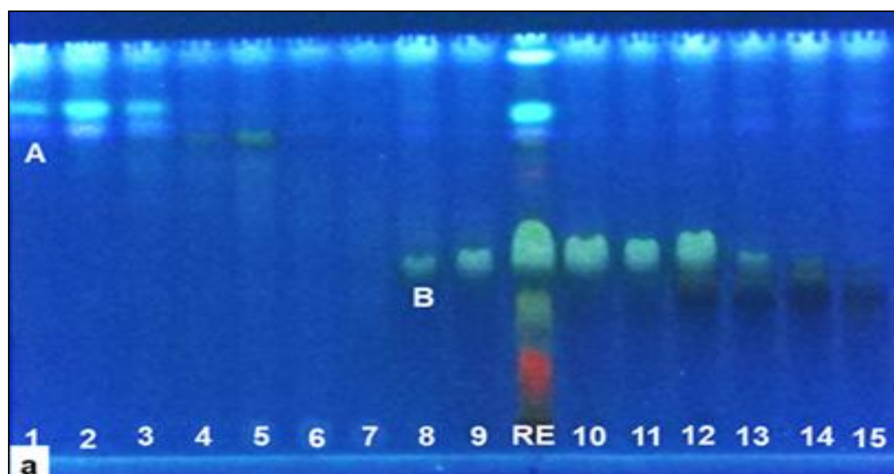
Fractionation of NRE using Column Chromatography: Results of preparative TLC analysis indicated that both NRE and IVHRE contain the band that has the same Rf value of standard garcinol. But both extracts also contained some additional fluorescent bands that might contribute to UV absorption. Hence a systematic study was undertaken to separate these bands by column chromatography, the separated fractions were subjected to TLC and spectrophotometric analysis and the ability of separated fractions to absorb UV radiations between 200 to 400 nm was studied.

Also, the fractions were further analyzed for their sunscreen activity. In the present study, only NRE was taken into consideration for further analysis. NRE was subjected to silica-gel column chromatography.

Column elution was started with 100 % hexane followed by successive gradients of hexane: ethyl acetate and then ethyl acetate: methanol 90:10 to 100 % methanol. A total of 27 fractions 2 ml each were collected and analysed for UV absorbing ability.

Separating UV-absorbing Compounds from Selective Column Fractions through Thin-Layer Chromatography and Evaluating their UV-Absorbing Ability using Different Sun Screening Parameters: All the fractions eluted on the chromatographic column were sequentially loaded (10 µl) on a TLC plate (20 ×10 cm) precoated with silica gel 60 F254 of 0.2 mm thickness.

Previously standardised solvent system hexane and ethyl acetate 70:30 were used for the resolution of UV absorbing compounds. Chromatograms developed on the TLC plate were visualized under ultraviolet light (365 nm) using CAMAG's TLC visualizer. TLC results are depicted in **Fig. 5A and 5B**.



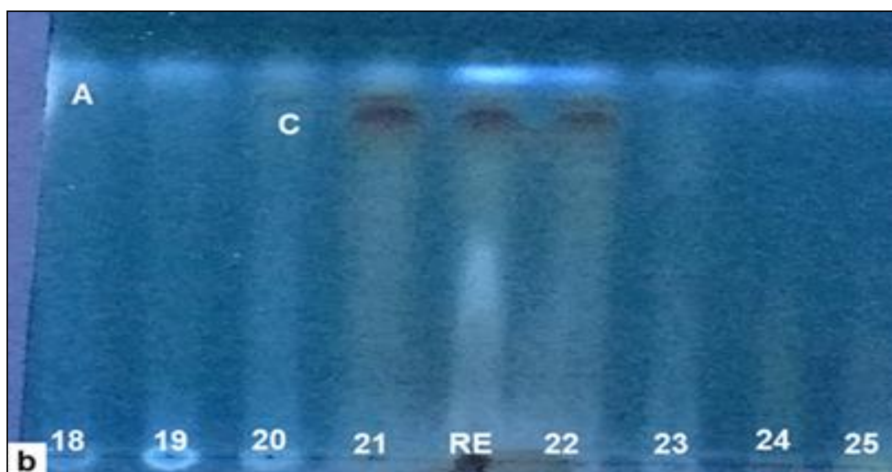


FIG. 5: PREPARATIVE TLC OF COLUMN FRACTIONS. A: FACTIONS 1-3: HEXANE 100%, HEXANE: ETHYL ACETATE (90:10, 80:20), 4-15: HEXANE: ETHYL ACETATE (70:30) SOLVENT SYSTEM, B: FRACTIONS FROM ETHYL ACETATE: METHANOL (90:10) SOLVENT SYSTEM. (RE: NATURAL ROOT EXTRACT)

The fractions were simultaneously analysed on a UV Vis-spectrophotometer and data represented in **Fig. 6 A-D**. The average UV A and UV B values, UV A/UV B ratio, SPF as well as the boot star rating of these fractions were calculated according to the Mansur equation and data are presented in **Fig. 6 E, F,** and **G**.

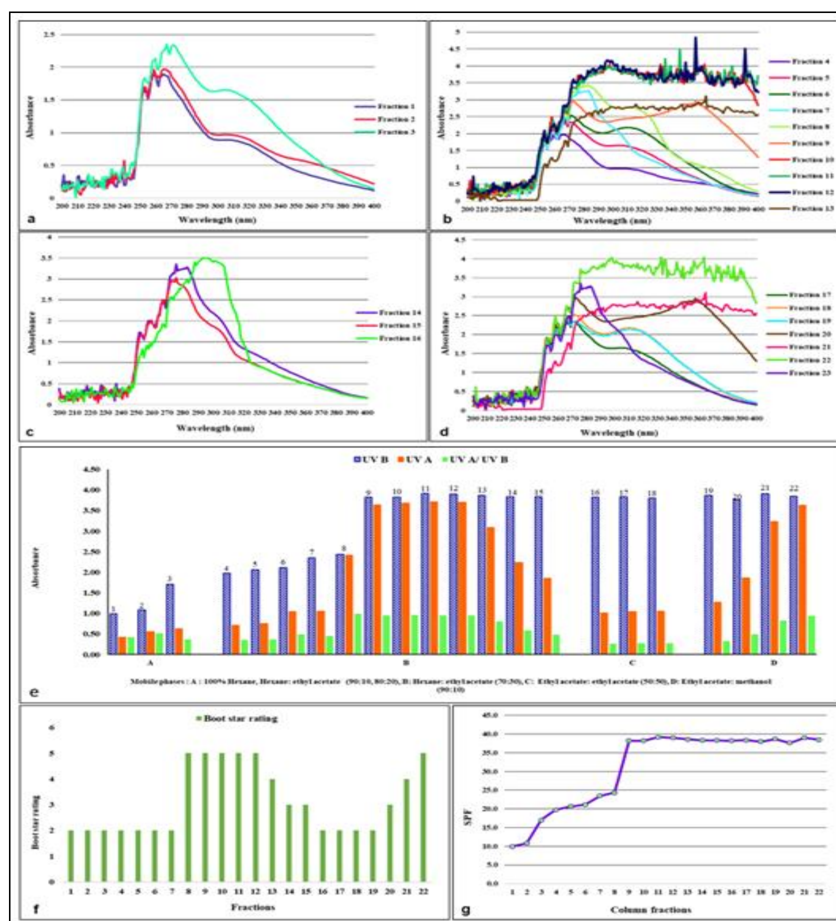


FIG. 6: UV ABSORBING PATTERN AND SUNSCREENING ABILITY OF COLUMN FRACTIONS. A: FRACTIONS FROM HEXANE (100%), HEXANE: ETHYL ACETATE (90:10, 80:20) SOLVENT SYSTEM B: FRACTIONS FROM HEXANE: ETHYL ACETATE (70:30) SOLVENT SYSTEM C: FRACTIONS FROM HEXANE: ETHYL ACETATE (50:50) SOLVENT SYSTEM, D: FRACTION FROM ETHYL ACETATE: METHANOL (90:10) SOLVENT SYSTEM, E:AVERAGE UV A AND UV B VALUES OF SELECTIVE COLUMN FRACTIONS, F:BOOT STAR RATING VALUES OF SELECTIVE COLUMN FRACTIONS, G: SPF VALUES OF COLUMN FRACTION

After analysing the TLC plate, it was observed that the fractions eluted initially at 100% hexane or when the concentration of ethyl acetate was low (hexane: ethyl acetate 90:10, 80:20) yielded a florescent blue colour band 'A' on the TLC plate of Rf value 3.7. Spectrophotometric analysis revealed that the first eluted fractions had the highest UV absorbance in the UV B region (290 nm to 320 nm) as illustrated in **Fig. 6A**. The average absorbance in the UV B region ranged from 0.99 to 1.70, with an average UV A absorbance between 0.42 and 0.63 **(Fig. 6E)**.

This indicates that these fractions primarily absorb UV B radiation and have a high SPF value **(Fig. 6 G)**. Additionally, the ratio of UV A to UV B absorption in these fractions **(Fig. 6 A)** is notably low, resulting in a 2 boot star rating **(Fig. 6 F, fractions 1 to 7)**. Upon conducting elution with hexane: ethyl acetate (70:30), the initial fractions solely exhibited the fluorescent blue band 'A'. However, as the fractionation proceeded, the fluorescent green band 'B' with an Rf value of 0.43 started to emerge alongside the blue fluorescent band 'A', as depicted in **Fig. 5 A**. Consequently, there was a gradual rise in the average UV A absorbance (0.71 - 3.71) and the average UV B absorbance (1.98 - 3.92) noted. This increase in average UVA values was also reflected in the enhanced UVA/UVB ratios **(Fig. 6 E)**.

As illustrated in the Bar Graph **Fig. 6 E**, the Green band B becomes visible from Fraction 8 and attains its peak intensity in Fraction 12. These fractions exhibit the greatest UV A/UVB ratios (1.0) and have been rated with 5 boot stars **(Fig. 6 E and Fig. 6 F)**. The SPF values of Fractions 8-12 were notably high, with a maximum SPF of 39 **(Fig. 6 G)**. Starting from fraction 13, the intensity of the green band reduced and gradually declined in the average UV A absorbance. This decline in UV A absorbance persisted in the fractions that were eluted from the solvent system of hexane: ethyl acetate (50:50). The fractions consistently exhibited high average UV B absorbance and a significantly high SPF (37-39) **(Fig. 6 E and Fig. 6 G)**. As the intensity of band B increased, the fractions displayed absorbance in both UV B and UV A regions **(Fig. 6 B, fractions 10-12)**. As observed in **Fig. 6 B**, fractions 4 to 7 exhibit relatively low UV A absorbance (below 1). However, as the fractionation process advances (fractions 9 to 12), the absorbance in both UV A and UV B regions becomes nearly equal to 3.5. Further fractionation results in a decline of UV A absorbance (drops to 1) while maintaining consistent UV B absorbance (3.5). Therefore, it can be inferred that fractions 8 to 12, which contain band B, are responsible for the broad-spectrum UV absorbance. Further elution of the column using a mixture of ethyl acetate and methanol (90:10) produced fractions with an additional band 'C' alongside band 'B' **(Fig. 5 B)**. These fractions also demonstrate broad-spectrum UV absorbance, which can be observed in fractions 20 to 22 of **Fig. 6 D**. These fractions exhibit significantly high average UV A absorbance along with the average UV B absorbance **(Fig. 6 E)**. The UV A/UV B ratios also increased with the boot star rating. Additionally, these fractions have high SPF values. When 100% methanol was added to the column at the end, all fractions exhibited absorption over the entire UV spectrum.

Characterization of anti-UV Compounds Present in NRE using HPTLC-MS Hyphenated Analytical Technique: In recent times, hyphenated procedures have gained significant focus as the leading approach to tackling intricate analytical issues. The potential of merging separation methods with spectroscopic procedures for both qualitative and quantitative identification of unknown substances in intricate extracts or fractions of natural products has been proven over time [36].

To determine the structural details that lead to the recognition of the substances present in a crud sample, thin-layer chromatography/high-performance thin-layer chromatography (TLC/HPTLC) is connected to a spectroscopic identification technique like mass spectrometry (MS), which has led to the development of HPTLC-MS. HPTLC-MS is an efficient and delicate method for separating and recognizing substances from a heterogeneous sample in a brief duration guaranteeing accurate identification of substances down to the nanogram scale and generating pure outcomes [43]. Using this technique, areas of interest are loaded into a mass analyzer for identification, delivering precise mass spectrometric information in less than sixty seconds [44].

In the present study, NRE was analysed by the HPTLC-MS technique to identify anti-UV compounds present in the green and blue colour fluorescent bands. For HPTLC analysis 10 mg of NRE and 0.5 mg of garcinol standard were separately reconstituted in 1ml of HPLC grade methanol. Sample (2 µl) and standard (2 µl) were loaded on a TLC plate precoated with silica gel 60 F_{254} of 0.2 mm thickness with a Linomat 5 semiautomatic applicator (CAMAG, Switzerland) fitted with 100 µl syringe. The developing solvent was Toluene: Ethyl acetate: Formic acid (4:1:0.5). The developed chromatogram was scanned under a CAMAG TLC scanner.

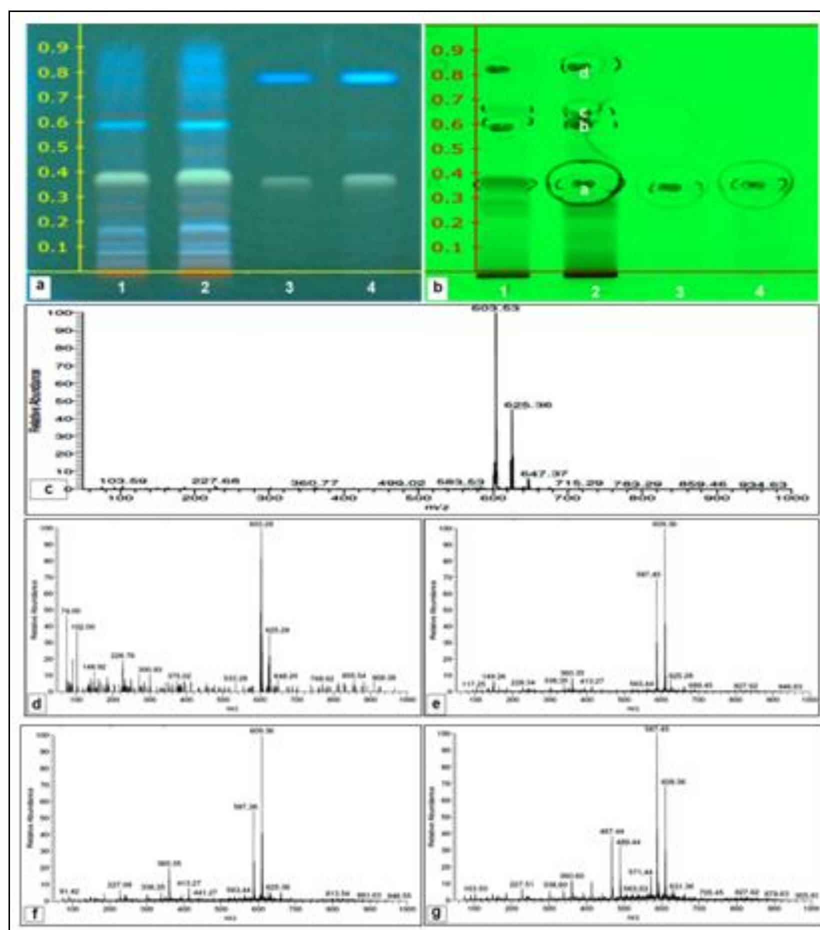


FIG. 7: HPTLC AND HPTLC-MS DATA OF NRE OF *G. INDICA*. A: HPTLC PLATE IMAGE OF NRE VISUALISED AT 366 NM (TRACK 1-2: NRE, TRACK 3-4: GARCINOL STANDARD), B: HPTLC REMISSION PLATE IMAGE AT 254 NM, C: TRACK 3A MASS SPECTRA OF GARCINOL STANDARD, D:TRACK 2A MASS SPECTRA (GREEN FLUORESCENT BAND), E: TRACK 2B MASS SPECTRA (BLUE FLUORESCENT BAND), F:TRACK 2C MASS SPECTRA (BLUE FLUORESCENT BAND), G: TRACK 2D MASS SPECTRA (BLUE FLUORESCENT BAND)

The detection of bands was done under a UV detector. NRE exhibited various bands with different RF values at 366 nm depicted in **Fig. 7A**. The green fluorescent band of NRE showed the Rf value of 0.345 which was similar to the Rf (0.373) value of the garcinol standard. Which again confirmed the presence of garcinol in NRE. Therefore, on the TLC plate interested bands were marked as a, b, c and d and targeted for HPTLC-MS analysis **(Fig. 7B)**. The mass spectra, representing each of the bands, are presented in **Fig. 7C-G**. The mass-to-charge ratios (*m/z*) of the most intense ions are listed in **Table 5**. The compound identification was done based on data available in the literature.

TABLE 5: UV ABSORBING COMPOUNDS IDENTIFIED IN NRE USING HYPHENATED HPTLC-MS

UV absorbing bands	Molecular mass (m/z)$^-$	Compound identified	Reference
Green fluorescent (a)			
1	603.53	Garcinol	[45]

2	625.28	7 epi-isogarcinol	[45]
Blue fluorescent (b, c, d)			
1	609.36	14-deoxy-7-epi-isogarcinol	[45]
2	587.45	14-Deoxy isogarcinol	[37]

The green band of track 2 marked as ‘a’ on the TLC plate yielded the most intense ion at *m/z* 603.28 **(Fig. 7 D)** which, according to literature corresponds to compound Garcinol [37]. This molecular mass also showed exact similarity with the most intense ion at *m/z* 603.53 exhibited by the green band of garcinol standard **(Fig. 7 C)**. A less intense ion fragment at *m/z* 625.28 from tack ‘2a’ corresponds to compound 7 epi-isogarcinol [45]. The presence of other small abundant peaks denotes the various xanthones **(Fig. 7 D)**. The intense ion corresponding to *m/z* 609.36 and m/z 587.45 are prominent in the mass spectrum of track 2b, c and d **(Fig. 7 E, F and G)**. These compounds exhibited a blue fluorescent band on the TLC plate. The ion at *m/z* 609.36 corresponds to 14-deoxy-7-epi-isogarcinol [45]. The ion fragment at *m/z* 587.45 corresponds to 14-Deoxy isogarcinol [37]. Therefore, the present analysis revealed that garcinol and 7 epi-isogarcinol are responsible for absorption in the UV A region. While 14-deoxy-7-epi-isogarcinol and 7 epi-isogarcinol are responsible for the absorption of UV B region.

Incorporation of NRE in Cosmetic Formulation and *In-vitro* SPF According to COLIPA Guidelines: According to earlier research, NRE canabsorb UV light across the UV A and UV B spectrums. In addition, adding root extract to cosmetic formulations has been claimed to boost their SPF value by five times [8]. Dike and co-workers, wanted to check the sunscreen potential of NRE, so they used only one concentration (5 %) of NRE. In the present investigation to find out the minimal concentration of NRE with good SPF, we incorporated lower concentrations (2%, 2.5%) of NRE along with the 5 % of NRE in cosmetic formulation. For comparison, 5% fruit extract (FE) was also incorporated into the cream. I*n vitro,* the SPF value was determined using a UV2000s transmittance analyzer. The results are interpreted in **Table 6**.

In the present study, the SPF of cosmetic formulation without any plant additive was 1.47 with no boot star rating. When different concentrations of (2%, 2.5% and 5%) NRE were added to cosmetic formulations increase in SPF with boot star rating was reported **Table 6**. An increase in SPF value was in a dose-dependent manner observed at tested concentrations of NRE. At 2 % concentration of NRE, SPF was found to be 5.22, at 2.5 % it was 5.52, at 5 % NRE the SPF was seen to be 6.25. Therefore, the SPF results suggest that lower concentrations of NRE also have excellent sunscreen potential and hence can be used in cosmetic formulation. However, SPF defines protection against UV B rays and not effectiveness against UV A rays. Therefore, in the present investigation in addition to SPF, critical wavelength and UV A/UV B ratios are taken into consideration to define the broad-spectrum UV protection ability of the formulation. The term broad spectrum defines protection from harmful UV A and UV B rays. UV B rays are the stronger of the two and can cause sunburns and most skin cancers. UVA rays are typically responsible for premature ageing but may also contribute to some skin cancers.

The “Critical Wavelength” (λc) is defined as the wavelength below which 90% of the area under the absorbance curve resides, hence only 10 % of rays are allowed to penetrate through the skin and such sunscreens are labelled as a broad spectrum. Sunscreens with a critical wavelength over 370 nm ensure broad-spectrum UV protection, especially protection from longer wavelength UVA rays. In the present study, the critical wavelength at 2 % of NRE concentration was noted to be 374 nm, at 2.5 % it was 380.53 nm, at 5 % it was 383 nm. Therefore, improvement in the critical wavelength of the cosmetic formulation was reflected in the Boot star rating. All the concentrations of NRE used in the present study exhibited broad-spectrum UV protection.

The same study demonstrated the UV A protection ability of NRE, which was evidenced by the ratio of UV A / UV B absorbance giving boots a star rating system developed in the UK. The UV A / UV B ratio of analysed concentrations of NRE was between 0.8 – 0.89, which yielded a 4 boots star rating.

TABLE 6: *IN-VITRO* SPF, UVA/B ACTIVITY, CRITICAL WAVELENGTH, AND BOOT-STAR RATING OF VARYING CONCENTRATIONS OF NRE AND 5 % OF FE

Formulations	Sun Screening Parameters			
	SPF	UV A/UV B	Critical wavelength	Boots star rating
Cream Base (Control)	1.47	0.218	356.73	No rating
Cream Base + 2 % NRE	5.22	0.699	374.00	***
Cream Base + 2.5 % NRE	5.52	0.743	380.53	***
Cream Base + 5 % NRE	6.25	0.823	383.00	****
Cream Base+ 5% FE	3.43	1.002	388.00	*****

Comparison of Sun Screening Ability of Root Extract with Fruit Extract using HPTLC Analysis: In the previous study by Dike and Deodhar [33],5% Ethyl acetate extract of fruit rinds of *G. indica* was reported to have SPF 3.43 and 5 boot star rating **Table 6**. Letter while screening various plant extracts of *G. indica* viz., leaf, seed stem, root and fruit extracts for UV protective activity Dike and co-workers [8] reported 5% root extract had maximum UV protective activity with SPF 7.19 and boot star rating 4. Whereas 5% fruit extract showed an SPF of 3.67 and a boot star rating of 4. In the present study also, we have noted 5 % root extract with an SPF value of 6.25 and a boot star rating of 4 **Table 6**. Therefore, to find out the difference between the composition of benzophenones in the fruit extracts and root extracts, 2µl (10mg/ml) of both fruit and root extracts along with the 2µl of standard garcinol were loaded on a TLC plate precoated with silica gel 60 F $_{254}$ of 0.2 mm thickness. The developing solvent was Toluene: Ethyl acetate: Formic acid (4:1:0.5). The developed chromatogram was scanned under a CAMAG TLC scanner. The results of the TLC plate are depicted in **Fig. 8**. As observed in fig. 8, the standard garcinol extract displays two prominent bands, with one band appearing green in colour at an Rf value of 0.373. This band has a mass-to-charge ratio of m/z 603.28, which corresponds to the compound Garcinol [37], as indicated in **Fig. 8E**.

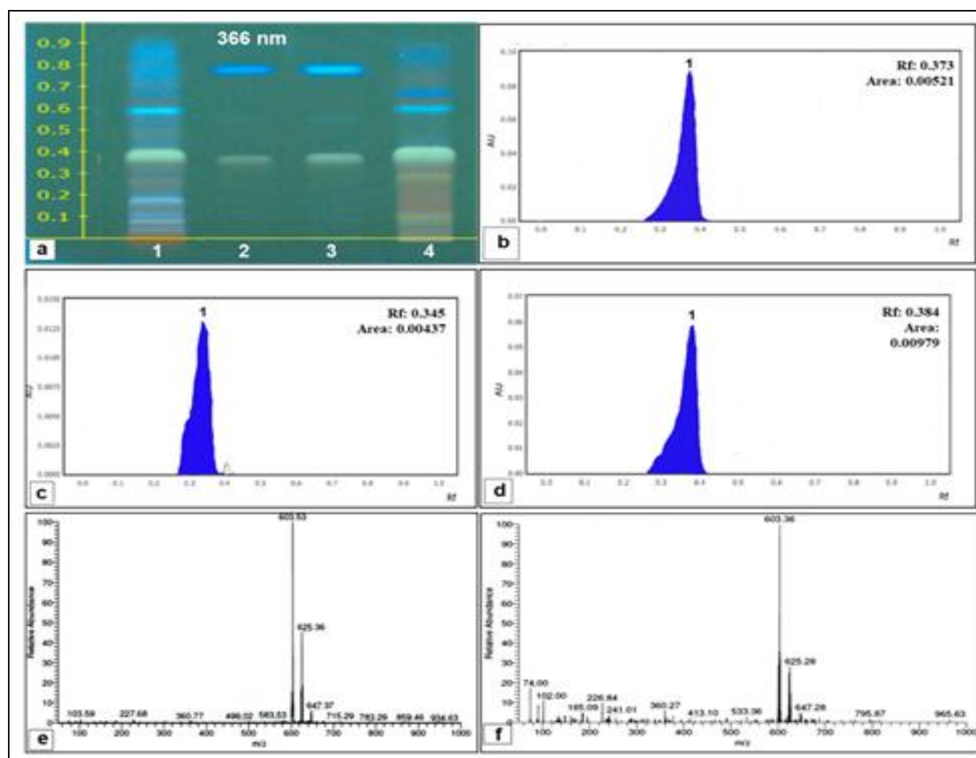


FIG. 8: COMPARATIVE HPTLC ANALYSIS OF NRE AND FRUIT EXTRACT OF *G. INDICA*. A: HPTLC PLATE IMAGE VISUALISED AT 366 NM AND 254 NM (TRACK 1: ROOT EXTRACT, TRACK 2 AND 3: STANDARD GARCINOL, TRACK 4: FRUIT EXTRACT), B: PEAK RESPONSE OF GARCINOL STANDARD, C: PEAK RESPONSE OF GARCINOL IN ROOT EXTRACT, D: PEAK RESPONSE OF GARCINOL IN FRUIT EXTRACT, E: MASS SPECTRA OF GARCINOL STANDARD, F: MASS SPECTRA OF A GREEN FLUORESCENT BAND IN FRUIT EXTRACT.

The ethyl acetate extracts from both the root and fruit of Garcinia also exhibit a similar band that corresponds to garcinol and has a comparable mass-to-charge ratio of m/z 603.28 (**Fig. 7 D** and **Fig. 8 F respectively**). In the fruit extract, the relative concentration of garcinol (9.3%) is higher compared to the root extract (4.1%). Additionally, when exposed to UV light at wavelengths of 366 nm, the root extract demonstrates greater fluorescence or activity in the blue band region with multiple distinct bands that are more prominent than those in the fruit extract.

Our previous research conducted (Data not yet published) on fruit extracts of *G. indica* suggests that garcinol is responsible for providing broad-spectrum UV protection in both the UV A and B regions, and it also contributes to the extract's high boot star rating. Previously, Bharte *et al.* [19] analyzed to quantify and validate two anticancer compounds, garcinol and isogarcinol, in ultrasound-assisted extracts of *Garcinia indica* fruits using high-performance thin-layer chromatography. They employed dichloromethane and methanol along with water to extract benzophenones from the dried fruit rinds of *G. indica*. For TLC development, they utilized a mixture of n-pentane, ethyl acetate, and formic acid (7:3:0.5, v/v). The plates were then visualized under white light after derivatization with an anisaldehyde reagent. In their chromatographic preparation, only two bands were found in Garcinia fruit rinds extracted with different solvents, and these bands corresponded to the standard garcinol and isogarcinol. Further identification of garcinol and isogarcinol was accomplished using LC/electrospray ionization (ESI)-MS/MS [46]. The highest quantity of garcinol (0.42 or 15.51%) was extracted in a 1:1 mixture of dichloromethane and methanol, while the amount of isogarcinol was 2.31% DWB.

In the current investigation, a mixture of Toluene, Ethyl acetate, and Formic acid (in a ratio of 4:1:0.5) was utilized, and the plates were observed under UV light. Upon comparing the peak area of the extracts from the roots and fruits with the peak area of the standard garcinol **(Fig. 8 B, C, and D)**, it was determined that the concentration of garcinol in the roots and fruits was 4.1% and 9.3% respectively. Additionally, the Ethyl acetate extracts of both the fruit and roots of *G. indica* exhibited additional fluorescent peaks at Rf 0.6 and 0.85. The compound that produced a blue fluorescent band on the TLC plate at Rf values 0.6 and 0.85 yielded an ion with a mass-to-charge ratio (m/z) of 609.36, corresponding to 14-deoxy-7-epi-isogarcinol [45]. The ion fragment at m/z 587.45 corresponds to 14-Deoxy isogarcinol [37]. As mentioned earlier, the compounds 14-deoxy-7-epi-isogarcinol and 14-Deoxy isogarcinol specifically absorb UV B light, which contributes to an increase in the SPF of the formulation. The higher SPF of the root extract is attributed to the higher concentration of these two compounds. In contrast, the fruit extract contains a greater amount of garcinol compared to the roots. The concentration of garcinol in the roots and fruits was determined to be 4.1% and 9.3% respectively, based on the dry weight of the samples. Since garcinol can absorb UV light across a broad spectrum, this is reflected in the higher critical wavelength, UV A/B ratio and boot star rating in the fruit extracts.

CONCLUSION: Currently, there is a growing demand for natural sunscreen ingredients instead of synthetic sunscreen due to the known negative effects of synthetic sunscreen. They are gaining significant attention as they are much safer, more cost-efficient, and have numerous advantages for the skin compared to synthetic sunscreens. The recent study revealed the existence of UV-absorbing benzophenones, specifically Garcinol (m/z 603.53) and 7 epi-isogarcinol (m/z 625.28), in the extracts of *in vitro* hardened roots and natural roots of *G. indica*. Both of these compounds were found to offer broad-spectrum UV protection. Additionally, two more UV-absorbing benzophenones, 14-deoxy-7-epi-isogarcinol (m/z 609.36) and 14-Deoxy isogarcinol (m/z 587.45), were identified, which only absorb in the UV B region. Hence, this research suggests that *in vitro* hardened roots have the potential to serve as an alternative source of UV-absorbing benzophenones. This would provide a promising avenue for further investigation into the development of herbal base sunscreening formulations.

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