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A Potential Antimicrobial Agent from *Cocos nucifera* mesocarp extract; Development of a New Generation Antibiotic

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Abstract

Cocos nuciferamesocarp has been used from time immemorial in the treatment of various skin ailments. Cocos nuciferamesocarp extract can also be used as an antimicrobial agent against clinical pathogens. The purpose of this study was to confirm the anti-bacterial effect of cocos nuciferamesocarp powder using escherichia coli and salmonella typhi. For this six different solvent extracts of cocos nuciferamesocarp powder were produced and the anti-bacterial activity was determined using disc diffusion method. The specific therapeutic compounds were isolated using thin layer chromatography (TLC) and qualitative analysis was performed by high performance liquid chromatogaphy (HPLC) and fourier transform infra-red spectroscopy (FTIR). The antimicrobial activity was found to be highest in case of benzene solvent against E.coli. In the case of salmonella typhi the antimicrobial activity was found to be highest with diethyl ether. TLC provided two fractions of the coconut shell extract powderwhichwere eluted further. FTIR graphs provided characteristic peaks which represented the components responsible for antimicrobial activity. HPLC helped to identify the active biocomponents as tocopherol, palmitoleyl alcohol, cycloartanol and β-sitosterol. The Cocos nuciferamesocarp powder can be utilized to develop indigenous antibiotics which can replace conventional antibiotics.

Keywords: *Cocos nucifera*mesocarp, *E.coli, S typhi,* TLC, HPLC, FTIR.

Introduction

Cocos nucifera L. (family *Arecaceae*), commonly known as coconut, is considered as an important fruit crop in tropical countries. Coconut is the most extensively grown and used nut in the world, playing a significant role in the economic, cultural, and social life of over 80 tropical countries. Currently, coconut is mainly an oil crop; rich in lauric acid, with a variety of other uses in addition to commercial oil production[1]. Coconut is a member of the monocotyledonous family *Arecaceae* (*Palmaceae*), subfamily *Cocoideae* and the monospecific genus *Cocos*. The existence of related genera of coconut in South America[2,3] and coconut's long history in the Eastern hemisphere has led to controversy over its centre of diversity. The main reasons for considering a Southeast Asian origin for coconut has been summarized[4] and Melanesia is considered as the most likely region for coconut domestication along the coasts and islands between Southeast Asia and the Western Pacific[1]. Coconut spread both west and east from this putative centre of diversity[5]. An alternative route for the evolution of coconut from a South American ancestor that could have been disseminated by ocean currents from South America to Polynesia has been suggested[3]. Coconut has been distributed to many different parts of the world including Central and South America, East and West Africa, Southeast Asia, East Asia and the Pacific islands.

Coconuts are unique in terms of their fruit (a drupe) morphology. The most interesting feature of the fruit is its wall. The fruit wall comprises of three layers exocarp, mesocarp and endocarp. Due to extensive cross linking between phenolics, lignin and polysaccharides, the mesocarp becomes hard and fibrous. Fibrous coconut fruit is not only edible but also suitable for multipurpose uses. As a traditional medicine in northeastern Brazil, coconut husks have been used for the treatment of diarrhea and arthritis. Antimicrobial activity of the water extract of coconut husk has already been demonstrated[6]. However, studies regarding the polyphenol content of the coconut fruit wall are limited[7]. The main bioactive constituents in coconut are fixed oil rich in tocopherol, fatty alcohol, triterpenealcohol, sterol, gum. The diuretic, astringent, antibiotic, antiseptic, antifungal activities of coconut have been reported already.

Medicinal uses have been reported for 104 neotropical palm species. Of these, uses in 19 different medicinal categories have been reported in the literature for *Cocos nucifera*, and other related species which are used medicinally to treat ailments in 15 different categories each. *Cocos nucifera, Oenocarpus bataua, Euterpeprecatoria* and *Socratea exorhiza* are the species with the most widespread use, being mentioned in references referring to 6–17 different countries or indigenous groups.

Material and Methods

Plant material: Ripe coconuts (*Cocos nucifera*) were procured from local market at Raipur, Chhattisgarh. The coconuts were broken open and were dried well after removal of the inner

white material. After drying well in direct sunlight for 48 hours, the coconut shells were removed from the exposure to direct sunlight and preceded for further studies. The shells were made sure of their dryness along with the absence of moisture.

Bacterial cultures: Two bacterial cultures, *Escherichia coli* (MTCC No. 1678) and *Salmonella typhi* (MTCC No. 733) were procured from IMTECH (Institute of Microbial Technology), Chandigarh, India. They were sub-cultured using the growth medium number respectively. They were carefully preserved to prevent any cross-infection.

Chemicals: Analytical grade chemicals were used in sample preparation and all the solvents for chromatographic purpose were TLC grade, purchased from Merck. Nutrient Agar media was bought from Hi-Media, India (Mumbai). Standard grade solvents acetone, benzene, chloroform, diethyl ether, ethanol and formaldehyde were bought from Merck, India. Deionised water for all procedures was obtained from MilliQ (USA) Millipore^{TM} water.

Spectroscopic Apparatus: The Perkin Elmer Spectrum FT-IR instrument consisted of globar and mercury vapor lamp as sources, an interferometer chamber comprising of KBr and mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm^{-1} was covered by this instrument. The spectrometer worked under purged conditions. Coconut shell extract samples were dispersed in KBr depending on the region of interest. This instrument has a typical resolution of 1.0 cm^{-1}. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible. The quantity of the sample used was 50mg.

Chromatographic apparatus: HPLC (High Performance Liquid Chromatography), (Shimadzu LC 10AT VP model) was performed for all the coconut shell extract samples. HPLC analysis was carried out using a Waters (waters, Milford, CA, USA) BREEZE^{TM} HPLC system consisting of a binary pump (Waters 1525) and a UV detector (Water 2487). Data were collected and analyzed in BREEZE^{TM} software (version 3.20) and elutes were monitored at 280 and 310 nm. A Phenomenex^{TM} Synergy 4 l Hydro-RP 80 C18 column (250 x 4.6 mm^2), coupled to a guard column Phenomenex^{TM} Security Guard^{TM} C18 ODS (4 x 3.0 mm^2) was used. A¨ KTAprime^{TM} low pressure chromatography system was equipped with a Sephadex LH-20 column and fraction collector of 65 tubes. Data were monitored using Prime-View^{TM} software.

Production of Bio-active *Cocos nucifera* Mesocarp crude extract: Empty dry *Cocos nucifera* mesocarp was taken, broken into pieces with pestle and mortar. Twenty five grams of the powder was weighed, made sure that it was free of any contamination and treated along with 100ml of solvents. Individual solvent extracts were obtained in the same fashion after allowing the powder to remain soaked inside the solvents for 48 hours in rotary incubator, which was maintained at room temperature and 120rpm speed. After the incubatory period was over the solvents containing the coconut shell powder were filtered using a pre-sterilized muslin cloth and separation of the coconut shell powder was affected from the solvents, producing bio-active *Cocos nucifera* powder.

Preparation of inoculum and Antimicrobial activity: Stock cultures were maintained at 4°C on slopes of nutrient agar. Stock cultures are the type cultures. Active cultures for experiments were prepared by transferring a loopful of cells fromthe stock cultures to test tubes of Nutrient agar broth and incubated without agitation for 24 hours at 37°C and 25°C, respectively. The cultures werediluted with fresh nutrient agar broth to achieve optical densities corresponding to 2.0 x 10^6 colony forming units (CFU/ml).

Antimicrobial Susceptibility test: The disc diffusion method was used to screen the antimicrobial activity[8]. The Nutrient agar plates were prepared by pouring 15 ml of molten media into sterile petri plates. The plates were allowed to solidify for 20 minutes, 0.1% inoculum suspension was spread uniformly, and the inoculums were allowed to dry for 5 min. Twenty micro liters of crude coconut shell extract were loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium, the compound was allowed to diffuse for 5 min, and the plates were kept for incubation at 37°C for 24 hours. Streptomycin disc was taken as positive control. At the end of incubation, inhibition zones formed around the disc were measured with a transparent ruler in millimeters.

Statistical analysis: All the experiments were done in triplicate. The triplicate data were subjected to an analysis of variance for a completely random design using statistical analysis software, SPSS 10.0. The significance level was fixed at 0.05 for all statistical analysis. Standard deviations were expressed in each table.

Results and Discussion

Extraction and isolation: Thin layer chromatography (TLC) analysis. Crude solvent extracts may contain thousands of compounds including amines, carboxylic acids, nitro-compounds and even alkenes. To establish a better characterization of the solvent extracts and to get a clear FTIR profile, purification through Column chromatography was carried out using Biogel^{TM}. As per TLC separation two fractions were obtained, frac1 and frac2.

Fourier Transform Infra-red Spectroscopy (FTIR) analysis: FTIR analysis of frac1 exhibits five major compounds in considerable concentration. In frac1 and frac 2 these five compounds were present (considerable concentration) along with some other compounds (not identified). In the present study we only focused on the identification of the five

compounds of the frac1 and frac 2. AFTIR profile of the frac1 is illustrated in figure-1. The identification of the compounds was tentatively made on the basis of TLC and Infra-Red spectral characteristics. FTIR absorption spectra peaks for frac1 at 3400, 2900, 1160, 800, 490 cm^{-1} wereused to identify the compounds as amines, alkanes, carboxylic acid, nitro compounds and phenyl ring substitution band respectively[9].

HPLC (High Performance Liquid Chromatography) analysis: The retention time and spectra of frac1 and frac 2 were compared with the authentic standards to confirm that the bioactive components are tocopherol, palmitoleyl alcohol, cycloartanol and β-sitosterol. Products were confirmed by HPLC comparing with authentic standards (data not shown).

Antimicrobial activity of the Coconut shell extract: The coconut shell extract showed strong anti-microbial activity in all the six solvents, acetone, benzene, chloroform, diethyl ether, ethanol and formaldehyde. The highest zone of inhibition against E.coli was formed in the case of benzene and highest zone of inhibition against S.typhi was formed in the case of diethyl ether as shown in table-1.

Cocos nucifera has been recognized as an entity with multi uses with every component being biologically active in one way or other. Extract of *Cocos nucifera* is used in treatment of wounds affected by leishmaniasis[10,11]. In the Indian subcontinent it is used as a rehydrating agent in cholera, diarrhea and dysentery; treatment of cancer; as a hair nutrient in alopecia. The use of microsatellite DNA markers to investigate the level of genetic diversity and populationgeneticstructure of coconut (*Cocos nucifera*) has been already reported[12]. The coconut shell extract powder has not been greatly explored by scientific means as per our knowledge. As per the available literature, the use of coconut oil, its wound healing nature, anti-allergic properties have only been studied and reported elaborately. The antimicrobial activity of thecoconut shell extract powder has not been reported by Indian andInternational researchers. Further research in this area can yield natural antibacterial components from coconut shell, replacing the conventional chemical antibiotics which produce numerous side effects[13].

Conclusion

We performed *in vitro* studies of antimicrobial properties of coconut mesocarp on common human pathogens which gave positive results. This showed the importance of coconut mesocarp which is generally regarded as a waste product thus highlighting its potential application in pharmaceutics, nutraceutics and cosmetic oils. We have identified the active biocomponents as tocopherol, palmitoleyl alcohol, cycloartanol and β-sitosterol in coconut mesocarp. With the latest developments in extraction techniques few more bioactive components can be sought from mesocarp of coconut.

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Table-1

Antimicrobial activity of various solvent extracts of Coconut shell powder. The antimicrobial action of coconut mesocarp extract in common organic solvents was examined by disc diffusion method. Two closely related foodborne pathogens- *E.coli* and *S.typhi* were studied. Coconut mesocarp extract showed maximum activity in Benzene in case of *E.coli* while for *S.typhi*, diethyl ether was more suited for the bioactivity of the extract.[a] Twenty microliter of extract was poured in each disc (6 mm).[b] Streptomycin (50 lg/ml)

Micro-organism	Zone of Inhibition in (mm)[a] in different solvents						
	Acetone	Benzene	Chloroform	Diethyl ether	Ethanol	Formaldehyde	Streptomycin[b]
E.coli	17±0.2	18±0.3	12±0.4	16 ±0.2	15±0.1	17±0.5	14±0.4
S.typhi	16±0.1	19±0.4	19±0.3	20±0.5	16±0.4	15±0.1	17±0.2

Table-2
HPLC data for fraction 1

Peak Number	Retention time	Area (mV.s)	Height (mV)	WO5 (min.)	Area (%)	Height (%)
1	4.007	1247.7753	54.0986	0.4067	53.3182	45.0777
2	4.260	318.5328	22.9027	0.2733	13.6111	19.0837
3	4.533	154.2854	14.8763	0.1733	6.5927	12.3957
4	5.280	2.3274	0.2718	0.1333	0.0995	0.2265
5	5.493	35.1312	2.2391	0.2200	1.5012	1.8657
6	6.107	6.5966	0.0935	0.0600	0.2819	0.0779
7	6.400	31.7101	2.1979	0.2400	1.3550	1.8314
8	6.973	3.0537	0.3008	0.1800	0.1305	0.2506
9	7.313	5.2352	0.4049	0.2000	0.2237	0.3373
10	7.887	7.4830	0.5180	0.2333	0.3198	0.4316
11	14.660	33.5234	3.5873	0.1733	1.4325	2.9891
12	15.027	494.5881	18.5208	0.4267	21.1339	15.4328
-	Total	2340.2424	120.0118	-	-	-

Table-3
HPLC data for fraction 2

Peak Number	Retention time	Area (mV.s)	Height (mV)	WO5 (min.)	Area (%)	Height (%)
1	3.513	2483.8703	149.9106	0.2533	83.0369	80.9637
2	3.967	146.8379	14.9345	0.1667	4.9089	8.0658
3	4.407	17.0414	1.6702	0.2000	0.5697	0.9020
4	4.920	4.5321	0.6169	0.1333	0.1515	0.3332
5	5.227	15.8060	1.4148	0.2000	0.5284	0.7641
6	5.467	41.8200	2.1563	0.1867	1.3981	1.1645
7	6.053	7.6382	0.1334	0.0600	0.2553	0.0720
8	6.347	92.3057	5.8011	0.2600	3.0858	3.1330
9	6.907	1.8522	0.2223	0.1400	0.0619	0.1201
10	7.280	6.0210	0.5279	0.1933	0.2013	0.2851
11	7.847	10.0720	0.7314	0.2200	0.3367	0.3950
12	14.553	27.9788	2.0050	0.2467	0.9353	1.0828
13	14.947	135.5082	5.0336	0.4467	4.5302	2.7187
-	Total	2991.2838	185.1578	-	-	-

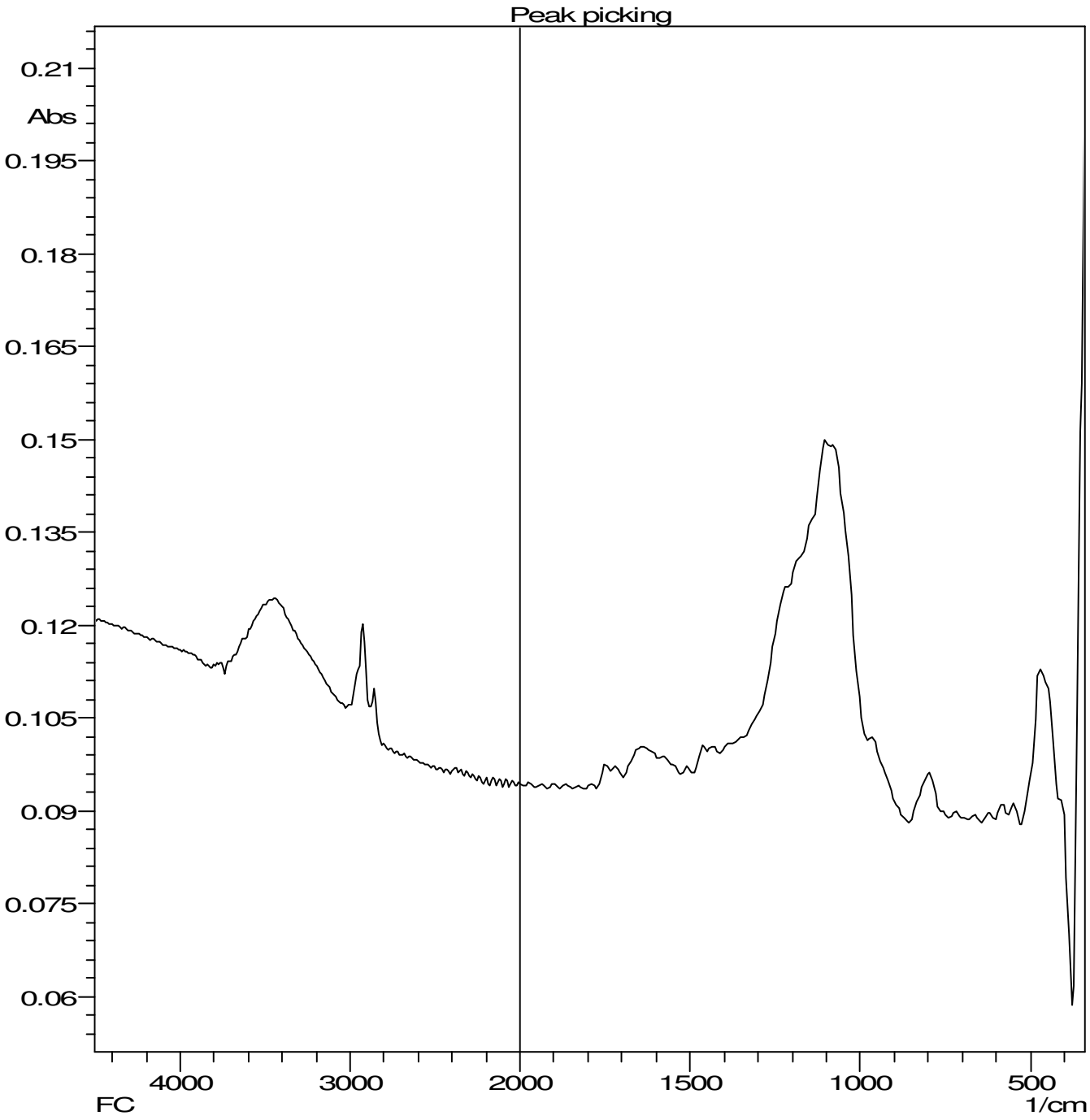

Figure-1
FTIR graph of fraction 1

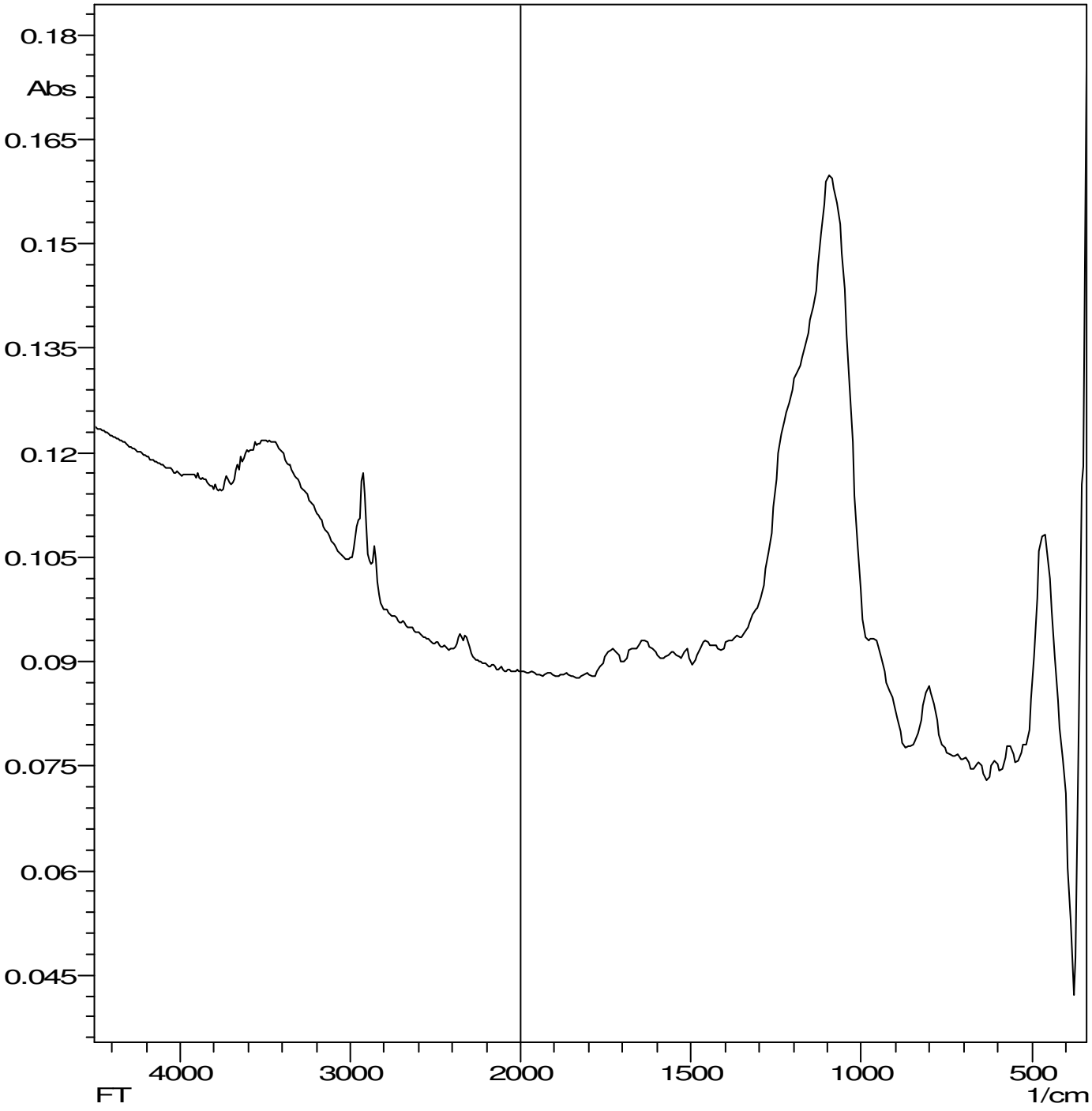

Figure-2
FTIR graph of fraction 2

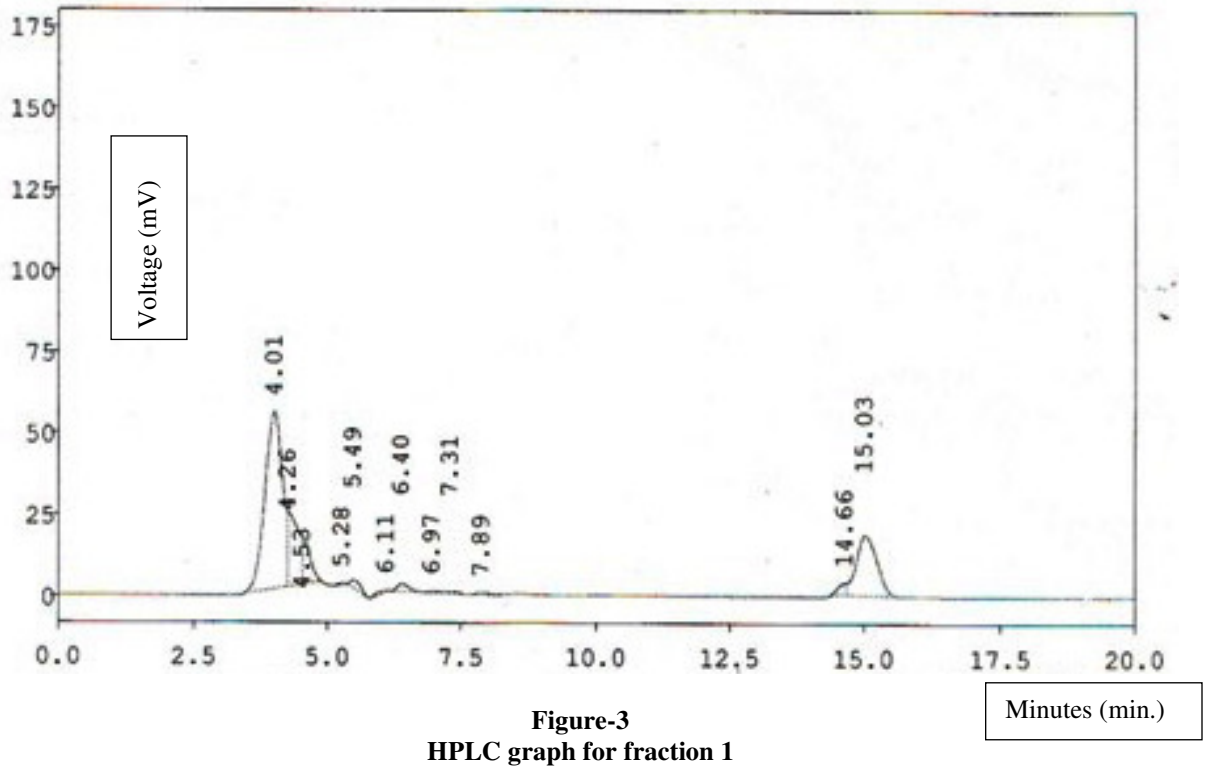

Figure-3
HPLC graph for fraction 1

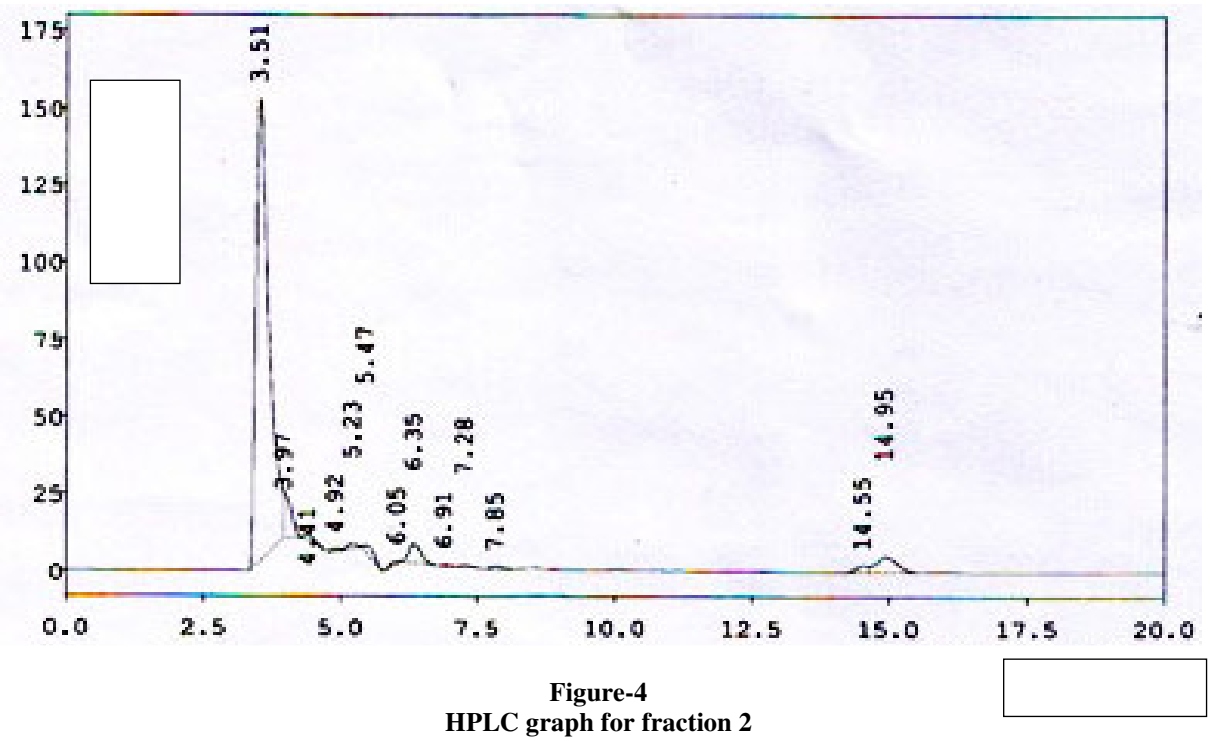

Figure-4
HPLC graph for fraction 2