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DETECTION OF FUNCTIONAL GROUPS AND PHYTOCHEMICAL CONSTITUENTS IN ULTRA-HIGH DILUTIONS OF ASTERIAS RUBENS USING ADVANCED SPECTROSCOPIC TECHNIQUES

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ABSTRACT

Homoeopathy is one of the most popular forms of medicine in which medicinal products from natural products are prepared through the process of dilution and potentization. Amongst many sources of homoeopathic ultra-high dilutions, the medicines derived from animals are least researched owing to their rich biochemical composition. Asterias rubens is a marine echinoderm also called starfish and has been used traditionally in homoeopathy for treatment of heart ailments, hormonal imbalance, haemorrhages and malignant diseases. The aim of this study is scientific comparison of functional groups and bioactive compounds of extract of Asteria rubens, mother tincture(Φ) and its homoeopathic ultrahigh dilutions (3X, 12C and 30C) by means of GC-MS and FTIR spectroscopic techniques. The functional groups have been identified using FTIR spectroscopy technique, whereas bioactive compounds have been detected and characterized using GC-MS analysis. FTIR technique has revealed presence of hydroxyl, carbonyl, aliphatic, ester and amine functional groups. Some of the biologically important compounds identified from Asteria rubens through GC-MS include fatty acids, steroidal derivatives, hydrocarbons and oxygenated compounds. These compounds are known to have antioxidant, antimicrobial and anticancer properties.

KEYWORDS: *Asterias rubens*; Ultrahigh dilutions: FTIR; GC-MS; Antioxidant activity; Antimicrobial activity; Anticancer activity; Marine bioactive compounds.

1. INTRODUCTION

Homoeopathy is recognized as the second largest system of medicine followed worldwide and is extensively used as a form of complementary and alternative therapy.(1) It is a therapy based on the three basic principles of 'similia similibus curentur' (like cures like), minimum dose, and individualized treatment.(2) Homoeopathic ultra-high dilutions are used both as curative and palliative therapy owing to their low cost and non-toxicity along with their holistic way of action.(3) Homoeopathic ultra-high dilutions are made from six main sources which are plants (herbs), animals or animal products, minerals, metals, nosodes (tissues or secretions of diseased organism) and sarcodes (tissues of healthy organism), and imponderabilia.(4) Medicinal agents from animal origin have received less attention although they contain a variety of bioactive agents with potential therapeutic activity.(5) Sea animals are considered as rich source of bioactive substances with different biological activities.(6)

Common starfish or Asterias rubens is a sea animal that belongs to the class Asteroidea. This sea creature bears typical star-like appearance, where it has a central disc with five arms symmetrically arranged. Secondary metabolites of this animal include steroids, steroidal glycosides, alkaloids, anthraquinones, phospholipids, peptides, and fatty acids.(7,8) Various biological actions have been observed in these substances such as cytotoxic, haemolytic, antiviral, antifungal, antimicrobial, antioxidant, and anticancer activities.(7,9) In homoeopathy, Asterias rubens are obtained from the preparation of starfish. This substance is usually prescribed in diseases of the heart, bleeding problems, hormone imbalance, mental disturbance, and malignancies.(10) It has been observed that this product contains high ash and calcium content with relatively low content of fats and crude proteins.(8,9) Though it is used clinically, there is lack of scientific evidence on Asteria rubens.

Phytochemical screening and instrumental characterization techniques such as UV–Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), and Gas Chromatography–Mass Spectrometry (GC-MS) play a vital role in

identifying and characterizing bioactive compounds present in medicinal substances.[8,9] Furthermore, *in vitro* antioxidant, antimicrobial, and anticancer assays provide valuable insight into the therapeutic potential of homoeopathic ultra-high dilutions beyond traditional clinical observations.[9,11]

The present study aims to scientifically compare the functional groups and bioactive compounds of *Asteria rubens* extract, mother tincture(Φ) and its homoeopathic ultrahigh dilutions (3X, 12C, and 30C) using GC-MS and FTIR. Spectroscopic characterization was carried out using Fourier Transform Infrared (FTIR) spectroscopy to identify functional groups, while Gas Chromatography–Mass Spectrometry (GC-MS) analysis was employed to detect and characterize bioactive compounds present in Asteria rubens.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Sample

The starfish Asterias rubens was obtained from the harbour beach (coordinates: 8.7561°N, 78.1790°E) located at Tuticorin, Tamil Nadu, India. The specimen obtained was rinsed properly with both seawater and distilled water to wash out sand particles, any foreign material, and epiphytes. Confirmation of species was done through the COI gene sequencing technique, and sequence comparison was done with GenBank sequences through BLAST analysis.

2.2 Preparation of Ethanolic Extract and Mother Tincture(Φ)

The cleaned Asteria rubens was dried under shaded condition in normal temperature and pulverized into coarse powder. Determined amount of moisture content (40 ml) using water bath. The conventional biological extraction method of Asteria rubens (solid - 44.365 gm, strong alcohol - 250 ml) has been carried out using Soxhlet apparatus for 12 cycles. Based on the Homoeopathic Pharmacopoeia of India volume 4 for the preparation of mother tincture(Φ) (method of Hahnemann, class – IV) using 100 gm of Asteria rubens, moisture - 360 ml and strong alcohol – 637 ml. The pulverized material is macerated in strong alcohol for 15 days with occasional shaking. Percolate obtained is filtered and concentrated to form mother tincture. As a result of decantation, 870 ml of mother tincture(Φ) has been obtained due to the absorption of menstruum, slight evaporation during preparation process and filtration and decantation loss which was preserved in tightly capped amber-coloured bottles.

2.3 Preparation of ultra-high dilutions:

Ultra-high dilutions were prepared using the method detailed in volume IV of the Homoeopathic Pharmacopoeia of India (HPI).

3X potency(10^{-3}): Drug strength – 1/10. One part (10 ml) of the 2X potency was mixed with nine parts (90 ml) of dispensing alcohol and was placed in a new amber glass vial. This glass vial was tightly stoppered. Succussion of the above mixture by the electric potentizer was done for 10 times in order to get 100ml of Asterias rubens 3X. This preparation was labelled and stored in a amber coloured glass bottle which protects it from heat and light.

12C Potency(10^{-24}): Drug strength is – 1/100. One part (1 ml) of the prepared 11C, 99 parts (99 ml) of dispensing alcohol was taken in a new amber glass vial and tightly stoppered. The mixture was then succussed ten times by the electric potentizer to produce 100ml of Asterias rubens 12C. The potencies ranging from 13C to 29C were all prepared on this scale.

30C potency(10^{-60}): Drug strength = 1/100. One portion (1 ml) of 29C preparation and 99 portions (99 ml) of dispensing alcohol was transferred to a clean amber glass vial. The vial was sealed. The mixture was agitated 10 times in one direction using potentizer machine to get 100 ml Asterias rubens 30C.

All Ultra-high dilutions were prepared under sterile conditions in the laboratory, put in sterilized amber glass bottles, and stored at room temperature protected from direct light until future analysis.

2.4 Gas Chromatography–Mass Spectrometry (GC-MS):

Gas Chromatography–Mass Spectrometry (GC–MS) analysis was carried out using a GC–MS system (Make: Shimadzu Corporation; Model: GCMS-QP2010 Plus, Kyoto, Japan) equipped with a capillary column. Gas Chromatography-Mass Spectrometry (GC-MS) was utilized to determine the chemical composition in the Asterias rubens extract, mother tincture(Φ) e, ultra-high dilutions - 3X, 12C and 30C. The sample solutions comprising Asterias rubens extract, mother tincture(Φ), ultra-high dilutions (3X, 12C and 30C) were filtered, concentrated, and diluted with HPLC-grade methanol before carrying out the test. GC-MS test was conducted by using capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm), helium carrier gas with a flow rate of 1.0 mL/min. One microliter of the sample was injected in split mode; chromatographic separation was done by using a programmable temperature between 60°C and 280°C. Mass spectra were collected in electron impact (70 eV) within the range of m/z 40-700. Detected compounds were identified using comparison with NIST and Wiley mass spectral library. Retention time, molecular formula, molecular weight, and peak area percentage were determined and used for characterization. All tests were done in triplicate.

2.5 Fourier Transform Infrared (FTIR):

FTIR spectroscopy of the sample was conducted through an IR Affinity-1 FTIR spectrometer (Make: Shimadzu corporation; Model: IR Affinity-1). Spectra of the samples were measured between 400 and 4000 cm^{-1} at 4 cm^{-1} resolution in % transmittance mode. The source was internal beam source, and mirror speed was 2.8 mm/s. Fourier Transform Infrared (FTIR) spectroscopy was used in the characterization of the functional groups in the Asterias rubens extract, mother tincture(Φ), and ultra-high dilutions (3X, 12C and 30C). The sample solutions comprising Asterias rubens extract, mother tincture(Φ), ultra-high dilutions (3X, 12C and 30C) were concentrated and dried, and a powdered residue was

obtained. About 1–2 mg of the dried substance was mixed with spectroscopic grade potassium bromide (KBr) and compressed to form a clear pellet. The FTIR spectrum was then measured within the wave number ranges of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} using an FTIR spectrophotometer. Analysis of the absorption bands was conducted and assigned to functional groups from standard spectra and scientific literature sources. All analyses were done in triplicate.

3. RESULTS AND DISCUSSION

3.1 GC–MS Analysis of *Asterias rubens*

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to identify the bioactive compounds present in extract, mother tincture(Φ) and ultrahigh dilutions (3X, 12C, and 30C) of *Asterias rubens* shown from **Figure 1-5.**

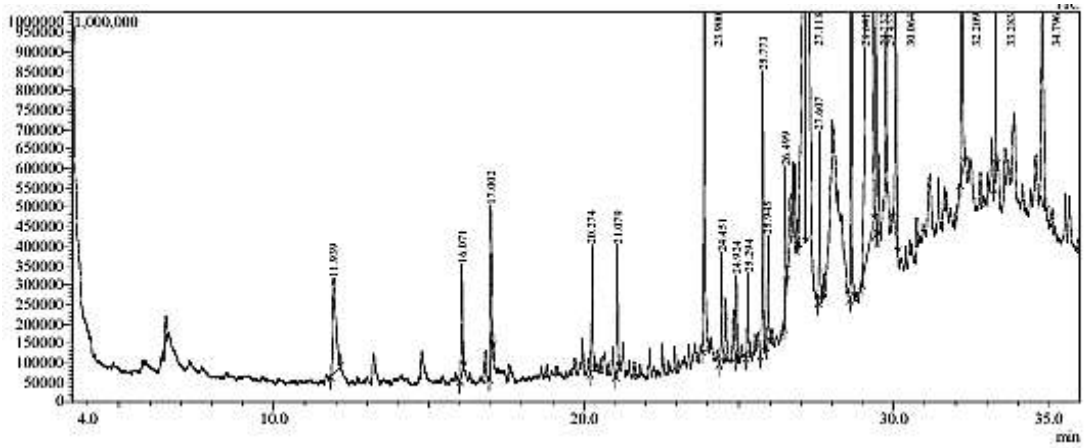

Figure 1: GCMS Chromatogram of Asterias rubens Crude Extract

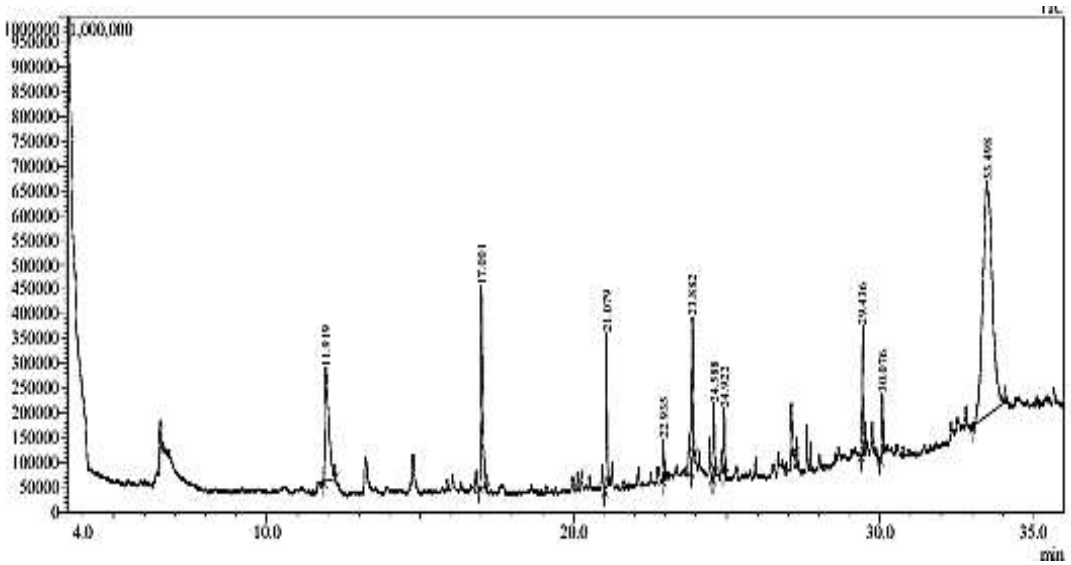

Figure 2: GCMS Chromatogram of Asterias rubens Mother Tincture

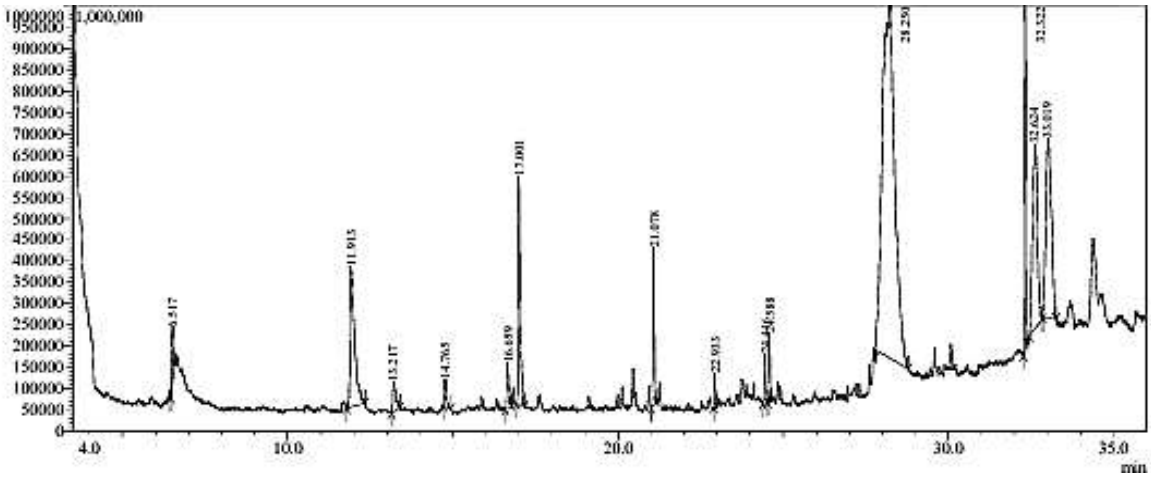

Figure 3: GCMS Chromatogram of Asterias rubens 3X

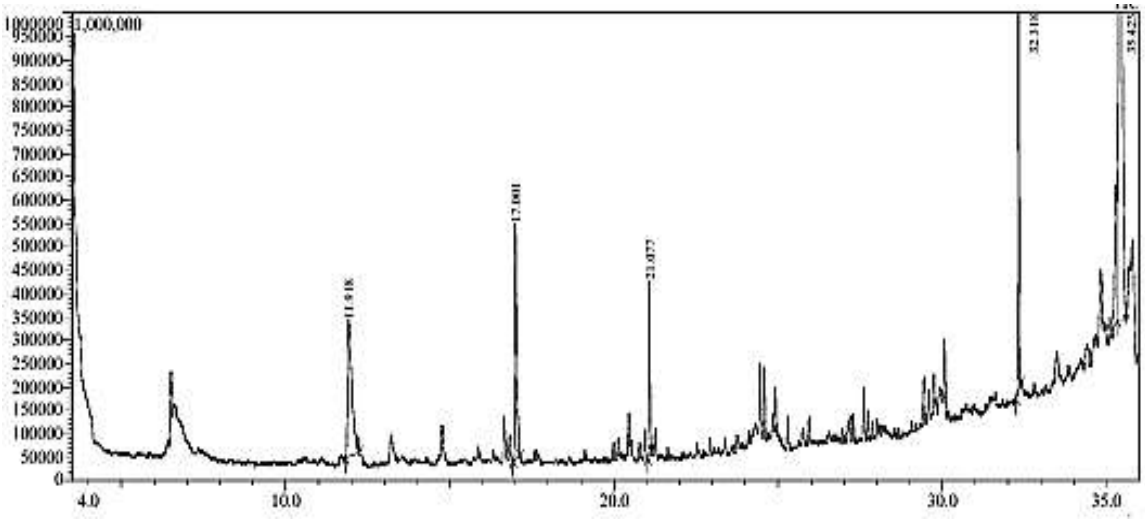

Figure 4: GCMS Chromatogram of Asterias rubens 12C

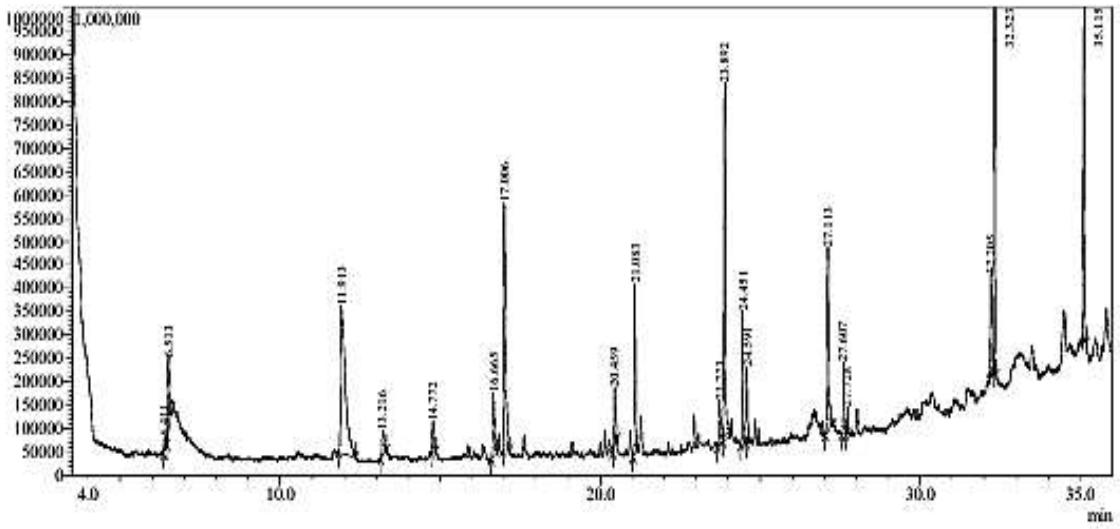

Figure 5: GCMS Chromatogram of Asterias rubens 30C

A clear decreasing trend in chromatographic complexity and peak intensity was observed across the samples from Extract > Φ > 3X > 12C > 30C. The GC–MS chromatogram shows multiple well-resolved peaks with varying retention times, indicating the presence of a complex mixture of bioactive compounds.

Chromatographic peaks observed in the extract was higher compared to others indicating the presence of more bioactive components in the form of a complex mixture. Among the main components found, there were long chain hydrocarbons (tetradecane, hexadecane, heneicosane), fatty acids (dodecanoic acid, tetradecanoic acid, n-hexadecanoic acid), fatty acid esters (hexadecanoic acid ethyl ester, octadecanoic acid ethyl ester), and sterol-like compounds.

Mother tincture (Φ) had retained most of the compounds found in the extract, which indicated an efficient transfer of active components in the hydroalcoholic solvent. Predominance of aromatic derivatives, fatty acids, and lipid-related compounds proves that the mother tincture(Φ) preserves the chemical composition of the starting material.

3X potency contained fourteen components, out of which the most significant were Eicosa-5,7-dien-3-ol (50.10%), Ergost-7-en-3-ol (11.30%), Cholesta-7,24-dien-3-ol (10.03%), tetradecane (7.19%), and hexadecane (4.94%). Steroids were the major component of the chromatographic analysis.

The sample of 12C potency contained less peaks than the extract and 3X potency. Still, characteristic compounds like tetradecane (14.39%), heptadecane (10.47%), bis(2-ethylhexyl) phthalate (18.07%), and cholesta-5,22-dien-3-ol (52.58%) were observed. Prevalence of cholesta-5,22-dien-3-ol (52.58%) could be detected. The presence of cholesta-5,22-dien-3-ol reveals that the steroidal ingredients are also present at higher levels of dilution.

The 30C potency revealed the following compounds with percentages of: tris(2,4-di-tert-butylphenyl) phosphate (59.72%), tetradecane (12.08%), hexadecanoic acid (10.26%), and bis(2-ethylhexyl) phthalate (22.67%).

The mother tincture (Φ) and lower dilutions (3X) exhibited a high concentration of peaks in terms of detectable components having peak intensity higher compared to higher potencies (12C and 30C). The reason for this was due to high dilution in 12C and 30C potencies. In addition, the bioactive compounds identified previously give a scientific backing for the biological activity exhibited by the drug.

GC MS analysis revealed various biologically active compounds including fatty acids, steroidal derivatives, hydrocarbons, and oxygenated compounds. Fatty acids like hexadecanoic acid and octadecanoic acid derivatives are known for their antioxidant, antimicrobial, and anti-inflammatory actions.[8,9] Steroid-like compounds identified in the sample support the

claim that starfish have steroidal glycosides with cytotoxic and anticancer properties.[7,12] Steroid-like compounds identified in the sample support the claim that starfish have steroidal glycosides with cytotoxic and anticancer properties.[9,11] The nitrogen containing compounds could be responsible for antiproliferative and enzyme inhibiting properties.[7,11]

3.2 FTIR Analysis of *Asterias rubens*

Fourier Transform Infrared (FTIR) spectroscopy was employed to identify the functional groups present in extract, mother tincture (Φ) and ultrahigh dilutions (3X, 12C, and 30C) of *Asterias rubens* shown from **Figure 6 -10**. The FTIR spectra revealed the presence of several characteristic absorption bands, indicating the complex biochemical nature of the marine-derived ultra-high dilutions.

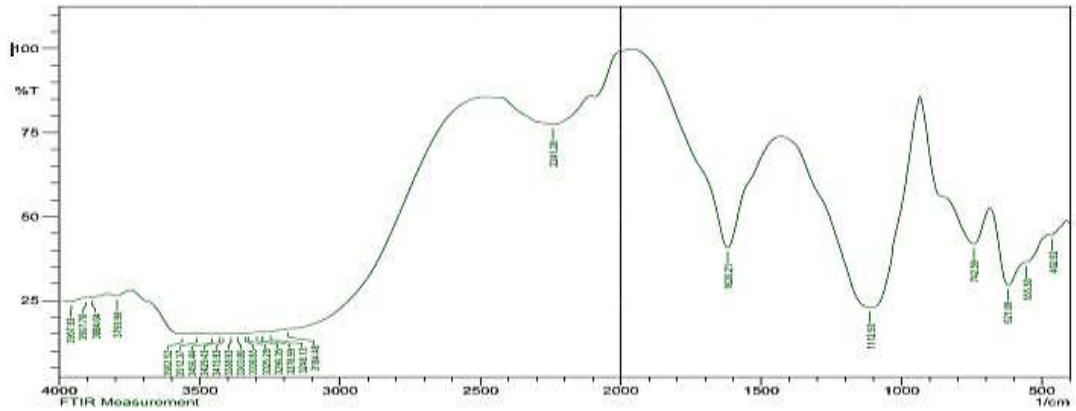

Figure 6: FTIR Spectrum of Asterias rubens Crude Extract

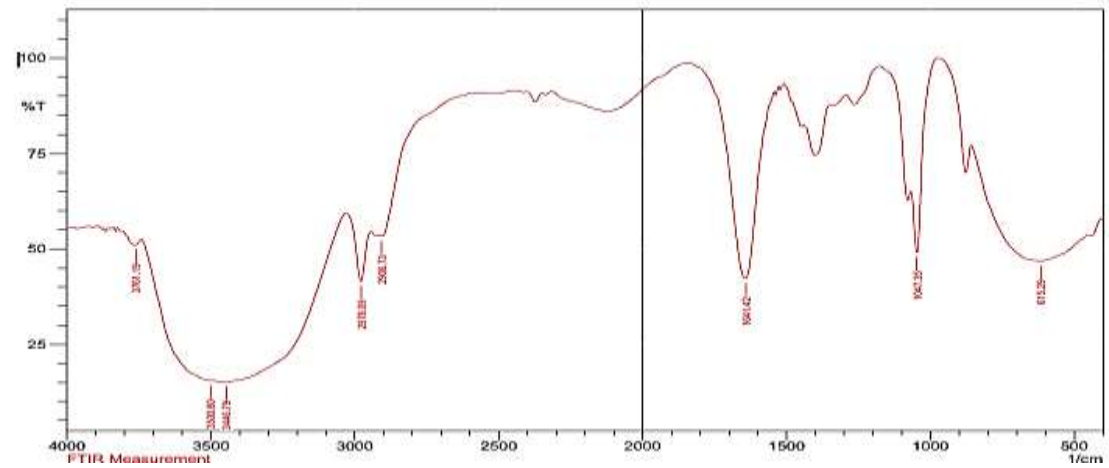

Figure 7: GCMS Chromatogram of Asterias rubens Mother Tincture

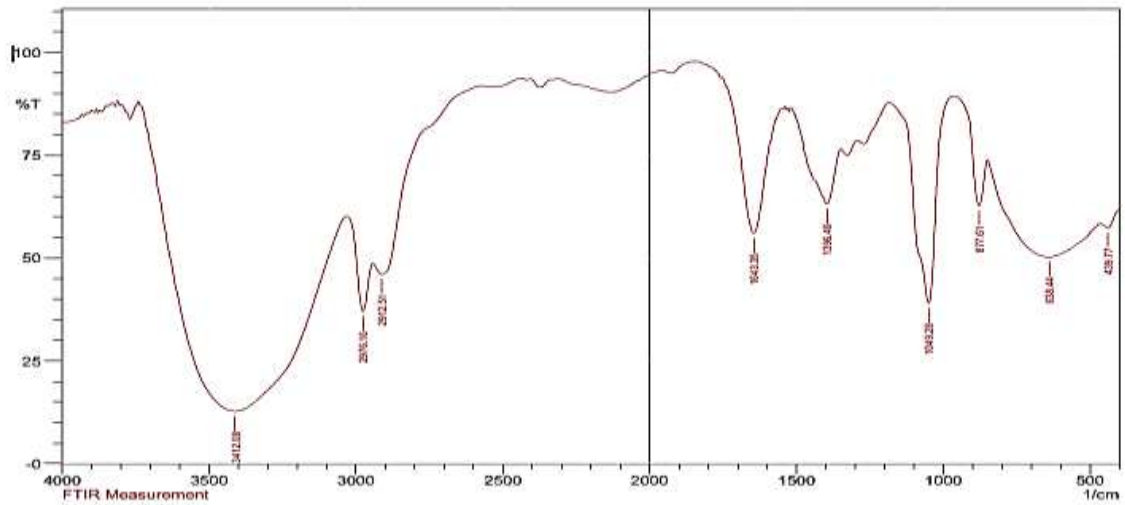

Figure 8: GCMS Chromatogram of Asterias rubens 3X

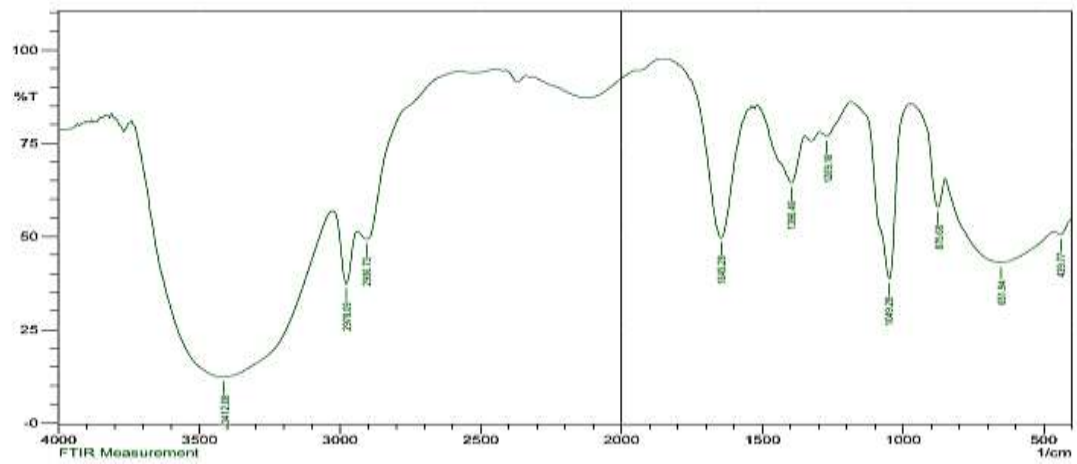

Figure 9: GCMS Chromatogram of Asterias rubens 12C

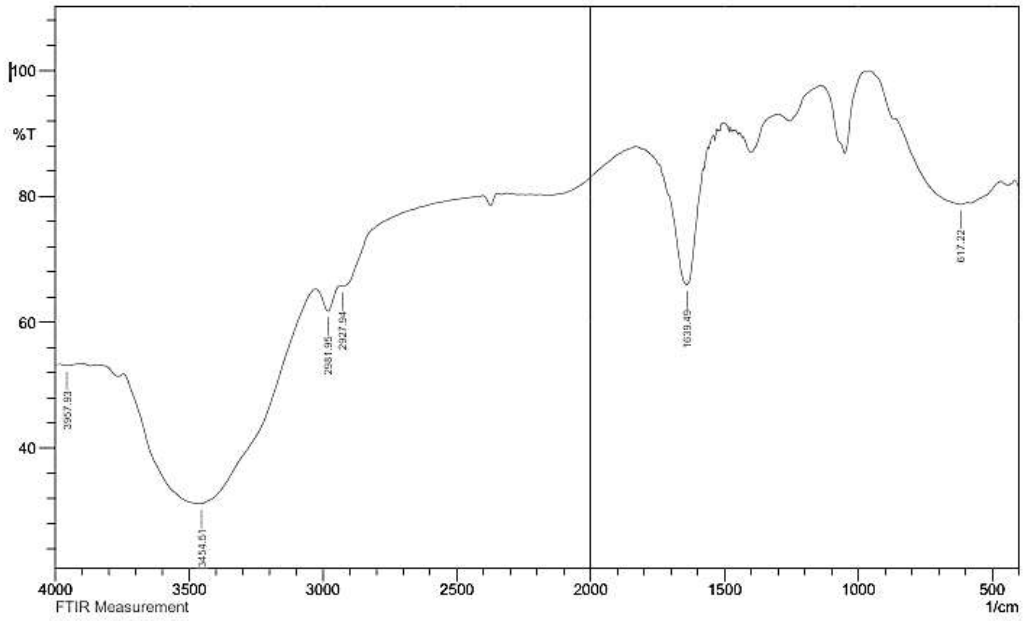

Figure 10: GCMS Chromatogram of Asterias rubens 30C

A progressive reduction in peak intensity is observed from Extract > Φ > 3X > 12C > 30C. Analysis of the FTIR spectra showed the presence of characteristic bands, which were linked to specific functional groups, that include O-H stretching vibration, C-H stretching of alkyl chain and C=O stretching vibrations (amides I and II bands). This has enabled the recognition of important phytochemical components and variations of those with different dilution concentrations.

Broad band seen in extract between 3200-3400 cm^{-1} is related to O-H stretching vibrations, which indicates the presence of alcohol, phenol and hydrogen bond functional group. Functional groups like this one have been reported to be found in biologically active marine metabolites and possess strong antioxidant activity.(11)

The peaks shown in mother tincture (Φ) between 2920-2850 cm^{-1} are attributed to C-H stretching vibration of aliphatic hydrocarbons. This means that there are lipids and fatty acid molecules in the samples. It is known that starfish contain a high amount of unsaturated fatty acids, which provide their biological activity.(8) Bands seen in the area of 1650-1600 cm^{-1} refer to C=O stretching vibrations, indicating carbonyl functional groups, such as ketones, aldehydes and amide bonds. Such functional groups can be found in steroid glycosides, peptides and proteins in Asteria rubens.(12)

Peaks seen in 3X in the range of 1450-1380 cm^{-1} are due to bending vibration of C-H bonds, thus indicating the presence of alkanes and methyl groups. Asteria rubens 12C peaks ranging from 1250-1050 cm^{-1} are the result of stretching vibration of C-O and C-N bonds, indicating the presence of esters, ethers, and amine compounds. They are well known for their antimicrobial and anticancer activity.(11,13) At higher potency levels (12C and 30C), even though there is reduced intensity of peaks, from 1220 – 1150 cm^{-1}, the presence of characteristic functional group peaks shows that imprinting and transfer of molecular information have occurred during potentization process.(13) Thus, the FTIR analysis confirms the presence of various functional groups in Asteria rubens responsible for its therapeutic effect (14-20).

The GC-MS analysis clearly supports the FTIR analysis in terms of identification of the presence of various functional groups and bioactive molecules even in ultrahigh dilutions of 3X,12C and 30C. The identified compounds are responsible for antioxidant, antimicrobial, and anticancer activities (21-25).

4. CONCLUSION

Analysis through GC-MS of ethanolic extract, mother tincture(Φ) and ultra-high dilution of Asteria rubens indicated that there are various bioactive chemicals present, such as fatty acids, steroid derivatives, hydrocarbons and oxygenated compounds, which have been found to possess antioxidative, antimicrobial and anticancer properties. FTIR analysis showed that there are different types of functional groups present, such as hydroxyl, carbonyl, aliphatic, ester and amine groups, proving the complex nature of chemistry of Asteria rubens. The above analytical data support the presence of important pharmaceutically active constituents in the source of the drug. The information provided above adds valuable experimental evidence to the traditional homoeopathic uses of this marine creature. The data encourages future advanced investigations, including in vivo experiments.

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