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Research Article

Isolation, Characterization and Antifertility Activity of Ethanolic Extracts of *Tabernaemontana divaricata* R.Br. ex Roem. & Schult

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Abstract

Because of the unequalled availability of chemical variety, natural products from medicinal plants, whether as pure compounds or as standardised extracts, provide limitless prospects for new therapeutic leads. The search for medicinal medications from natural products has increased the demand for chemical variety in screening programmes, which has led to a rise in interest in culinary plants in particular. Medicinal botanicals and herbal medicines contain a variety of bioactive chemicals. Worldwide, ethnic medical systems use Tabernaemontana divaricata (T. divaricata Apocynaceae) because of its significant therapeutic value. The goal of this work was to separate and identify the bioactive components of T. divaricata's ethanol fraction. Using two different experimental animal models, the isolated fraction's anti-fertility efficacy was assessed: 1) Ethinyl estradiol was used as the standard to measure the estrogenic activity in immature female rats. Modifications in uterine weight and uterine histology are among the evaluating criteria. 2) Female Wistar rats experienced anti-implantation and early abortifacient action. Using column chromatography and gradient elution with several mobile phases, the isolation was carried out. The isolated chemical was analysed using spectroscopy. On the basis of spectrum analyses (UV, IR, 1H NMR, and MASS), the structure was determined. The extracted substance from the ethanol fraction of the root was identified as 25-Methyl-25-norethyl-Stigmast-5-en-3-one and 19, 20-dimethyl-betulinic acid-3-yl-3'-hydroxyhexadecanoate based on spectrum characteristics. The uterine weight and height of stoma and epithelial cells in the estrogenic model are significantly higher than those in the ethanolic fraction of T. divaricata, however the tumour size is significantly smaller in the anti-implant model. This is the first time that the molecule 25-Methyl-25-norethyl-Stigmast-5-en-3-one and 19, 20-dimethyl-betulinic acid-3-yl-3'-hydroxyhexadecanoate from the ethanol fraction of T. divaricata roots has been reported. These compounds have potential as sources for the treatment of a number of disorders.

Keywords: T*abernaemontana divaricata*, Estrogenic activity, Anti-fertility effect, Anti-implantation activity, Abortifacient effect, Isolation

INTRODUCTION

In nations like China [1] and India [2], where traditional medicine has been practised for thousands of years, plants have moulded its foundation. It is well known that many other civilizations use plants in their traditional medicine. The World Health Organisation estimates that approximately 80% of the world's population still relies primarily on traditional medicines for their primary healthcare, although plant products also play a major role in the health care systems of the remaining 20% of the population, who are primarily concentrated in developed countries [3]. These plant-based technologies are still very important in the medical field. In Asia, T. divaricata (Apocynaceae) is a plant that is extensively dispersed. It is used to treat bacterial, fungal, parasitic, and inflammatory illnesses in the traditional medical systems of several Asian nations. One of the plants utilised in Ayurveda, Chinese, and Thai traditional medicine is T. divaricata. T. divaricata has been used traditionally in many parts of the world to treat a wide range of conditions, including ulceration, arthralgia, asthma, diarrhoea, epilepsy, eye infections, fever, fractures, headache, inflammation, leprosy, mania, piles, paralysis, and rheumatic pain. It is also employed as an emmenagogue, purgative, anti-poison, anti-hypertensive, anti-helmintic, aphrodisiac, diuretic, anti-hypertensive, tonic for the brain and liver, and promoter of hair growth. There is mounting evidence that this plant has therapeutic effects and that its extracts may be employed as pharmacological interventions in a variety of disorders. A minimum of 66 indole alkaloids, as well as non-alkaloidal components such enzymes, flavonoids, hydrocarbons, phenolic acids, phenyl propanoids, steroids, and terpenoids, are found in this plant, according to phytochemical research on different portions. The primary secondary metabolites that have a wide range of physiological and pharmacological effects on live cells include alkaloids, flavonoids, and terpenoids [4]. Three, four, fourteen, and nineteen-tetrahydro-olivacine, 11-methoxy-N-methyl dihydropericyclivine, apparicine, isovoacangine, isovoacristine, tabernaemontanine, tabersonine, voaphylline, N-1-methyl-voaphylline, and vobasine are all found in T. divaricata flowers. Numerous T. divaricata alkaloids and their derivatives have not yet had their pharmacological effects

thoroughly investigated. Numerous methods of contraception have been utilised to support family planning, however due to the terrible side effects caused by synthetic steroidal contraceptives, emphasis is now being paid to native plants for potential contraceptive properties. The need to create a reliable and secure contraceptive pill derived from medicinal plants has arisen despite the popularity and effectiveness of contraceptives containing oestrogen and progesterone. Therefore, it is necessary to look for a suitable product made from local medicinal plants that might be used in place of pills [5]. An alternate method of birth control to the ones now used is the study of plant components having anti-fertility qualities. It would be extremely beneficial as a fertility regulating agent if it could be demonstrated that an oestrogen from a local source is active in people. Anti-fertility medications are known to function by interfering with or desynchronizing pre-ovulatory and pre-implantation processes. Anti-estrogenic action can also cause anti-fertility effects, which are frequently caused by estrogenic activity. In addition, certain plants, such as anti-ovulatory plants, interceptory and abortifacient plants, uterine tonus-inducing plants, and uterine stimulants, can operate as anti-fertility agents [6]. However, a lot of current medications are created using the information gleaned from phytochemicals. The isolation, characterisation, and determination of the structure of the new isolated compound from T. divaricata are covered in this article. The newly isolated compound's capacity to prevent conception was evaluated.

MATERIAL AND METHOD

Plant materials

T. divaricata roots were obtained from various localities in Bhopal (M.P.). Senior botanist Dr. Saba Naaz, a professor in the department of botany at Safia College of Arts and Science in Peer Gate, Bhopal, identified the sample. Specimen library of Safia College of Arts and Science, peer gate Bhopal, received a herbarium of plants that was donated. T. divaricata's specimen voucher number is 256//Saif/Sci/Clg/Bpl.

Chemical reagents

The Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India), and SRL Pvt. Ltd. (Mumbai, India) provided all the chemicals utilised in this work.The solvent and compounds utilised in this study were all of analytical grade.

Defatting of plant material

T. divaricata plant material was ground into a powder and air dried at room temperature. The material from the shade-dried plants was coarsely ground up and extracted with petroleum ether using the soxhlation process. The extraction process was continued until the material had been sufficiently defatted.

Extraction by soxhlation process

T. divaricata defatted powder was thoroughly extracted with ethanol using the soxhlation process. Over their boiling temperatures, the extract was evaporated. To determine the extractive yield, the dried crude concentrated extract was weighed. It was then placed to glass vials (6×2 cm) and kept in a refrigerator (4°C) until it was utilised for analysis [7].

Isolation of compounds from ethanol fraction of *T. divaricata*

To isolate the bioactive components, a T. divaricata ethanol extract was treated to silica gel column chromatography. Chromatography was performed using a vertical glass column constructed of borosilicate material. Before packing, the column was thoroughly dried after being cleaned with acetone. Silica gel (# 60-120) was used as the adsorbent during the wet packing procedure used to pack the column. Hexane was used to make the slurry, which was then put into the column. After combining them with a small bit of silica gel on top of the column, 1gm of extract was added. A pet was used to elute the column completely. ethanol (E4), water (E1), ether (E1), chloroform (E2), and ethyl acetate (E3).and the quantity of elutes were gathered. To determine the existence of a certain drug, TLC analysis was done on the concentrated fractions/elutes that had been collected. Using TLC, different phytocompounds were tested for in the fractions/elutes of the T. divaricata ethanolic extract obtained using silica gel column chromatography. The phytocompounds with identical Rf values were gathered into one fraction.

Thin layer chromatography

On TLC plates of silica gel 60 F254 pre-coated with layer thickness of 0.2 mm, thin layer chromatography was performed using various solvent systems, including toluene: ethyl acetate: formic acid (5:4.5:0.5), toluene: methanol (9:1), and n-hexane: ethyl acetate (8:2). Spots were manually applied using a capillary tube, plates were air dried using an air blower, and TLC chambers were produced with the appropriate solvent solutions at room temperature. Sulphuric acid solution spraying and UV light imaging were used to see spots on TLC plates. Values for Rf were computed.

Spectroscopic characterization

In ethanol, UV spectra of the isolated compounds were captured throughout a 200-800 nm scanning range, and the maximum absorbance of the compounds was calculated. A Shimadzu 1700 twin beam UV-VIS spectrophotometer was used to record the spectra. At SAIF, Chandigarh, a Waters Micromass Q-Tof Micro mass spectrometer was used to record EIMS (electron impact mass spectrum) in the positive mode. A pellet was formed using the isolate and 200 mg of FT-IR grade KBr. The sample pellet was put into the sample holder, and SAIF, Chandigarh, used a Model RZX (Perkin Elmer) FT-IR spectrometer to record spectra in the range of 375-7500 cm-1. At SAIF, Chandigarh, India, 1H NMR spectra were captured using an FT-NMR Cryomagnet Spectrometer 400 MHz (Bruker) and TMS as an internal standard. Methanol and DMSO were the solvents employed. Chemical shifts are displayed with TMS serving as an internal reference in ppm levels. Silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany) was used for column chromatography. Prior to use, chromatography solvents were distilled. Using TLC plates (Silica Gel G-60), thin layer chromatography (TLC) was carried out. **Animal used**

The animals used in this investigation were female Swiss albino mice (18–22 g), Wistar albino rats (150–200 g), and immature female Wistar albino rats (21–23 days old; 40–60 g). The mice were housed in polypropylene cages and acclimated for ten days under laboratory conditions, including a 12:12 h light/dark cycle at a room temperature of (25 10) oC. Standard rat pellets (Gold Mohur Lipton India Ltd.) were freely available to the animals, and water was always available under stringent hygiene guidelines. Animals were kept separate for each experimental group, and precautions were taken to make sure the same animals weren't used for more than one response. Prior to the experimental protocol, animals were acclimated to laboratory conditions for 48 hours to reduce any non-specific stress. The institutional animal ethical committee (IAEC) approved the use of animals in research. The Institutional Animal Ethical Committee is registered under the number (Reg. No. 1824/PO/RcBi/S/15/CPCSEA), and the animal experiment proposal is registered under the number IAEC-PBRI/IAEC/PN-21028.

Acute oral toxicity

The Ministry of Social Justice and Empowerment, Government of India, conducted the acute oral toxicity tests in accordance with the Organisation for Economic Co-operation and Development's (OECD-423) recommendations [8, 9].

Antifertility study

The estrogenic, anti-implantation, and abortifacient effects of plant extracts were used to assess their antifertility potential [10].

Estrogenic pharmacological screening model

Wister strain immature female rats, weighing 40–60 g, were used. They were 21–23 days old. Six groups each had six different animals. The following was done to the various groups:

Group I: control (saline solution) p.o.

Group II: reference standard (ethinyl estradiol 0.02 mg/kg, p.o.)

Group III: ethanolic fraction of *TD* (200 mg/kg, p.o.)

Group IV: ethanolic fraction extract of *TD* (400 mg/kg, p.o.)

All of the animals were decapitated, and the uteri were removed, cleaned of sticky tissue, wiped on filter paper, and immediately weighed on a sensitive balance. The therapy was given for six days, beginning 24 hours after the last treatment. The tissues were fixed in Bouin's fixative for 24 hours before being dehydrated in alcohol and embedding in paraffin. The paraffin blocks were divided into 6 sections and stained with hematoxylineosin solution (H and E Stain) for histological examinations.

Anti-implantation pharmacological screening model

Rats with proven fertile males were kept in a 2:1 ratio with female rats in the proestrus. The female rats were examined the following morning for indications of copulation. Male partners were isolated from animals whose vaginal smears contained thick masses of spermatozoa. Only rats with conventional estrous cycles were used in the investigation. Three groups of six animals each were formed from the animals. The corresponding groups received the following care:

Group I: control (saline solution) p.o.

Group II: ethanolic fraction of *TD* (200 mg/kg, p.o.)

Group III: ethanolic fraction extract of *TD* (400 mg/kg, p.o.)

Statistical data

The mean SEM is used to express all values. One-way analysis of variance (ANOVA) was used to statistically analyse the means, and values of P 0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

The T. divaricata root component of the raw ethanol is used for bioassay orienting. Silica 60 gel and column chromatography were used to extract the root extract from the ethanol (0.063-0.2mm match size). Using a chromatographic extraction method, crude ethanol was extracted from a column using ether (E1), chloroform (E2), ethyl acetate (E3), ethanol (E4), and water. The chromatographic column extracted from the crude ethanol root of T. divaricata, collected in separate flasks (TLC), was examined. This was carried out using the ethanol/chloroform cell compound (1: 4) on silica gel plates (Merck, 60 F254). The features of each element were represented by dots that could be seen and identified using ultra violet light with a wavelength of 254 nm. Finally, evaporators with the same content are combined and concentrated using a rotating evaporator Table 1. All fractions were gathered, with the exception of fractions 14 and 16, and thin layer chromatography was performed on all of them. After thin layer chromatography, all fractions, barring fractions 14 and 16, displayed two or more spots in ethanolic solvent. The fact that fractions 14 and 16 only have one point each indicates that they were picked for additional analysis and categorization. Thin layer chromatography was used to identify the active ingredients after a chemical examination of the T. divaricata root was completed. Petroleum ether, chloroform, ethyl acetate, ethanol, and water were used to elute the column.

Table 1: Yield of the ethanolic extract and its fractions

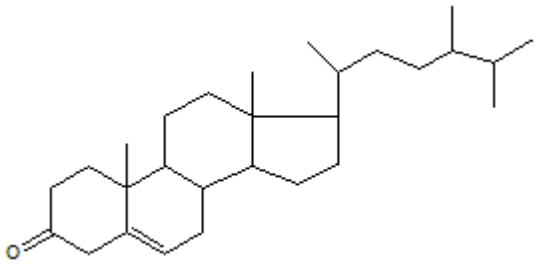
S. No.	Fraction	Description	Yield (% w/w)
1	Pet. Ether Extract	Reddish Brown Residue	12.3
2	Chloroform Fraction	Green Residue	0.534
3	Ethyl Acetate Fraction	Dark Green Residue	4.246
4	Ethanol Fraction	Reddish Brown Residue	5.167
5	Fraction 1	Light Green Powder	0.0547
6	Fraction 2	Light Green Powder	0.00733
7	Fraction 3	Light Green Powder	0.0413
8	Fraction 4	Light Green Powder	0.0861
9	Fraction 5	Light Green Powder	0.825
10	Fraction 6	Light Green Powder	0.66
11	Fraction 7	Brown Powder	1.938
12	Fraction 8	Brown Powder	0.166
13	Fraction 9	Brown Powder	0.821
14	Fraction 10	Brown Powder	4.7
15	Fraction 11	Brown Powder	0.44
16	Fraction 12	Brown Powder	0.92
17	Fraction 13	Brown Powder	2.82
18	Fraction 14	Brown Powder	0.342
19	Fraction 15	Brown Residue	0.529
20	Fraction 16	Green Powder	0.356
21	Fraction 17	Green Powder	0.0437
22	Aqueous Fraction	Brown Residue	62.77

Fraction 14

Fraction 16

Unknown compound 1

The substance has an easy dissolution rate in methanol, ethyl acetate, and chloroform, and it has a brown solid with a melting point of 170 °C. The chemical formula is C28H46O1, and the molecular weight was found to be 398. TLC tests using a variety of solvent systems were carried out to confirm the compound's purity.



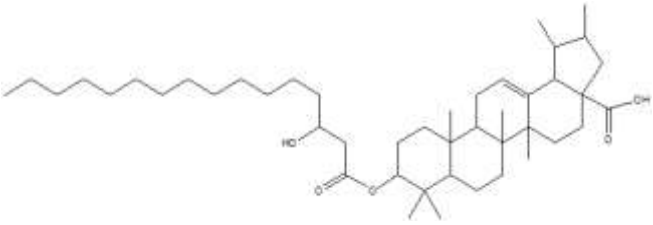
25-Methyl-25-norethyl-Stigmast-5-en-3-one

Spectral Characterization

Data from the molecule's UV, FTIR, 1H-NMR, 13C-NMR, and mass spectra were examined to determine its structure. According to UV analysis, it increases to UVmax: 260 nm. The presence of OH groups was indicated by the absorption bands in Vmax3500, 3025, 1736, 2925, and 2859 in the FTIR spectrum, as well as by aromas such as keto, methyl, and methylene from the 1H spectrum and the NMR. (7.26, 1H, s), a sweet group (5.34, 1H, s), five methyl groups (1.92, 3H, d), 1.48, 3H, s), (1.12, 3H d), (1.02, 3H d), (1.02, 3H, s), and 0.86, 3H, s), and a seven-membered ring (7.26, 1H, s). Scented Groups 124, C-2, 77, C-1, 76, C-2, 46, C-3, 39, C-4, 29, 27, 25, 24, and 24 techniques for 13C NMR Methyl and methylene carbons' mass spectra, which represent high values of m/z 887 mean high ion cell and ion separated by 613, 585, and 543 peaks, respectively, and 371 and 159. Compound 1 is designated as 2-8-4, 5-dihydroxy-6-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl by the IUPAC. -2,5,6-trimethoxy-4-oxo-3,4-dihydro-2H-chromen-7-yl) 3',4'-dihydroxyphenyl -6 hydroxymethyl -7-ethoxy-5-hydroxy-8-3-hydroxy-6 -4H-chromen-4-one (-4,5-methoxy-tetrahydro-2H-pyran-2-yl).

Unknown compound 2

It was possible to create colourless crystals with a melting point of 196–197°C. In DMSO (dimethyl sulfoxide), it was readily soluble. The molecular weight and chemical composition were discovered to be 696 and C45H76O5, respectively. To verify the purity of the compound, TLC tests were conducted utilising a range of solvent systems.



19, 20-dimethyl-betulinic acid-3-yl-3'-hydroxyhexadecanoate

Spectral Characterization

The structure of the molecule was determined by analyzing its UV, FTIR, 1H-NMR, 13C-NMR, and mass spectrum data. UV detection was detected at a wavelength of 271 nm. The absorption bands in Vmax3649, 3392, 3136, 2921, and 1772 in the FTIR spectrum showed the presence of OH, - NH, aromatic, keto, and methyl groups, respectively, from 1H-NMR spectra showed the presence of a single OH. group (7.26), two fragrant groups (5.37), (5.09, 1H, s), and six methyl groups (2.07, 3H, s), (2.04, 3H, d), (1.9, 2H d), (1.8, 3H, d), (1.2, 3H, s), and (1.0, 2H, d), and two methylene groups (0.86, 3H, d) and two methylene groups. The signal for the C-2 keto group, signal 124, and the 13C NMR signals for the aromatic groups 77, C-1, 78, C-2, 48, C-3, 38, C-4 methyl and methylene, carbon 29, 26, 24, and 23 are all displayed. The ion cell height is indicated in the mass spectrum by a height of m/z 754. It was discovered that Compound 2's IUPAC name is 2. -7- (5-ethoxy-4-oxo-2-phenylchroman-7-yloxy) -5-hydroxy-8-6-(hydroxymethyl) (2,4-diethoxy-5-hydroxyphenyl) -4H-chromen-4-one, 5-methoxy-tetrahydro-2H-pyran-2-yl).

Table 2: Functional groups detection by FTIR

	Frequency	Functional groups
Compound 1	3500	- OH
	3023	Aromatic
	1736	Keto
	2925	Methyl
	2859	Methylene
Compound 2	3649	-OH
	3392	-NM
	3136	Aromatic
	2921	Methyl
	1772	Keto

Table 3: ^{1}H and ^{13}C NMR Chemical Shifts for unknown compound 1 and 2

	^{1}H NMR		^{13}C NMR	
	Chemical Shift (δ)	Spin spin Splitting	Chemical Shift (δ)	Functional group
Compound 1	7.26	1H, s	124	Keto
	5.34	1H, s	77	Aromatic
	1.92	3H, d	76	Aromatic
	1.48	3H, s	46	Aromatic
	1.12	3H, d	39	Aromatic
	1.02	3H, s	29	Methyl
	0.86	3H, s	27	Methylene
Compound 2	7.62	1H, s	206	Keto
	5.37	1H, s	114	Aromatic
	2.07	3H, t	120	Aromatic
	1.9	3H, d	135	Aromatic
	1.0	2H, d	144	Aromatic
	0.96	3H, t	30	Methyl
	0.87	3H, t	39	Methyl
	0.82	3H, m	40	Methylene

Table 4: Effect of *T. divaricata* ethanolic fraction on uterine weight of immature female rats

Group	Extracts / Drug	Dose (mgkg^{-1})	Uterine weight (mg)
I	Control (vehicle)	--	219.57 ± 22.79
II	Ethynilestradiol (Standard)	0.02	289.34 ± 23.50*
III	Ethanolic fraction	200	299.19 ± 09.98***
IV	Ethanolic fraction	400	314.39 ± 10.52**

Values are mean±SEM *(n=6).* *P < 0.05, **P < 0.01, ***P < 0.001 as compare to control group.

Table 5: Effect of *T. divaricata* ethanolic fraction on anti-implantation and early abortifacient activity in rats ($x \pm s$, $n = 6$)

S. No.	Treatment/dose	% Anti-implantation activity	% Early Abortifacient activity	% Anti-fertility activity
1	Vehicle control	0	0	0
3	Ethanolic extract 400 mg/kg, p.o.	41.98 ± 0.29	4.32 ± 2.24***	64.17 ± 0.39
5	Aqueous extract 400 mg/kg, p.o.	44.71 ± 0.14	9.09 ± 4.11***	44.68 ± 0.19

** *P* <6 0.01, ****P* < 0.001 *vs* vehicle control

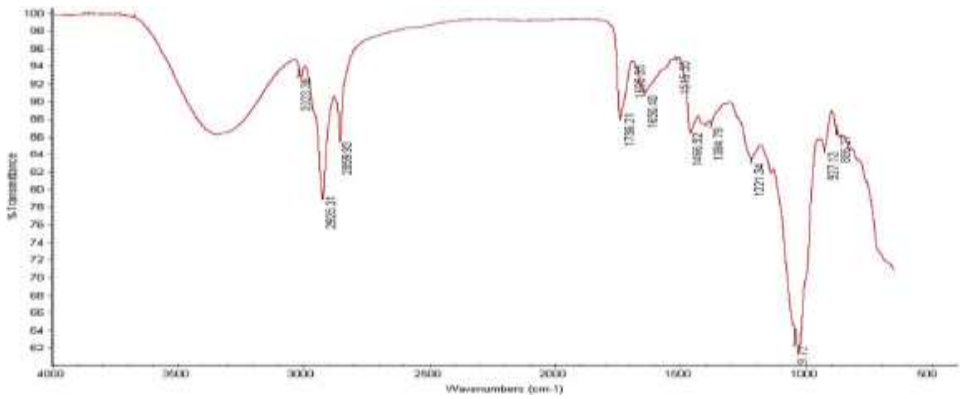


Figure 1: FTIR spectrum of unknown compound 1

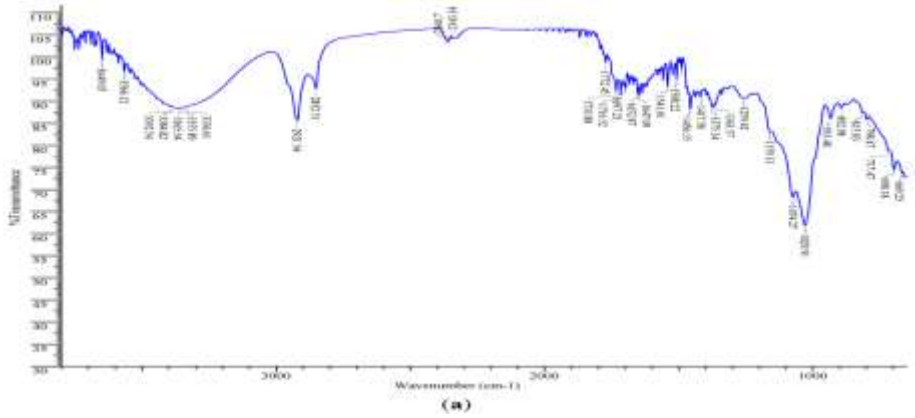


Figure 2: FTIR spectrum of unknown compound 2

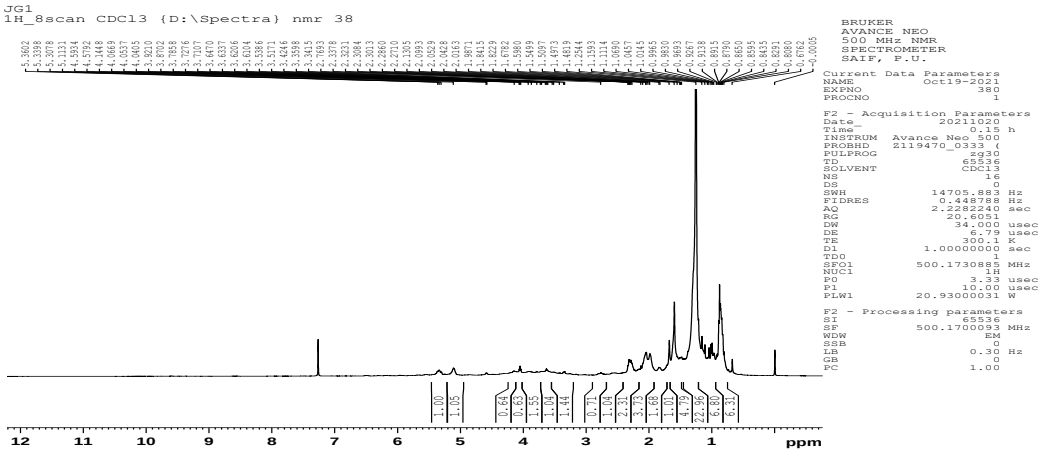


Figure 3: ^{1}HNMR spectrum of unknown compound 1

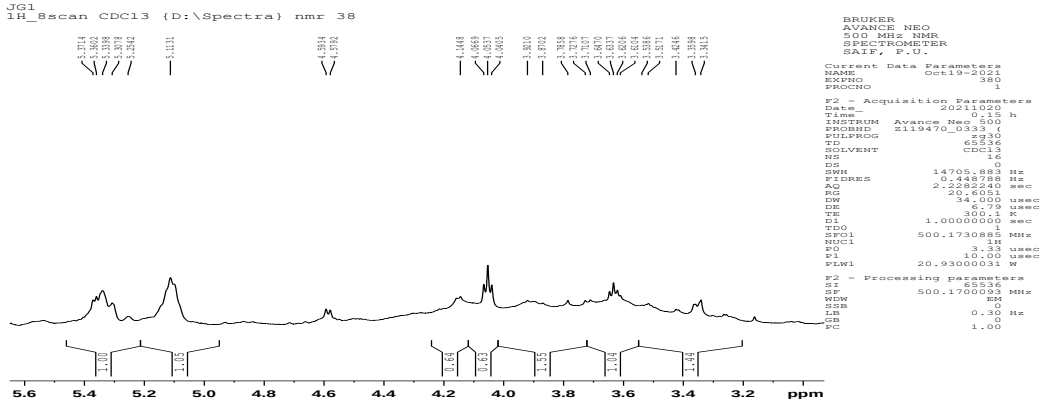


Figure 4: ^{1}H NMR spectrum of unknown compound 2

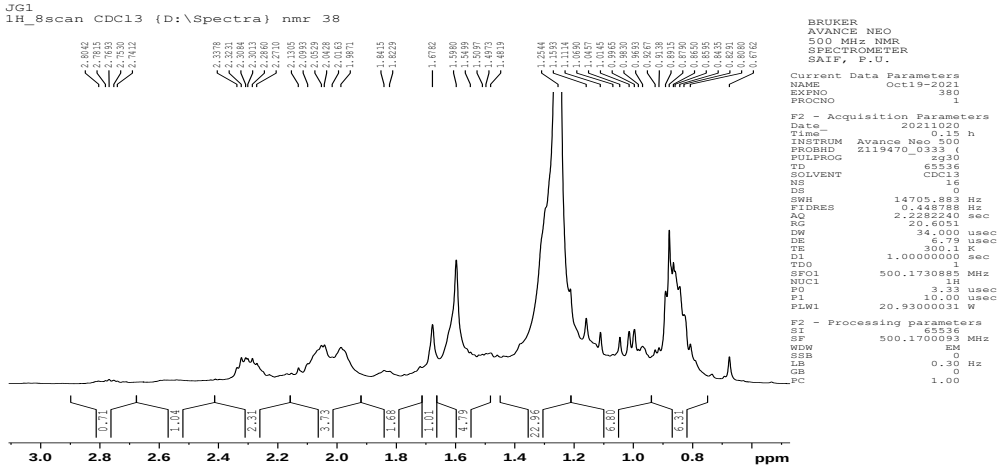


Figure 5: ^{13}C NMR spectrum of unknown compound 1

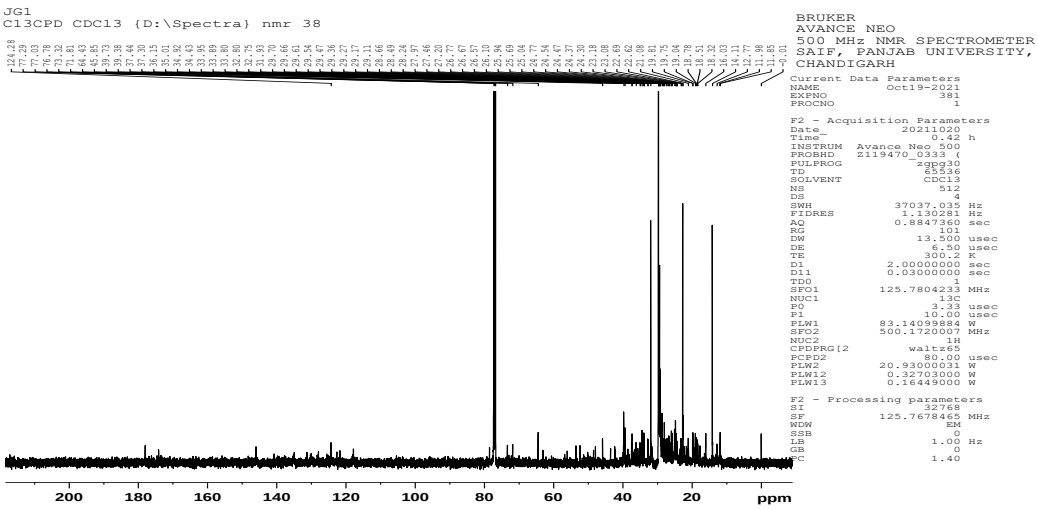


Figure 6: ^{13}C NMR spectrum of unknown compound 2

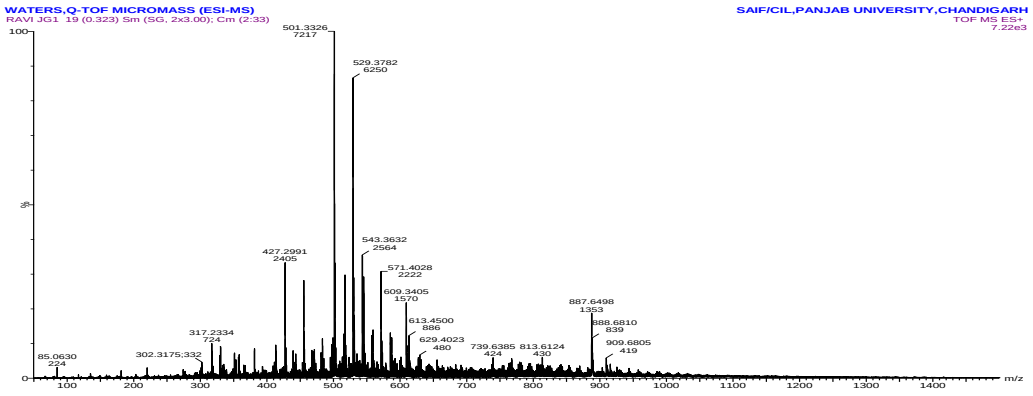


Figure 7: Mass spectrum of unknown compound 1

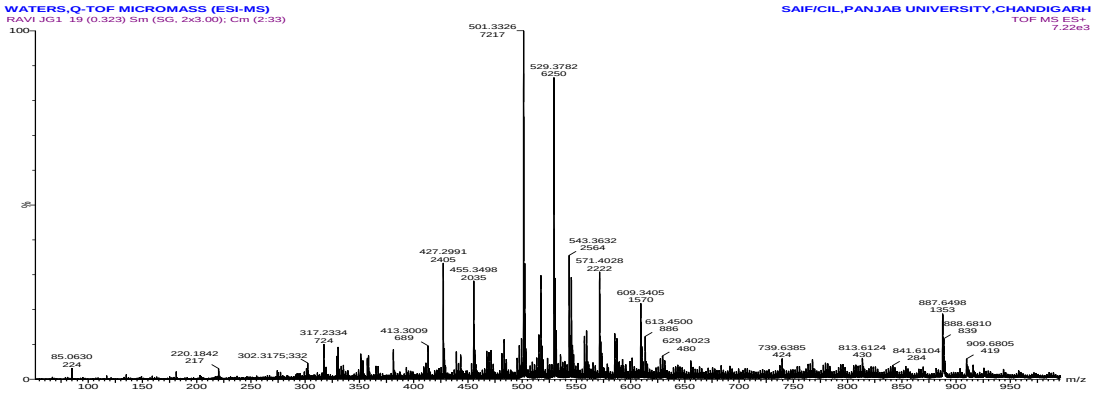


Figure 8: Mass spectrum of unknown compound 2

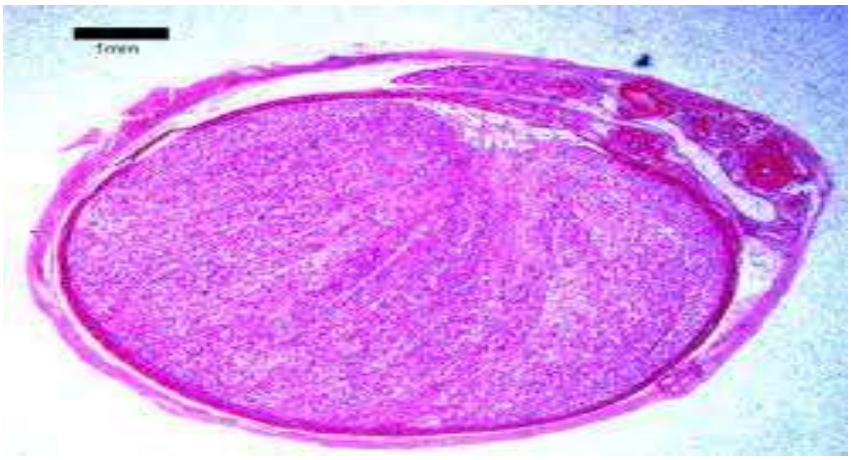


Figure 9: Photograph showing section of uterus indicating surface epithelium with no secretary activity (Control group)

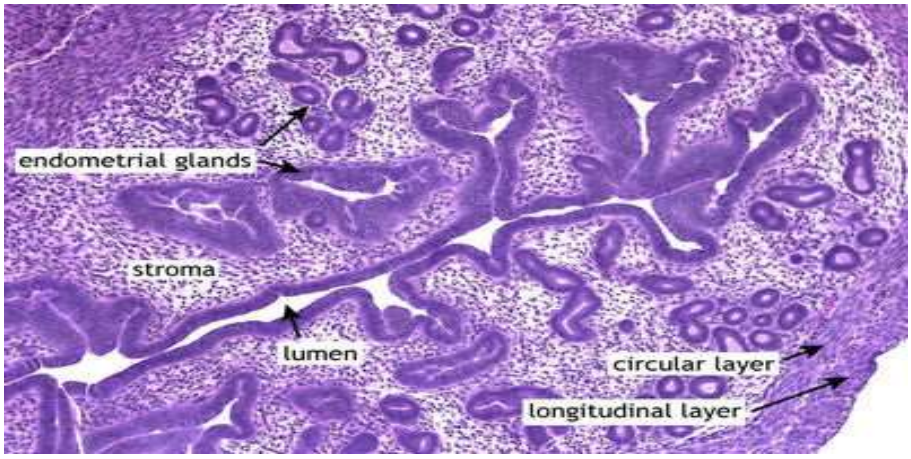


Figure 10: Photograph Ssowing section of uterus indicating increasing height of luminal epithelium (Ethynil estradiol)

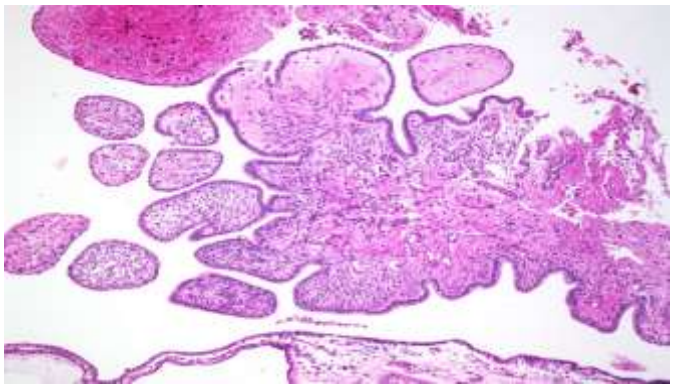


Figure 11: Photograph showing section of uterus indicating moderate increase in height of luminal epithelium with moderate stimulation of uterine weight (ethanolic fraction 200 mg/kg)

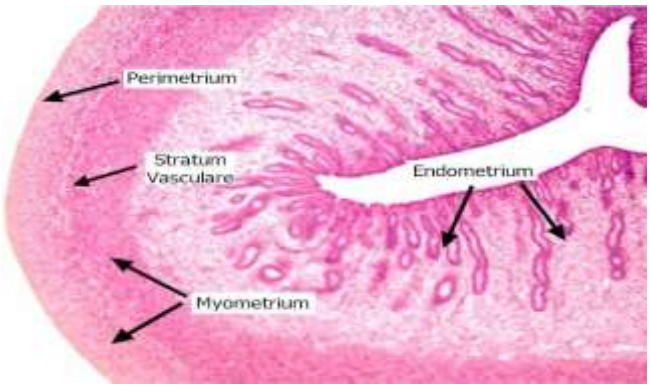


Figure 12: Photograph showing section of uterus indicating moderate increase in height of luminal epithelium with stimulated uterine glands (ethanolic fraction 400 mg/kg)

CONCLUSION

The ethanol fraction of the root of the apocynaceous plant T. divaricata was successfully used for the phytochemical analysis. It was determined that compounds 1 and 2 are 25-Methyl-25-norethyl-Stigmast-5-en-3-one and 19, 20-dimethyl-betulinic acid-3-yl-3'-hydroxyhexadecanoate from these physical, chemical, and spectral data. We might infer from these preliminary findings that the ethanolic fraction of T. divaricata exhibited notable anti-fertility activity.

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