ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Isolation and Characterization of Stigma-5,22dien-3-O-b-D-Glucopyranoside from the ethanolic root extract of *Operculina turpethum*

Veena Sharma*[1], Manu Singh[2]

1. Department of Bioscience and Biotechnology, Banasthali University, Banasthali 304022, Rajasthan, India.

2. Department of Bioscience and Biotechnology, Banasthali University, Banasthali 304022, Rajasthan.

Manuscript Info

Manuscript History:

Received: 14 September 2013
Final Accepted: 23 September 2013
Published Online: October 2013

Key words:
Chromatography, Glycoside, Isolation, *Operculina turpethum***,** Stigma-5, 22dien-3-O-b-D-Glucopyranoside, HPLC

Corresponding Author

Abstract

Secondary metabolites play very crucial role in the treatment of various ailments in human body. Steroid Glycoside represents essential group of secondary metabolites which exhibits a broad spectrum of pharmacological profile. Current study was undertaken for the isolation and characterisation of an important glycoside from *Operculina turpethum*. The ethanolic root extract of the plant was analysed by Thin Layer Chromatography and Column Chromatography. The isolated compound was crystallised from the desired fraction and was subjected to HPLC (High Performance Liquid Chromatography) and spectroscopic studies.The structural characterization of the isolated compound was done using IR ,^{1}H NMR,UV and LCMS techniques which confirmed the structure of compound. Acid hydrolysis was carried out using 5% H_2SO_4 and the identity of the compound was confirmed. Roots of the plant *Operculina turpethum* yielded a pharmacologically important Glycoside, Stigma-5,22dien-3-O-b-D-Glucopyranoside which can be further utilised for several health formulations.

Introduction

Phytosterols are the sterols of plant origin, which have been shown to possess cholesterol lowering (Jong et al., 2003) as well as anticancer property (Von et al., 1998). Sterols are known to reduce the blood plasma cholesterol. These sterols are believed to interfere the esterification of cholesterol. It has been reported that these sterols usually bind with the intestinal mucosal cell and interfere the flow of cholesteryl ester through an interaction with lipoprotein towards the blood vessels (Pamela et al., 1994). Since the morbidity and mortality from cardiovascular disease have been dramatically reduced by the use of cholesterol lowering drugs (statins), the interest in plant sterols lies in their potential to act as a natural preventive dietary product. The sterols have anticancer and immune-modulating properties (Ling and Jones, 1995; Pegel, 1997; Moreau et al., 2002; Bouic, 2001). Sterols in foods exist as free sterols, glycosides, fatty acid esters and acylated steryl glycosides (Grunwald and Huang, 1989). It was suggested that up to 80% of total phytosterols are glycoslyated in foods (Jonker et al., 1985).

Glycosides play numerous important roles in living organisms.The use of glycoside containing plants for medicinal purposes was first reported in ancient texts more than 1500 years ago. Sterol glucosides have also been added to dietary supplements designed to improve lipid metabolism and immune function (Bouic et al., 1999). Glycoside is a molecule in which sugar is bound to another functional group via a glycosidic bond. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside. The glycone and aglycone portions can be chemically separated by hydrolysis in the presence of acid (Brito and Marco, 2007). New findings within the past five years have revealed these compounds to be involved in complex cell-signal transduction

mechanisms, resulting in selective control of human tumors but not normal cellular proliferation and they represent a promising form of targeted cancer chemotherapy.

Operculina turpethum which is commonly known as *trivit,* belongs to the family Convolvulaceae. It is widely grown throughout India and it is occasionally cultivated in gardens as an ornament. It has been used as a folk medicine in many countries to treat constipation, jaundice, rheumatism, chronic gout, piles and tumors,obesity and many other diseases. The bark of the plant contains a glycosidic resin, which has the insoluble glycoside turpethein (Sharma and Singh, 2012). The plant contains various secondary metabolites including saponins, flavonoids, glycosides, phenolics and it also contains some amount of essential oil, glucose and fructose (Sharma and Singh, 2012). Upon literature survey it was found that Four new dammarane-type saponins, operculinosides A–D (1–4), were isolated from the aerial parts of *O. turpethum* (Ding et al., 2011). *Operculina turpethum* when consumed may add to the antioxygenic potential and hence may prove useful in protection against oxidative stress caused by a large number of xenobiotics including carcinogens.

There has been an increasing trend towards replacement of synthetic pigments with natural plant products in the last 20 years. Population rise, traditional inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for various diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. The use of natural products with therapeutic properties is as ancient as human civilisation and, for a long time, plant products were the main sources of drugs. The reasons for this is that pure compounds are easily obtained and structural modifications to produce potentially more active and safer drugs can be easily performed. Several bioactive compounds have been isolated from the plant sources such as digoxin, digitoxin, quercetin, morphine, taxol etc. (Ghani, 1998) which have different useful pharmacological properties. Hence the aim of the study was to isolate the secondary metabolite from the plant *Operculina turpethum*.

Experimental Methodology

Collection of the plant

Operculina turpethum was collected from Pharmacological garden of CCSHAU Hisar, Haryana, India in the month of November 2012. The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra Rohtak, Haryana, India.

Preparation of Ethanolic Extract

The freshly collected *Operculina turpethum* roots were dried in shade and coarse powder was extracted. Dried powdered material was placed in the Soxhlet thimble with ethanol in 500 ml flat bottom flask. Further refluxed for 18 h at 80^{o}C for two days . Collected solvent was cooled and poured in a glass plate. The marc was dried in hot air oven below 50^{o}C for 48 h and kept in dissector for 2 days (Sharma and Singh, 2012). The yield of the extract was 12.5% w/w of powdered plant material for further exploration. Collected dried extract was stored at 50^{o}C in air tight containers.

Chromatographic Separation

Chromatography is an analytical method that is widely used for the separation, isolation, identification, and quantification of components in a mixture. Components of the mixture are carried through the stationary phase by the flow of a mobile phase. Separations are based on differences in migration rates among the sample components (Harwood and Moody, 1989).

TLC

Thin layer chromatography (TLC) was employed in this study to analyze the compounds present in the crude plant extract using the solvent system of mobile phase as Chloroform : Methanol (18:1;15:1;12:1;9:1;7:1;4:1, 4:2;3:2) with increasing polarity which was chosen by trial and error and previously published reports. The mobile phase with the maximum number of spots was selected. All TLC separations were performed at room temperature, i.e. 18-23°C. After sample application the plates were placed vertically into a solvent vapour saturated TLC chamber. The spotting line was about 0.5 cm from the developing solution. After the mobile phase had moved about 80% from the spotting line, the plate was removed from the developing chamber, dried and developed with iodine (Reich and Schibli, 2007).

Column Chromatography

Column chromatography is generally used as a purification technique to isolate the desired compounds from a mixture. Here, the crude extract was employed to get the pure fraction from it. Column chromatography of crude ethanolic extract was conducted using silica gel (Mesh 60-120).

a) Adsorbtion of the extract: The crude ethanolic extract for fractionation was adsorbed on stationary phase in ratio 1:1.

b) Packing the Column : The column was packed with silica gel by wet packing method wherein a padding of cotton was placed at the bottom of the column and then it was filled with eluting solvent of the lowest polarity (hexane). Then the required amount of stationary phase (silica gel) was poured into the column to form a bed of silica. The extract was then poured on to the bed of silica, a layer of cotton covered it again and more amounts of solvents were poured over it. The extract was then subjected to column chromatography using different solvent systems. Silica gel was used as stationary phase. Column chromatography was done by using a glass column. The dimension of the column was 5x15 cm in height and 4 cm in diameter.

c) Saturation of the column*:* The charged column was left for 1h for complete saturation and removal of air bubbles to make the bed static.

d) Elution: The charged column was then eluted with different mobile phases with gradual increase in polarity.

Isolation

Various fractions of extract were collected and further chromatographed, to know the no. of constituents present. The solvent was recovered from the collected fractions by simple distillation. All the concentrated fractions were subjected to TLC for the identification of the desired bands. The different solvents used were Hexane, Petroleum ether, Toluene, Diethyl ether, Chloroform and methanol with different solvent concentration ratios based on increasing polarity.

HPLC

An isocratic HPLC (Shimadzu HPLC Class VP series) with one LC-10 AT VP, pump (Shimadzu), variable wavelength UV-Visible Detector SPD-10A VP, Grace Smart RP 18 column (250 X 4.60 mm) was used. The mobile phase components Methanol:water (70:30) were filtered through 5 micron membrane filter before use, and pumped from the solvent reservoir at a flow rate of 1 ml/min.

Test for Glycosides and Steroids

The presence of the glycoside was analysed with the various tests including Legal's test, Baljet's test, Keller-Killiani test, Borntrager's test whereas the test used for steroid was Salkowski test (Harborne, 1998).

Spectroscopic Analysis

The identification of the plant constituent once it has been isolated and purified is achieved by determining its description, solubility, melting point and spectral properties.

UV: The crude extract of the plant was analysed with UV spectrophotometer for the absorption wavelength. UV absorption spectra was recorded on a Perkin-Elmer LAMBDA 35 UV system. The wavelength of the maxima of the absorption spectrum so obtained was recorded (in nm). UV spectral measurement is important in the identification of many plant constituents, for monitoring the eluates of chromatographic columns during purification of plant products and for screening crude plant extracts for the presence of particular classes of compounds.

IR: Characterization of the chemical structure was done by using Fourier transform infra red (FTIR) technique. The specific chemistries and orientation of the structure will be known from the IR spectrum. The infra red spectra was measured using the Perkin Elmer FTIR model 2000 spectrophotometer. The absorption spectrum was recorded in the wave number range from 4000 cm^{-1} to 650cm^{-1} and the presence of functional groups in the isolated compound was determined.

NMR: Spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with matter. NMR spectroscopy is the study of interaction of radio frequency (RF) of the EMR with unpaired nuclear spins in an external magnetic field to extract structural information about a given sample. Proton NMR is a plot of signals arising from absorption of RF during an NMR experiment by the different protons in a compound under study as a function of frequency (chemical shift). ^{1}H NMR spectra was obtained using a JEOL JNM-ECA400 (400MHz).

ARAS-LCMS: Liquid chromatography coupled with mass spectrometry (LC/MS) is a powerful technique for the analysis of complex botanical extracts. HPLC is efficient in separating chemical compounds in a mixture, and MS provides abundant information for structural elucidation of the compounds. The LC/MS analysis provides the molecular weight information for the components of the extract. MS dissociations give further structural information on the target compounds (Chen et al., 2007). Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable.

Acid Hydrolysis

Acid hydrolysis cleaves the glycosidic bond and has been used by some researchers to analyze total sterols in foods (Jonker et al., 1985). To a solution of each compound (250 μg) in MeOH (1 mL) was added 1 mL of 5% H_2SO_4 and the mixture was refluxed for 8 h. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 5 mL) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugar (glucose) using the TLC systems EtOAc/pyridine/ethanol/H2O (8:1:1:2), EtOAc/*n*butanol/ water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1) (Bedir et al., 2001; Huan et al., 1998).

Results and Discussion

Prior to the practical analysis, a proper study of the botanical information, chemical background, and ethnobotany of the investigated plants was carried out. The isolation of the compound started with extracting of the mixture of secondary metabolites present in the plant material. The preliminary phytochemical analysis of the crude extract was carried out to determine the presence of different phytochemicals.

TLC

The maximum number of spots were observed with the mobile phase as Chloroform: Methanol (9:1) The chromatograms when developed in iodine chamber yielded four spots respectively at R_f (0.48, 0.67, 0.76, 0.96) with light brown colour indicative for steroidal nucleus. The spot with the R_f of 0.48 was considered to be an indication for the presence of glycoside in the crude extract, as this R_f corresponds to glycosides with the same mobile phase as reported from previously published reports (Khatun et al., 2012).

Column Chromatography

The column was run with mobile phase Chloroform : Methanol (9:1) which gave 12 fractions. These were further analysed by TLC using different mobile phases of increasing polarity and the best spots were observed in the fraction number 4 which was further re-chromatographed with the solvent system Chloroform : Methanol (4:1). It gave 6 different fractions, which were further analysed using TLC. The fraction number 2 was selected with the single spot and Rf value of 0.49 which was comparable with the previously published reports. The fraction was run on TLC with different polarity mobile phases to confirm the singularity of the isolated product.

HPLC

HPLC of isolated compound from ethanolic extract as well as the crude extract was carried out to confirm its nature by analyzing HPLC chromatograms. The HPLC detection chromatogram of crude extract is shown in (Figure 1). As shown in the figure, the crude extract used in present study is highly complex, containing a number of compounds. The sharpness of peaks, its retention time (Retention time), height and percent area were recorded as shown in (Figure 1 and 2).

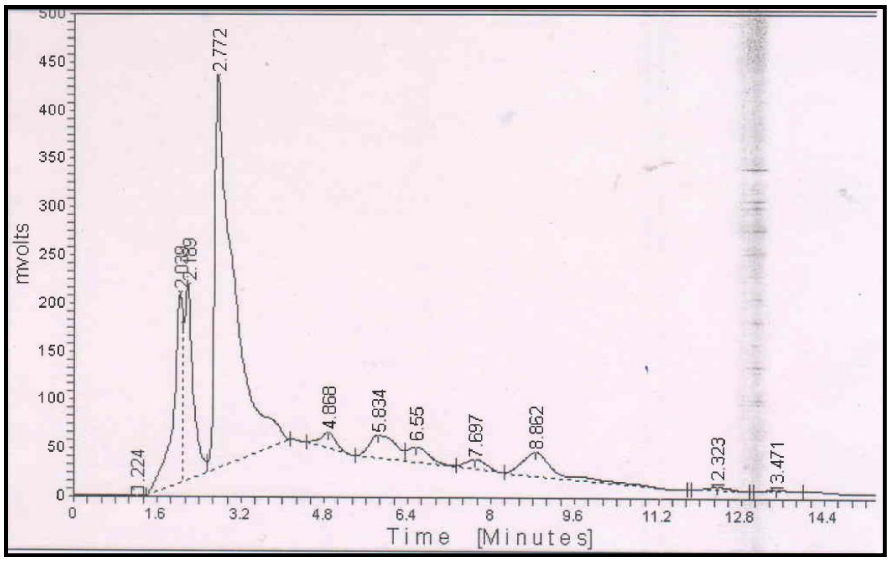

Figure -1. HPLC chromatogram of the crude ethanolic root extract obtained by UV detection at 270 nm.

The most prominent peak with 56.0 % area and 44.965% height was observed in the crude extract at the retention time 2.772 (Retention time), which was similar to that observed in the chromatogram of isolated compound as a singular isolated peak with 2.936 (Retention time), 99.415% purity area and 93.881% purity height, indicative of the purity of the isolated compound. The other prominent peaks were recorded with retention time 2.039 and 2.189 (Retention time) in the crude extract respectively.

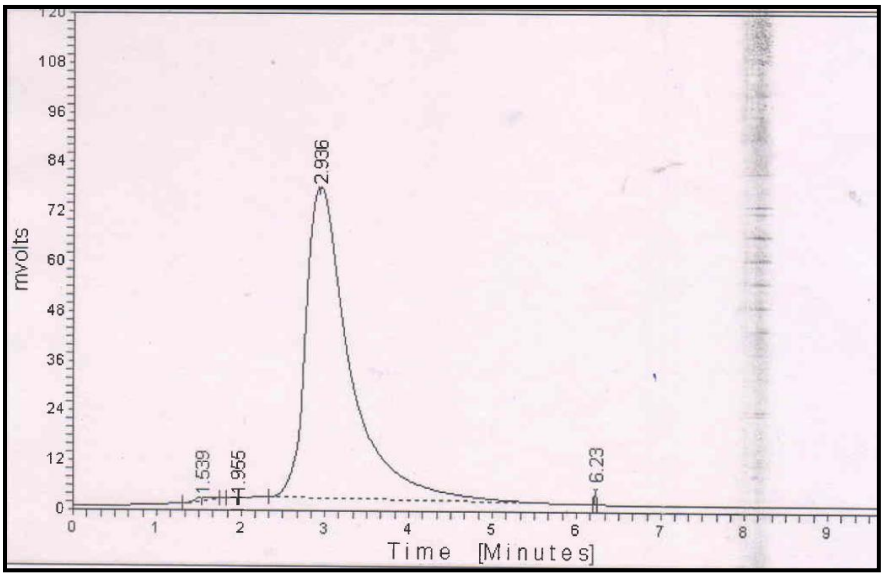

Figure 2. HPLC chromatogram obtained by UV detection at 270 nm of the isolated Stigma-5, 22dien-3-O-b-D-Glucopyranoside.

Description, solubility and melting point

The isolated plant product gave the positive tests for the presence of glycoside and steroids confirming its nature as a steroidal glycoside. Crystallisation from the fraction provided the isolated compound with the yield of 120mg.It is pale white crystalline powder with the melting point of 285- 295^{o}C, soluble in chloroform and alcohols.

UV absorbance

The absorption maxima of Stigma-5,22dien-3-O-b-D-Glucopyranoside under UV was observed and was found to be at 270nm. (Figure 3.)

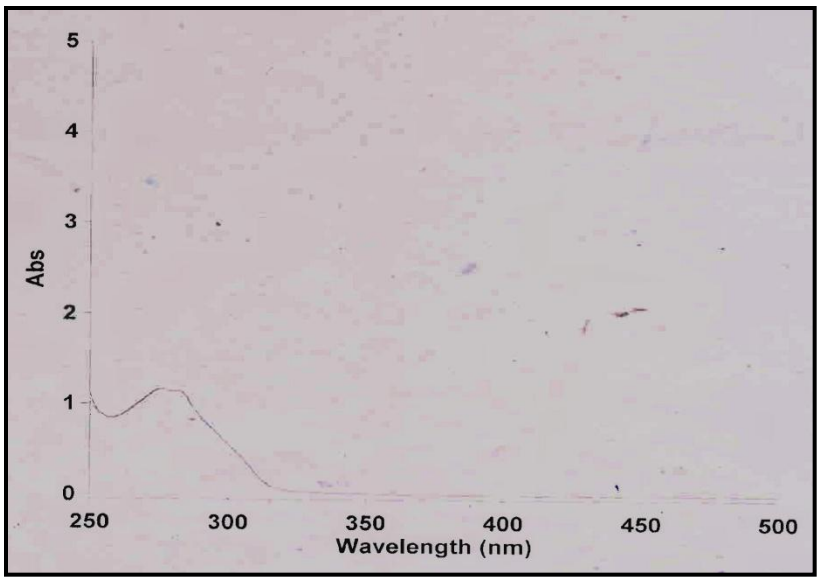

Figure- 3. UV absorbtion spectra of the ethanolic extract of *Operculina turpethum*.

From the positive tests for steroid and glycosides it was assumed that the isolated product can be a steroid glycoside. On subjection to IR spectroscopic analysis, the observed absorption bands are 3550 cm^{-1} that is characteristic of O-H stretching. (Figure 4.) Absorption at 2933 cm^{-1} and 2967 cm^{-1} is due aliphatic C-H stretching. Other absorption frequencies include 1652 cm^{-1} as a result C=C stretching, at 1450 cm^{-1} is a bending frequency for cyclic $(CH_2)_n$ and 1367 cm^{-1} for $–CH_2(CH_3)_2$. The absorption frequency at 1027 cm^{-1} signifies cycloalkane.

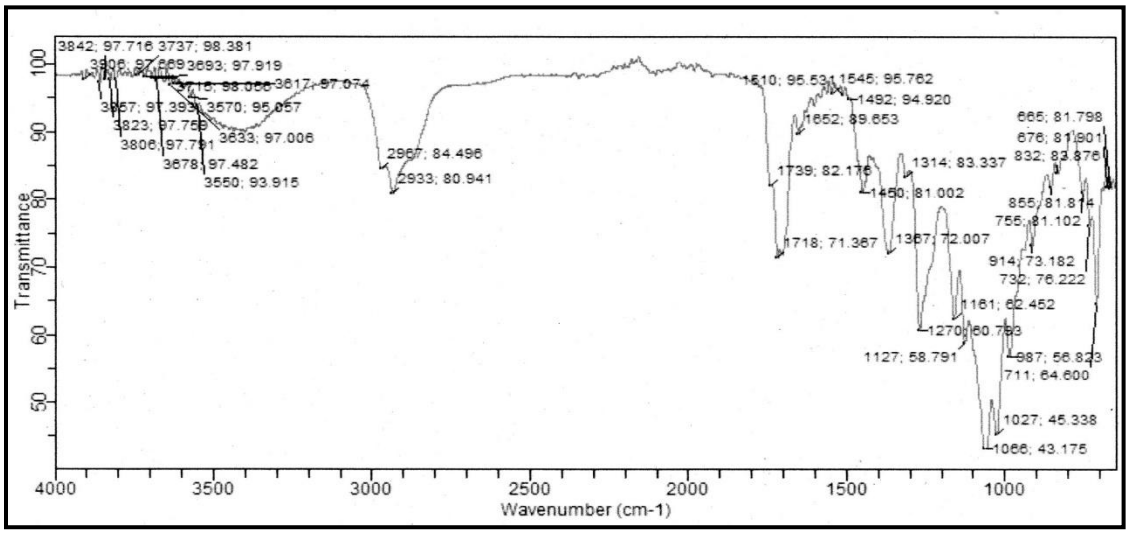

Figure- 4. IR spectra of the isolated compound (Stigma-5,22dien-3-O-b-D-Glucopyranoside).

The isolated compound was subjected to ^{1}HNMR analysis that determined the number of hydrogen atoms present in the compound. The area under the plots provides information about the number of protons present in the molecule, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal

or geminal) protons (Farlane, 1972). The ^{1}H NMR data showed the presence of the oxymethine proton at δ3.66 and two tertiary methyl at δ 0.85(Me) ,δ 0.99(Me) and δ 0.91(Me). (Figure 5.)

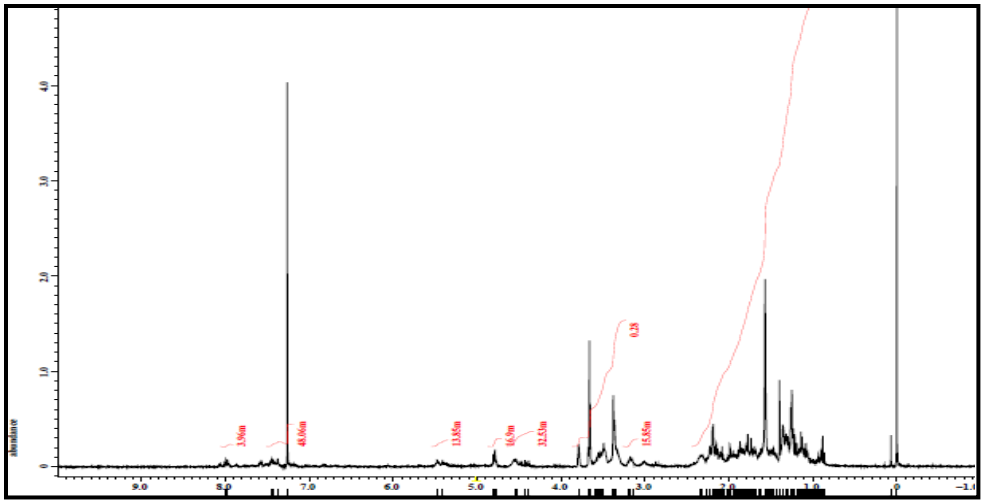

Figure- 5. NMR spectra of the isolated compound (Stigma-5,22dien-3-O-b-D-Glucopyranoside).

The protons corresponding to sugar moiety showed the peaks at δ 4.39, δ 3.17, δ 3.38, δ 3.65. It also showed the presence of olefinic protons at δ 4.79 and δ 4.80. Stigma-5,22dien-3-O-b-D-Glucopyranoside was identified by examining the IR spectra in comparison with the authentic data (Paul and Singha, 2010). The ARAS- LCMS spectral data of the compound gave a molecular formula $C_{35}H_{51}O_6$ with the molecular ion, m/z 567.7 $[(M)-C]^{+}$ and the base peak at 305.5(100%) (Figure 6.). Energy imparted by the electron impact can cause the molecular ion to split into fragment ions, cleavage may take place through rearrangement reactions causing the change in the m/z value of the molecular ion.

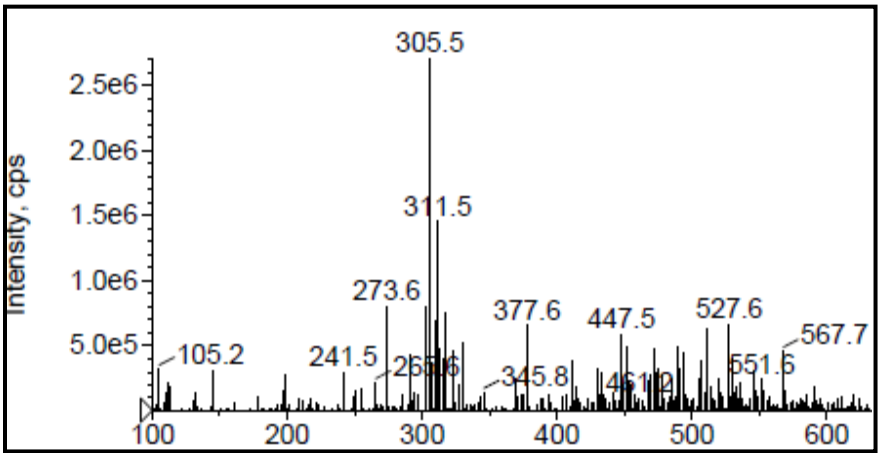

Figure- 6 . ARAS LCMS spectra of the isolated compound.

These ^{1}H NMR, IR and LC/MS observations are in consideration with the observed data and hence it proposes the structure of the isolated compound (Figure 7).

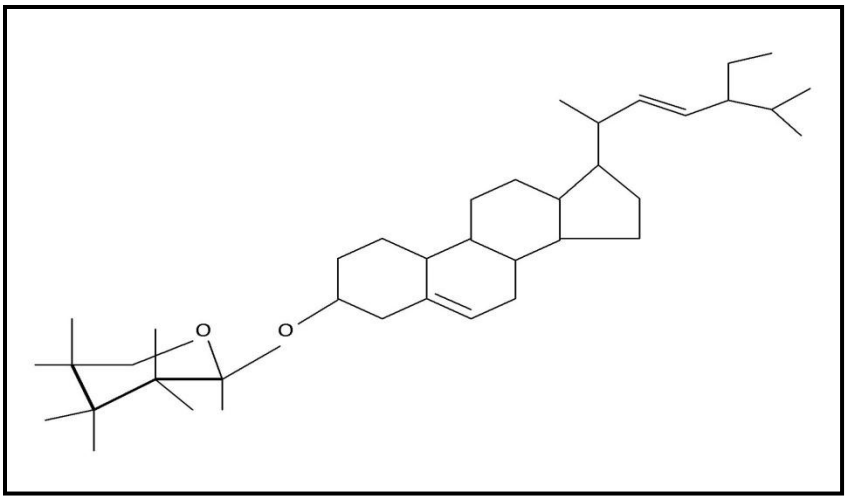

Figure- 7. Chemical Structure of Stigma-5, 22dien-3-O-b-D-Glucopyranoside as proposed from the spectroscopic analysis.

Acid hydrolysis was performed on the isolated product with 5% H_2SO_4 as described above and the sugar residue obtained from the aqueous extract was identified by direct comparison with authentic sugar (glucose) using the TLC systems EtOAc/pyridine/ethanol/H2O (8:1:1:2), EtOAc/*n*-butanol/ water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1). From the hydrolysis results, it was found that the isolated compound has comparable retention factor value as the standard sugar glucose.

The beneficial medicinal effects of plant materials typically results from the combinations of secondary products present in the plant, so that the medicinal actions of plants are unique to particular plant species or groups as the combinations of secondary products in a particular plant are taxonomically distinct. The consumption of plant extracts in herbal medicine for the treatment of diseases and prevention of infections has grown with tremendous recognition, giving rise to the need to identify the specific compounds that could impart such therapeutic benefits. Different strategies will result in a herbal medicine or in an isolated active compound. Phytoconstituent compounds comprise a large group of molecules derived from a variety of plant sources. It is well known that the secondary metabolites, are widely distributed in the plant kingdom and that they are sometimes present in surprisingly high concentrations. Although plant secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has shown a pivotal role of these chemicals in the eco-physiology of plants. Each secondary metabolite family has some specific chemical characteristics which implies that specific extraction and analysis methods should be developed to study each family in detail. Using their polarity properties, it was possible profile the constituent present in the ethanolic root extract of *Operculina turpethum* by using different analytical systems. To obtain isolated active compounds, the plant extracts are first qualitatively analysed by thin layer chromatography (TLC) and/or other chromatographic methods and screened to determine the biological activity or to obtain a general evaluation of biological activities. Steroids are apparently involved in the regulation of large number of biological activities like anti-inflammatory, oxytocic, cytotoxic, antibacterial, hypocholesterolemic and antioxidant functions. The occurrence of steroidal glucoside in *Operculina turpethum* can contribute to be the reason for its use as a herb in phytomedicine against various ailments.

Conclusion

As the conclusion, it can be stated that the present study reveals the presence of steroidal glycoside in *Operculina turpethum* roots which was further confirmed by various characterization analysis. Here, the structure elucidation, mainly undertaken by means of spectroscopic and chemical evidence, provided unambiguous information about the aglycone skeletons and structures, the position of the glycosidic linkage, and the sequence of the monosaccharides in the sugar moiety. In addition, it can be stated that steroidal glycosides are one of the active ingredients of *Operculina turpethum* and they can be further analysed for their potential pharmacological importance, providing an insight for the use of the plant in various formulations.

Acknowledgements
The authors are thankful to the authorities of Banasthali University for providing support to the study.

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