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Extraction, isolation and identification of bioactive compound from extract of *Euphorbia thymifolia* Linn

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***Abstract*---**Natural products are stored in plants, animals, and microorganisms, which are referred to as "natural sources generating compounds." Strong therapeutic substances are developed with the help of medicinal plants. As a source of several beneficial secondary metabolites that work as a plant's defensive system against predators including microorganisms, insects, and herbivores and have been shown to be potentially active chemicals, plant-based medicines provide a significant addition to current treatments. Common names for Euphorbia Thymifolia Linn (E. Thymifolia) include "duddi" and "Laghu didhika" and "Raktavindaka," respectively, in Sanskrit. It is a member of the Euphorbiceae family. This plant is used as a diuretic, laxative, and thermogenic. It is bitter, acrid, and sweet. This plant is commonly used in ayurveda to treat a variety of illnesses, including leprosy, helminthiasis, and ringworm skin infections. The current study's objective is to analyze the entire E. thymifolia Linn plant in order to isolate and identify bioactive components and determine its phytochemical profile. An established test technique that is documented in the literature was used to determine the qualitative analysis of different phytochemical elements. From a methanolic extract of E. thymifolia, a bioactive component was isolated and characterized. Several techniques, including TLC, column chromatography, and preparative TLC, were used to separate the bioactive substance from methanolic extracts. By using FT-IR, 1HNMR, and mass, the isolated bioactive molecule is discovered. The resulting chemical is next subjected to the TLC that comes before. The bioactive substance found in the plant was validated by the 1HNMR spectrum. The presence of alkaloids, glycosides, phenols, flavonoids, and tannins was discovered by phytochemical examination. The

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pharmaceutical industry, pharmacologists, and phytochemists will all benefit from the study's findings.

***Keywords*---**Extraction, Isolation, Bioactive Compound, *Euphorbia thymifolia* Linn.

Introduction

Plants, animals and micro-organisms represent a reservoir of natural products, the so called “natural sources deriving compounds”. Particularly, the plant kingdom offers a variety of species still used as remedies for several diseases in many parts of the world such as Asia, Africa and South America. Even if, as reported by World Health Organization, traditional medicines represent the primary health care system for the 60% of the world’s population, the plant species with possible biological activity remain largely unexplored. Natural products, such as plants extract, open a new horizon for the discovery of new therapeutic agents [1]. The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed and about 80% of the world’s population relies on herbal medicines [2]. Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operation included steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. If the plant was selected on the basis of traditional uses, then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional ‘herbal’ drug. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. The goal of this study is to extract, isolate, screen for phytochemicals, and identify bioactive components from a ethanolic and chloroform extract of E. Thymifolia whole plant extract using thin layer chromatography. Several studies, including FT-IR, 1HNMR, and mass were performed to determine the structure of the bioactive molecule.[3-8]

Materials and Methods

Collection and authentication of plant material

The *Euphorbia thymifolia* L. aerial parts were collected from hill area of *western ghat* near Bhor city district Pune Maharashtra. The first Specimen sample authenticated by Botanist Dr. P. B. Kamble, Head of Botany Department A. T. College Bhor, dated 29th September 2019. Fresh plants of *E. thymifolia* were collected from Bopdev Ghat near under 10 km, area Pune Maharashtra India. and deposited the specimen for identified and authenticated at Botanical Survey of India Western Regional Office Pune. The Voucher specimens N / No. BSI/WRC/100-1/Tech./2020/102 by Scientist Mrs. Priyanka A. Ingale for Comparison and authentication of plant species *Euphorbia thymifolia* was used.[9-12]

Extraction Procedure

The selected plant material (crude drugs) *Euphorbia thymifolia* L. was collected from local place and dried in the shade. Then the dried material is pulverized in grinder. The powdered material was passed through 120 mesh sieves to remove fine powder and course powder was used for extraction. Continuous Soxhlet extraction method used for extraction. Each powered batch (200 gm. powder) loaded for extraction.[13]

Further for Preparations of Hexane extract**:** Weighed accurately crude drug course powder extracted with an adequate quantity of hexane using a soxhlet extractor at 60°-70°C. (Boiling point: 69°C) Likewise extraction was performed with Chloroform, Ethyl acetate, Ethanol and After ethanol extraction, the air-dried marc of crude drug was macerated with distilled water containing chloroform (2.5ml/1000ml) for 48 hours with stirring. Then the resultant extract was filtered through a muslin cloth and the marc was separated. The filtrate was evaporated to dryness on hot plate at 45°C to get an aqueous semisolid extract.[14]

Qualitative analysis of Phytochemicals

Phytochemical analysis of all the solvent extracts was performed for the detection of active secondary metabolites or different constituents such as Terpenoids, Steroids, Glycosides, Saponins, Alkaloids, Flavonoids, Tannins, Proteins, Free Amino Acids, Carbohydrate and Vitamin C. Qualitative chemical test used to identify drug quality and purity. The identification, isolation and purification of active chemical constituents are depending chemical methods of evaluation. [15-17]

UV-visible spectroscopy

UV-visible spectroscopy can be performed for qualitative analysis and for identification of certain classes of compounds in both pure and biological mixtures. Preferentially, UV-visible spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. Natural compounds can be determined by using UV-visible spectroscopy. The UV-Vis spectroscopy was used to determine flavonoids extract (325nm).

Linearity by UV spectroscopy

Preparation of Standard Solutions

A standard stock solution was prepared by dissolving 100 mg of crude extract in 100 ml volumetric flask containing mobile phase, then sonicated for about 10 minutes and made upto 100 ml with mobile phase to get the primary standard stock solution containing 1000 µg/ml. Working standard solutions were prepared by further dilution with mobile phase. (2-10 µg/ml)

Fractionation of the extract by thin layer and column chromatography [18-21]

Thin layer chromatography

Preparation of TLC plate:
Prepared the slurry of adsorbent media (silica gel-G) in distilled water and poured the slurry on the TLC glass plates to obtain a thin layer.

Activation of TLC plate:
TLC plate was activated by heating in oven for 30min at 105^{O}C.

Sample application:
Dipping the capillary into the solution to be examined and applied the sample by capillary touched to the thin layer plate at a point about 2cm from the bottom. Air-dried the spot.

Chamber saturation:
The glass chamber for TLC should be saturated with mobile phase. Mobile phase was poured into the chamber and capped with lid. Allowed saturating about 30 min

Chromatogram development:
After the saturation of chamber and spotting of samples on plate, it was kept in chamber. The solvent level in the bottom of the chamber must not be above the spot that was applied to the plate, as the spotted material will dissolve in the pool of solvent instead of undergoing chromatography. Allowed the solvent to run around 10-15cm on the silica plate

Visualization:
Plates were removed and were examined visually, under UV and suitable visualizing agent (Vanillin-H_2SO_4, Methanolic $FeCl_3$ solution) after that R_f was calculated by formula
RF (retention factor) values of the fractions were measured by applying formula of Brimley and Barrett as:

$$Rf = \frac{\text{Distance travelled by the solvent}}{\text{Distance travelled by the solute}} \quad \text{Formula 1}$$

Column chromatography

The separation of phytoconstituents was done by column chromatography technique. For that clean and dried glass column was used. The silica gel for column chromatography mesh size #60-120 was activated at 110°C. The column was filled with silica gel (Stationary phase) and solvent (mobile phase) without development of any air bubbles. The silica gel (Stationary phase) was then allowable to stabilize in the column. The sample was prepared by energetically (vigorously) mixing the extract with Stationary phase and mobile phase. The solvent was then evaporated so as to form a free flowing material. This dried extract material was charged in the column. The sample was eluted in gradient manner and fractions were collected. Solvent was recovered by simple distillation. Fractions giving same separation pattern on TLC plates were mixed together and forward for further studied.

In preliminary phytochemical test of ethanol extract of *Euphorbia thymifolia* L. show presence of flavonoids, steroidal and phenolic compounds. So flavonoid constituents from ethanolic extract were need to isolate using column chromatography. About 10 gm of Semisolid ethanolic extract mixed with silica and subjected to column chromatography to obtain separated fractions.

HPTLC Quantification of the extracts

The quercetin content of various extracts was determined by comparing the area of chromatogram with the calibration curve of concentration of standards. The Rf value of standard quercetin (0.65-0.70) was compared with the Rf value of the extracts. Calibration parameters were as follows: calibration mode- single level, statistics mode-cv, evolution mode- peak height. The average content of the quercetin in different extracts was expressed in percentage.

The sample was spotted in the form of bands of width of 6 mm with space between bands of 8.0 mm, with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F254 (5 cm ×10 cm) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The slit dimensions 5 mm × 0.45 mm and scanning speed of 20 mm/sec was employed.

The linear ascending development was carried out in 10 cm×10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using ethyl acetate: acetic acid: formic acid: water solvent in 10:0.5:0.5:1.0 (v/v) as mobile phase. The optimized chamber saturation time for mobile phase was 15 min. The length of chromatogram run was 8 cm and development time was approximately 20 min. TLC plates were dried in a current of air with the help of a hair drier. After development densitometric scanning was performed on CAMAG thin layer chromatography scanner at 263 nm operated by WINCATS software version 1.4.2.

Standard Quercetin Solution Preparation

Standard solution of Quercetin was prepared by dissolving 10 mg of Quercetin RS in methanol to get 1000 µg/ml solution. From this 1 ml was further diluted to 10 ml to get 100 µg/ml solution. This solution was applied on TLC plate (2, 4, 6, 8, 10, 12 µl, 14 µl, 16 µl and 18 µl) to get concentration range from 200- 1800 ng/spot)

Sample Solution Preparation

Sample was dissolved in 10 ml of methanol. 2 µl volume was applied on the plate.

Isolation and structural elucidation of the compound

The Structure elucidation of isolated fractions A4, A5 fractions was carried out by using different spectroscopic analytical methods as FTIR, 1HNMR and LCMS to find out the active component responsible for the pharmacological action. [22]

Results and Discussion

The crude extracts so obtained after the maceration process, extracts was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction.

Table 1. Successive extractive values of the powdered leaves of *Euphorbia thymifolia* L

Sr. No.	Extraction solvent used	Yield%
1.	Hexane extract	08.61 %
2.	Chloroform extract	15.15 %
3.	Ethyl acetate extract	07.47%
4.	Ethanol extract	17.16 %
5.	Aqueous (Water) extract	03.22%

To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from sample using chloroform, ethyl acetate, methanol and water as solvents are depicted in the Table 1.

Table 2. Preliminary phytochemical screening of extracts

Extracts	Hexane	Chloroform	Ethyl acetate	Ethanol	Aqueous
Tests for carbohydrates					
Molish Test	-	+	-	-	-
Fehling Test	-	+	-	+	-
Benedict Test	-	+	-	+	-
Test for Monosaccharide					
Barfoed's Test	+	-	-	-	-
Test for Non-reducing polysaccharides					
Iodine Test	-	-	-	-	-
Test for Proteins					
Biuret test	-	+	-	-	-
Millions test	-	-	-	-	-
Tests for Steroids					
Salkowaski reaction	-	+	+	+	-
Libermann Burchard reaction	+	-	-	+	-
Libermann reaction	-	-	-	-	-

Tests for Terpenoids	+	+	-		-
Test for Glycosides					
Borntrager's Test	-	-	-	+	-
Killer- Killani Test	-	+	-	-	-
Test for Saponin					
Foam test	-	-	-	+	+
Tests for Flavonoids					
Shinoda test	-	-	+	+	-
Lead acetate Test	-	-	-	+	-
Sod-hydroxide Test	-	-	+	-	-
Tests for Alkaloids					
Meyers Test	-	+	-	+	-
Wagner's Test	-	-	-	-	+
Hager's Test	-	-	-	-	-
Dragendorff Test	-	+	+	+	-
Test for Tannins & Phenolic compounds					
$FeCl_3$	-	+	-	-	-
Lead acetate	-	+	-	+	-

+ Indicates presence of phytoconstituents, - Indicates absence of phytoconstituents

The all extracts were screened for the presence of various constituents. The result of this preliminary phytochemical examination is shown in above. The result of phytochemical study on *Euphorbia thymifolia L.* revealed presence of primary metabolites as well as secondary metabolites such as carbohydrates, lipids, alkaloids, steroids, flavonoids, tannins and terpenoids.

Determination of λmax

Selection of Analytical Wavelength of crude extract scanned for absorption maxima, the observed wavelength used for analysis. The extract showed maximum absorption at 325nm.

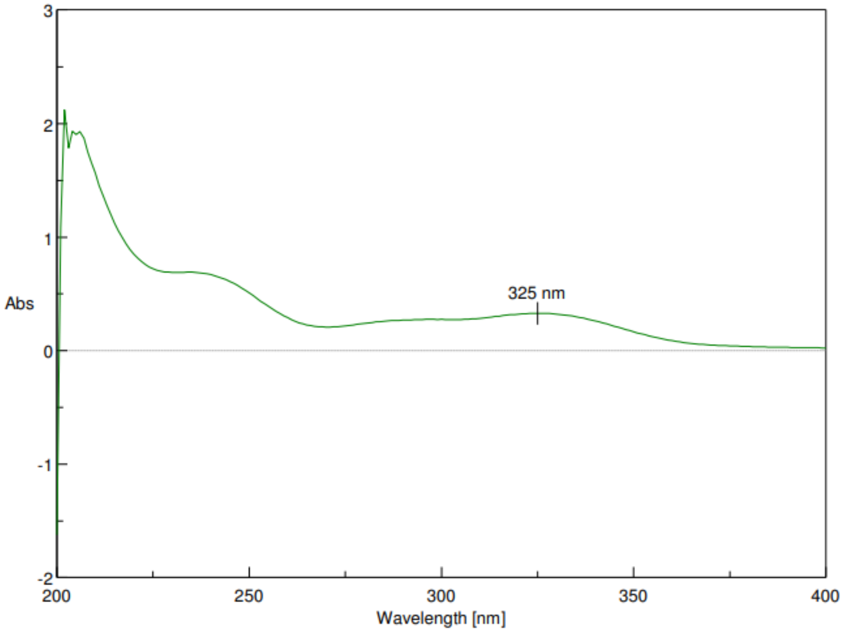


Figure 1. Maximum absorption at 325nm (λmax)

Linearity by UV spectroscopy

Preparation of Standard Solutions

A standard stock solution was prepared by dissolving 100 mg of crude extract in 100 ml volumetric flask containing mobile phase, then sonicated for about 10 minutes and made upto 100 ml with mobile phase to get the primary standard stock solution containing 1000 µg/ml. Working standard solutions were prepared by further dilution with mobile phase. (2-10 µg/ml)

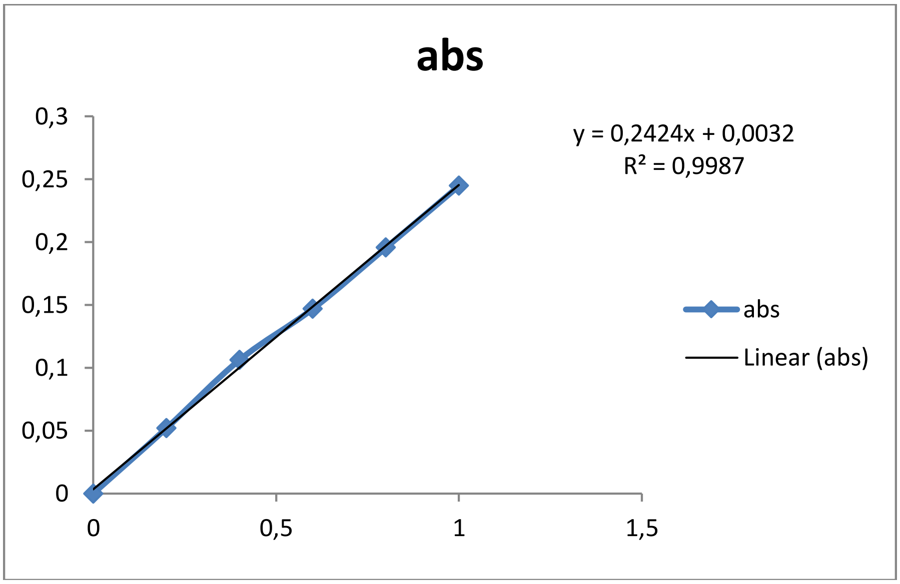


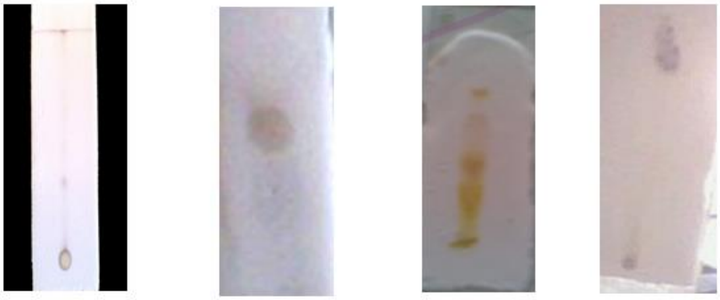
Figure 2. Linearity curve of crude extract.

TLC- characterization of bioactive extract:
After pharmacological evaluation all extracts, bioactive ethanol extract further evaluated by thin layer chromatography for determination of phytocomponents by following ways.

Table 3. TLC- Characterization of bioactive extract

Sr. no.	Chemical constituent	Mobile Phase	Visualization / Detection	Observed Rf- value	Standard reference Rf value
1.	Alkaloids	n- butanol: Ethyl acetate: Formic acid : Water (30:50:10:10)	UV -365nm	0.79	0.60
2.	Glycoside	Ethyl acetate : Methanol : Water (100 : 16.5 : 13.5)	UV -365nm	0.40	0.52
3.	Flavonoid	Toluene : Ethyl acetate : Glacial acetic acid :Water (100:11:11:26)	Anisaldehyde – Sulfuric acid. UV -365nm	0.62	0.60
4.	Tannins	Ethyl acetate: Formic acid : Acetic acid : Water (100:11:11:26)	5% $FeCl_3$ in 0.1N HCl	--	0.55
5.	Steroids	Ethyl acetate : Methanol : Water (70 : 20 : 10)	Vanillin – Sulfuric acid.	0.52	0.54
6.	Terpenoids and	Cyclohexane : Ethyl acetate (75 : 25)	UV- 268nm	0.45	0.60

	Carotenoids	Petroleum ether : Benzene (9 : 1)	UV- 254nm	0.44	0.62
7.	Triterpenoids	Chloroform : Glacial acetic acid Methanol :Water (60 : 32 : 12 : 8)	--	0.45	0.65
		Ethyl acetate : Glacial acetic acid : Formic acid : Water (100 : 11: 11 : 26)	Anisaldehyde – Sulfuric acid. UV – 254nm, 365nm	0.52	



TLC of Steroid TLC of Terpenoid TLC of Flavonoids TLC of Triterpenoid

TLC of Steroid TLC of Flavonoids TLC of Triterpenoid

Figure 3. Thin Layer Chromatography of *Euphorbia thymifolia L.*

The column chromatographic isolation was targeted toward the isolation of bioactive secondary metabolite from bioactive ethanol extract. To isolate phytocomponents from bioactive ethanol extract mobile phase solvent used from range nonpolar to polar nature.

Table 4. Details of Gradient solvent system in column chromatography for the ethanolic extract

Solvent used	Ratio	Fraction Code	Colour	% Yield
Hexane	100%	A1	Dark brown	1.4
Hexane: Ethyl acetate	7:3	A2	Brown	1.6

Hexane: Ethyl acetate	5:5	A3	Brown	1.6
Hexane: Ethyl acetate	2:8	A4	Brown	1.7
Ethyl acetate	100%	A5	Brown	2
Ethyl acetate: Methanol	7:3	A6	Yellowish-brown	2.1
Ethyl acetate: Methanol	5:5	A7	Yellowish-brown	2
Methanol	100%	A8	Yellow	1.7
Methanol: Water	5:5	A9	Yellow	0.7
Water	100%	A10	Faint yellow	0.5

HPTLC Quantification of the extracts

The HPTLC procedure was optimized with a view to quantify the samples extract. Initially ethyl acetate: acetic acid: formic acid: water in varying ratio was tried. The mobile phase ethyl acetate: acetic acid: formic acid: water solvent in 10:0.5:0.5:1.0 (v/v) gave good resolution with Rf = 0.65-0.70. Well defined spots were obtained when the chamber was saturated with mobile phase for 20 min. at room temperature. The TLC plates were visualized under UV light at 254 nm after derivatization. The identity of the quercetin bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. The Rf's obtained for the said plant extracts closely replicated the Rf's found for standard quercetin, thus making it a significant fingerprint parameter. The chromatogram of standard quercetin is shown in figure 5 and 6 and that of quercetin identified.

Table 5. HPTLC Quantification of the extract and Standard

Track No	Name	Volume Applied	Conc (ng/band)	Start Position	Start Height	Max Position	Max Height	End Position	End Height	Area AU
1	Quercetin	2 µL	200	0.61 Rf	3.1 AU	0.63 Rf	149.5 AU	0.67 Rf	0.1 AU	2312.0
2	Quercetin	4 µL	400	0.60 Rf	1.8 AU	0.63 Rf	273.0 AU	0.67 Rf	1.7 AU	4221.6
3	Quercetin	6 µL	600	0.59 Rf	3.2 AU	0.62 Rf	545.2 AU	0.67 Rf	3.6 AU	5530.2
4	Quercetin	8 µL	800	0.59 Rf	3.2 AU	0.62 Rf	401.2 AU	0.67 Rf	3.5 AU	7045.5
5	Quercetin	10 µL	1000	0.58 Rf	2.4 AU	0.62 Rf	451.2 AU	0.67 Rf	1.5 AU	7785.6
6	Quercetin	12 µL	1200	0.57 Rf	1.2 AU	0.62 Rf	469.5 AU	0.67 Rf	3.2 AU	9021.6
7	Quercetin	14 µL	1400	0.59 Rf	3.2 AU	0.65 Rf	570.0 AU	0.70 Rf	0.1 AU	10509.1
8	Quercetin	16 µL	1600	0.58 Rf	6.9 AU	0.64 Rf	694.0 AU	0.68 Rf	1.9 AU	11931.2
9	Quercetin	18 µL	1800	0.60 Rf	0.9 AU	0.65 Rf	599.8 AU	0.69 Rf	0.3 AU	12235.6
10	Sample	2 µL	X	0.59 Rf	9.3 AU	0.62 Rf	247.2 AU	0.67 Rf	0.2 AU	4312.3

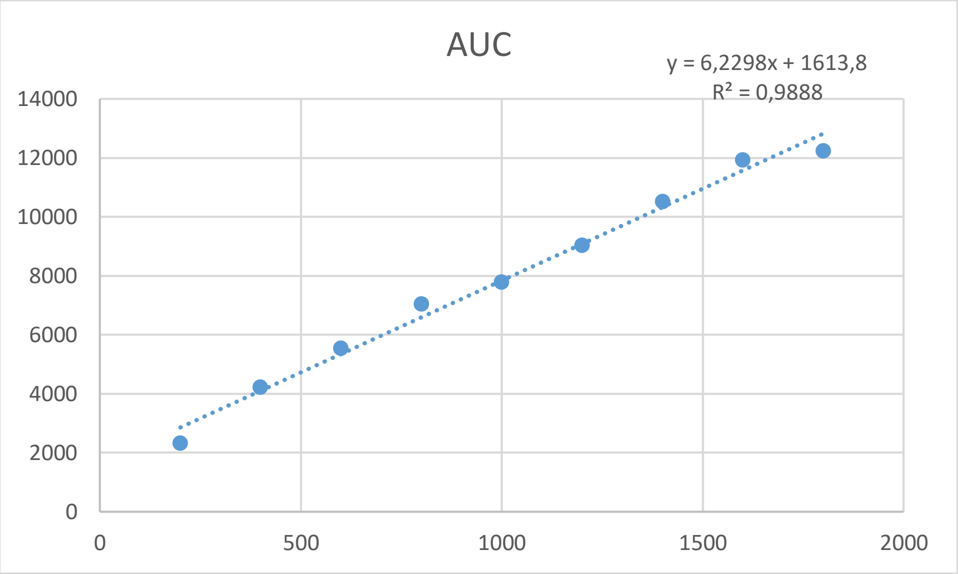


Figure 4. Plot of Area against Quercetin Concentration

Quantification:

2 µL of sample applied. It contains 433.15ng of Quercetin through linear equation. Therefore 10 ml (10000 µL) of diluted sample contains = 4081.710ng = 0.4332µg of Quercetin.

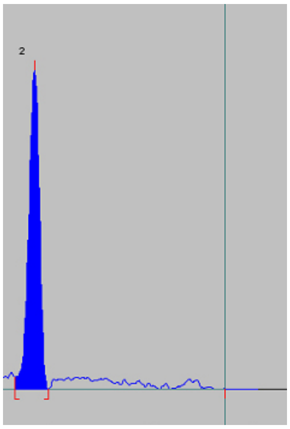


Figure 5. HPTLC chromatogram of quercetin.

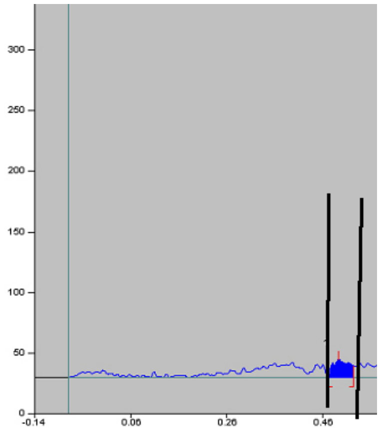


Figure 6. HPTLC chromatogram of sample.

Spectral Characterisation

Spectral analysis of all fractions were done by using FTIR, H^1 NMR spectroscopy and Mass spectroscopy. The isolated extract after performing TLC, were found that steroids and flavonoids are the main constituents showing promising antidiabetic activity. The spectral characterisations are described below,

Compound 1-
Predicted Molecular Formula of the compound 1- $C_{15}H_{12}O_8$
Mol. wt. 320.25
3-(3,4-dihydroxyphenyl)-7,8-dihydroxy-6-methoxy-1,4-benzodioxin-2(3*H*)-one

A. FT-IR SPECTRA

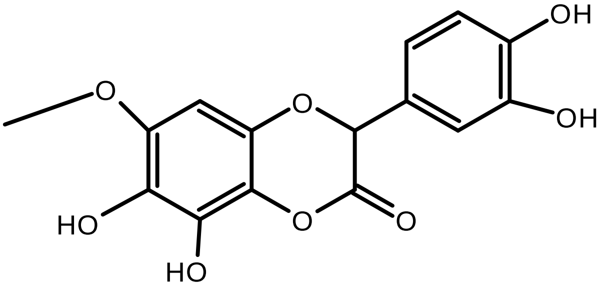


Figure 7. Predicted Structure -1

Table 6. Vibrational frequency of predicted structure

Vibrational frequency	Functional group
3163	(OH)
2928.17	Methoxy
1660.20	(C=0)
1500.46	(Phenolic C-O)
840.08	(strong aromatic absorption).

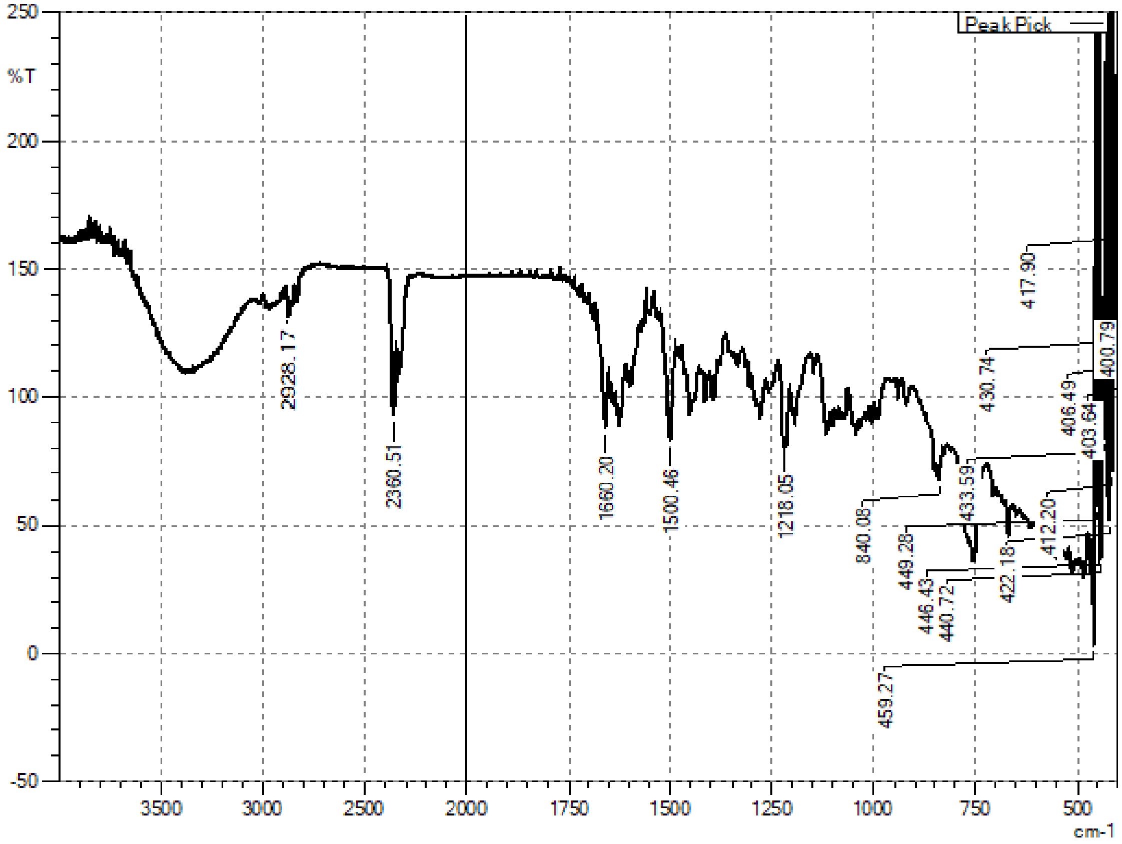


Figure 8. IR Spectra of predicted compound

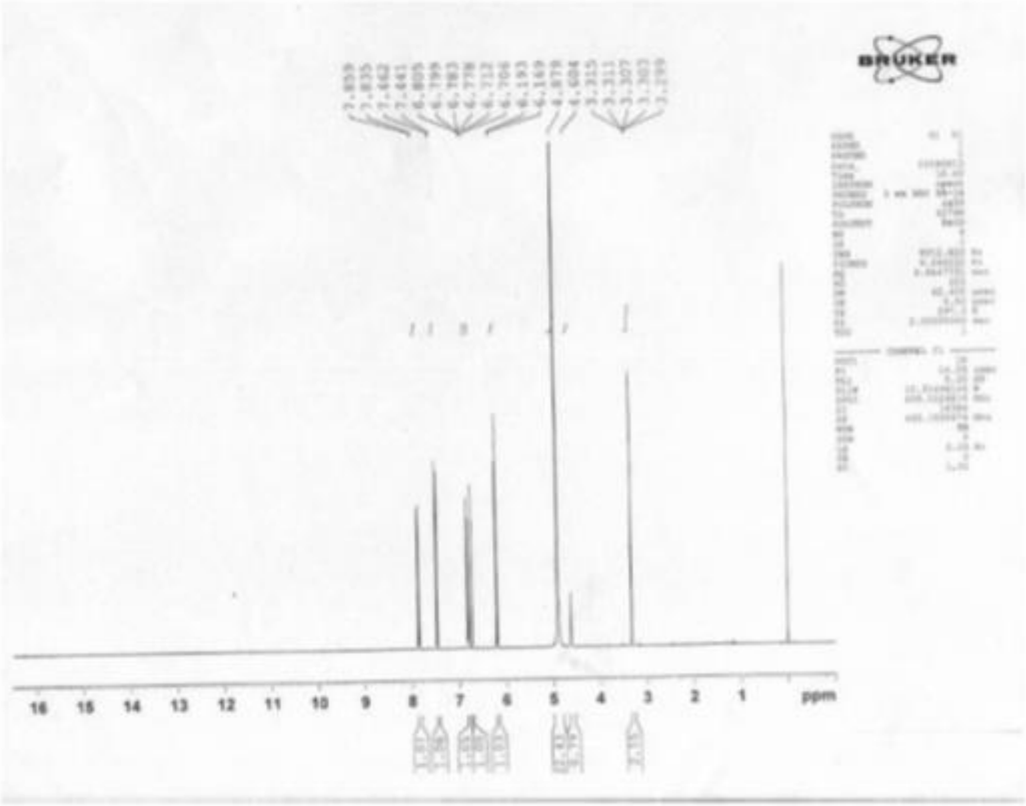
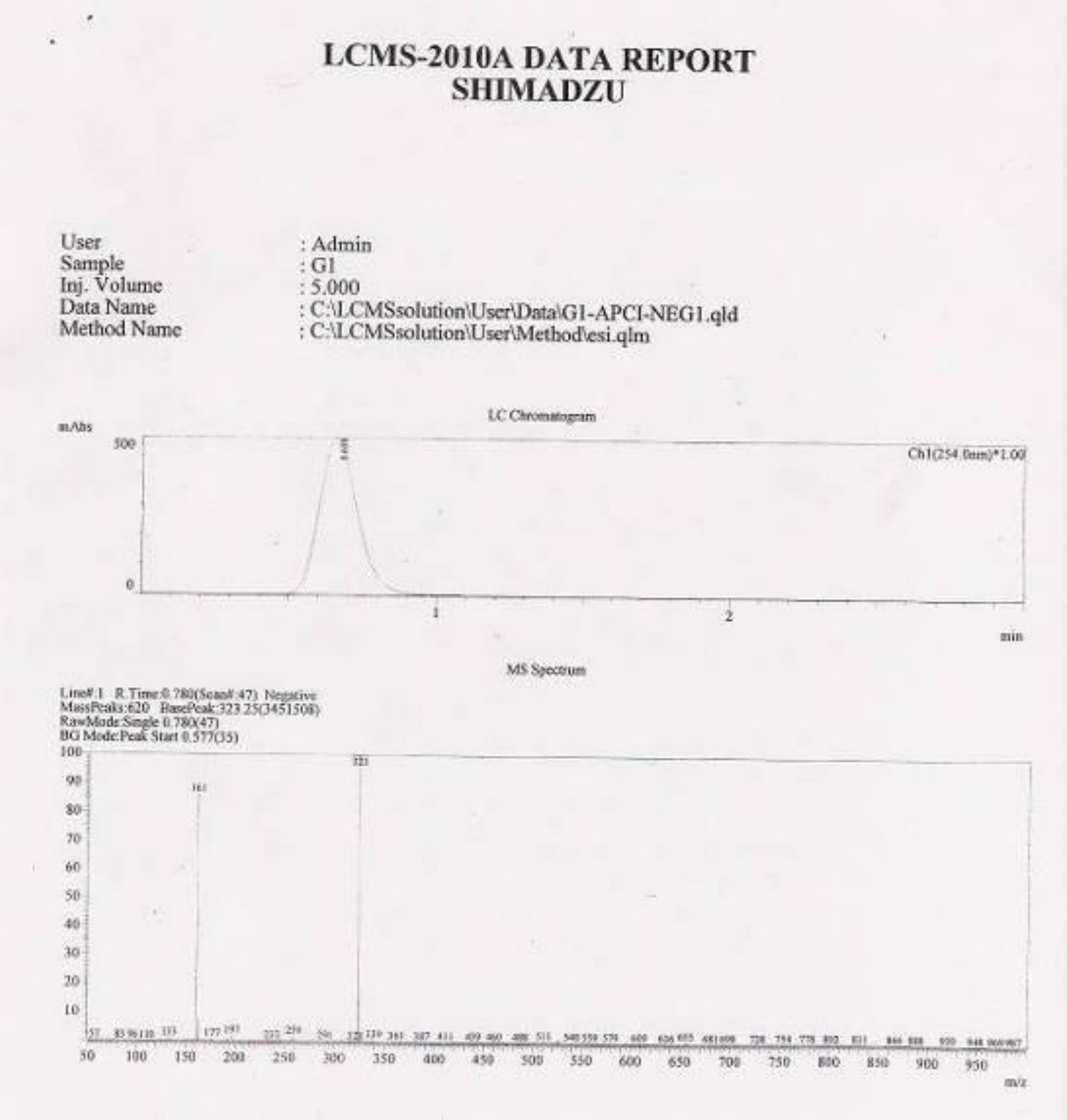


Figure 9. 1HNMR graph of predicted compound.

The 1HNMR spectrum of the isolated compound I showed nine prominent signals. A peak at

1. 163.7 was assigned to the corner function (C-2) of the compound.
2. At C-7, a downfield signal
3. 163.1 revealed the presence of a Hydroxyl function. C-8, C-3, C-4 A, C-6, C- 5, C-4 and C-8
4. 103.4, 112.3, 113.1, 114.5, 130.6, 146.0 and 157.2 respectively.



LCMS-2010A DATA REPORT
SHIMADZU

User : Admin
Sample : G1
Inj. Volume : 5.000
Data Name : C:\LCMSsolution\User\Data\G1-APCI-NEG1.qld
Method Name : C:\LCMSsolution\User\Method\esi.qlm

Line#:1 R.Time:0.780(Scan#:47) Negative
MassPeaks:620 BasePeak:323.25(3451508)
RawMode:Single 0.780(47)
BG Mode:Peak Start 0.577(35)

Figure 10. LCMS spectra of predicted compound

The LC MS showed a molecular ion peak at m/z 323 (M]+ and was in match with the proposed structure with $C_{15}H_{12}O_8$. Hence, it was concluded through spectroscopic studies that the compound I could be characterised as flavonoid derivative.

Conclusion

Since E. thymifolia contains more secondary metabolites than other plants, it may be inferred from the results that the entire plant offers therapeutic benefits. From ethanol and the whole-herb extract of E. thymifolia, a bioactive component (Total

1) was extracted, identified, and submitted to spectrum analysis, including IR, 1H-NMR, and mass spectroscopy. These substances have been discovered for the first time in this plant and might be an effective standardizing tool. Since ethanol extracts provide positive findings for the presence of phytoconstituents, these plants may be used directly to make medicines or to create new treatments for a range of pathological conditions. It may be necessary to conduct further study on the potential health benefits of this plant's phytochemicals. The HPTLC procedure was optimized with a view to quantify the samples extract. Initially ethyl acetate: acetic acid: formic acid: water in varying ratio was tried. The mobile phase ethyl acetate: acetic acid: formic acid: water solvent in 10:0.5:0.5:1.0 (v/v) gave good resolution with Rf = 0.65-0.70. The identity of the quercetin bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. The Rf's obtained for the said plant extracts closely replicated the Rf's found for standard quercetin, thus making it a significant fingerprint parameter.

Conflict Of Interest

The authors declare no conflict of interest.

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