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Evaluation of chemical constituents of *Lyonia ovalifolia* (wall.) Drude (1897) leaves extract

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Abstract

Lyonia ovalifolia has been used in a folk medicine for the treatment of wounds, cuts, burns, and scabies by different communities in Nepal. The present study is intended to evaluate the phytochemical constituents present in the leaf of plant and to subject methanol and hexane extract of plant leaf to phytochemical screening, GC-MS analysis, HPLC and FTIR analysis. Phytochemical screening of methanol extract revealed the presence of phenolic compounds, flavonoids, glycosides, tannins, xanthoprotein, quinones and saponins. Hexane extracts showed the presence of resins and quinones. The bioactive compounds of *L. ovalifolia* leaves have been evaluated using GC-MS, HPLC and FTIR. Fourteen chemical constituents have been identified through GC-MS analysis, among that the major constituents are Cyclopental [c] pyran-1(3H)one, hexahydro4,7-dimethyl (27.88%), Cyclopentene, 1-(2-propenyl) (16.52%), Diglycerol (14.99%) and others. The compounds identified through GC-MS analysis were probably helpful for the cure of many infections. The mass spectra of the compounds formed by the methanol extract are matched with NIST library.

Keywords: *Lyonia ovalifolia*, bioactive compound, HPLC analysis, FTIR analysis

1. Introduction

Lyonia ovalifolia is a deciduous or evergreen, shrubs or trees, ranging up to 4.5 m tall. Distributed throughout Bangladesh, Bhutan, Cambodia, India, Japan, Laos, Malaysia, Myanmar, Nepal, Pakistan, Sikkim, Thailand and Vietnam. The tree has brown bark, peeling in narrow strips. Ovate leaves are leathery, short-stalked, acute or long-pointed, having length of 8-15 cm. Small white flask-shaped flowers are born in almost horizontal clusters in leaf axils, constricted at the mouth and are finely hairy.

L. ovalifolia is a plant of ethno medicinal relevance used to cure wounds, burns and scabies by the practitioners of traditional medicine in Nepal^[1, 2]. Some previous studies have suggested its in-vitro antibacterial activities^[3, 4]. The cyclic adenosine monophosphate (cAMP), formed from ATP, regulates several biological process. In human body, cAMP have impact on higher order of thinking, neurogenesis, memory, emotional disorder and cognitive function. The intracellular concentration of cAMP is regulated by two membrane-bound enzymes, adenylate cyclase and phosphor-diesterase. The plant may induce the paralysis of nerve centers and motor nerve terminals, the cAMP regulation activity of the compounds isolated from this plant was evaluated by Alpha-Screen assay. Two compounds (secorhodomollolides A and D) found in *L. ovalifolia* significantly decrease the cAMP level at a concentration of 50µm in N1E-115 neuroblastoma cell indicating a neuropharmacological efficiency of this plant^[5].



Fig 1: Leaves of *Lyonia ovalifolia*

The tender shoot of this plant is highly toxic to live stock especially to goat. However the toxicity of shoot gradually decreases with maturation [6]. Upon injection of this plant causes bloating of stomach and severe purgation, which often leads to fatality, if not treated promptly. This indicates the toxic effect of the plant.

2. Material and Methods

2.1. Plant collection and preparation of leaf extract

Plant sample collected area is situated at 27°42" North latitude and 85°43" East longitudes which lies at 1323 meter from sea level. It was ensured that the plant was healthy and uninfected. Leaves were washed under running water to remove any traces of soil particles and other dirt. Then washed with distilled water, air dried and cut in to small pieces and dried for 10-15 days in shade.

The leaves were powdered using mixer grinder and sieved to get fine powder. 100 grams of leaf powder was taken in separating funnel, 400 ml of hexane was added and kept 48 hours. The extract was filtered and supernatant was collected. The residue was again subjected to extraction with methanol solvent. After 7 days methanol extract was then filtered. The collected extract was dried by first distillation, then evaporation under boiling point. Again methanol extract was soaked with chloroform, ethyl acetate, and butanol and their respective extracts were obtain.

2.2. Phytochemical screening

Phytochemical analysis of the methanol and hexane extracts were undertaken using standard methods as described by Edeoga [7], Trease and Evans [8], Harborne [9], Daniel [10] and Prasthith [11].

2.2.1. Test for alkaloids (Dragendroff's test)

In 1 ml of extracts solution, few drops of Dragendroff's reagent were added and the color developed was noticed. Appearance of orange color indicates the presence of Alkaloids.

2.2.2. Test for terpenoids

In a test tube containing 1 ml of extracts, a few drops of thionyl chloride were added. Appearance of pink color indicates the presence of terpenoids.

2.2.3. Test for coumarins

1 ml extract and 1 ml 10% sodium hydroxide was added. Formation of yellow color indicates the presence of Coumarins.

2.2.4. Test for tannins

To the few mg of powder, 10% alcoholic ferric chloride was added; formation of dark blue or greenish black color shows the presence of Tannins.

2.2.5. Test for flavonoids

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid indicates the presence of flavonoids.

2.2.6. Test for phenol

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenol.

2.2.7. Test for volatile oils

To 2 ml of extracts, 0.1 ml of dilute NaOH and small amount of dilute HCl acid were added and the formation of white precipitates indicates volatile oils.

2.2.8. Test for quinones

To 1ml of extract 2 drops of concentrated hydrochloric acid was added. Formation of red color indicates the presence of Quinones.

2.2.9. Test for sugar

To 1 ml of extract, Fehling's solution was added. Appearance of red color indicates the presence of sugar.

2.2.10. Test for carbohydrates

- Molisch's test: Filtrates were treated with 2 drops of alcoholic α -naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates.
- Benedict's test: filtrates were treated with Benedict's reagent and heated gently. Orange red colored precipitate indicates the presence of reducing sugars.
- Fehling's test: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitates indicates the presence of reducing sugar.

2.2.11. Detection of glycosides

Extracts were hydrolyzed with dil. HCl, and then treated with Ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose- pink color in the ammonical layer indicates the presence of anthranol glycosides.

2.2.12. Detection of saponins

- Froth test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minute. Formation of 1 cm of foam indicates the presence of saponins.
- Foam test: 0.5 gm of extracts was shaken with 2 ml of water. If foam persists for ten minutes it indicates the presence of saponins.

2.2.13. Test for fixed oil (Spot test)

A small quantity of extracts was pressed between two filter papers. Formation of grease spot indicates the presence of fixed oils and fats.

2.3. GC-MS analysis

The methanol extract of *L. ovalifolia* was analyzed by GC-MS-QP2010 ultra fitted with capillary column RTX-%MS. The column temperature was programmed from 100°C to 280°C at a rate of 15°C/min with the lower and upper temperature being held for 1, 2 and 10 min respectively. The GC injector and MS transfer line temperatures were set at 280°C. GC was performed in split less mode. Helium was used as a carrier gas at a flow rate of 3mL/min. For MS detection, the electron ionization mode with ionization energy of 0.80kV was used, with a mass range at m/z 30 to 600. An Injection volume of 1 μ L was used for the methanol extract. The components were identified by their retention time and based on the commercially available spectral data of National institute of Standard and technology (NIST) library.

2.4. HPLC analysis

The HPLC system binary gradient Shimadzu LC-20 AD with a UV detector with UV detector was used for determination of qualitative analysis of methanol and hexane extracts of *Lyoniaovalifolia*. Reverse phase chromatic analysis was carried out in isocratic condition using column, C-18 phenomenex at 40°C. Instrumental parameters setup for

analysis was: injection volume 2 μ L; Solvent system were methanol (HPLC grade) and water (40:60); flow rate; 1 mL/min. The chromatogram was monitored at 180 nm to 800nm for both methanol and hexane extracts. Samples were filtered through an HPLC filter membrane using nylon 6, 6 filter paper.

2.5. Fourier transform infrared spectrophotometer (FTIR) analysis

The chloroform, butanol and ethyl acetate fraction of methanol extract of *L. ovalifolia* were loaded in FTIR spectrometer (SHIMADZU IR Prestige- 21) with a scan range

from 5000-400 cm^{-1} . Thus obtained FTIR spectra were analyzed.

3. Results and Discussions

3.1. Extraction: Hexane and methanol extract of young leaf of *L. ovalifolia* were prepared using cold percolation method. During the process 1.09g hexane extract and 19.56g of methanol extract were obtained.

3.2. Phytochemical screening analysis

The result of phytochemical screening of leaf extract of *L. ovalifolia* is presented in Table 1.

Table 1: Phytochemical screening of leaf extract of *Lyonia ovalifolia*

S.N.	Phytochemical constituents	Hexane	Methanol
1	Phenols	+	+
2	Flavonoids	+	+
3	Alkaloids	-	-
4	Carbohydrates	-	-
5	Glucosides	-	+
6	Tannins	-	+
7	Xanthoprotein	-	+
8	Resin	+	+
9	Quinone	+	+
10	Emodin	-	+
11	Saponin	-	+

(+)= Present, (-) = Absent

The methanol extract detected the presence of phenols, flavonoids, tannins, glycosides, xanthoprotein, resin, quinone, emodin and saponins. The hexane extract showed the presence of phenols, flavonoids, resin and quinone.

From the results, it was confirmed that the plant *Lyonia ovalifolia* has a large number of chemical constituents which may be responsible for many pharmacological actions and medicinal properties. Flavonoids found in plant are therapeutically applicable for some biological actions and pharmacological effects like anti-oxidant, anti-inflammatory, anti-cancer, anti-diabetic, anti-viral, anti-allergic and diuretic activities [12].

Saponins detected during analysis are reported to have numerous properties which includes pharmacological activities such as anti-microbial, anti-inflammatory as well as sweetness and bitterness, foaming and emulsifying properties. These properties suggest that saponins act as a chemical barrier against potential pathogens in plants [13, 14].

Tannins are found to possess spasmolytic activity, free radical scavenger properties and anti-oxidant activities.

Plants rich in phenolic contents have been reported to have effective beneficial effects such as, anti-microbial, anti-inflammatory, anti-viral, anti-mutagenic, anti-tumor [15], anti-oxidant activity [16], and chemo protective effects.

3.3. GC-MS analysis

GC-MS chromatogram of the methanol extract of *L. ovalifolia* (Figure 2) showed 14 peaks which indicated the presence of fourteen major phytochemical constituents. The identification of the phytochemical compound was confirmed based on the peak area, retention time and molecular formula. The active principles with their Retention time (RT), Molecular formula, Molecular weight (MW) and peak area in percentage are presented in table 2. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library.

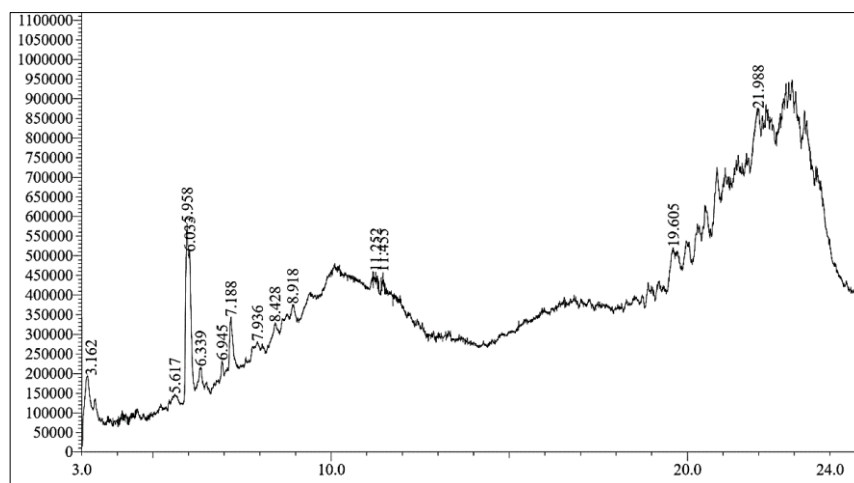


Fig 2: GC-MS chromatogram of methanol extract of *L. ovalifolia*

GC-MS analysis of methanol extract of *L. ovalifolia* identified the presence of carboxylic acid compounds, alcohol

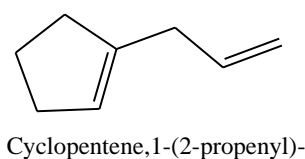
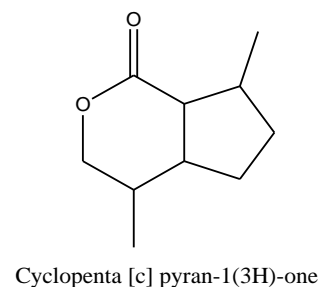
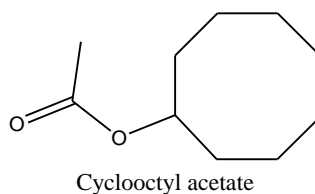
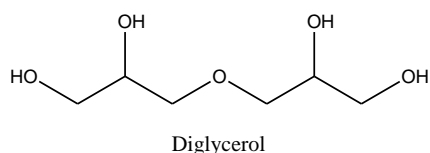
compound, diterpenes, Fatty acid ester compound and ester compound.

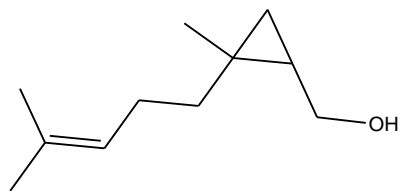
Table 2: GC-MS showed phytochemicals with their molecular weight and molecular formula

Peak	RT	Area %	Name	MW	Mol. Formula	Base m/z
1.	3.162	14.99	Diglycerol	166	C ₆ H ₁₄ O ₅	61.0
2.	5.617	0.79	Cyclooctanol	170	C ₁₀ H ₁₈ O ₂	43.05
3.	5.958	27.88	Cyclopenta[c]pyran-1(3H)one, hexahydro-4,7-dimethyl	168	C ₁₀ H ₁₆ O ₂	113.05
S4.	6.033	16.52	Cyclopentene,1-(2-propenyl)	108	C ₈ H ₁₂	67.05
5.	6.339	1.32	Cyclopropanecarboxylic acid,3-(3butenyl)-2,2-dimethyl	168	C ₁₀ H ₁₆ O ₂	81.05
6.	6.945	1.44	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl	180	C ₁₁ H ₁₆ O ₂	111.05
7.	7.188	6.76	1,3-Di(cyclohexyl)but-1-ene	220	C ₁₆ H ₂₈	124.1
8.	7.936	0.21	3-Nonyn-1-ol	140	C ₉ H ₁₆ O	67.05
9.	8.428	0.75	3-Tetradecyn-1-ol	210	C ₁₄ H ₂₆ O	81.05
10.	8.918	1.91	Cyclopropanemethanol,2-methyl-2-(4-methyl-3-pentenyl)	168	C ₁₁ H ₂₀ O	69.05
11.	11.252	0.10	Ethyl 3-cyclopropylpropanoate	142	C ₈ H ₁₄ O ₂	67.05
12.	11.455	0.22	Octanoic acid, 8-(octylthio)	288	C ₁₆ H ₃₂ O ₂ S	55.0
13.	19.605	13.78	(E)-13-Docosenoic acid	338	C ₂₂ H ₄₂ O ₂	323.25
14.	21.988	13.33	7-Tetradecenal, (Z)	210	C ₁₄ H ₂₆ O	323.20
	Total	100				

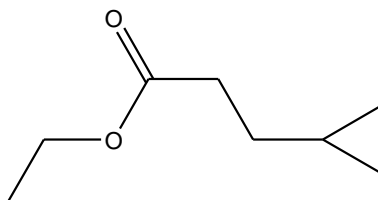
Table 3: Comparison of spectroscopic data of chemical constituents of cold methanol extract of *L. ovalifolia* with NIST library compounds

S.N.	RT	Name	Spectral data (MS) of plant compound	Spectral data (MS) of NIST library compound	Common peak
1	3.162	Diglycerol	30,31,43,61,75,97,117,133,147,165,177,193,207	31,43,61,75,93,103,117,135	31,43,61,75,117
2	5.617	Cyclooctanol	31,41,43,67,81,95,109,139,152,170	27,41,43,67,82,94,110,112,127,152	41,43,67
3	5.958	Cyclopenta[c]pyran-1(3H)one, hexahydro-4,7-dimethyl	30,41,55,67,81,95,111,113,126,153,168	39,55,67,81,95,109,113,126,153,168	55,67,81,113,126,153,168
4	6.033	Cyclopentene,1-(2-propenyl)	30,39,51,67,82,93,107,121,138	27,39,51,67,79,93,108	39,51,67,93
5	6.339	Cyclopropanecarboxylic acid,3-(3butenyl)-2,2-dimethyl	30,41,43,55,67,81,95,111,126,155	27,41,55,67,81,95,108,113,139,153	41,55,67,81,95
6	6.945	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl	30,41,43,67,81,95,111,124,137,152,165,180	27,41,43,67,79,95,111,124,137,152,165,180	41,43,67,95,111
7	7.188	1,3-Di(cyclohexyl)but-1-ene	30,41,55,69,82,95,109,124,137,149	39,41,55,67,81,96,109,124,137	41,55,109,124,137
8	7.936	3-Nonyn-1-ol	31,39,54,67,69,93,109,124	27,41,54,67,81,109	54,67,109
9	8.428	3-Tetradecyn-1-ol	30,41,55,67,81,93,111,121	27,41,55,67,79,97,107,121,135	41,55,67
10	8.918	Cyclopropanemethanol,2-methyl-2-(4-methyl-3-pentenyl)	31,41,55,69,81,84,111,124,140	27,41,55,69,81,95,109,125,150	41,55,69,81
11	11.252	Ethyl 3-cyclopropylpropanoate	30,51,67,73,97,110,129,151	41,55,69,70,88,99,115,127,142	-
12	11.455	Octanoic acid, 8-(octylthio)	37,55,57,83,95,98,113,129,145,181	27,41,55,69,83,87,98,124,145,157,288	55,83,98,145
13	19.605	(E)-13-Docosenoic acid	31,41,55,69,81,95,113,137,152,323	27,41,56,69,81,97,98,112,137,152	41,55,69,81,137,152
14	21.988	7-Tetradecenal, (Z)	30,41,55,69,81,95,109,113,135,149,164,321,323	27,41,55,67,81,97,121,135,149,192	41,55,81,135,149

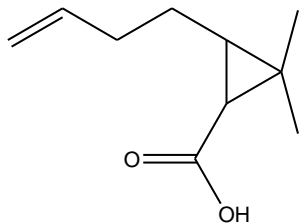




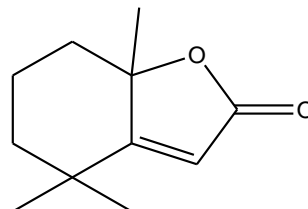
Cyclopropanemethanol,2-methyl-2-(4-methyl-3-pentenyl)



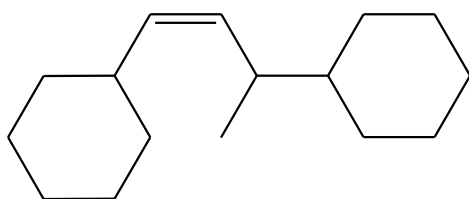
Ethyl 3-cyclopropylpropanoate



Cyclopropanecarboxylic acid, 3-(3-butenyl)-2,2-dimethyl



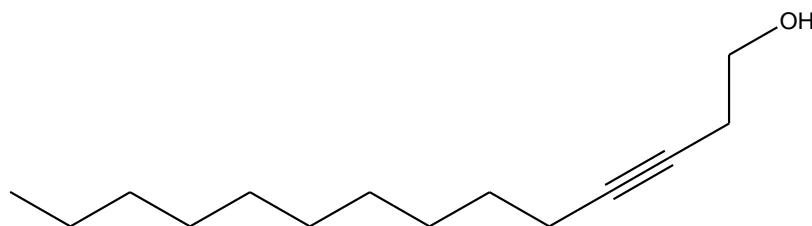
2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl



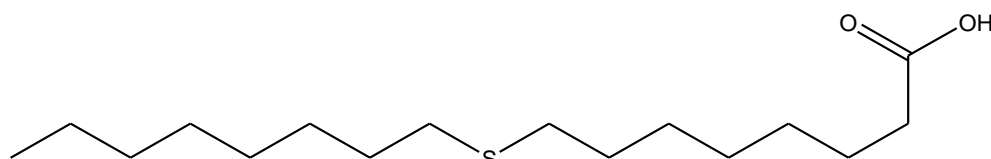
1,3-Di(cyclohexyl)but-1-ene



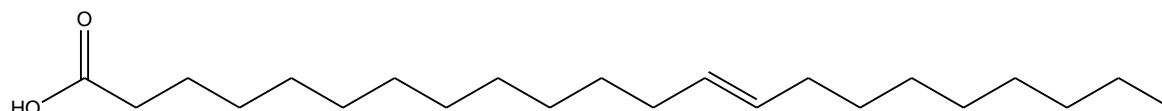
3-Nonyn-1-ol



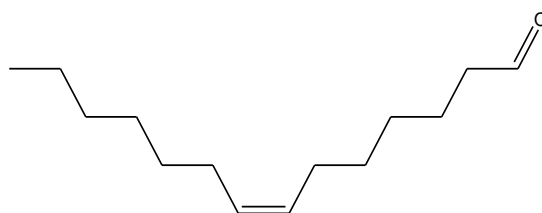
3-Tetradecyn-1-ol



Octanoic acid, 8-(octylthio)



(E)-13-Docosenoic acid



7-Tetradecenal, (Z)

Fig 3: Structure of compound of NIST library which are closely match with spectral data from plant extract

3.4. HPLC analysis

The HPLC fingerprints of methanolic extract of *Lyonia ovalifolia* showed major peaks at the retention times (min.) 0.62, 0.73, 1.615, 1.757, 2.316, 2.901, 3.317, 3.441,

3.441 respectively at wavelength of 190 nm (figure 4) whereas the hexane extract hexane extract showed major peaks at the retention times (min.) of 0.714, 1.727, 2.229 respectively at wavelength of 254nm (figure 5).

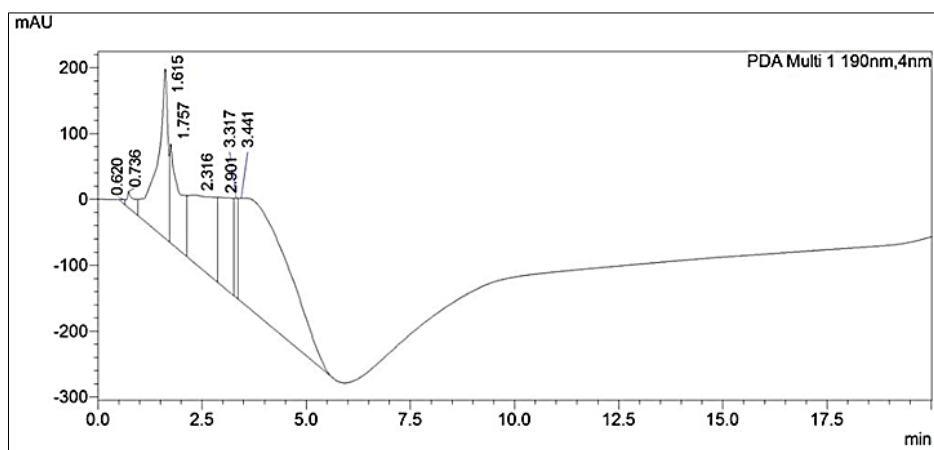


Fig 4: HPLC fingerprint profile of methanol extract of *L. ovalifolia*

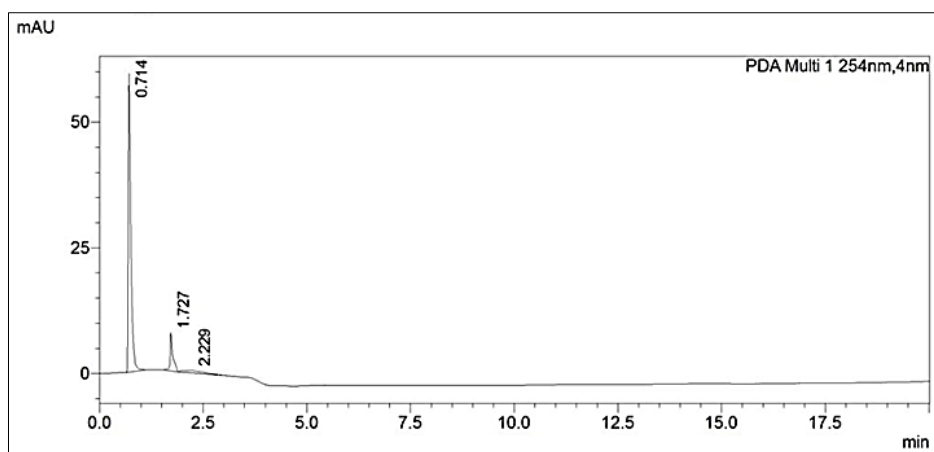


Fig 5: HPLC fingerprint profile of hexane extract of *L. ovalifolia*

3.5. FTIR analysis

The FTIR spectrum of leaf extracts (prepared in different solvents) of *L. ovalifolia* are given in Fig 6, 7 and 8. The data on the peak values and the probable functional groups (obtained by FTIR analysis) present leaf extracts prepared in Chloroform, Ethyl Acetate and Butanol of *L. ovalifolia* are

presented in tables 4, 5 and 6 [17, 18].

3.5.1. Chloroform extract

The characteristic absorption bands were exhibited at 3363 cm^{-1} , 2924 cm^{-1} , 1689 cm^{-1} , 1458 cm^{-1} , 1373 cm^{-1} , 1041 cm^{-1} .

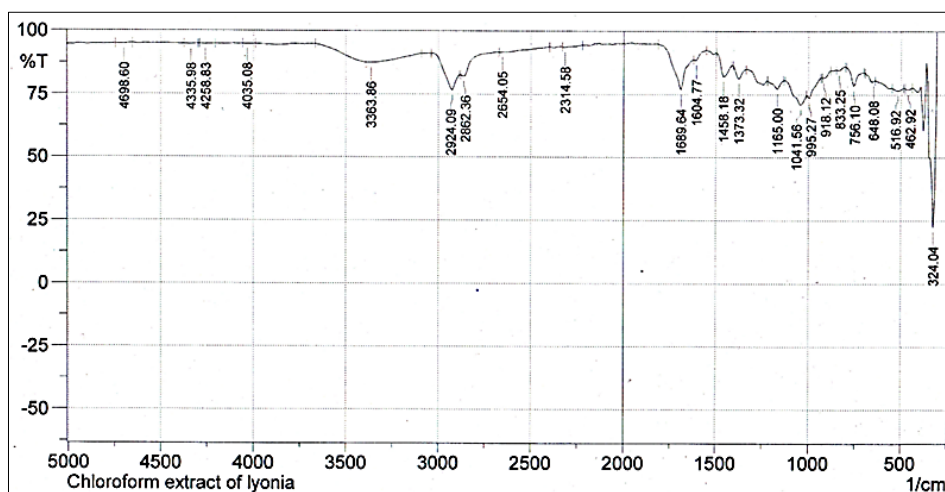


Fig 6: FTIR spectra of chloroform extract of *L. ovalifolia*

The spectral data analysis of chloroform extract of *L. ovalifolia* indicates the presence of –OH bond at 3363 cm⁻¹, C-H stretching at 2924 cm⁻¹, carbon to carbon unsaturated (with double bond) at 1689 cm⁻¹, C-C saturated at 1458 cm⁻¹, cyanide at 1373 cm⁻¹, and finally R-O functional group at 1041 cm⁻¹.

3.5.2. Ethyl acetate extract

The Ethyl acetate extract showed the characteristic absorption

bands at 3286 cm⁻¹, 2924 cm⁻¹, 1674 cm⁻¹, 1026 cm⁻¹, 516 cm⁻¹.

Result indicated the peaks at 3286 cm⁻¹, 2924 cm⁻¹, 1674 cm⁻¹, 1026 cm⁻¹ and 516 cm⁻¹ hints the presence of –OH bond, C-H bond, carbonyl functional group and alkoxide functional groups respectively.

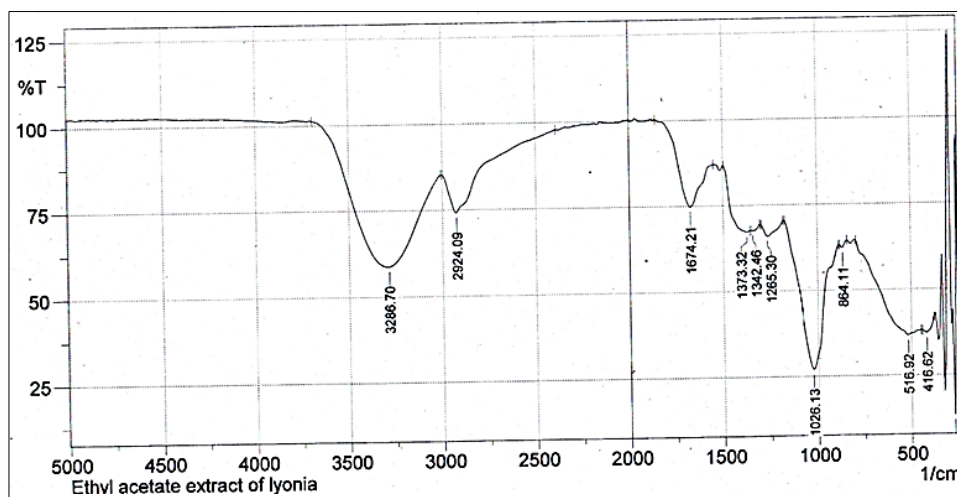


Fig 7: FTIR spectra of ethyl acetate extract of *L. ovalifolia*

3.5.3. Butanol extract

2-Butanol extract exhibited a characteristic band at 3348 cm⁻¹,

2931 cm⁻¹, 1689 cm⁻¹, 1165 cm⁻¹, 1049 cm⁻¹, 995 cm⁻¹, 910 cm⁻¹, 817 cm⁻¹, 779 cm⁻¹.

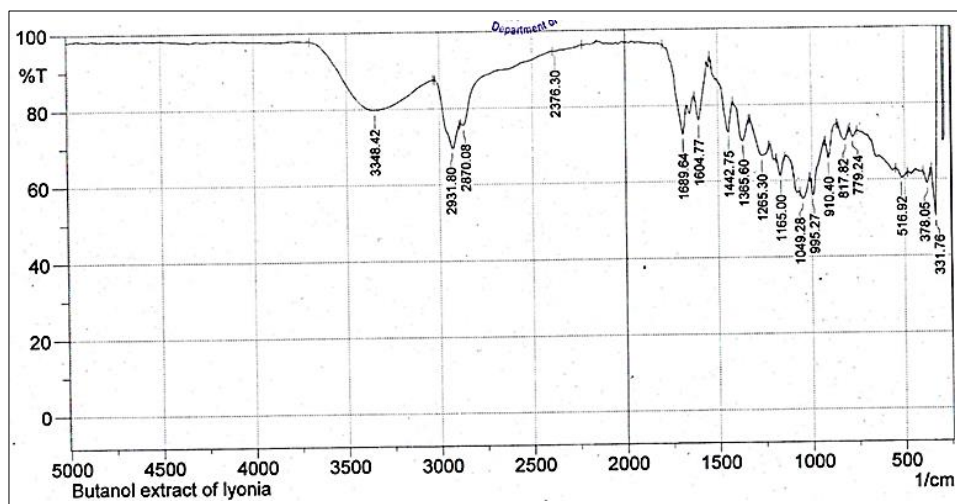


Fig 8: FTIR spectra of butanol extract of *L. ovalifolia*

Table 4: FTIR spectral peak values and functional groups obtained for the leaf extracts of *L. ovalifolia*

S.N.	Peak values (cm ⁻¹)			Functional groups
	Chloroform	Ethyl acetate	Butanol	
1	3363	3286	3348	Alcohol
2	2924	2924	2931	C-H Stretching
3	1689	-	1689	C=C
4	-	1674	-	C=O
5	1458	-	-	C-H group
6	-	-	1165	C-O
7	1373	-	-	C-N
8	1041	1026	1049	R-O
9	-	-	995	C-O
10	-	-	817	C-H
11	-	-	779	C-Cl

Result revealed the presence of –OH functional group, C-H stretching, C=C bond, C-O bond, R-O bond and C-Cl bond at wavenumbers 3348 cm^{-1} , 2931 cm^{-1} , 1689 cm^{-1} , 1165 cm^{-1} , 1049 cm^{-1} , 995 cm^{-1} , 779 cm^{-1} and 817 cm^{-1} respectively.

From all spectral data obtained from these three (chloroform extract, ethyl acetate extract and butanol extract) it can be concluded that the extract prepared in polar solvent showed more functional group than the extract obtained by using less polar solvent. Hence it can be said that, with increase in polarity of solvent, increase in chemical constituents takes place.

4. Conclusion

Phytochemical screening of methanol and hexane extract of *L. ovalifolia* recorded the presence of phenols, flavonoids, glycosides, tannins, xanthoprotein, quinone, emodin, saponins and resin. Phytochemical constituents such as tannins, flavonoids, and several aromatic compounds or secondary metabolites of plants serve as defense mechanism against predation by many micro-organisms.

The GC-MS chromatographic analysis of the methanol extract showed the presence of 14 different peaks areas at retention time 3.162 (Diglycerol, M/Z- 61, P.A- 14.99%, Mol. Formula- $\text{C}_6\text{H}_{14}\text{O}_5$, Mol. Wt.- 166) to 21.988 (7-Tetradecenal, M/Z-323, P.A-13.33, Mol. Formula - $\text{C}_{14}\text{H}_{26}\text{O}$, Mol. Wt.-210). Presence of various compounds probably support various biological activity of plant.

HPLC analysis of methanol extract of the plant showed eight major peaks at retention time from 0.62 min. to 3.441 min. whereas hexane extract showed three major peaks at retention time 0.741 to 2.229 min.

FTIR spectroscopy of chloroform, butanol and ethyl acetate extracts indicated the presence of alcohol, alkane, ketone, alkene, alkyl halide and cyanide compound. Among the functional groups observed in the extracts, OH group was found to be present in all the extracts of plant probably indicates the higher potential towards biological activity.

5. Reference

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