RESEARCH ARTICLE

Isolation and Characterization of Phytoconstituents from the Plant *Excoecaria agallocha* Linn

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ABSTRACT

Background: Marine resources are currently widely studied because academic and industrial researchers are striving to extract lead molecules from the interior of oceans. Marine resources are increasingly the subject of significant research. *Excoecaria agallocha* Linn., one of the plants with several pharmacological effects such as anti-inflammatory, analgesic, antiulcer, anticancer, etc., is one of the phytoconstituents in mangroves that have been widely used in folk medicine to treat disorders.

Objective: The objective of present study to perform a comprehensive phytochemical investigation of milky mangrove. The plant *E. agallocha* were collected, extracted, isolated, and analyzed in an attempt to better comprehend the various phytochemical aspects of the plant.

Material and method: The isolated product was analyzed by different spectroscopic method.

Results: The isolated product is characterized as steroidal moiety containing two double bond at 6 and 22-position and alcoholic group at 3-position of cyclopentanoperhydrophenanthrene in to the isolation of (1,2,3,4,5,10,11,12,14,15,17-dodecahydro-3-hydroxy-4,4,10,13,14-pentamethyl17-((E)- 5,6-dimethylhept-3-en- 2- yl-)9-H-cyclopenta[a]phenanthren-6,16-dione.

Conclusion: The compound's actual structure is made known using all of the UV, IR, NMR, and mass spectrometry evidence.

Keywords: *Excoecaria agallocha,* Mangroves, Folk medicine, Ultraviolet-visible, Nuclear magnetic resonance.

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INTRODUCTION

One of the world's 25 richest and the most endangered eco-regions is found in India. Various types of forests, including tropical and subtropical, which comprise around 20% of the country's total land area, are home to India's unique plant species. Mangrove plants can just grow under certain conditions, thus limits their range. The mangrove ecosystem's plants have evolved to cope with extreme salinity, significant tidal movement, and winds. The wide coasts and mangrove vegetation along them play a crucial role in preserving coastal biodiversity.[1]

*Excoecaria agallocha*Linn belongs to the family Euphorbiaceae has a prosperousworth in folk remedy and is commonly used in ayurvedic medicine. The plant has been reported to have antiulcer, analgesic, cancer treatment, anti-reverse transcriptase, antimicrobial, antihistamine-release and antitumor shielding activities, etc. The bark is used in the management of rheumatism, leprosy, emetic, and paralysis and roots in the treatment of toothache and swellings.[2]

The existence of diverse phytoconstituents had been reported by a number of authors and the essential phytoconstituents in a different part of the plant are B-amyrenone, cycloartenol, diterpenoids, agallowchaexcoerin, agallochin, excoecarins, exoagallochaols A-D, secoatisanediterpenoids, agallochaols G-J, K-P, phorbol ester, diterpenoids, excoecarins, secoatisane, diterpenoids, agallochaols, phorbol ester, etc.[3]

MATERIAL AND METHODS

Extraction of Crude Drug: Extraction of *E. agallocha* Linn

Leaves were dried under shade, powdered, and passed through a mesh sieve, weighed, and then used for extraction. In 500 gm of powdered drugs was positioned in a thimble produced using cellulose filter paper and extracted through soxhlet equipment for defatting by petroleum ether. The powdered defatted drug, was continuously extracted in soxhlet apparatus for 72 hours using ethanol.[4]

Preliminary Phytochemical Study

E. agallocha crude extract (ethanolic extract) undergone phytochemical screening. Following the prescribed procedures,

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phytochemical tests were carried out on plant extracts to identify various chemical constituents responsible for the biological activity.[5]

Collection of Eluting Sample and Purification

Column chromatography was used in the elution process. Each fraction included about 100 mL of elute, which was collected at a rate of 20 drops per minute. TLC was done to each fraction utilizing silica gel G. To produce pure compounds or a blend of 1-2 compounds, fractions of the same Rf were collected and concentrated.

The ethanolic extract's soluble ethyl acetate fraction was loaded into a column. Hexane was utilized to start the elution with in the silica gel column, and then ethyl acetate was added gradually at a rate of 10 to 100%. The portions (100 mL) were taken from the column then mixed together based on respective TLC profiles. A silica gel column was used for re-chromatography of the column's mixed crude fractions.[6]

Compound-I isolation

Hexane was used to initiate the isolation of ethyl acetate fraction of *E. agallocha* Linn, then ethyl acetate was added to increase polarity. Based on their TLC profiles, the elution upto to hexane: ethyl acetate (5:5) was combined to produce a fraction (A). With an increasing concentration of DCM in hexane, that portion was concentrated and rechromatographed. Di-ethyl ether was added to an elution until to hexane: DCM (7:3), which was merged and refrigerated overnight to obtain the compounds (1A,1B) as white and yellowish powders, respectively. Further elution up to hexane: DCM (2:8) yielded compound (2A, 2B), which was recrystallized as yellow & dark brown mass, respectively with acetone.

Characterizations of Isolated Phytoconstituents from Leaves of *E. agallocha* Linn. Isolated Compound-I (From Ethyl Acetate Fraction)

HPTLC and HPLC study: studies of isolated compound-I

HPTLC study was carried out using silica gel 60 F_{254} plates as per stationary phase and acetonitrile-methanol (90:10), by means of solvent system. Acetonitrile and methanol are employed as the solvent system in HPLC, and the flow rate varies between 0.001 to 9.999 mL/min.

UV-vis spectrometry of compound

For UV-vis spectroscopy UV spectrophotometer: Shimadzu 1700 double beam spectrophotometer is used. Isolated compound-I 5 µg/mL dissolved in chloroform is used as a sample and the UV spectra were taken from 200 to 600 nm which show the λ_{max} of isolated compound.

GC-MS of compound-I

GC-MS analysis was performed at National Chemical Laboratory, Pune. The GC-MS condition established for compound-I analysis is as specified. That compound-I was processed to GC-MS analysis in a Shimadzu GCMS-QP2010 Ultra. Analysis of spectra was done using NIST08 library.

FTIR spectrometry and NMR of compound-I

Shimadzu FTIR spectrophotometer 8400S is used in the characterization using ^{13}C-NMR and ^{1}H- Spectra: Bruker AVANCE-II (100 MHz for 13C and 400 MHz for 1H) spectrometer. The NMR analysis of the compound was performed at SAIF, Punjab University, Chandigarh.

LC-MS analysis and CHN analysis of compound-I

LC-MS Agilent Technology was used for characterization. The LC-MS analysis was performed at the Centre for Food Testing, Pune. Thermo Finnigan instrument was used for CHN analysis.

RESULTS

Using the continuous hot extraction, the *E. agallocha* Linn leaves were extracted with ethanol, and the fractionation was done. The characteristics of extract and fraction are given in Table 1.

Phytochemical screening of ethanolic extract of *E. agallocha* showed the presence of protein, tannins, phenolic compounds, alkaloids, flavonoids, steroids, terpenoids, and carbohydrates. The results of phytochemical screening are shown in Table 1.

TLC study is particularly important for qualitatively identifying small constituent amounts. Rf values of separated phytoconstituents of extract were determined (Tables 2 and 3).

Table 1: Phytochemical screening of ethanolic extract of *E. agallocha* Linn.

Sr. No.	*Test*	*Particulars*
1	Carbohydrates	+
2	Protein/amino acid	+
3	Fats/ waxes	-
4	Flavonoids	+
5	Saponins	-
6	Glycosides	-
7	Steroids	+
8	Alkaloids	+
9	Terpenes	+
10	Tannins/Phenolics	+

Where; (+) = Present; (-) = Absent

Table 2: TLC Studies of Ethanolic extract and Ethanolic fraction of *E. agallocha* Linn

Fraction/ Extract	*Solvent system*	*No of spots*	*TLC profile*	
			R_f value	*Color*
Ethanolic extract	Toluene:Ethyl acetate:Methanol: Water(7:6:5:2)	9	0.94;0.90;0.81; 0.74;0.69;0.61; 0.59;0.45;0.38	Dark green, green, green, faint green, pale green, yellow, light yellow, light green, brown
Ethanolic Fraction	Ethyl acetate: methanol: toluene: water (5:4:6:5)	5	0.90;0.86;0.73; 0.54;0.32	Dark green, green, light green, light yellow, brown

Table 3: TLC studies of Hexane fraction and chloroform fraction of *E. agallocha* Linn

Fraction/ Extract	*Solvent system*	*No of spots*	*TLC profile*	
			R_f value	*Color*
Ethyl acetate fraction	*Hexane: Ethyl Acetate (03:07)*	4	0.76;047;0.41 0.22	Dark yellow, dark brown, dark brown, light brown
Chloroform fraction	Hexane: Ethyl acetate: methanol (10:3:7)	5	0.70;0.64;0.58; 0.47;0.39	Light green, very light green, light brown, light yellow, light brown

The ethanolic extract of *E. agallocha* Linn. was processed for fractionation. Fractions of ethyl acetate and ethanol obtained better yield and were further selected for isolation study. The isolated phytoconstituents structures were determined using spectral and chromatographic details. The nature of phytoconstituents identified is steroid and alkaloid**.**

Compound-I Characterization

The isolated compound I was processed for HPTLC analysis in Methanol: Toluene (5.5:4.5) and compound is resolved at R_f 0.21 (Figure 1) and the chromatogram shows the sample is substantially pure for subsequent structural analysis.

The compound I was analyzed using HPLC with Acetonitrile: Methanol as mobile phase (90:10) and separated compound has shown a retention time of 1.724 minutes (Figure 2). The HPLC chromatogram shows compound is not a mixture and is partly pure for the elucidation of chemical structure.
When the compound-I in chloroform was diluted, a UV-vis spectrophotometer exhibited two distinct peaks at 262 and 360 nm (Table 4, Figure 3).

Predominant peaks observed in the FTIR spectra are 771.55, 1049.31, 1228.70, 1330.93, 1701.27, 2731.29, 2953.12, 3209.66, 3227.02, 3427.62 cm^{-1}. Table 5 shows a connection between the IR frequencies and the likely functional groups (Figure 4).

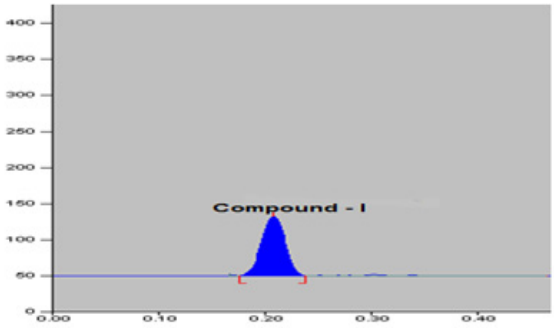

Figure 1: HPTLC Chromatogram of Compound-I

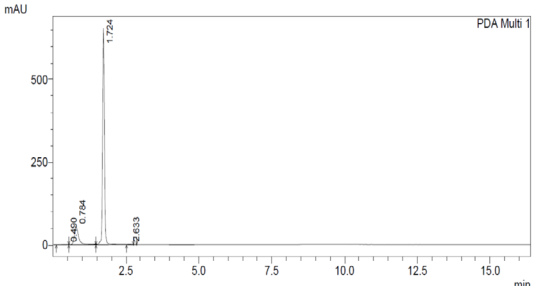

Figure 2: HPLC profile of isolated compound-I

Shimadzu GCMS-QP2010 Ultra was used to conduct the GC-MS analysis of compound I using consistent GC-MS parameters. The spectra obtained were analyzed by the NIST08 library.

The GC-MS peak shows the mass spectrum from the small mass (m/z) 50 and high mass (m/z) 503. The molecular ion peak is seen at 468 and the peak at 496 may be spurious. Taking the molecular mass as 468, the molecular formula works out to be $C_{31}H_{48}O_3$. After the side chain removal, there are losses of methyl groups The steroidal ring system is broken and interpretation of fragments becomes difficult (Table 6 and Figure 5).

^{13}C-NMR and ^{1}H- and Spectra: Bruker AVANCE-II (100MHz for ^{13}C and 400 MHz for 1H) spectrometer (Tables 6 and 7).

Table 4: Results of UV-vis spectroscopy

Absorbance Maxima (λ_{max}) nm	*Specification*
262	Unsaturated carbonyl group
360	-OH Group connected with the double bond

Table 5: Results of FTIR

Frequency (cm^{-1})	*Functional Group*
771	C = C-H Bend
1049	C- O Stretch
1701	C = 0 Stretch
2753	CH2, CH3 Stretch
3209	C-H stretch attached to C=C
3427	C-OH

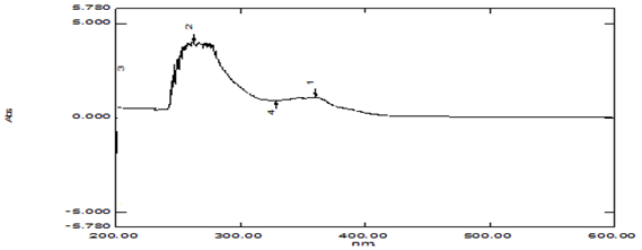

Figure 3: UV-VIS Profile of Isolated Compound-I

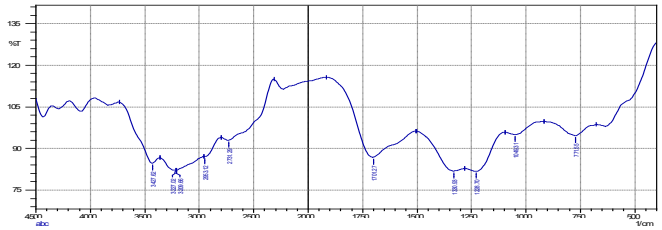

Figure 4: FTIR spectrum of compound-I.

Table 6: Fragmentation pattern

Sr. No.	*Fragments (m/z)*
1	468 (M^+ + 1) Positive ionization
2	344 (-Side Chain)
3	330 (M^+ - CH_3) Loss of methyl
4	316 (M^+ - CH_3) Loss of methyl

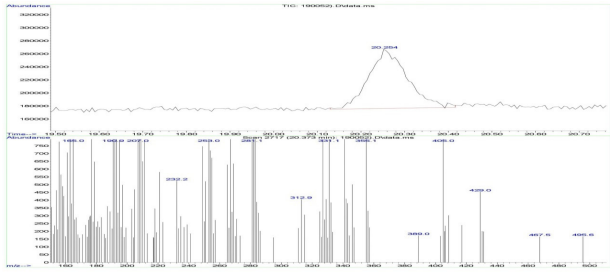

Figure 5: GC-MS of compound-I

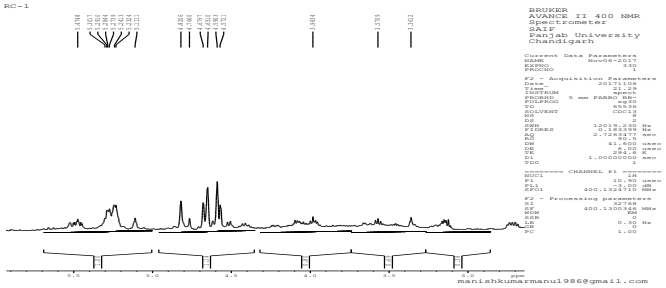

Figure 6: Proton ^{1}H- NMR spectra of compound-I

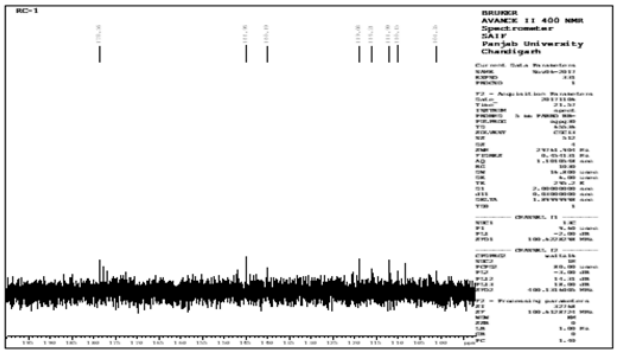

Figure 7: ^{13}C-NMR spectra of compound-I

Chemical shifts δ in ppm was recorded with TMS as a reference marked as ^{1}H-NMR was recorded in $CDCl_3$ solvent (400 MHz) and ^{13}C-NMR was recorded in $CDCl_3$ solvent (100 MHz). The chemical shift of ^{13}C-NMR and ^{1}H-NMR of the compound-I (Figures 6 and 7) (Table 8).

There is only one major compound having a retention time at 1.27 minutes. Utilizing all the data of separated compound is shown in Figures 8 and 9.

DISCUSSION

The plant *E. agallocha*'s active moieties were separated, purified, and analyzed in an attempt to better understand the several phytochemical aspects of the plant. Phytochemical screening of ethanolic extract of *E. agallocha* discovered the presence of tannins, alkaloids, Phenolic compounds, steroids, terpenoids, flavonoids and tannins.

Table 7: NMR details of isolated compound-I

Sr. No.		*δ(H)*	*δ(C)*
1	Hα - C(1)	1.95 (m)	23.78
	Hβ - C(1)	1.78-2.08 (m)	--
2	Hα - C(2)	5.11-5.47 (m)	76.33
	Hβ - C(2)	--	--
3	Hα - C(3)	--	36.70
	Hβ - C(3)	--	--
4	Hα - C(4)	4.57-4.82 (m)	50.44
	Hβ - C(4)	--	--
5	Hα - C(5)	--	21.68
	Hβ - C(5)	--	21.68
6	Hα - C(6)	1.56 (bs, *J*=2.8, 1H)	29.77
	Hβ - C(6)	1.39-1.7 (m)	29.77
7	Hα - C(7)	--	178.56
	Hβ - C(7)	--	178.56
8	Hα - C(8)	--	177.00
	Hβ - C(8)	--	177.00
9	Hα - C(9)	1.24 (m)	50.44
	Hβ - C(9)	--	50.44
10	Hα - C(10)	--	22.51
	Hβ - C(10)	--	22.51
11	Hα - C(11)	--	49.89
	Hβ - C(11)	--	49.89
12	Hα - C(12)	--	46.31
	Hβ - C(12)	--	46.31
13	Hα - C(13)	1.06 (dd, *J*=10.9, 4.5, 1H)	28.90
	Hβ - C(13)	1.08-1.12 (m)	28.90
14	Hα - C(14)	0.97 (m)	24.60
	Hβ - C(14)	0.99 (m)	24.60
15	Hα - C(15)	1.18 (m)	33.08
	Hβ - C(15)	1.23 (m)	33.08
16	Hα - C(16)	0.90 (m)	27.77
	Hβ - C(16)	0.92 (m)	27.77
17	Hα - C(17)	0.89 (m)	46.19
	Hβ - C(17)	--	46.19
18	Hα - C(18)	0.87 (m)	37.69
	Hβ - C(18)	--	37.69
19	Hα - C(19)	0.86 (m)	36.33
	Hβ - C(19)	0.85 (m)	36.33
20	Hα - C(20)	0.84 (m)	24.98
	Hβ - C(20)	0.83 (m)	24.98
21	Hα - C(21)	0.82 (m)	39.78
	Hβ - C(21)	0.80 (m)	39.78
22	Hα - C(22)	0.79 (d, *J*=7.3,1H)	38.35
	Hβ - C(22)	--	38.35
23	Hα - C(23)	0.75(bs, *J*=3.2, 3H)	22.94
	Hβ - C(23)	--	22.94

24	Hα - C(24)	0.73 (s, 3H)	18.01
	Hβ - C(24)	--	18.01
25	Hα - C(25)	0.73 (s, 3H)	19.70
	Hβ - C(25)	--	19.70
26	Hα - C(26)	0.71 (s, 3H)	36.26
	Hβ - C(26)	--	36.26
27	Hα - C(27)	0.70 (s, 3H)	21.61
	Hβ - C(27)	--	21.61
28	Hα - C(31)	--	144.96
	Hβ - C(31)	--	144.96
29	Hα - C(32)	2.24-2.26 (m, 3H)	28.69
	Hβ - C(32)	--	28.69
30	Hα - C(34)	0.67 (s, 3H)	13.78
	Hβ - C(34)	--	13.78
31	Hα - C(35)	0.57 (dd, *J*=9.4, 2.1, 3H)	13.12
	Hβ - C(35)	--	13.12
32	Hα - C(36)	1.01 (m, 2H)	10.97
	Hβ - C(36)	--	10.97

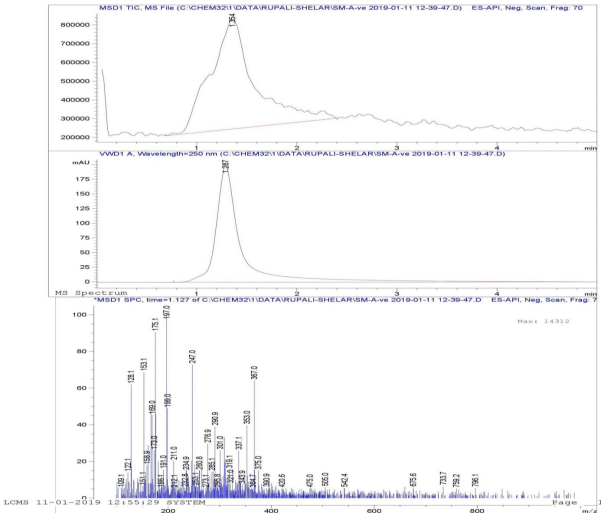

Figure 8: Shows the LCMS analysis report of compound-I and separated peaks showing the fragment's molecular weight.

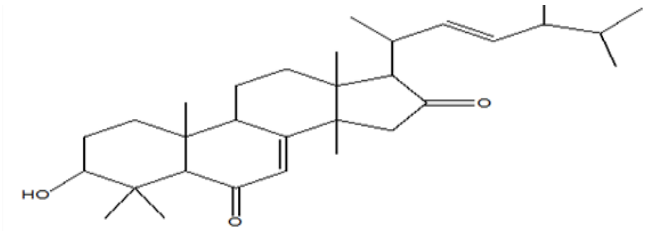

Figure 9: Structure of Isolated compound-I

Ethanolic extract of *E. agallocha* Linn. was processed for fractionation. The ethyl acetate and ethanol obtained better yields and were further selected for isolation study. The isolated

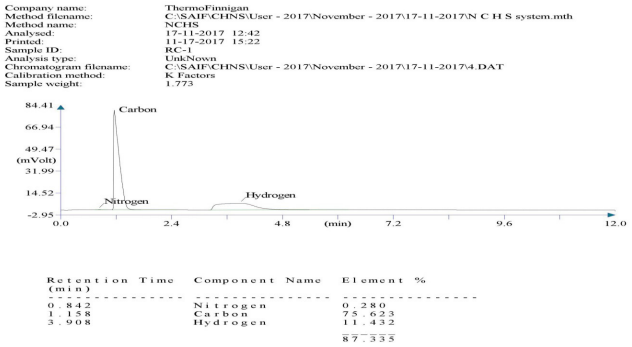

Figure 10: CHN analysis of isolated compound-I

Table 8: CHN analysis of isolated compound-I

Molecular Formula	*Particulars of Element*	*Theoretical Result (%)*	*Experimental Result (%)*
$C_{31}H_{48}O_3$	Carbon	79.48	75.63
	Hydrogen	10.25	11.43
	Oxygen	10.25	12.66
	Total	99.98	99.72

phytoconstituent structures were determined using spectral and chromatographic details. The isolated compound I was processed for HPTLC analysis and the compound is resolved at R_f 0.21.

The compound I was analyzed by using HPLC with acetonitrile: Methanol as mobile phase (90:10) and separated. Then, further characterization of the isolated compound is done using various spectroscopic techniques. UV-vis spectrophotometric study shows unsaturated carbonyl group and -OH group connected with double bond while functional group C = C-H Bend, C-O Stretch, C=O Stretch, CH_2, CH_3 Stretch, C-H stretch attached to C= C, C -OH has been revealed using FTIR. Taking the molecular mass as 468 using GC-MS, the molecular formula works out to be $C_{31}H_{48}O_3$ and further NMR characterization is done to confirm the final structure (Figure 10).

CONCLUSION

Mangroves are a gigantic source of various phytochemicals. Because of the diversity of the compounds, it has been difficult for researchers to identify and analyze these bioactive chemical compounds from herbal origins. *E. agallocha* extracts can be quantified using such a blend of HPTLC and HPLC methods. This strategy is incredibly simple, cheap, rapid, and exceptionally quick. The leaf, stem, and root parts of this mangrove were identified as natural sources due to the existence of this valuable bioactive component and can be used to build isolation strategies for both this plant's bioactive compound and other herbal raw materials.

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