RESEARCH ARTICLE

Extraction, Isolation and Fingerprinting of Phenolic Compound from Hydroalcoholic Extract of *Cordia macleodii* Hook Bark

Kadoo B. M.*, Dhaswadikar S. R., Meshram S. S., Ghagare A. S., Badnale A. B., Itankar P. R.*

Department of Pharmaceutical Sciences R.T.M. Nagpur University, Nagpur-440033. Maharashtra, India

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ABSTRACT

Natural products in plants extract are usually found in complex form with numerous phytoconstituents of different polarities and thus it becomes big challenge to isolate them into pure components. In addition to fingerprinting of isolated component by HPLC and spectroscopic methods, an effort is made to develop applicable column chromatographic approach for the separation and detection of phenolic compound from the fraction of hydroalcoholic extract of *Cordia macleodii* bark (CMB). Hydroalcoholic extract (60:40) of *Cordia macleodii* bark was prepared by cold maceration method and partitioned into different fractions based on increasing polarity of solvents using separating funnel. Most productive ethanolic fraction thus obtained was undertaken for column chromatographic study using ethyl acetate: methanol (20:80) as mobile phase. HPLC study performed for identification of bioactive compound and diverse spectral studies for structural elucidation. Yield of ethanolic fraction obtained from CMB hydro alcoholic extract was found to be 84.33% w/w. HPLC data recognized gallic acid in fraction since the RT of fraction matches with standard gallic acid RT of 5.080. By using spectroscopic techniques like UV, IR, 1H & 13C NMR, and MS, the structure of the isolated compound was identified as 3,4,5 trihydroxy benzoic acid, gallic acid. Ethyl acetate: methanol (20:80) is best suited mobile phase for separation and isolation of phenolic compound from the complex mixture of fraction of extract. Gallic acid confirmed by chromatographic and spectroscopic analysis may support the pharmacological relevance of its use and substantiate traditional benefits.

Keywords: Hydroalcoholic extract, *Cordia macleodii* bark, HPLC, Spectroscopic methods, gallic acid.

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INTRODUCTION

Native to India, *Cordia macleodii* Hook, is a folklore medicinal plant belongs to family-Boraginaceae which has been historically used for mouth sores, jaundice and aphrodisiac purposes [1]. There are about 300 different species of deciduous blooming trees and shrubs commonly known as Phankhi / Shikari found in tropical regions. Locally it is referred as "Dahiman" or "Dahipalas," [2]. It is a prominent drug distributed over India's Deccan, Carnatic region and in the forests of Madhya Pradesh, Chhattisgarh, Karnataka, and Bihar. The medium-sized, polygamous, evergreen Cordia macleodii Hook tree grows to a height of 8 to 10 meters and has white, hairy limbs (**Figure 1**). This uncommon and endangered kind of medicinal plant may be found in both dry and humid mixed deciduous forests [3,4]. While the fruit, bark (**Figure2**), seeds, and leaves of *Cordia macleodii* have all been shown to have significant ethnomedicinal value, the leaves were most frequently used to treat a wide range of illnesses, including respiratory conditions, stomach discomfort, wounds, inflammation, myalgia, cough, dysentery, and diarrhea [5]. Numerous pharmacological properties, including antioxidant, anti-inflammatory, larvicidal, hepatoprotective, analgesic, antibacterial, and antidiabetic are documented for several *Cordia* species extracts and their isolated chemicals [6]. The leaves of various species are decocted and used as a tonic or remedy for illness, fever, colds, menstrual cramps, headaches, and snakebite. The mucilaginous fruits of this plants are used as demulcent, blood purifier and therapy for kidney, spleen, and lung conditions [7]. Brazilians mostly prepare a paste by using leaves of *C. americana, C. corymbose,* and *C. macleodii*, which is then practiced as a painkiller and antibacterial to treat bruises and wounds [8]. While Mexicos utilizes leaves of *C. americana, C. boissieri, C. cylindrostachya,* and *C. linnaei* in the form of decoction to cure lung and stomach illnesses [9]. Phenolic compounds in *Cordia species* prevent the unfavorable effects caused by free radicals by neutralizing them, thereby limits the inflammation process in the body [10]. Phytochemical screening of *C. macleodii* bark extract reveals existence of terpenoids, flavonoids, phenolics, saponins, tannins, glycosides, alkaloids, carbohydrates, resins, and reducing sugar. By Nadiya et al. (2014), three sterol compounds were discovered namely, campesterol, Stigmasterol and Cholesterol-5-EN-3-OL(3-Beta)-carbonyl chlorinated [11,12]. Soni and Bodakhe (2014) evaluated the anti-venom potential of an ethanolic extract derived from the bark of *Cordia macleodii* in relation to the pharmacological effects produced by Naja venom. The ethanolic bark extract of *C. macleodii* efficiently counteracts the defibrinogenating and coagulant effects of Naja venom in an equivalent dose of around 400 to 800 mg/kg in experimental species [13].

**Author for Correspondence: prakashitankar@hotmail.com*

Figure 1: *Cordia macleodii* tree

Figure 2: *Cordia macleodii* fresh bark

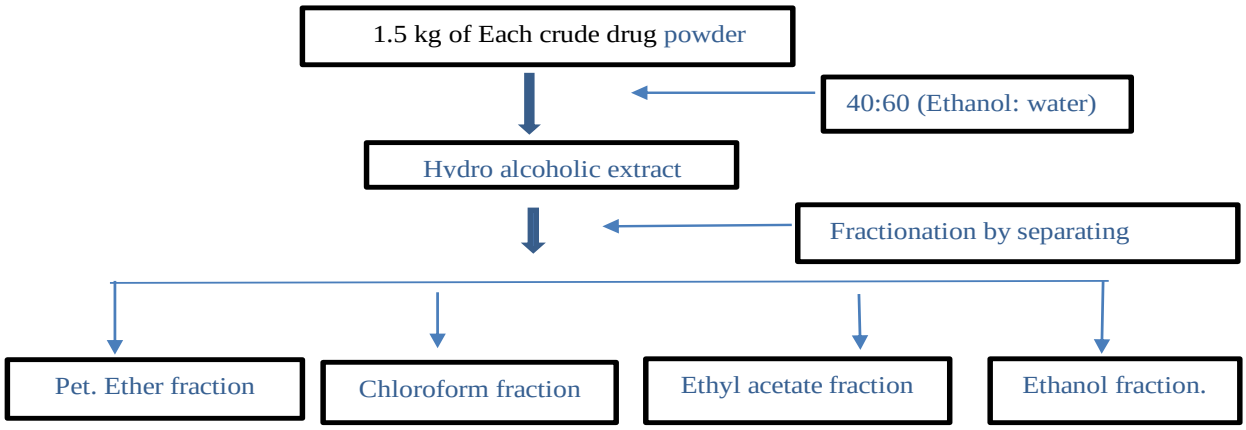


Figure 3: Schematic diagram for extraction and fractionation

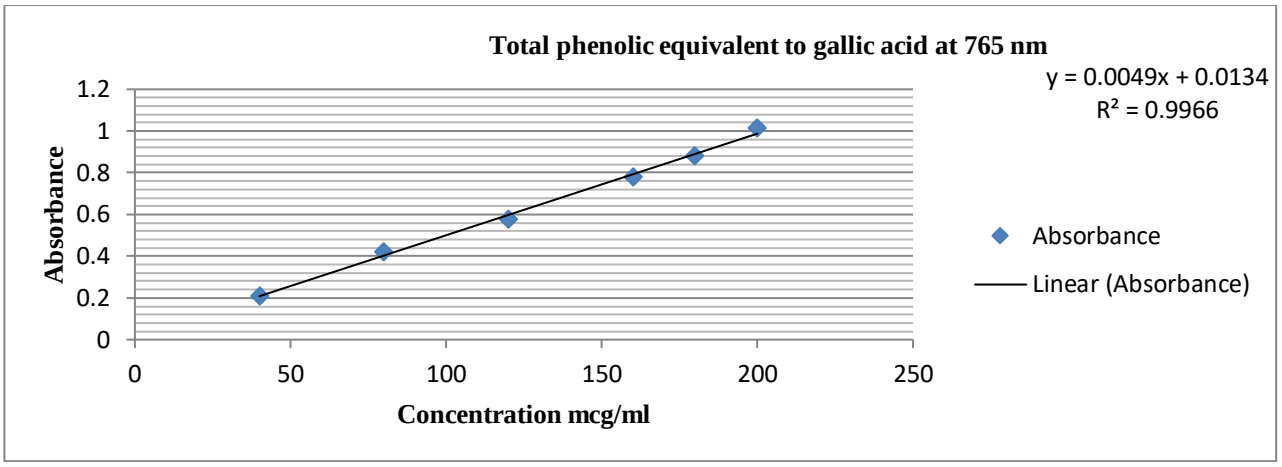
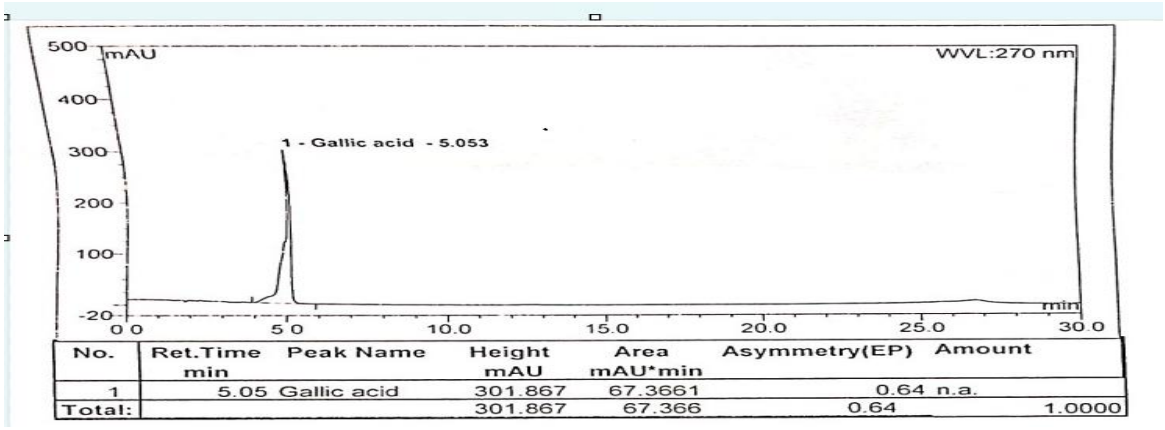


Figure 4: Gallic acid calibration curve

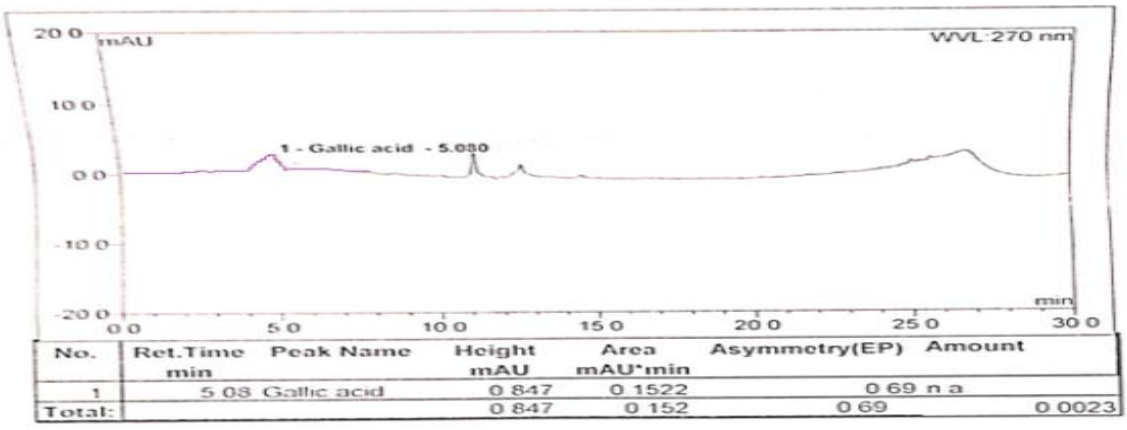
Similar to this, the antivenom, anti-inflammatory and antimyotoxic potentials of phenolic components that were discovered in leaves, flowers, fruits and barks of eleven species in the genus *Cordia*, including *C. dentate, C. verbenacea, C. americana, C. latifolia,* and, *C. dichotoma* were evaluated [14].

A phenolic compound called rosmarinic acid was extracted from *C. verbenacea* demonstrated notable antivenom and antimyotoxic properties against snake venom. According to reports, rosmarinic acid mostly interacts with Lys49-PLA2BthTX-I, which in turn increases the effectiveness of polyvalent antivenom against separated phospholipase A2 and crude venom [15]. Despite its historic usage, this plant's phytochemical composition and biological actions are well documented in scientific literature [16]. Using column chromatography and spectroscopic analysis techniques namely Ultraviolet, Infrared, NMR and Mass spectroscopy, the current work aims to isolate the most therapeutic pure phenolic molecule.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Asymmetry(EP)	Amount
1	5.05	Gallic acid	301.867	67.3661	0.64	n.a.
Total:			301.867	67.366	0.64	1.0000

Figure 5: Standard HPLC chromatogram of gallic acid



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Asymmetry(EP)	Amount
1	5.08	Gallic acid	0.847	0.1522	0.69	n.a.
Total:			0.847	0.152	0.69	0.0023

Figure 6: The CMB fraction's HPLC Chromatogram

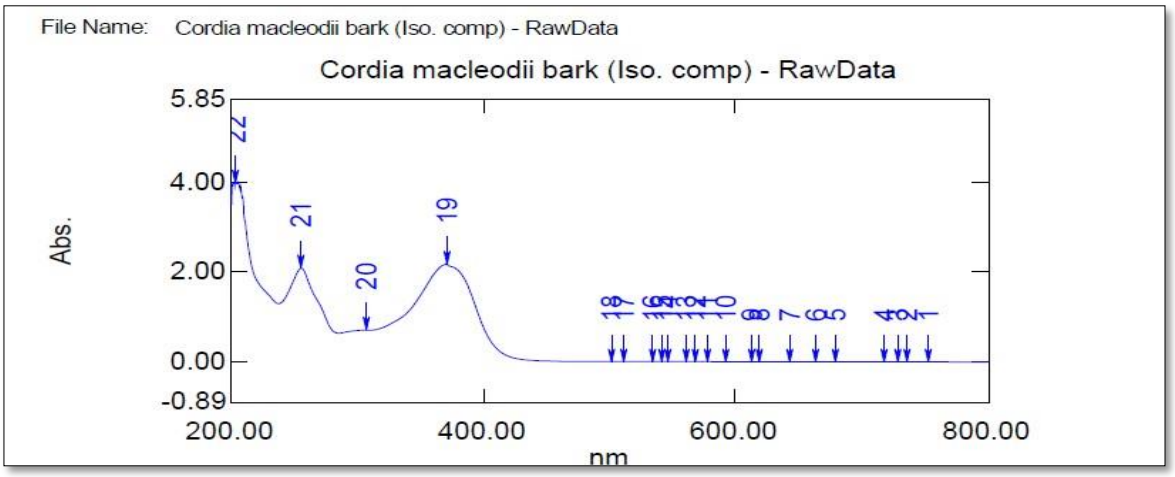


Figure 7: UV spectrum of isolated compound from ethanolic fraction of CMB extract

MATERIALS AND TECHNIQUES

Reagents and reference drugs

Every requirements and solvents include petroleum ether, silica gel G, ethyl acetate, methanol, vanillin- H_2SO_4 spray and chloroform were procured from Merck India Limited, Bombay and all of them were of analytical quality.

Acquisition of Plant resource

Barks of *Cordia macleodii* was acquired from the forest of village Pimpalgaon, Tahsil- Lakhandur Dist-Bhandara (Maharashtra) in November 2020 and dried in shade. Collected bark of *Cordia macleodii* Hook. was identified and authenticated by Dr. S. M. Bhuskute, Taxonomist and former Principal, Bhawbhuti Mahavidyalaya Amgaon-441902, District-Gondia (Maharashtra). The verified specimens have been preserved in herbarium museum of Department of Botany with vide no. BMV704 for future references.

Preparation of plant extract

After washing 1.5 kg of fresh Cordia macleodii bark, it was dried for about two weeks at 30 ^{0}C temperature and made converted into a coarse powder. Bark's coarse powder was then extracted by cold maceration process using Methanol:

Table 1. Phytochemical analysis of *CMB* hydroalcoholic extract

Sr. no.	Phytoconstituents	Chemical test	*Cordia macleodii* bark extract
1	Carbohydrates	Fehling test Molish test	-- --
2	Fats and fixed oil	Spot test	++
3	Alkaloids	Mayers test Wagners test Dragendorffs test	-- ++ ++
4	Glycosides	Saponin glycoside test	--
5	Flavonoids	Shinoda test Lead acetate test	++ ++
6	Steroids and Terpenoids	Liebermann-Burchard reaction Salkowski test	++ ++
7	Phenolic compounds and Tannins	Iodine test FeCl3 test	++ ++
8	Resins	Resin test	--
9	Amino acids	Ninhydrin test	++

Water (40:60) solvent. 1 kg coarse powder of bark was soaked in 6 litres of hydro-alcoholic mixture for 24 hours. Soaked crude drug mixture was then heated at 80 ^{0}C for 4 hours in reflux condenser [17]. Extraction was repeated twice and combined extract were evaporated to dryness by rotary evaporation at 40 ^{0}C. A sticky, dark black crude extract yielding 8.70% w/w was produced. The semisolid extract was refrigerated in preparation for more research [18].

Fractionation of hydroalcoholic extract

About 15 g of hydro alcoholic extract of CMB was partitioned into various fractions by sequential fractionation according to rising polarities of solvents using separating funnel [19]. Thus, individual fractions were obtained as a separate segregate which then completely dried under high vacuum on rotary evaporator and kept in desiccator. The fraction in ethanolic solvent was found as most productive portion and hence considered it as active fraction for further studies. The Schematic diagram for extraction and fractionation is shown in **Figure 3**.

Table 2. Fractions of hydroalcoholic Cordia macleodii bark extract

Sr. No.	Fractions	Color	Fraction obtained	% Yield
1	Petroleum ether fraction	Sticky black	0.180 g	1.66 %
2	Chloroform fraction	Sticky black	0.350 g	2.33 %
3	Ethyl acetate fraction	Sticky black	0.200 g	1.33 %
4	Ethanol fraction	Sticky black	12.700 g	84.66 %

Table: 3: Shows the TPC of the ethanolic fraction of hydroalcoholic extract of CMB

Extract	Total phenol content		
	Average (mg/g)	Standard Deviation	% Relative SD
Ethanolic fraction of CMB extract	0.3717 ± 0.137	0.51	0.1372

Determination of phenolic content

The total phenolic content was determined using the Folin-Ciocalteau procedure [20]. Method relies on reaction of phenolic compounds with FCR under basic condition (transfer of electron) to form blue chromophore. The total phenolics were reported as the gallic acid equivalent in milligrams per gram of extract and absorption was measured at 765 nm. Sodium carbonate solution (20%) about 1.5 ml and approximately 0.5 ml of 2 N Folin Ciocalteau reagent were mixed to a test tube containing hundred milligrams of extract that had been solubilised in 100 ml of methanol and diluted five times. Using methanol, make up the final amount to 10 ml, and keep the mixture in the dark for two hours. Three duplicates of each determination were made.

Isolation by Column chromatography

After fractionation of hydroalcoholic extract of CMB, the ethanolic fraction with maximum yield was exposed to silica gel column chromatography for the separation of phenolic compound. The adsorption affinities of the targeted component for the surface of adsorbents and mobile phase varied, which determined the separation by column chromatography [21]. To separate the components, a simple vertical glass column (30 mm in width and 600 mm in length) with a sintered disc Teflon rota flow stop cock composed of borosilicate material was employed. The column was completely dried and cleaned with acetone before packing. Sea sand (particle size: 50–70 πm) was applied on glass wool up to a height of one centimetre. After shutting the stopcock, two hundred gm of silica gel with a mesh size about 60–120 was added to column to help in correct packing. A slurry was also made. Petroleum ether was then poured into the column up to the 3/4th level.

Ten grams of the ethanolic fraction of the bark extract of Cordia macleodii were permitted to column chromatography. The fraction then added to a little quantity of pet. ether, poured from the top down the column's sides, and then cleaned using the same solvent. Maintaining a solvent level around 6 cm above the extract prevented the column from drying out. Following the loading of the extracts onto the stationary phase, the eluent (mobile phase) was pumped through the column. A wide range of solvents were used in varied proportions to fractionate the components of variable solubility using the gradient elution

Table 4. Column chromatography of productive Ethanol fraction from CMB extract

Fractions	Mobile phase used	Part	Column chromatographic elutes	TLC observation
Ethanol fraction	Pet. ether	100:0	1-5	Clear (No spot)
	Pet. ether**:** $CHCl_3$	50:50	6-9	Clear (No spot)
	Pet. ether**:** $CHCl_3$	20:80	10-15	Clear (No spot)
	$CHCl_3$	100:0	16-21	Clear (No spot)
	$CHCl_3$**:** Ethyl. acetate	50:50	22-27	Clear (No spot)
		20:80	28-33	Clear (No spot)
	$CHCl_3$**:** Ethyl. acetate	100:0	34-36	Clear (No spot)
		50:50	37-40	Clear (No spot)
	Ethyl. acetate	**20:80**	**41-51**	**Red color (Spot**)
	Ethyl acetate**:** Ethanol	100:0	52-57	Clear (No spot)
	Ethyl acetate: Ethanol			
	Ethanol			

technique. After increasing the flow rate to 0.48 ml/min, around 40 ml of eluent was collected in each portion. The mobile phase used in the separation procedure was ethyl acetate to ethanol (20:80).

HPLC analysis

Thermo Fischer Ultra 3000 HPLC was used to assess the ethanolic fraction of Cordia macleodii bark extracts using a RP C18 column (About 5 μm, 250mm × 4.6 mm). Employing octa decylsilyl-silica gel as a stationary phase, the phenolic content of the extracts was found. Water with 0.05 percent glacial acetic acid was utilized as the mobile phase in a solvent solution that included acetonitrile. For the purpose of identifying phenolic acid in ethanolic fraction of CMB extract, reference of gallic acid was employed, by comparing retention period under comparable experimental settings. UV detectors operating at 270 nm were employed for the analysis [22]. *Preparation* of *test solution:* 0.4 g of extract reflux in 25 ml of methanol in water bath, cool and filter. Fill the volume to the mark after transferring filtrate to a 100 ml volumetric flask. *Reference solution*

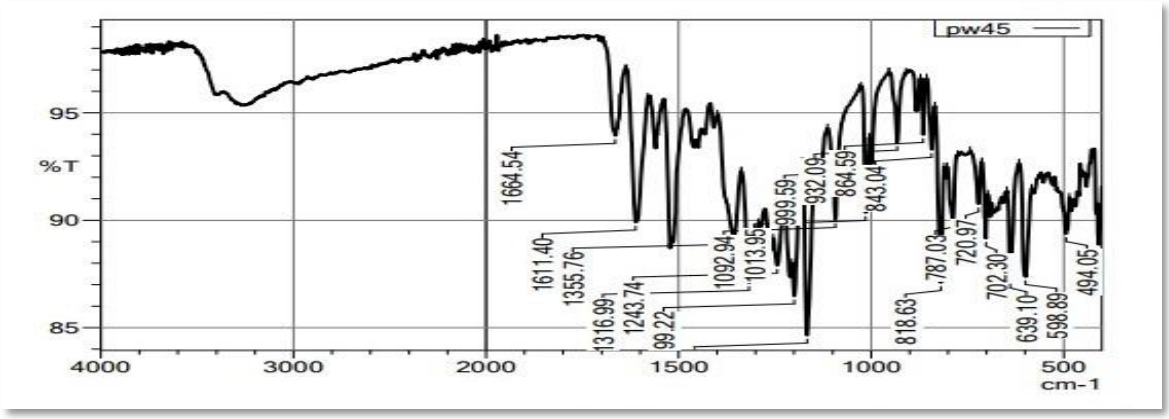


Figure 8: IR spectrum of isolated compound from ethanolic fraction of CMB extract

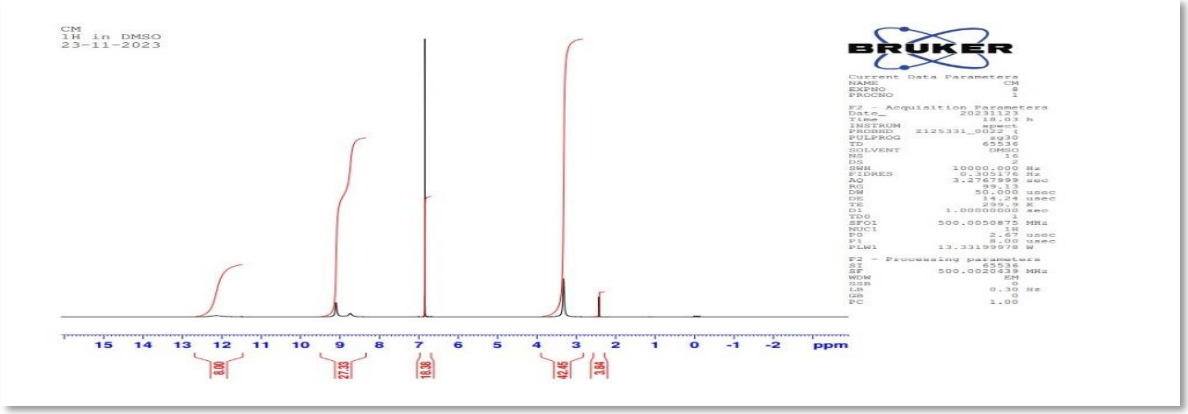


Figure 9: ^{1}H NMR spectrum of isolated compound from ethanolic fraction of CMB extract

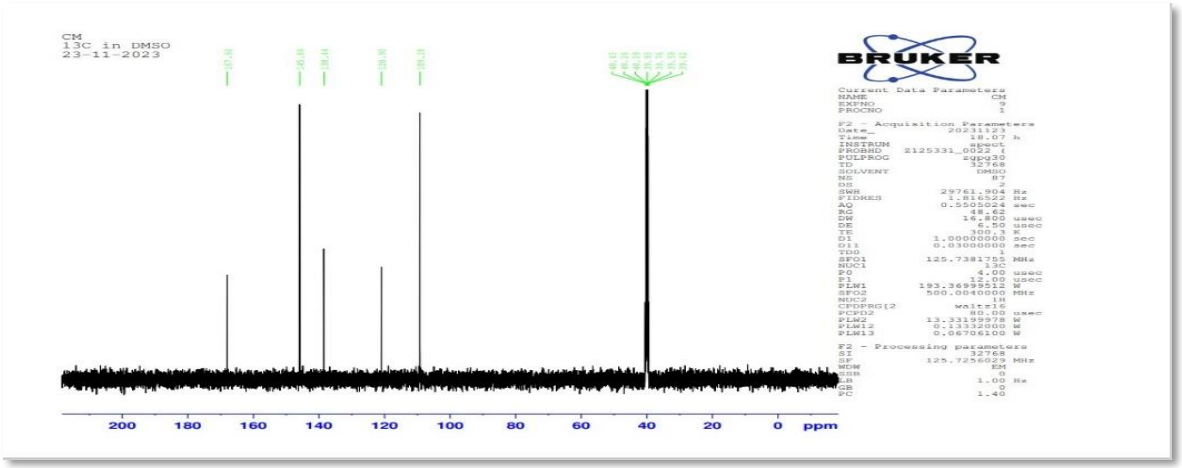


Figure 10: [13] C NMR spectra of separated component from the CMB extract's ethanolic fraction

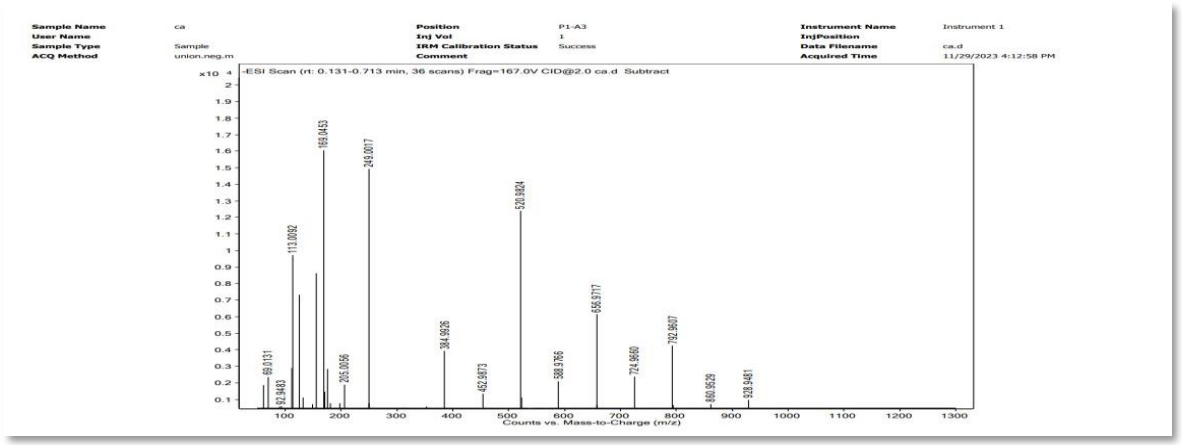


Figure 11: Mass spectrum of isolated compound from ethanolic fraction of CMB

preparation: To prepare the standard solution, dissolve ten milligram of gallic acid standard to about 50 millilitre of methanol using volumetric flask. Next, add methanol to the capacity until it reaches 100 ml.

Characterization of isolated compound

For characterization of isolated compound, several spectroscopic methods like UV, IR, NMR and Mass spectra were used. These techniques included comprehensive structural elucidation, multiple bond and ring detection, hydrogen and carbon arrangement, and functional group identification [23-25]. A UV-Vis Spectrophotometer (Shimadzu, UV-1900-Series) in methanol was used to assess the UV absorption of the isolated component from the Cordia macleodii bark active extract throughout a scanning range of 200 to 800 nm. After the substance was dissolved in methanol, a spectrum was captured [26]. One of the best useful technique for determining the functional groups included in isolated components is infrared spectroscopy. The separated constituent's infrared absorption spectra were analyzed using an FTIR-Bruker (Alpha, made in Germany). Wave numbers (4000-400 cm -1) representing absorption peaks were found [27]. A Bruker Ascend-500 spectrometer was used to obtain 1H NMR spectra, and a Bruker Ascend 500 MHz (ADVANCE III HD-500, Switzerland) was used for 13C NMR experiments. Tetramethylsilane was used as the internal reference standard and solvent DMSO. Data was analysed by Top spin software. ^{1}H-NMR was employed to find out kind of hydrogen existing in the isolated compound and Radiolabeled ^{13}C NMR is operated to identify the class of carbon exist in separated compound [28]. Mass spectroscopy was performed on the isolated component using a quadrupole time-of-flight mass spectrometer (Q-ToF LC/MS 6540 series, Agilent Technologies, USA) coupled to an Agilent 1200 series LC system. The isolation component was characterized using Agilent's mass hunter collection software. It is possible to ascertain the molecular weight of the material using the MS spectrum. The structural elucidation of organic molecules is the primary use of this approach [29]. The National Institute of Pharmaceutical Education & Research in Hyderabad, India, provided the mass spectra.

Results

Phytochemical screening

The hydroalcoholic extract (60:40) of CMB that contains an ethanolic fraction indicates the presence of flavanoids, terpenoids, amino acids, tannins, phenolic compounds, and alkaloids as shown in **Table 1**.

Table 5. Retention time, area and height in mAU for Std. and CMB fraction				
Sr. No	Std/Sample	Retention time (min)	Area	Height (mAU)
1	Gallic acid	5.035	67.3661	301.867
2	CM-sample	5.080	0.152	0.847

Fractionation of hydroalcoholic extract

15 g of hydroalcoholic crude extract of CMB was partitioned in different fractions based on varying polarity of solvents which gives respective % yield of respective fraction as given in (**Table 2**).

Phenolic content in fraction

Table 3 shows the phenolic content of ethanolic fraction of CMB extract as resolved by the Folin-Ciocalteau method. The determination of TPC values was based on the calibration curve **(Figure 4)** $y = 0.0049 \pm 0.0134$ with $R^2 = 0.9966$, where the concentration of the gallic acid solution (µg/ml), stated as mg GAE/g of the ethanolic component of CMB, is represented by y and the absorbance by x.

Isolation by Column chromatography

The most fruitful fraction extracted from the ethanolic solvent was separated and the biological components it contained were purified using silica gel column chromatography. The intended component was separated using ethyl acetate: ethanol (20:80) as the mobile phase. Elutes (41-51) aliquotes of 25 ml each in above solvent system was collected and same was preceded to TLC study to see the any difference in a specific property of individual aliquotes. Samples were collected up until the point on the TLC plate when the sample compound was no longer visible.

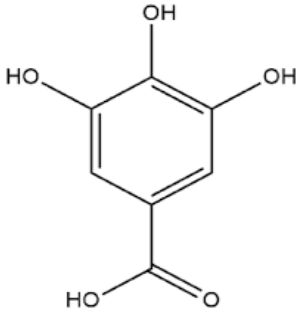
HPLC analysis of active fraction of CMB extract

The separated component was injected into the Thermo Fischer Ultra 3000 HPLC system. The gradient system in the HPLC system is Acetonitrile: Water with 0.05% glacial acetic acid. The column, RPC18 (250 x 4.6mm), was used, and the room and column temperatures were maintained at 30°C. 1.5 ml/min was added to the phase flow rate adjustment. Concurrently, each fraction used a 20 µl inject volume. Thus, in order to determine the presence of gallic acid in a fraction, we get the retention time, area chromatogram, and height mAU from active fractions (**Table 5**). HPLC chromatogram of Gallic acid and CM-sample are shown in **Figure 5** and **Figure 6.**

Spectral analysis and identification of the isolated compound

The isolated hydroalcoholic CMB extract molecule exhibits spectral maxima at 260 nm and 210 nm, as well as a λ max at 260 nm, in its UV spectrum (**Figure 7**). Phenolic acid is present in the isolated compound's UV-Vis spectra at 260 nm. Peaks in the IR spectra at 3600 cm-1 show (**Figure 8**) that there is a carboxylic acid hydroxyl group present. Another band displays hydroxyls bending at 1355 cm^{-1}. Band at 1664.54 cm^{-1} and 1611.40 cm^{-1} suggest the existence of carboxylate ion. ^{1}H NMR spectra of isolated compound (**Figure 9 and Figure 10**) show singlete at δ12.4 ppm indicates proton of carboxyl acid. Further doublet peak at δ 9.20 ppm. show the hydroxyl proton. Peak at δ 6.9 indicate proton of aromatic ring. Another singlet at δ 2.4 ppm and δ 3.4 ppm indicate another hydroxyl proton. ^{13}C NMR spectra in DMSO shows peak at 167.92 indicate presence of carbonyl carbon. Another peak at 145.86 ppm, 138.44 ppm, 120.19 ppm and 109.18 ppm suggest carbon skeleton of aromatic ring. Mass spectra of isolated compound **Figure 11** at m/e 169.04 indicate M-1 peak, which reveals the molecular weight is 170. This peak is match with molecular weight of Gallic acid.

Structure elucidation: Gallic acid



Gallic acid

It is white color compound, soluble in DMSO solvent and having m.p. in the range of 260-265^{0}C.

DISCUSSION

Cordia macleodii bark was extracted by maceration process using Methanol: Water (40:60) as extraction solvent. It gives dark black extract with yield of 8.70 % w/w, which further processed for fractionation using variety of solvent of different polarities. The portion of the CMB extract that was subjected to phytochemical screening, according to the research findings, included alkaloids, glycosides, phenolic compounds, tannins, phytosterols, carbohydrates, amino acids, and flavanoids. The phenolic molecule, which is believed to have therapeutic properties, had a moderate yield of 0.3717 ± 0.137 mg/g, according to quantitative measurements. Using silica gel column chromatography, several solvents with varied polarity, were used to further isolate and purify the isolated component. There were 57 fractions in all, each eluting 25 millilitres. Fractions 41–51 provide a white, 0.31 g residue that is soluble in DMSO solvent and has a melting point between 260-265^{0}C. Spectral data from spectral analysis was used to identify the isolated compound as gallic acid. This study indicates that *Cordia macleodii* bark, comprises medicinal compound such as gallic acid which may be leading component for the development of novel drug. These results support the use of Cordia macleodii bark as a medicinal plant and suggest that it might be a source for novel medications against a variety of illnesses.

CONCLUSION

From the research findings, it is concluded that phenolic acid present in active fraction of CMB extract. Overall, investigation demonstrated the confirmation of gallic acid after the examination of its physical properties such as color, solubility and m.p. determination. It is also inferred that hydroalcoholic extract of *Cordia macleodii* bark could be a great source of phenolic acid like gallic acid and it may

be very important with regard to potential pharmacological effects.

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