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Assessment of Phytochemicals and Characterisation of a Polyherbal Formulation

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Abstract

Objectives: The present study aims to isolate and characterize bioactive compounds from a polyherbal formulation (PHF). **Methodology:** A PHF was prepared by homogenously mixing specific proportions of the following medicinal plants: *Justicia adhatoda*, *Glycyrrhiza glabra*, *Withania somnifera*, *Cassia angustifolia*, *Zingiber officinale*, *Elettaria cardamomum*, *Terminalia chebula*, *Nigella sativa*, *Curcuma longa*, *Piper nigrum*, *Moringa oleifera*, *Cuminum cyminum*, *Phyllanthus emblica*, *Tribulus terrestris*, *Andrographis paniculata*, *Cinnamomum verum*, *Tinospora cordifolia*, *Gymnema sylvestre*, *Abies webbiana*, *Vetiveria zizanioides*, *Ocimum sanctum*, *Centella asiatica*, *Trigonella foenum-graecum*, *Solanum trilobatum*, and *Azadirachta indica*. Preliminary phytochemical screening of the PHF was conducted using both qualitative and quantitative analyses to identify key phytoconstituents. The optimization of solvent systems was performed using Thin Layer Chromatography (TLC), followed by compound separation via column chromatography using a gradient of high to low polarity solvents. The eluted fractions were further analysed using a series of advanced characterization techniques like GC-MS to identify bioactive compounds based on retention time, peak area, and molecular weight. LC-MS to determine the composition and specific compounds present in the fractions. FT-IR analyses to detect the functional group present in the compound, H1NMR analyses to know the type of proton present. **Findings:** Phytochemical analysis confirmed the presence of tannins, steroids, alkaloids, flavonoids, quinones, and saponins. Quantitative analysis of the methanolic extract revealed 46.3 mg AE/g of alkaloids, 45.3 mg QE/g of flavonoids, and 47.5 mg GAE/g of phenolic compounds. GC-MS analysis identified 1,1-bis(hydroxymethyl)cyclopropane as a major component. LC-MS analysis revealed the presence of 3-hydroxy-N-methylcoclaurine, an isoquinoline alkaloid. FT-IR to detect functional groups, where the highest peak at 1020.34 cm^{-1} indicated a C–N (amine) stretch. ^{1}H-NMR to identify proton environments, with the highest peak at δ 3.227 ppm corresponding to alkyl protons. **Novelty and Conclusion:** These findings highlight the pharmacological potential of the PHF, suggesting it is a rich source of bioactive natural compounds. The identified constituents could serve as promising candidates for the development of novel drug delivery systems and therapeutic agents.

Keywords: Polyherbal formulation; GC-MS; LC-MS; FT-IR; TLC; Column Chromatography; ^{1}H-NMR; Phytochemical Screening

1 Introduction

Previously, the plant parts were directly used as such for the treatment, but now-a-days, the active principles are identified, isolated in pure form, also synthetically produced with the help of advanced techniques. In the development of new synthetic drugs, the chemical structures derived from these phytoconstituents can be utilized as models. Identification of phytoconstituents in the plant material helps predict the potential pharmacological activity of that plant[(1)]. Polyherbal formulations are effective in promoting the wound-healing process. They can stimulate a variety of physiological functions that accelerate the healing process. These formulations merit further investigation in clinical trials, and production upscaling will aid in the creation of a new horizon of polyherbal wound- healing products[(2)].

The polyherbal blend 18KHT01 has shown promising anti-obesity properties. Comprised of specific proportions of *Quercus acutissima* (acorn jelly powder), *Camellia sinensis* (dry leaf buds), *Geranium thunbergii* (dry aerial parts), and *Citrus limon* (fruit juice), but further research is necessary to elucidate its detailed molecular mechanism of action[(3)].

Iron content of RK and its ability to prevent phenylhydrazine-induced hemolytic anaemia provides that the RK (Raktavardhak Kadha), PHF has beneficial effect against both iron deficiency as well as haemolytic types of anaemia[(4)].

Polyherbal formulation has many benefits over single herb. Herbal remedies have contributed a great deal of the world's most effective medications to the enormous supply of pharmaceuticals that modern medicine has at its disposal, both as pure chemicals and in their raw form organized[(5)].

Phytochemicals are plant-based bioactive compounds produced by plants for their protection. These phytochemicals possess strong antioxidant activities and exhibit antimicrobial, antidiarrheal, anthelmintic, antiallergic, antispasmodic, and antiviral activities. They also help to regulate gene transcription, enhance gap junction communication, improve immunity, and provide protection against lung and prostate cancers[(6)].

A polyherbal formulation (PHF) was developed by homogeneously mixing a specific proportion of *Justicia adhatoda, Glycyrhiza glabra, Withania somnifera, Cassia angustifolia, Zingiber officinale, Elletaria cardamomum, Terminalia chebula, Nigella sativa, Curcuma longa, Piper nigrum, Moringa oleifera, Cuminum cyminum, Phyllanthus emblica, Tribulus terrestris, Andrographis paniculata, Cinnamomum verum, Tinospora cordifolia, Gymnema sylvestre, Abieswebna, Vettiveria zizanoides, Ociumum sanctum, Centella asiatica, Trigonellafoenum-graecum, Solanum triblobatum, Azadirachta indica.* We performed a simultaneous comparative phytochemical analysis of using four different polar and non-polar solvents for this study. Fourier Transformed Infrared Spectrum (FTIR), Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectroscopy (LCMS), Nuclear Magnetic Resonance (H1NMR) analysis was performed to identify the multiple metabolites present in the eluted fraction. Since these plants are used traditionally for many diseases, further research can lead to a promising discovery of new compounds which is useful to mankind.

2 Methodology

2.1 Preparation and Extraction of Polyherbal Formulation (PHF)

The polyherbal formulation (PHF) is composed of dried plant powders. It is created by combining equal amounts of various herbs commonly utilized as dietary supplements. Sample PHF were procured from Siddha doctor, Madurai, Tamil Nadu. Phytochemical profiling qualitative and quantitative analysis, isolation of bioactive Compound by Column chromatography technique. The isolated compound was investigated using the GC-MS, LCMS technique, FT-IR, H1NMR. **a. Standardization:** The PHF powder is subjected to extraction with different solvents to identify the most effective extract. Standardization is a critical aspect of polyherbal formulations, as it allows for the evaluation of drug quality based on the concentration of active principles. **b) Prototypic Study:** A sample of PHF powder (2g) was immersed in several solvents, including ethanol, methanol, aqueous solutions, hexane, chloroform, and benzene, and was maintained in a shaker for 72 hours. Subsequently, the solvent extracts were filtered, and the filtrates were stored at 40 degrees Celsius for future applications. A qualitative analysis was conducted to determine which solvent extract contained the highest concentration of phytoconstituents**. c) Bulk Preparation (30g):** Utilizing the Soxhlet extraction method, the solid material intended for extraction is placed in a thimble made from a material that retains solids while allowing liquids to pass through, functioning similarly to filter paper. This thimble is then positioned in the extractor. An organic solvent is heated in reflux, causing vapor to rise and subsequently condense in the condenser, which fills the thimble. This cycle continues until the extraction of all desired materials from the solids is complete. The solvent extracts are then condensed using a rotary vacuum evaporator and air-dried. Following air drying, the sample is macerated in boiling water for two hours[(7)] . Finally, the yield percentage of the extract is Extract yield (%) = (weight of extract obtained/weight of plant material used) x 100.

2.2 Assessment of Phytochemical Profiling

The protocol for phytochemical screening and prospection, investigating the presence of saponins, anthraquinones, flavonoids, alkaloids, phenols, steroids, and glycosides. The qualitative analysis was based on precipitation reactions and the formation of foamy layers [8].

2.3 Quantitative Assessment of PHF Extracts

*2.3.1. **Quantitative Estimation of Flavonoid:***

Total flavonoid content was determined by Aluminium chloride method using Quercetin as a standard. 1ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as Quercetin equivalents (mg Quercetin/g dried extract) [9].

*2.3.2. **Quantitative Estimation of Phenolic Compounds:***

The total phenolics content in different solvent extracts was determined with the Folin-Ciocalteu's reagent (FCR). In the procedure, different concentrations of the extracts were mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min 4 ml of sodium carbonate solution was added. The final volume of the tubes was made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using gallic acid solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of gallic acid per gram of dry weight and the standard graph. [10]

*2.3.3. **Quantitative Estimation of Alkaloids:***

To 1ml of test extract 5ml pH 4.7 phosphate buffer was added and 5ml of BCG solution and shake a mixture with 4 ml of chloroform. The extracts were collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470nm against blank prepared as above but without extract. Atropine is used as standard material and compared the assay with atropine equivalents. [11]

2.4 Isolation and purification of bioactive compound

2.4.1. Purification of sample by column chromatography:

Active extracts of PHF(20 g) was subjected to column chromatography on silica gel of 100–200 mesh size (Merck) packed in a cylindrical glass column. Sample was passed in the pre-packed column and eluted with mixture of solvents based on polarity (low to high polarity) as mobile phase to separate and collect fractions. The elution starts with 100% Hexane and then increased the polarity using Chloroform, Ethyl acetate and Ethanol and Methanol in the ratio of 90:10, 80:20, 70:30 and 50:50. Extract was fractionated via gradient separation of mobile phase, and each elute was carefully collected and labelled for further analysis. All the fractions were pooled specific fractions were further purified using preparative TLC. [12]

2.4.2. Thin layer chromatography (TLC)

The eluate fractions from column chromatography were subjected to thin-layer chromatography (TLC) for the separation of active compounds. The fractions were run on a silica gel 60 F254 pre-coated aluminum plate with a thickness of 0.2 mm. The optimal solvent for identifying the compounds was determined by varying the ratios of solvents to develop the solvent system. Due to capillary action, the solvent travels up the plate along with the sample, resulting in the separation of various components of the sample. The formation of bands was observed under UV light. p-Anisaldehyde stain, ninhydrin for amino acids, and cerium sulfate for alkaloids were sprayed over the TLC plate and visualized under UV light to identify complex compounds. The TLC procedure was followed until good resolution was achieved [13].

2.5 Characterization of Phytochemicals

2.5.1. Gas Chromatography-Mass Spectroscopy (GCMS) Analysis:

Gas Chromatography-Mass Spectrometry (GC-MS) is crucial for analysing unknown components of plant origin. The methanolic extract of PHF underwent GC-MS analysis using a GC Clarus 500 Perkin-Elmer system with an AOC–20i

autosampler. Key analysis conditions included: An Elite-1 fused silica capillary column (30 m × 0.25 mm ID × 1μm film thickness). Electron impact mode at 70 eV, using helium (99.999%) as the carrier gas at 1 ml/min and a 2μl injection volume (split ratio of 10:1). Injector temperature at 250 °C and ion-source temperature at 280°C. The oven temperature was programmed from 110 °C (isothermal for 2 min), increasing to 200 °C at 10 °C/min, then to 280 °C at 5 °C/min, with a final 9-minute isothermal period at 280 °C. Mass spectra were recorded at 70 eV, with a scan interval of 0.5 seconds, covering fragments from 45 to 450 Da. The total run time was 36 minutes. Relative percentages of each component were determined by comparing average peak areas, and mass spectra interpretation utilized the NIST library. The results, including names, molecular weights, and structures of the components, were summarized in a table.[14]

2.5.2. Liquid Chromatography-Mass Spectroscopy (LCMS) Analysis:

An Agilent 6400 series Triple Quad (QQQ) LC-MS instrument equipped with an electro spray ionization source (ESI) was employed to analyse the composition of fractions. The optimized detection parameters were as follows: Chromatographic conditions were set up for the identification of an isolated compound. Full wavelength scanning was performed from 190 to 400 nm, the flow rate was 0.4 ml/min, sampling volume was 5μl, and the column temperature was 30°C[15]. Mass spectrometry conditions: negative ion mode, atomization gas pressure-40 psi, dry gas velocity-9 l/min, drying temperature -350°C, ionization voltage3,000V, electro spray ionization (ESI), detection of anion way-auto Msn, scanning range -200 800 m/z.

2.5.3. Fourier Transformed Infrared Spectrum (FT-IR):

Fourier-transform infrared (FTIR) spectroscopy is a valuable tool for identifying the functional groups present in plant extracts. This technique aids in the identification and structural determination of molecules. There are several methods for preparing samples for FTIR analysis. For liquid samples, one of the simplest approaches is to place a drop of the sample between two plates of sodium chloride, allowing the drop to form a thin film. For solid samples, they can be mixed with potassium bromide (KBr) and then compressed into a thin pellet for analysis. Alternatively, solid samples can be dissolved in a solvent such as methylene chloride. A few drops of this solution can then be placed onto a single High Attenuated Total Reflectance (HATR) plate to record the spectrum in terms of percentage transmittance. The peaks observed at specific wave numbers are assigned to various bonds and functional groups based on references found in FTIR instrument manuals.[16]

2.5.4. H1 NMR (Nuclear Magnetic Resonance):

Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. One dimensional technique is routinely used but the complicated structure of the molecules could be achieved through two dimensional NMR techniques.H1NMR is used to find out types of hydrogen are present in the compound and 23 to find out how the hydrogen atoms are connected. Hydrogen-NMR spectra of the extracts was performed using Bruker Biospin Avance 500-MHz NMR spectrophotometer with a 5 mm broad inverse probe head, equipped with shielded z-gradient accessories. Hydrogen NMR spectral analysis of the extract was carried out using one-pulse sequence by dissolving the extract in 500ul of DMSO (Dimethyl Sulfoxide) in 5 mm NMR tubes[17]. A re-usable, sealed capillary tube containing 30ul of 0.375% of trisodium phosphate in deuterium oxide was inserted into the NMR tube before recording the spectra. Tri-sodium phosphate served as chemical shift reference as well as internal standard for quantitative estimation.

3 Results and Discussion

The analyses of PHF show that the isolated compound 1,1-Bis(hydroxymethyl)cyclopropane is Alkaloid family. Generally, alkaloids are valued for their therapeutic effect like antimalarial, anticancer, antibacterial, antiasthma properties.[18]

3.1 Evaluation of Phytochemical Profiling

The presence of saponins, flavonoids, alkaloids, phenols, steroids, and terpenoids was examined in the four solvent extracts. Precipitation processes, foamy appearance, and colour change—all characteristics of the PHF powder's chemical components—as well as the presence or absence of secondary metabolites, as indicated in Table 1 were the main focus of the qualitative assessment. With the exception of glycosides, the majority of the phytochemicals were detected in the methanol extract. With the exception of hexane extract, all of the extracts showed the presence of alkaloids, flavonoids, and phenols.

Table 1. Preliminary Investigation of Phytochemical Constituents in Various Extracts of the Formulation

Test	Ethanol	Methanol	Chloroform	Hexane
Tannin	+	+	-	+
Flavonoid	+	+	+	-
Steroid	+	+	+	-
Alkaloid	+	+	+	-
Quinone	+	+	-	-
Coumarin	-	-	+	+
Terpenoids	-	-	-	-
Glycosides	-	-	-	-
Phlobatannin	-	-	-	-
Saponin	+	+	-	+
Carbohydrate	+	+	-	+
Phenol	+	+	+	-

+ presence; - absence

3.2 Bulk preparation(30g)

From Qualitative analysis of PHF results that most of the phytoconstituents present in ethanol and methanol extracts. This shows that bulk preparation of PHF extract can be done by Soxhlet extraction method in which the solid material (PHF powder) is packed in a filter paper allows only liquid to pass through it. Then it is placed in a thimble. As methanol extract showed good results ,500ml of methanol is then heated in reflux due to which the vapours generated starts boiling and as the vapor rises up they are further condensed by the condenser which further fills up the thimble. This process is repeated until all the materials which is to be extracted from the solids is done. The extract was reconstituted with distilled water for experimental purposes. The yield of extract is 8g.

3.3 Determination of the Total Phenolic, Flavonoid Content, Alkaloid Content in Various Extracts of the Formulation

From methanolic extract of PHF, the total phenolic, flavonoid content determined in Polyherbal Formulation. The total flavonoid content of the extract was expressed in terms of quercetin equivalents (QE) using the standard curve equation in ($y = 0.0479x - 0.9352$) with a correlation coefficient (R2) of 0.874 (Figure 1a). The content of flavonoid was quantified in the methanolic extract of PHF was found to be 45.3mg QE/g. The phenolic content was expressed in terms of gallic acid equivalents (GAE) using the standard curve equation ($y = 0.0469x + 1.0036$) with a correlation coefficient (R2) of 0.6445 (Figure 1b). The content of Phenol was quantified in the methanolic extract of PHF was found to be 47.5 mg GAE/g. The alkaloid content was expressed in terms of alkaloid equivalents (AE) using the standard curve equation ($y= 0.0728x + 0.2691$) with a correlation coefficient (R3) of 0.973 (Figure 1c). The content of alkaloid was quantified in the methanolic extract of PHF was found to be 46.3mg AE/g.

3.4 Column Chromatography

The PHF (Polyherbal Formulation) extract was subjected to silica gel column chromatography, a classical technique widely used for the separation of phytochemicals based on polarity. Elution was carried out using a gradient of solvents ranging from low to high polarity, allowing the sequential release of compounds from the column. A total of 45 fractions were collected and each was analysed using Thin Layer Chromatography (TLC) to detect the presence of bioactive constituents (Figure 2). Among the eluted fractions, the 32nd fraction exhibited distinct TLC banding and was selected for further characterization based on its phytochemical profile. This approach aligns with standard practices in natural product isolation and phytochemical profiling. [19]

3.5 Thin Layer Chromatography

Using a variety of solvent systems, Thin Layer Chromatography (TLC) profiling of successive PHF extracts was carried out to find phytochemical components (Figure 3). The solvent mixture of methanol, chloroform, and acetic acid in the ratio of 1

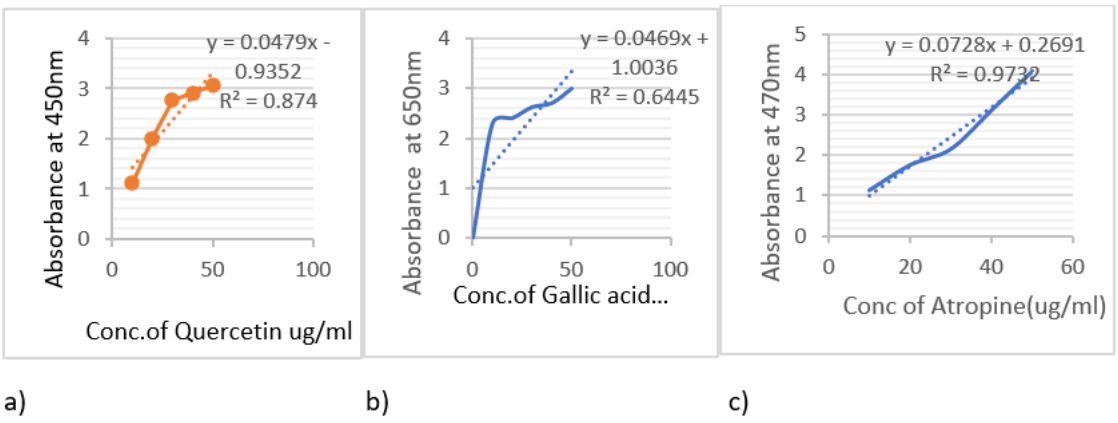

Fig 1. Estimation of phytochemical content in various extracts of the formulation: (a) calibration curve for the estimation of the total flavonoid content in various extracts of the formulation using quercetin as a standard compound (b) calibration curve for the quantification of the total phenolic content in various extracts of the formulation using gallic acid as a standard compound, (c) calibration curve for the estimation of the total alkaloid content in various extracts of the formulation using atropine as a standard compound

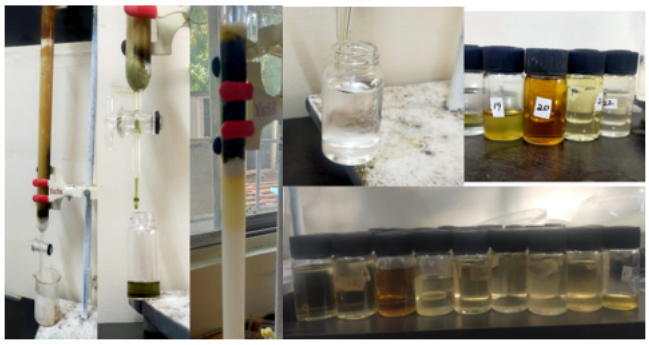

Fig 2. Column chromatography and their elution

mL:0.9 mL:100 μL was determined to be the most successful in obtaining distinct band separation and resolution among the studied combinations. Several different bands were seen after being exposed to UV light, suggesting the existence of a variety of phytocompounds with different polarity. For first phytochemical screening, TLC is a quick and affordable method that enables the comparison and visualization of secondary metabolites in various solvent fractions. [20]

3.6 Gas Column Chromatography (GCMS)

GC-MS results revealed a total of 15 effective compounds were identified from the chromatogram (Figure 4). The bioactive compounds were predicted by their retention time (RT), peak area percentage (%) and molecular weight Cyclopentadecanone,2hydroxy(2Hydroxycyclopentadecanone),1(hydroxymethyl)cyclopropyl] ethanol, trimethylsilyloxy formate(Performic acid, trimethylsilyl derivative), Spiro[2.4]hepta-4,6-diene,4,5,6,7,8,9-hexahydrocycloocta[d][1,3]thiazole(4,5Hexamethylenethiazole),Undecane, bis(2-methylpropyl) benzene-1,2-dicarboxylate), Epi-inositol(cyclohexane- 1,2,3,4,5,6-hexol), trans-3-Ethoxy-b-methyl-b-nitrostyrene(1-ethoxy-3-[(E)-2-nitroprop-1enyl]benzene), 3,5Dimethylbenzaldehydethiocarbamoylhydrazone[(E)(3,5dimethylphenyl)methylideneamino] thiourea),2-Ethylacridine(2-ethylacridine),1,4-Benzenedicarboxylicacid,bis(2ethylhexyl) ester(bis(2-ethylhexyl) benzene-1,4- dicarboxylate), Cyclotrisiloxane, hexamethyl-(2,2,4,4,6,6-hexamethyl-1,3,5,2,4,6-trioxatrisilinane),Benzo[h]quinoline,2,4-dimethyl-(2,4 dimethylbenzo[h]quinoline),Tetrahydrorhombifoline((1S,2R,9R)-11-but-3-enyl-7,11-diazatricyclo[7.3.1.02,7]tridecan6-one).Among the isolated compounds 1,1 Bis(hydroxymethyl)cyclopropane has high highest peak which indicates highest concentration. The isolated compounds were confirmed by comparison with mass spectral library of NIST. As 1,1-

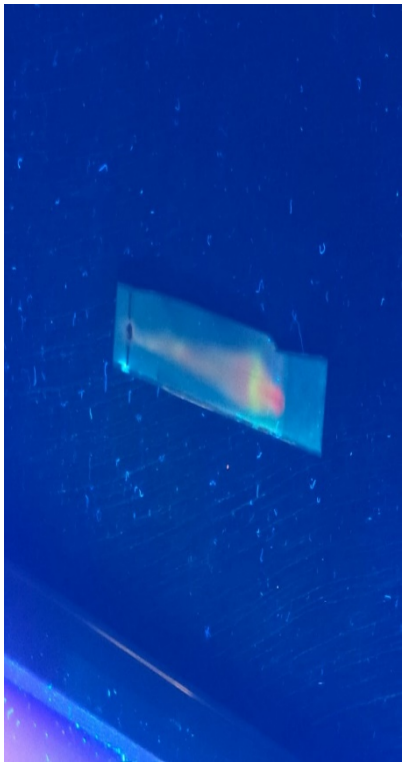

Fig 3. TLC profiling of PHF extract

Bis(hydroxymethyl)cyclopropane ([1-(hydroxymethyl)cyclopropyl]methanol) shows highest peak in GCMS Chromatogram which indicates that the methanolic elution of PHF contains high Concentration of 1,1-Bis(hydroxymethyl)cyclopropane (Table 2) (Figure 4).

RT= Retention time

1,1-Bis(hydroxymethyl)cyclopropane's considerable abundance suggests that it significantly contributes to the phytochemical profile of the PHF extract. Despite being less frequently found in conventional herbal extracts, this chemical has been investigated for its structural resemblance to cyclic compounds based on alcohol that may have cytoprotective and antioxidant effects.[21]

3.7 Liquid Chromatography (LCMS)

LCMS is done to know the composition of fractions present in the methanolic elution .From LCMS 19 fractions were identified based on mass and charge ratio(m/Z).From previous study the reference of LCMS of alkaloid compounds is used to detect the methanolic elution of the highest peak of RT 5.92 indicates 3 hydroxy N methylcoclaurine also known as isoquinoline alkaloid having a tetrahydroisoquinoline core with 3,4-dihydroxybenzyl,methoxy and hydroxy groups at the 1-,6- and 7-positions respectively. Some studies shows that this fraction of compound have antiproliferative activity. The Compound isolated from GCMS and LCMS are of same group of family Alkaloid **(Figure 5)**.[22]

3.8 Spectroscopic study

The FT-IR spectral technique is typically employed to detect the different functional groups in the plant extract and is confirmed by comparing it with the standard IR. The FT-IR spectrum of PHF displayed a broad band around 1020.34Cm^{-1}, demonstrating the presence of C-N stretch with amine groups. The peaks at 2829.57m^{-1} and 3319.49 cm^{-1} revealed the skeletal N—H stretch of amine salt and secondary amine. The spectrum also confirmed the presence of active Secondary alcohol and alcohol with C-O and O-H functional motifs, attributed to the stretching peaks around 1114.86 and 3743.83cm^{-1}, respectively. The symmetric stretching of the N=C=S stretch emerged around 2042.62 cm^{-1}. The less intense peaks around 665.4 cm^{-1} revealed the alkene C=C bend in the PHF. The prevalence of these chemical stretches and linkages for various functional groups raises the possibility

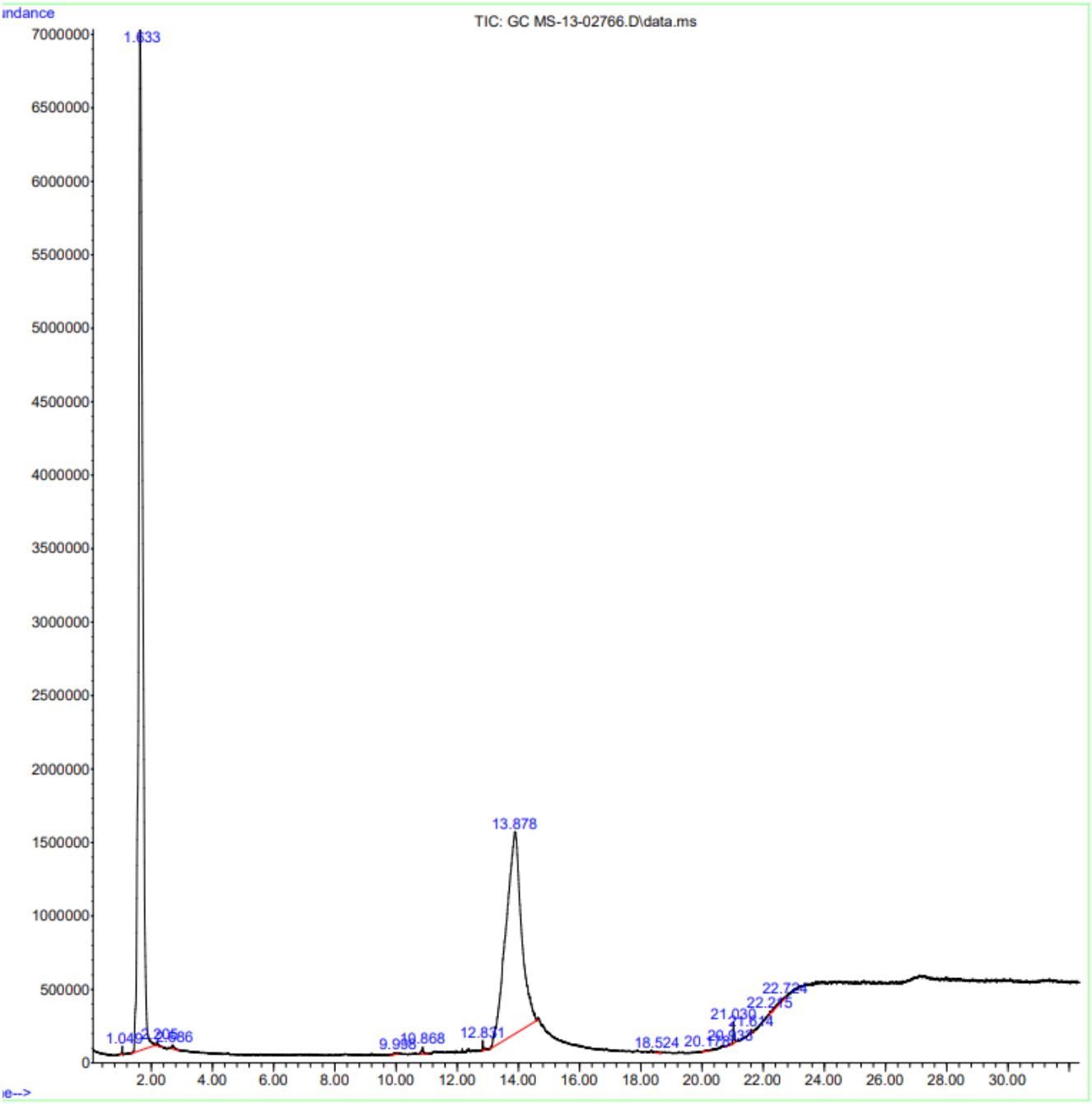

Fig 4. Chromatograms of identified phytochemical constituents profile in the methanolic extract of the PHF using the GCMS technique

Table 2. Phytochemical Compounds Identified in the Methanolic Extract of the PHF Using the GC—MS Technique

SI no	Compound label	Molecular weight	RT	DB Formula	Hits (DB)
1	Cyclopentadecanone, 2-hydroxy(2-Hydroxycyclopentadecanone)	240.38g/mol	1.049	$C_{15}H_{28}O_2$	3
2	1,1-Bis(hydroxymethyl)cyclopropane ([1-(hydroxymethyl)cyclopropyl]methanol)	102.13g/mol	1.633	$C_5H_{10}O_2$	3
3	trimethylsilyloxy formate(Performic acid, trimethylsilyl derivative)	134.21g/mol	2.205	$C_4H_{10}O_3Si$	3
4	Spiro[2.4]hepta-4,6-diene	92.14g/mol	2.686	C_7H_8	3
5	4,5,6,7,8,9-hexahydrocycloocta[d][1,3]thiazole(4,5-Hexamethylenethiazole)	167.27g/mol	9.998	$C_9H_{13}NS$	3
6	Undecane	156.31g/mol	10.868	$C_{11}H_{24}$	3
7	bis(2-methylpropyl) benzene-1,2-dicarboxylate (	278.34g/mol	12.831	$C_{16}H_{22}O_4$	3
8	Epi-inositol(cyclohexane-1,2,3,4,5,6-hexol)	180.16g/mol	13.878	$C_6H_{12}O_6$	3
9	trans-3-Ethoxy-b-methyl-b-nitrostyrene (1-ethoxy-3-[(E)-2-nitroprop-1-enyl]benzene)	207.23g/mol	18.524	$C_{11}H_{13}NO_3$	3
10	3,5-Dimethylbenzaldehyde thiocarbamoylhydrazone [(E)-(3,5-dimethylphenyl)methylideneamino]thiourea)	207.3g/mol	20.178	$C_{10}H_{13}N_3S$	3
11	2-Ethylacridine(2-ethylacridine)	207.27g/mol	20.933	$C_{15}H_{13}N$	3
12	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester(bis(2-ethylhexyl) benzene-1,4-dicarboxylate)	390.6g/mol	21.030	$C_{24}H_{38}O_4$	3
13	Cyclotrisiloxane, hexamethyl-(2,2,4,4,6,6-hexamethyl-1,3,5,2,4,6-trioxatrisilinane)	222.46g/mol	21.614	$C_6H_{18}O_3Si_3$	3
14	Benzo[h]quinoline, 2,4-dimethyl-(2,4-dimethylbenzo[h]quinoline)	207.27g/mol	22.215	$C_{15}H_{13}N$	3
15	Tetrahydrorhombifoline((1S,2R,9R)-11-but-3-enyl-7,11-diazatricyclo[7.3.1.02,7]tridecan-6-one)	248.36g/mol	22.724	$C_{15}H_{24}N_2O$	3

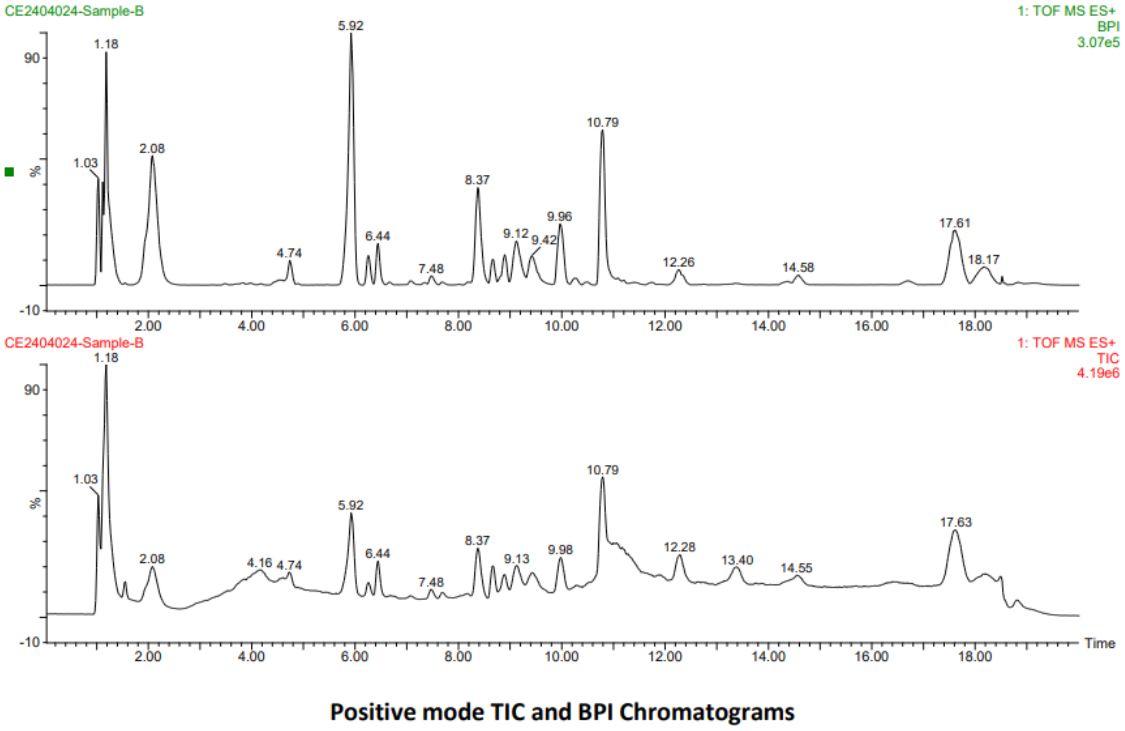

Fig 5. LCMS Chromatogram of methanolic extract of PHF

that the extract components may bind to the target proteins that trigger the apoptotic signalling cascade in antitumor activity. A FT-IR investigation on methanolic extract of PHF was related to the molecular docking of volatile chemicals based on GC-MS [23] (Table 3) (Figure 6).

Table 3. All Potential Bands, Corresponding Functional Groups Identified in the methanol Extract of PHF Using FT-IR Spectroscopy

Peak Position	Spectroscopic Assignment	Functional Group
665.4	C=C bend	alkene
1020.34	C-N stretch	amine
1114.86	C-O stretch	secondary alcohol
2042.62	N=C=S stretch	isothiocyanate
2829.57	N-H stretch	amine salt
2943.37	C-H stretch	alkane
3319.49	N-H stretch	secondary amine
3743.83	O-H stretch	alcohol

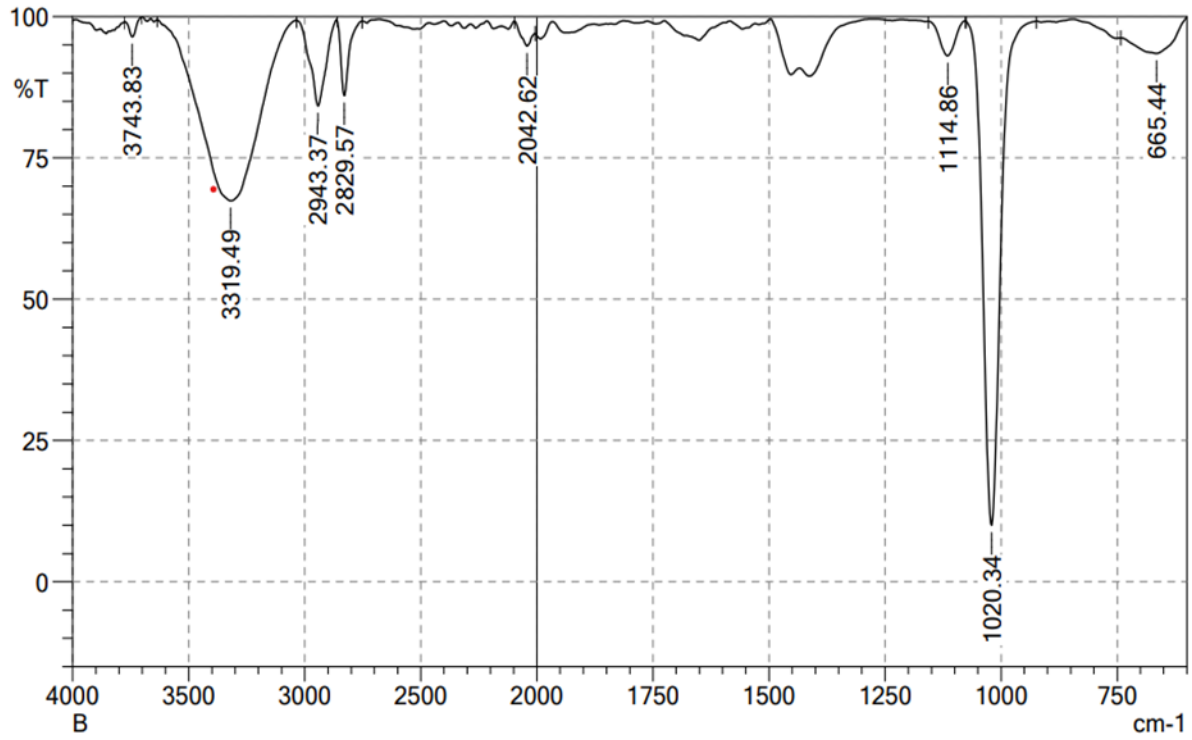

Fig 6. FT-IR spectrum representing potential bands in the methanol extract of PHF

3.9 H1 Nuclear Magnetic Resonance (H1NMR)

The identification of the Hydrogen atom present in methanolic elution were characterized using H1 Nuclear Magnetic Resonance (H1 NMR). Based on Chemical shift (ppm) the Hydrogen atom is compared with spectral library (Table 4) (Figure 7).

Previous studies suggest that similar compounds exhibit anticancer properties, indicating the potential of this compound for anticancer activity. LC-MS analysis revealed that the highest peak, with an RT value of 5.92, corresponds to isoquinoline, a member of the alkaloid family known for its antiproliferative properties [24]. Alkaloids are naturally occurring compounds containing carbon, hydrogen, nitrogen, and sulfur.

Table 4. Type of proton present in PHF

Type of proton	Chemical shift (ppm)
(alkyne grp)	4.656
	4.342
	3.227

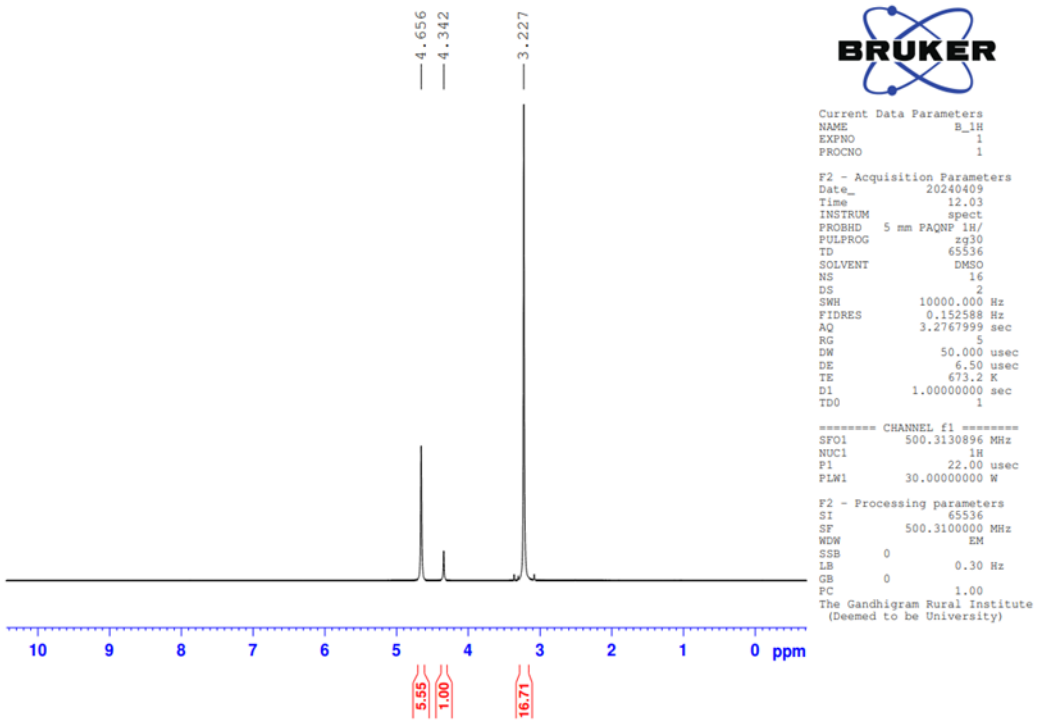

Fig 7. H1NMR spectral analysis of methanol extract PHF

4 Conclusions

In this study, the medicinal potential of PHF and its pharmacologically active compounds has been explored. Qualitative analysis revealed the presence of various phytochemicals, including tannins, flavonoids, alkaloids, phenols, quinones, saponins, and steroids in the extracts of PHF. FTIR analysis confirmed the presence of functional groups, with a strong C-N stretching peak at 1020.4 cm^{-1}. Additionally, ^{1}H-NMR analysis provided insights into the type and stability of hydrogen or protons present in the compound based on chemical shift (ppm) values.

These findings suggest that the isolated compound can be further purified using LC-LCMS and HPLC until a pure form is obtained. Subsequent studies can then evaluate its anticancer activity, paving the way for potential drug development in future research.

The methanolic elution of the polyherbal formulation was found to contain multiple bioactive compounds, as characterized by GC-MS, LC-MS, FTIR, and ^{1}H-NMR analyses.

GC-MS analysis identified a prominent peak at a retention time (RT) of 1.633, corresponding to 1,1-bis(hydroxymethyl)cyclopropane, a precursor of alkaloids.

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