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ORIGINAL PAPER

GC–MS Based Metabolomics Strategy for Cost-Effective Valorization of Agricultural Waste: Groundnut Shell Extracts and Their Biological Inhibitory Potential

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Abstract

Groundnut shells (GNS) make up about 20% of the weight of a dried peanut pod, indicating a substantial amount of shell residue after groundnut processing. In primary screening, bioactive metabolites present in the various GNS extract solvents like methanol, ethanol, acetone, ethyl acetate, hexane and petroleum ether were analyzed. Further, in the quantitative analysis, total phenol and tannin content have been analyzed. Major metabolites present in the GNS extracts are Octadecane (65%), Palmitic acid (23.53%), Oleic acid (10.41%), and Lupeol (21.44%). Methanol exhibits stronger antioxidant property than other extracts due to polarity and the phenols abundance. It was reflected in IC_{50} results of DPPH (789.36 µg/mL) and ABTS (480.11 µg/mL) radical scavenging assays. Identical results were found in antimicrobial potential against *Aeromonas hydrophila, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Staphylococcus aureus*. Higher antibacterial activity was obtained in methanolic extracts compared to other exrtacts. Minimum inhibitory concentration (MIC) was determined against the organisms tested, in which methanol exhibited lower MIC value at 250 µg/mL, whereas other solvent extracts showed at 500 µg/mL of GNS extracts. Further, the antimicrobial ability was confirmed by analyzing growth of microorganisms with the obtained MIC and Sub-MIC range of the extracts. At MIC range, bacterial growth was completely inhibited. This research is being implemented in order to develop a zero-waste production system by converting waste into valuable bio-products, including the use of GNS as a potential antioxidant and antibacterial agent, which is cost effective when compared to other pharmaceutical agents.

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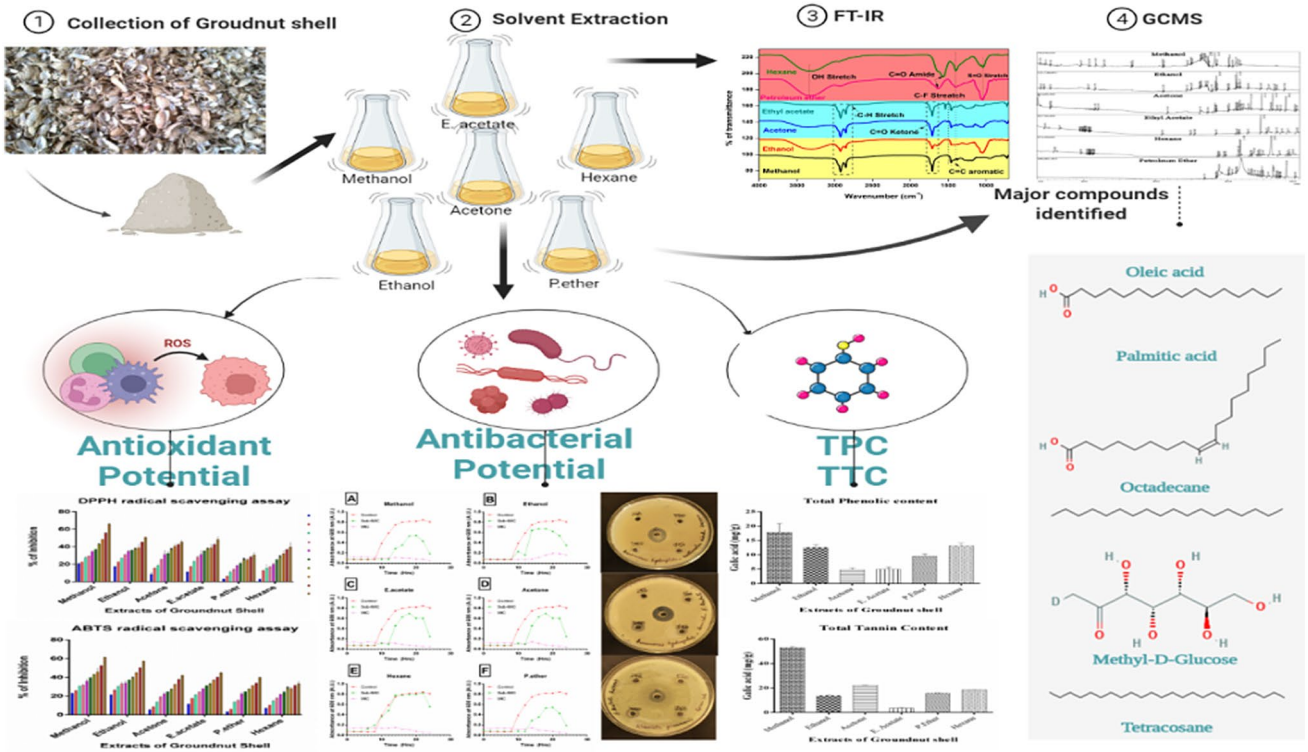
Graphical Abstract



Keywords Waste management · Groundnut shell · Phytochemicals · Scavenging activity · Minimum inhibitory concentration

Statement of Novelty

Groundnut shell is generally perceived as a waste biomass, and millions of tonnes are being dumped into the environment every year. To attain zero waste disposals, this waste can be recycled into a useful biomedical product. The current study is unique that focuses on recycling groundnut shell waste and evaluating its biomedical potential. Furthermore, it reduces the significant disposal challenges as well as its potential for serious pollution. This paper discusses the use of renewable resource in an innovative strategy to identify the prospective anti-microbial and anti-oxidant compounds. Novelty of the study is to use an environmentally friendly approach and concerted efforts for the development of recycling agricultural waste into a profitable venture. Furthermore, "Waste to Wealth" will create revenue for the farmers as a findings of the research.

Introduction

Natural sources provide abundant therapeutic and pharmaceuticals substances for decades. Especially, plant-based conventional medicine still plays an essential role in human and animal health care [1]. Plant metabolites are vital for capturing and neutralising free radicals, also acting as protective mechanism against serious health issues.The hunt for antioxidant and antimicrobial compounds predominantly in plants, is a decisive approach in fighting against diseases in widespread nature [2].Groundnuts (*Arachis hypogaea* L.), a common nutritional leguminous crop cultivated mostly for seed and oil, providing a high quality protein with improved health benefits [3].

Around 29−30 million metric tonnes of groundnuts are produced every year worldwide, in which China, United States and India are the major contributors [4]. Groundnut shells (GNS) is a waste product formed after extracting the groundnut seed from its pod. Its an agro-based byproduct that takes a long time to degrade in environmental condition [5]. However, GNS contain variety of metabolites and beneficial compounds for humanitarian benefit. It includes

a great deal as a feedstock, foodstuff, fertiliser filler, and bio-filter carrier. Unfortunately, the isolated GNS wastes are normally burnt or buried and thus results in environmental pollution leads to the emission of green house gases causing harmfulness to the human health [6]. Therefore, novel technologies are required to develop and achieve zero waste production, i.e., transform waste products into useful products of paper, and bio-energy industries [7–9].

Various attempts to recycle GNS waste have been tried over the years. For example, a small portion of groundnut shells are incorporated as animal feed, mainly as cattle feed [10]. Collins et al. [11], conducted experiments on shells as a dietary fiber for human beings. Its usage in pulp manufacture was further explored in experimental research [12], feedstock for the manufacturing of bioethanol [13–15], board material [16], and activated charcoal [17]. Composting wet materials, wastewater treatment, metal casting, pesticide medium, activated carbon, and the manufacture of plastics, wardrobes, and insulation board are some of the other applications of GNS. The main drawback of GNS waste is its greater lignin content, which inhibits biodegradation under regular environmental circumstances and creates digestion problems in ruminants.

Since it is non-polluting and ecofriendly, bio-diesel, which is generated from vegetable oil or animal fat, is a popular fuel [18]. Udeh. [19] used the fungus *Aspergillus niger* to manufacture bio-diesel from lipase-catalyzed GNS using fermentation. Thota et al. [20] studied the manufacture of cellulases by individuals and synergistic fungal communities.

Biowaste of groundnut shells or hulls is used for various industrial, agricultural, and scientific fields. Activated carbon from groundnut shell acted as better absorbant to remove pollutants from industrial effluents [21, 22]. Groundnut shell activated carbon (GSAC) removed methylene blue dye found in the water with moderate micro biostatic activity. GNS waste is also used as a binder in sandcrete blocks (1:8 ratios) instead of cement [23, 24]. Kutshik et al. [25] conducted a study to manufacture single-cell protein via *Saccharomyces cerevisiae* using agricultural biomass such as bagasse and groundnut shell as source media. Omidi et al. [26] investigated the ability of GNS as an alternative peat composite for decorative plant growth.

Experiments have been carried out on poultry birds fed with raw and boiled GNS along with the commercial feed. It has been declared that the low concentration of commercial feed supplemented with higher concentration of boiled groundnut shells drastically improves the weight gain in poultry birds [27]. In Bali cow, corn and GNS concentrate usage increase the commercial feed replacing 50% rice bran [28]. Interestingly, in vitro digestibility of GNS and other agro-byproducts is higher than in vivo digestibility [29]. Alu et al. [30] findings suggest that the alkali-treated groundnut shell meal can be used to increase poultry production for superior quality and growth of meat. Alkali-treated GNS improves the ruminal blood and serum glucose concentration [31].

With the improvement of the nutrient quality of GNS by fermentation, it can be used for the livestock production. Armayanti et al. [32] conclude that 5% of fermented GNS helps in weight gain, feed conversion ratio, and improves the performance of the broilers. In rabbits, GNS treated with different doses of urea and soyabean meal improves feed intake, nutrient digestibility, weight gain, and nitrogen retention resulted lower feed costs [33]. Plants provide a more reliable source of phytoconstituents, which are utilised to treat a variety of diseases. Generally, natural antioxidants like phenols, flavonoids, alkaloids and saponins rich plant material tend to act against the reactive oxygen species (ROS) related diseases like cancer, diabetes and liver disorder [34]. Ethnopharmacological data-based phytochemical research is largely regarded as a successful strategy for discovering novel, effective medications from plant extracts.With this outline, this study is designed to evaluate biomedical applications such as the antibacterial and antioxidant capacity of agricultural waste like GNS. It focuses on examining the content of phytochemicals such as total phenols and tannin and qualitative analysis of the metabolites by GC–MS analysis of GNS waste, which provides insight into the utilization of waste and its recycling. Further, this research paves the way to obtain nutritional and pharmaceutical benefits from agricultural wastes.

Materials and Methods

Collection of GNS Wastes and Preparation of Extracts

Groundnut shell wastes were collected from Alangudi, Pudukkottai district in Tamil Nadu, India. The collected wastes were shade dried at 37 °C and coarsely powdered. The powder was then sieved with 0.2 mm sieve plates and kept in an air-tight container at − 20 °C for further analysis. The extracts were prepared from powdered GNS waste using the cold maceration method with six solvents: methanol, petroleum ether, hexane, ethyl acetate, ethanol and acetone (10:90 W/V) [35]. Solvents were chosen based on the polarity, the polar solvents, methanol and ethanol were used for polar compound extraction, whereas non-polar solvents such as hexane and petroleum ether were used for non-polar compound extraction. The acetone and ethyl acetate are the mid-polar solvents (Partially polar/non-polar), which were used to elute mid-polar compounds. The extract was filtered using Whatman No.1 filter paper before being concentrated at 40 °C using rotary vacuum evaporator under decreased

pressure until agglomerates were produced. The extracts were desiccated to eliminate excess solvents before being stored at 4 °C for further research.

Phytochemical Analysis

Each extract was tested to identify the presence of saponins, anthroquinone, terpenoids, steroids, alkaloids, tannins, flavonoids, phenols and reducing sugars.

Steroids

250 µL concentrated H_2SO_4 was added slowly to 0.5 mL of GNS extracts and combined with 2 mL chloroform. The upper layer coloured red, while the H_2SO_4 layer turned yellow and fluoresced green [36].

Terpenoids

2 mL chloroform was added to 1 mL of GNS extracts followed by 2 mL of concentrated H_2SO_4 was cautiously added and gently shaken. The existence of the steroidal ring, i.e. the glycone part of the glycoside, was displayed by a reddish-brown tint [36].

Reducing Sugar

In a test tube, 2 mL of the extract's aqueous solution was added to a 5 mL mixture of equal volumes of Fehling's solutions I and II and heated in water bath for 2 min. The existence of reducing sugar is indicated by a brick-red precipitate [37].

Alkaloids

1 mL of extract was dissolved with 2 mL of diluted HCl solution and filtered. In 2 mL of filtrate was added with drops of Hager's reagent. Alkaloids were detected by the presence of a brilliant yellow precipitate [38–40].

Flavonoids

2–3 mL of extract filtrate were treated with a strip of magnesium ribbon and 1 mL of concentrated HCl. Flavonoids presence was confirmed by the pink-red/crimson colouring of the solution [41].

Saponins

In a test tube, 5 mL of distilled water was mixed with 1 mL of crude extract. The mixture was agitated for 30 s. The presence of saponins was determined by the production of stable foam after 30 min shaking [36].

Tannin

After adding a few drops of newly prepared ferric chloride to 1 mL of the extract, a dark blue or greenish-black colour was detected, confirms the existence of tannins [36].

Phenol

The presence of phenol was determined by placing a drop of 5% lead acetate to 1 mL of GNS extract, which resulted in a yellow colour precipitate [42].

Anthraquinones

In a water bath, 5 mg of powdered extract were heated with 10% HCl for 5 min. Then the reaction mixture was filtered and cooled down, an equivalent volume of $CHCl_3$ was added to the filtrate. A few drops of 10% NH_3 were added, then the mixture was heated gently. The presence of anthraquinones was detected by the development of a pink tint [37].

Characterization of the GNS Extracts

FT-IR Analysis

The Fourier transform infrared (FT-IR) spectrophotometer (Perkin Elmer, USA) was used to identify the functional groups involved in GNS extracts by adopting KBr pellet method in the spectral range of 4000–500 cm^{-1}.

GC–MS Analysis of GNS Extracts

Gas Chromatography—Mass Spectrometry (GC–MS) investigation of GNS extracts was performed using a Shimadzu (QP2020) instrument integrated with a mass spectrometer. A 30 m long SH-Rxi-5Sil-MS capillary column with 0.25 mm inner diameter and 0.25 m film thickness was adopted in the instrument, which was coated with 100(%) polydimethylsiloxane. The oven temperature was 50 °C intially and it was gradually increased to 280 °C accounted by 6 °C min^{-1} with final hold duration of 2 min. The maintained injector temperature was 250 °C and pressure at 68.1 kPa, helium was used as carrier gas, with flowing rate of 1.2 mL/min (linear velocity of 39.7 cm/s). 100 µL of filtrate dissolved in methanol, ethanol, acetone, ethyl acetate, hexane and petroleum ether solvents were filtered by using syringe filter (0.25 µM) to remove the impurities. The prepared sample was injected into GC with a 1:10 split ratio. The mass spectrum obtained in electron ionisation mode maintained at 70 eV. The temperature of ion source was kept constant at 200 °C. The mass spectra of metabolite obtained in GNS extract was compared and matched with standard spectra in

NIST 2005 MS collection library to interpret the information about compounds and in literature [43]. The average peak area to total area ratio was calculated for relative percentage of each compound.

In Vitro Antioxidant Activity

DPPH Scavenging Assay

Prepared GNS extracts were tested for their ability of radical scavenging activity when exposed to 2, 2-diphenyl-1-picryl-hydrazile (DPPH) using the procedure of Brand-Williams et al. [44]. On brief, 100 µL of prepared GNS extracts at various concentrations (200, 400, 600, 800, and 1000 µg/mL) were prepared in 96 well plates. Ascorbic acid (Vitamin-C) was used as reference standard. To each well, 100 µL of freshly prepared DPPH (1 mM) suspension was added. The mixture kept in dark condition and incubated for 30 min at room temperature. The inference of violet to yellow color of the solution showed that the free radicals had been scavenged, and it was detected at 517 nm in Synergy HT Multimode Reader (Biotek, Winooski, USA). Subsequently, the following equation was used to calculate the percentage of scavenging capability of the GNS extracts.

$$\% \text{ Scavenging} = [\text{Ac} - \text{As} \div \text{Ac}] \times 100 \quad (1)$$

where Ac–OD value of blank, As–OD value of GNS extracts.

ABTS Radical Scavenging Assay

ABTS radical cation decolorization assay was used to determine the antioxidant properties of various GNS extracts [45]. 7 mM ABTS was dissolved in deionized water with 88 µL of 140 mM ($K_2S_2O_8$) potassium persulphate solution. The mixture was allowed to stand at 37 °C in dark condition for 14 h. The ABTS mixture was diluted in ethanol (1:89 v/v) to obtain an absorbance at 734 nm for each concentration in triplicate run.Then, 100 µL of various concentrations (200, 400, 600, 800, and 1000 µg/mL) of solvent extracts were added to 200 µL of freshly prepared ABTS + solution respectively. After a 10 min incubation period, the reaction absorbance of solution mixture was measured at 734 nm. As control, ascorbic acid was employed. Equation was used to calculate ABTS radical scavenging activity (1).

Determination of Total Phenol and Total Tannin Content of the GNS Extracts

Total phenolic contents (TPC) of the GNS extract were determined by the slight modification in the Klompong and Benjakul's [46] method. Briefly, 900 µL of deionised water was added to 100 µL of the extracts. Then, 500 µL of Folin-Ciocalteu's phenol reagent was added to it. The mixture was incubated for 5 min, followed by the addition of 10 mL of 7.5% Na_2CO_3 and mixed thoroughly. The mixture was kept undisturbed for 30 min at 37 °C. After the incubation, UV visible spectrophotometer Synergy HT Multimode Reader (Biotek, Winooski, USA) was used to measure the absorbance at 765 nm. The total phenolic content of the extracts were determined by milligrams gallic acid equivalent (GAE)/100 g using gallic acid as standard reference.

The total tannin content (TTC) of the different GNS extracts were estimated by Folin-Ciocalteu's phenol reagent, as described by Amorim et al. [47]. In a test tube, 100 µL of the extract was mixed with 8.3 mL of double-distilled water, then by 0.5 mL of Folin-Ciocalteu phenol reagent and maintained at 37 °C for 5 min. After the incubation period, 1 mL of 35% Na_2CO_3 solution was mixed in the test tube. The mixture was held at 25 ± 2 °C for 30 min after being well shaken. The absorbance was then measured at 725 nm. As control, dis.H_2O was used. Tannic acid as standard reference and total tannin content of the extracts were reported as milligrams tannic acid equivalent (TAE)/100 g.

Assesment of Antibacterial Activity

Agar Well Diffusion Method

Antibacterial efficacy of prepared GNS extracts was examined by agar well diffusion method [48] against selected pathogenic bacteria, namely, *Aeromonas hydrophila*, *Pesudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. These strains were procured from Microbial Type Culture Collection, Chandigarh, India. Streptomycin (1 mg/mL) was used as the standard antibiotic to compare the zone of inhibition of prepared GNS extracts diluted in DMSO at doses of 250, 500, 750, and 1000 µg/mL in a progressive manner. Tested microbes were inoculated in 10 mL nutrient medium and incubated at 37 °C for 24 h to yield working culture. The zone of inhibition (diameter) was used to measure the antibacterial effect of the prepared GNS extracts.

Determination of Minimum Inhibitory Concentration (MIC)

Sterile 96-well microtiter plate with resazurin as cell growth indicator was used to acquire MIC values for all GNS extracts [49]. In a sterilized laminar air flow chamber, 96 well microtiter plate filled with 100 µL of nutrient broth and 100 µL of various extracts (10 mg/mL) in 10% (v/v) DMSO was added into the third row of the plate and serial dilutions were performed; first two rows were acted as a control (without GNS extracts). In each well, 10 µL of resazurin solution was added and followed by 10 µL of bacterial

inoculum (5×10^6 cfu/mL). The microtiter plate was then gently wrapped in aluminium wrapper to prevent culture dehydration, and the plates were maintained at 37 °C for 24 h. The wells were visually examined for colour change, and shifts from purple to pink (positive) or colorlessness were deemed negative. MIC range was obtained by identifying the lowest concentration of extract to kill the bacteria completely (colour change occurred). Each experiments were performed in triplicates to determine the exact MIC value of the GNS extracts.

Effects on Growth of the Various GNS Extracts on Microbial Cultures

Growth of *A.hydrophila*, *P.aeruginosa*, *S.aureus*, and *K.pneumoniae* were analyzed with respect to determined MIC and sub-MIC (1/2 MIC) concentrations of various GNS extracts with modified procedure of Qayyum et al. [50] method. Microbial cultures were seeded into tubes to obtain final inoculum of 1.6×10^4 CFU mL^{-1} followed by the addition of extracts based on obtained MIC and sub-MIC values (in triplicates). The cultured tubes were maintained at 37 °C. The bacterial growth was observed spectrophotometrically in UV–Vis spectrophotometer (Synergy HT Multimode Reader, Bioteck instrument, Winooski, VT, USA) by measuring OD at 600 nm for 24 h with 2 h time intervals. Untreated microbial cultures acted as control.

Statistical Analyses

The findings were presented as mean ± standard deviation (SD) and analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the triplicate results. There were significant changes between the experimental and control groups and it was determined at $p < 0.05$. Graphs were plotted using Origin Pro 9.0 (OriginLab corp. Northampton, US) and visualized using Graphpad prism (GraphPad Software, San Diego, California USA).

Results and Discussion

Primary Phytochemical Screening

The phytochemical evaluation of the various extracts of GNS is shown in Table 1. These extracts contain phenols, tannins, saponins, alkaloids, flavonoids, reducing sugars, terpenoids, and steroids. Both phenols and tannins are present in all the extracts; flavonoids are present in methanol, ethanol, and acetone. Alkaloids are present in methanol, ethanol, and hexane, whereas terpenoids are present in all solvent extracts except hexane. These metabolites play a crucial role for medicinal purposes [34].Tannins are used to heal wounds and they may act as anti-septic, anti-inflammatory [51]. Flavonoids are used to treat cancer due to their potential antioxidant properties [52]. Terpenoids inhibits the Ca^{2+} influx in the vascular smooth muscle or by reducing reactive oxygen species (ROS) and stimulating the production of nitric oxide (NO) in cardio vascular smooth muscle [53]. Saponins may trigger mucus membrane coping mechanisms, reducing congestion in the stomach's mucus layer leads to reduction of acidity [54]. These bioactive chemicals are found naturally and have been shown to have antimicrobial or antifungal activities against the human infections examined [55]. The reasons for the inhibitory effect of the secondary metabolites are the disruption of the biochemical pathways, droping of protein synthesis, and membrane deterioration [56].

Table 1 Preliminary phytochemical screening of various extracts of Groundnut Shell

S. no	Name of the phytochemicals	Methanol	Ethanol	Hexane	Acetone	Petroleum ether	Ethyl acetate
01	Steroids	−	−	−	+	−	−
02	Terpenoids	+	+	−	+	+	+
03	Reducing sugar	+	+	−	+	−	+
04	Alkaloids	+	+	+	−	−	−
05	Flavonoids	+	+	−	+	−	−
06	Saponins	+	+	+	+	+	+
07	Tannins	+	+	+	+	+	+
08	Phenolic content	+	+	+	+	+	+
09	Anthraquinone	−	−	−	−	−	−

(+ Presence, −Absence)

FT-IR Analysis

FT-IR is a spectral analysis to identify the potential functional groups available in the prepared extracts of GNS. Figure 1 shows the FT-IR spectra of extracts acquired in the range from 4000 to 500 cm^{-1}. Methanol, ethanol, acetone and ethyl acetate extracts showed strong peak with high intensity band around 3391.11 cm^{-1} (OH stretching alcohols and phenols), 2920.39 cm^{-1} & 2851.86 cm^{-1} (=C–O stretching aldehydes), 1710. 11 (C=O stretching ketones) and 1603.15 cm^{-1} and 1482.57 cm^{-1} for stretching bend of C–H (alkanes), N–H (amide and amines). The peaks in the range of 1441.55 cm^{-1}, 1085.84 cm^{-1}, 1034.43 cm^{-1}, and 1037.06 cm^{-1} were corresponding to alkane (–C–H bend), aromatic amines (C–N stretching), aliphatic amines (C–N stretch), and sulfoxide (S=O stretching), respectively. The remaining peaks at 921.75 cm^{-1} indicated O–H bend (carboxylic acids), 765.51 cm^{-1} represented C–H bend (alkynes) and 652.11 cm^{-1} represented C–Br stretch (alkyl halides), whereas petroleum ether and hexane extracts of the groundnut shell showed all the peaks related to other extracts except 3391.11 cm^{-1} (OH stretching alcohols and phenols). For detecting bio molecule composition, FTIR spectroscopy has proved to be a reliable and sensitive approach [57]. The presence of the functional groups indicated the phytochemical metabolites like phenols, alkaloids, tannins, saponins, and flavonoids. These study results correlated with the antioxidant and antibacterial potential of the extracts due to the presence of these functional groups [58, 59]. Alkane groups were found in the waxy portion of numerous species, which protect the water loss of plants and prevent the leaching of minerals to act against pathogens [60]. Carboxylic acids are responsible for the fat metabolism and hydroxyl groups intercede for their cell strengthening, which is influenced by scavenging free radicals [61, 62]. Biological inhibitory potential of the extracts may be assigned by the single or multiple group of compounds [63]. It is important to observe minute alterations in primary and secondary metabolites, identify the concrete structure of some biologically active compounds, and identify the functional groups responsible for plant therapeutic characteristics [64].

GC–MS Analysis of Various GNS Extracts

Gas Chromatography Mass Spectrometry (GC–MS) chromatogram detected the existence of bioactive compounds in various GNS extracts (Fig. 2). In this study, around 120 bioactive metabolites were identified: hexane (30), acetone (30), petroleum ether (25), methanol (30), ethyl acetate (30), ethanol (30). The major compounds present in hexane extract was octadecane (65%) posses antioxidant, and anticancer properties [65], Hexatriacontane (9.47%), 2-Methylhexacosane (3.40%) reveals anti-diabetic activity [66]; in acetone extract was palmitic acid (7.14%), hexatriacontane (9.07%) exhibits anti-inflammatory potential [67], octadecane (9.26%), and tetracosane (9.42%) explicit cytotoxic potential [68]; in petroleum ether extract was palmitic acid (30.14%), eicosane (8.72%) posses antifungal activity [69],

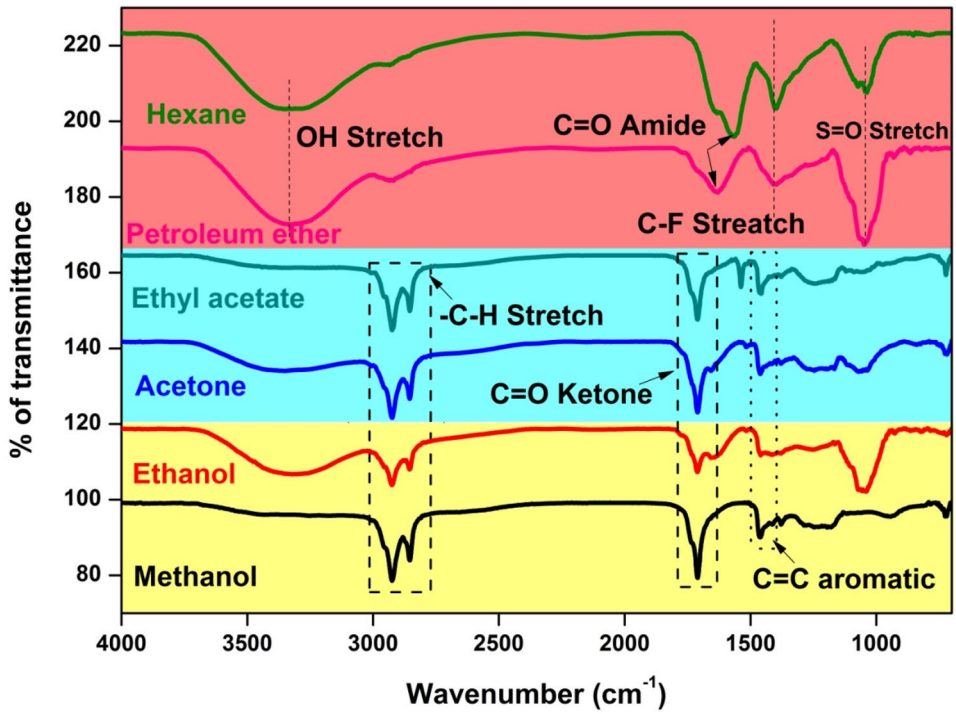


Fig. 1 FT-IR spectrum of Groundnut shell extracts

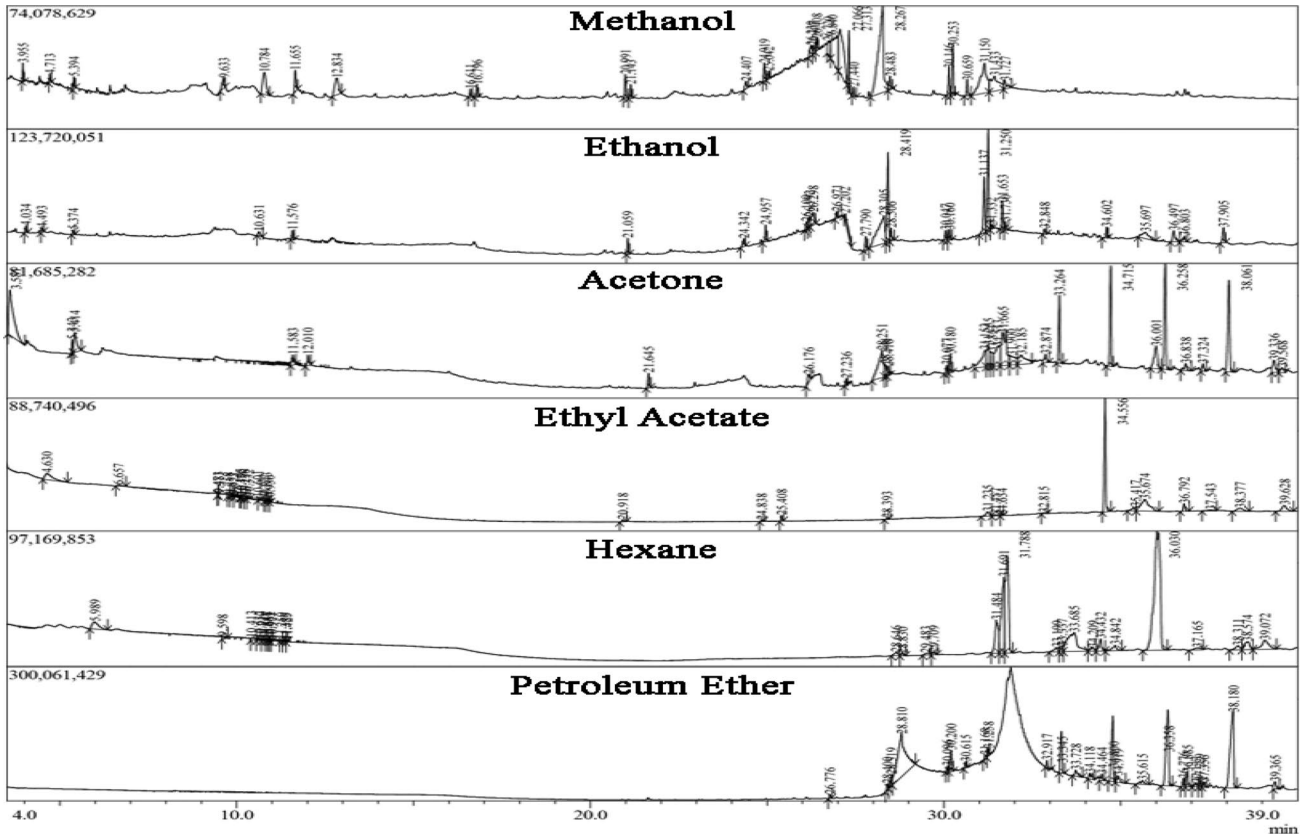


Fig. 2 GC–MS chromatogram of the various GNS extracts

and octadecane (13.56%); in methanol extract was Methyl-D-Glucose (9.46%) playing crucial role in glucose regulatory metabolism [143], mome inositol (12.03%) shows antialopethic, anticirrhotic, cholesterolytic and anti-neuropathic properties [70], palmitic acid (23.53%) have the tendency to act against bacteria and cancer [71], and oleic acid (10.41%), a long chain unsaturated fatty acids shows antibacterial activity [72]; in ethyl acetate extract was cyclohexanone (10.96%) shows antibacterial activity [73] and lupeol (21.44%) exhibits anti-inflammatory and anticancer activity [74, 75]; and ethanol extract was palmitic acid (18.97%), ethyl palmitate (14.35%) posses anti-cancer activity [76], and ethyl oleate (14.54%) exhibits anti-analgesic property [66]. The most of these compounds have been shown to have considerable biological action, and these are originated from a variety of chemical classes.These bioactive compounds function as antimicrobial, antioxidant, antifungal, anti-inflammatory, larvicidal agents and glucose homeostasis (Table 2, 3, 4, 5, 6, 7). This disparity in bioactive compounds could be caused by the volatile nature of the compounds in the solvent extraction [77]. The GC–MS results from the various GNS solvent extracts revealed that they all contained similar compounds with varying concentration percentage due to the solvents' polarity [78].

In Vitro Antioxidant Activity

DPPH Scavenging Assay

DPPH scavenging assay reveals the antioxidant potential of the various GNS extracts. The obtained IC_{50} value indicates the scavenging ability of the different GNS extracts (Table 8), (Fig. 3a). The natural products capability to donate electrons can be assessed using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) purple-colored solution method. This mechanistic approach works by scavenging DPPH by adding antioxidant agents to the DPPH solution, which decolorizes it. The amount of color change is dependent on antioxidant content and potential [173]. The phenolic concentration of the methanolic extracts is greater compared to other extracts, which can give a hydrogen to a free radical to scavenge it. Methanol and ethanol have relative polarities of 0.762 and 0.654, respectively [174]. Rezaei et al. [175] reported that methanol appeared to be more effective solvent for polyphenol and flavonoid extraction and phenol solubility than ethanol. Kwon et al. [176] reported that, a study on pharmacological activity of plant metabolites, antioxidants such as flavonoids, isoflavones, flavones, anthocyanin, catechin, and isocatechin were linked to the main protective

Table 2 GC–MS analysis of GNS hexane extract

S. no	Name of the compound	Molecular formula	Molecular Weight	Retention time	Peak area (%)	Biological applications	References
1	6-oxabicyclo[3.1.0]hexan-3-one	$C_5H_6O_2$	98.10	5.989	2.42	Antiviral potential against RNA viruses including corona, coxsakie, influenza and polio virus	[79]
2	Diallyl oxalate	$C_8H_{10}O_4$	170.16	9.598	0.33	–	–
3	N,N-Dipyrrolidinyl-dithiocarbamic acid	$C_9H_{17}N_3S_2$	231.4	10.413	0.47	–	–
4	4-(2-{[(2-Aminoquinolin-7-Yl)methyl]amino}ethyl)benzonitrile	$C_{19}H_{18}N_4$	302.4	10.632	0.29	–	–
5	N,N-Dipyrrolidinyl-dithiocarbamic acid	$C_9H_{17}N_3S_2$	231.4	10.710	0.23	–	–
6	Butanenitrile	C_4H_7N	69.11	10.842	0.14	Antibacterial activity	[80]
7	1,1-Difluoro-2-(trans-1-propenyl)cyclopropane	$C_6H_8F_2$	118.12	10.881	0.06	–	–
8	Butanenitrile	C_4H_7N	69.11	10.952	0.15	Antibacterial activity	[80]
9	Butanenitrile	C_4H_7N	69.11	10.997	0.11	Antibacterial activity	[80]
10	Butanenitrile	C_4H_7N	69.11	11.250	0.12	Antibacterial activity	[80]
11	Butanenitrile	C_4H_7N	69.11	11.385	0.10	Antibacterial activity	[80]
12	Butanenitrile	C_4H_7N	69.11	11.429	0.04	Antibacterial activity	[80]
13	Tetratriacontane	$C_{34}H_{70}$	478.9	28.646	0.64	Anticancer activity	[80]
14	Tetratriacontane	$C_{34}H_{70}$	478.9	28.830	0.28	Anticancer activity	[80]
15	Tetratriacontane	$C_{34}H_{70}$	478.9	29.483	0.53	Anticancer activity	[80]
16	1-Iodohexacosane	$C_{26}H_{53}I$	492.6	29.709	0.39	–	–
17	Hexatriacontane	$C_{36}H_{74}$	507.0	31.484	5.94	Antimalarial/larvicidal activity	[81]
18	Octadecane	$C_{18}H_{38}$	254.5	31.691	*12.05*	Antifungal agent	[82]
19	Octadecane	$C_{18}H_{38}$	254.5	31.788	*16.44*	Antifungal agent	[82]
20	Octacosane	$C_{28}H_{58}$	394.8	33.199	0.98	Mosquitocidal activity	[82]
21	Tetrapentacontane	$C_{54}H_{110}$	759.4	33.337	0.89	Antioxidant and antimicrobial property	[83]
22	Hexatriacontane	$C_{36}H_{74}$	507.0	33.685	9.47	Antimalarial/larvicidal activity	[81]
23	Celidoniol, deoxy	$C_{29}H_{60}$	408	34.209	0.66	Antibacterial, Anti-inflammatory,Chemical communication especially in *Anopheles stephensi* mosquito, Pheromone of *Orgyia leucostigma*	[84–86]
24	2-Methyloctacosane	$C_{29}H_{60}$	408.8	34.432	1.68	Found in cuticles of various insects spp. (chemical communication)	[87]
25	3-Methyloctacosane	$C_{29}H_{60}$	408.8	34.842	1.20	Found in cuticles of various insects spp. (chemical communication)	[88]
26	Octadecane	$C_{18}H_{38}$	254.5	36.030	*37.06*	Antifungal agent	[82]
27	Tetratriacontane	$C_{34}H_{70}$	478.9	37.165	0.73	Anticancer activity	[80]
28	4-Methyltetradecane	$C_{15}H_{32}$	212.41	38.311	0.98	Antibacterial activity	[88]
29	Hexatriacontane	$C_{36}H_{74}$	507.0	38.574	2.22	Antimalarial/larvicidal activity	[81]
30	2-Methylhexacosane	$C_{27}H_{56}$	380.7	39.072	3.40	Antimicrobial activity and reduces the blood cholesterol	[89]

–Not available

Higher peak area % compounds were given in italic

Table 3 GC–MS analysis of GNS Acetone extract

S.no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
1	5-Methoxy-2-Pentanone	$C_6H_{12}O_2$	116.16	3.581	*10.70*	-	-
2	2,2-Dimethyl-1,3-dioxolane-4-methanol	$C_6H_{12}O_3$	132.16	5.343	0.76	Anxiolytic, Antidepressant, anti-nociceptive activity	[90]
3	2,2-Dimethyl-1,3-dioxolane-4-methanol	$C_6H_{12}O_3$	132.16	5.414	3.05	Anxiolytic, Antidepressant, anti-nociceptive activity	[90]
4	Azulene	$C_{10}H_8$	128.17	11.583	1.13	Chemical used in cosmetic	[91]
5	R-3-Hydroxy-4,5-dimethyl- 2(5H)-furanon	$C_6H_8O_3$	128.13	12.010	0.89	-	-
6	Diphenylamine	$C_{12}H_{11}N$	169.22	21.645	1.10	Anti-inflammatory drug	[92]
7	Esculin	$C_{15}H_{16}O_9$	340.28	26.176	0.91	Antidiabetic, antioxidant, anti-inflammatory activity, antiradical activity	[93, 94]
8	Methyl Palmitate	$C_{17}H_{34}O_2$	270.5	27.236	0.50	Antiradical activity, Anticancer activity	[95, 96]
9	Palmitic acid	$C_{16}H_{32}O_2$	256.42	28.251	7.14	Anti-inflammatory activity, Anti-cancer activity	[97, 98]
10	2-(2-Nitro-1-p-tolyl-ethyl)-cyclohexanone	$C_{15}H_{19}NO_3$	261.32	28.341	0.99	–	–
11	Ethyl palmitate	$C_{18}H_{36}O_2$	284.5	28.418	0.87	Larvicidal and insecticidal agent	[99]
12	Methyl linoleate	$C_{19}H_{34}O_2$	294.5	30.077	0.54	Antioxidant activity	[100, 101]
13	Methyl elaidate	$C_{19}H_{36}O_2$	296.5	30.180	1.45	Cytotoxic property	[102]
14	Ethyl linoleate	$C_{20}H_{36}O_2$	308.5	31.153	4.06	Cosmetic chemical, anti-inflammatory	[103]
15	Ethyl oleate	$C_{20}H_{38}O_2$	310.5	31.255	2.97	Cosmetic oil, emulsifier	[104]
16	4,5-Diphenyl-1,7-octadiene	$C_{20}H_{22}$	262.4	31.353	2.03	–	–
17	2-Hexyldecanoic acid	$C_{16}H_{32}O_2$	256.42	31.513	5.40	–	–
18	Hexatriacontane	$C_{36}H_{74}$	507.0	31.665	*9.07*	Antimalarial/larvicidal activity	[81]
19	Oleic acid	$C_{18}H_{34}O_2$	282.5	31.900	2.80	Antischistosomal activity, anti-inflamatory	[105, 106]
20	2,6,10,14,18-Pentamethylicosane	$C_{25}H52$	352.7	32.185	3.63	–	–
21	Stearyl alcohol	$C_{18}H_{38}O$	270.5	32.874	0.96	Skin conditioning agent used in cosmetic	[107]
22	Eicosane	$C_{20}H_{42}$	282.5	33.264	4.36	Leishmanicidal activity, anti-inflammatory, antioxidant, antipyretic activity	[108, 109]
23	Tetracosane	$C_{24}H_{50}$	338.7	34.715	7.33	Antimicrobial activity	[110]
24	Hexatriacontane	$C_{36}H_{74}$	507.0	36.001	3.81	Antimalarial/larvicidal activity	[81]
25	Octadecane	$C_{18}H_{38}$	254.5	36.258	*9.26*	Antifungal agent	[82]
26	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	36.838	0.93	Antimutagenic activity	[111]
27	2-Methylpentacosane	$C_{26}H_{54}$	366.7	37.324	0.72	Antimicrobial activity and reduces the blood cholesterol	[89]
28	Tetracosane	$C_{24}H_{50}$	338.7	38.061	*9.42*	Antimicrobial activity	[110]

Table 3 (continued)

S.no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
29	Celidoniol, deoxy	$C_{29}H_{60}$	408	39.336	2.11	Antibacterial, Anti-inflammatory, Chemical communication especially in Anopheles stephensi mosquito Pheromone of *Orgyia leucostigma*	[84–86]
30	3-Methylhexacosane	$C_{27}H_{56}$	380.7	39.568	1.09	Antidiabetic, anticancer activity	[112]

–Not available

Higher peak area % compounds were given in italic

benefits of secondary metabolites. Our results provide evidence that phenolic chemicals exhibit antioxidant properties and agreed with the findings of Lefahal et al. [177], who found that extracts with higher antioxidant potential also have higher phenol content. GC–MS results also reveals the presence of the compounds like octadecane [65]; 1-Nonadecene [138, 139];Lupeol [74]; Mome inositol [70] are the key compounds of GNS extracts exhibits antioxidant potential. The methanolic extract of the GNS has a higher antioxidant potential compared to the other extracts due to the phenolic contents present in the particular extracts [178]. According to Annapandian and Rajagopal [179] the extracts' proton-donating capacity stabilizes free radicals in connection with multiple hydroxyl groups, resulting in increased DPPH scavenging activity.

ABTS Radical Scavenging Assay

The ABTS radical scavenging activity of prepared GNS extracts was investigated. Based on the IC_{50} value (Table 8), the scavenging ability of various GNS extracts was determined (Fig. 3b). The plant extracts have the ability to reduce the cation in the ABTS assay. Michalak [180] reported that the existence of hydroxyl groups and disulphide bonds in chemical structure of GNS active components may be linked to their ability to scavenge free radicals and prevent oxidation processes. The ABTS radical scavenging capability of the GNS extracts was identified by determining the IC_{50} value listed in Table 8. Methanol has the least IC_{50} value followed by ethanol, acetone, hexane, ethyl acetate, and petroleum ether. These results correlate with the phenol content, which was obtained higher in the methanolic extract [181, 182]. Because phenolics are made up of many aromatic rings with many hydroxyl groups, it has the ability to absorb free radicals. Dudonne et al. [183] reported that the phenoxyl radicals with resonance stabilization in majority of extracts expressed with the total phenolic content (TPC) and antioxidant activity. Our results are in agreement with previous results that phenolic contents are responsible for the antioxidant potential [184, 185].IC_{50} values obtained for ABTS was lower than DPPH due to variations in the redox potential of the GNS extracts [186]. Moreover, the compounds like Eicosane [187]; Methyl linoleate [188]; 3beta-Acetoxystigmasta-4,6,22-triene [189] are responsible for the antioxidant capacity of the GNS extracts.

Determination of Total Phenol and Total Tannin Content of the GNS Extracts

Total Phenol Content

The polarity of the solvents may alter the extraction of the phenol component. From the obtained results, methanol has the highest amount of phenolics, followed by ethanol, petroleum ether, hexane, ethyl acetate, and acetone given in Fig. 4B and its standard gallic acid equivalents (GAE) in Fig. 4A. In our study, Table 9 shows the total phenol content obtained in prepared GNS extracts using the regression equation ($y = 0.0019x + 1.668$, $r^2 = 0.98$). The primary components of total phenol content (TPC) are phenolic acids, flavonoids etc. Furthermore, discrepancies in total phenol extraction processes, which involved in usage of organic solvent, time, temperature, and procedures (e.g., ultrasound) in some cases, or earlier treatments such as lyophilization and radiation, could be the reason for these variations [190]. Plant extracts contain phenolic compounds that function as natural antioxidants, scavenging free radicals, and decreasing oxidative stress [2]. The phenolic content of the plant would be varying due to genetic and environmental factors [191]. Phenolic compounds are made up of one phenol unit and an aromatic ring that is bordered by a hydroxyl substitution and can donate protons [192]. Antioxidant agents are phenolic groups that have a tendency to accept electrons and create phenoxy stable radicals, causing the oxidation reaction to be disrupted [193]. Furthermore, the presence of

Table 4 GC–MS analysis of GNS Petroleum ether extract

S. no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
1	Heneicosane	$C_{21}H_{44}$	296.6	26.776	0.40	Antimicrobial activity	[113]
2	Ethyl palmitate	$C_{18}H_{36}O_2$	284.5	28.400	0.81	Larvicidal and insecticidal agent	[99]
3	Hexadecane	$C_{16}H_{34}$	226.44	28.519	1.82	Induces hyper Keratinization in tested rodents	[114]
4	Palmitic acid	$C_{16}H_{32}O_2$	256.42	28.810	*30.14*	Anti-inflammatory activity, Anti-cancer activity	[97, 98]
5	Methyl linoleate	$C_{19}H_{34}O_2$	294.5	30.096	0.56	Antioxidant activity	[100, 101]
6	1,54-Dibromotetrapentacontane	$C_{54}H_{108}Br_2$	917.2	30.200	3.01	Anticancer activity, Semiochemical involved in insect metabolism	[115, 116]
7	Methyl stearate	$C_{19}H_{38}O_2$	298.5	30.615	0.63	Nematocidal activity in plants	[117]
8	7,10-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.5	31.168	0.55	Anti-inflammatory, anxiolytic activity in rats	[118]
9	Ethyl oleate	$C_{20}H_{38}O_2$	310.5	31.258	0.82	Cosmetic oil, emulsifier	[104]
10	(Z)-9-Tricosene	$C_{23}H_{46}$	322.6	32.917	0.65	Pheromone compound	[119]
11	Eicosane	$C_{20}H_{42}$	282.5	33.345	5.30	Leishmanicidal activity, anti-inflammatory, antioxidant, antipyretic activity	[108, 109]
12	Methyl 18-methylnonadecanoate	$C_{21}H_{42}O_2$	326.6	33.728	1.40	Antiulcerative, oxidoreductase inhibitor, gastrin inhibitor	[120]
13	(Z)-13-Octadecenal	$C_{18}H_{34}O$	266.5	34.118	1.52	Insect sex pheromone	[121]
14	Hexyl palmitate	$C_{22}H_{44}O_2$	340.6	34.464	1.08	–	–
15	Eicosane	$C_{20}H_{42}$	282.5	34.800	*8.72*	Leishmanicidal activity, anti-inflammatory, antioxidant, antipyretic activity	[108, 109]
16	Tetracosamethyl-cyclo-dodecasiloxane	$C_{24}H_{72}O_{12}SI_{12}$	889.8	34.919	1.66	-	-
17	Amyl elaidate	$C_{23}H_{44}O_2$	352.6	35.615	1.33	Insecticidal activity	[122]
18	Octadecane	$C_{18}H_{38}$	254.5	36.358	*13.56*	Antifungal agent	[82]
19	Methyl behenate	$C_{23}H_{46}O_2$	354.6	36.776	1.25	–	–
20	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$		36.885	2.37	Antimutagenic activity	[111]
21	trans,trans-9,12-Octadecadienoic acid, propyl ester	$C_{21}H_{38}O_2$	322.5	37.133	0.79	Antidepressant activity in mice	[123]
22	Amyl elaidate	$C_{23}H_{44}O_2$	352.6	37.229	0.85	Insecticidal activity	[122]
23	2-Methylpentacosane	$C_{26}H_{54}$	366.7	37.356	0.71	Antimicrobial activity and reduces the blood cholesterol	[89]
24	Pentadecane, 2,6,10,13-tetramethyl-	$C_{19}H_{40}$	268.5	38.180	*18.67*	–	–
25	Dotriacontane, 1-iodo-	$C_{32}H_{65}I$	576.8	39.365	1.40	Anticancer, antioxidant and antimicrobial activity	[124]

–Not available

Higher peak area % compounds were given in italic

Table 5 GC–MS analysis of GNS Methanol extract

S.no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
1	3-Furanmethanol	$C_5H_6O_2$	98.10	3.955	0.87	Antioxidant activity	[125, 126]
2	Oxime-, methoxy-phenyl-	$C_8H_9NO_2$	151.16	4.713	0.43	–	–
3	1,2-Cyclopentanedione	$C_5H_6O_2$	98.10	5.394	0.74	Anti-inflammation property, antimicrobial, antioxidant potential	[127, 128]
4	Melamine	$C_3H_6N_6$	126.12	9.633	1.23	Nitrogen containing organic compound widely used in poultry and livestock feed preparation in small quantities	[129]
5	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.12	10.784	4.18	Antioxidant, anti-inflammatory activity	[130]
6	Naphthalene	$C_{10}H_8$	128.17	11.655	1.74	Antialzheimer's agent	[131]
7	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11	12.834	3.47	Antibiofilm, Antivirulence activity	[132]
8	1-Tetradecene	$C_{14}H_{28}$	196.37	16.611	0.58	Antiparasitial activity	[133]
9	Tetradecane	$C_{14}H_{30}$	198.39	16.796	0.97	Antimicrobial activity	[134]
10	1-Hexadecene	$C_{16}H_{32}$	224.42	20.991	1.58	Antimicrobial, antioxidant property	[135]
11	Heptadecane	$C_{17}H_{36}$	240.5	21.143	0.93	Antinflammation, Antibactericidal	[136, 137]
12	Myristic acid	$C_{14}H_{28}O_2$	228.37	24.407	0.77	Antimicrobial activity	[134]
13	1-Nonadecene	$C_{19}H_{38}$	266.5	24.919	1.26	Antioxidant, Antibacterial activity	[138, 139]
14	Heneicosane	$C_{21}H_{44}$	296.6	25.042	0.59	Antimicrobial activity	[113]
15	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.40	26.239	1.19	Anticancer activity	[140]
16	6-Methyl[1,2,4]triazolo[4,3-b][1,2,4]triazin-7(8H)-one	$C_5H_5N_5O$	151.13	26.307	0.80	Antibacterial, antifungal, anti-inflammatory	[141]
17	9-Heptadecanone	$C_{17}H_{34}O$	254.5	26.408	0.93	Anticancer activity	[142]
18	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194.18	26.720	2.15	Involved in glucose regulatory mechanism	[143]
19	3-*O*-Methyl-d-glucose	$C_7H_{14}O_6$	194.18	26.840	*9.46*	Involved in glucose regulatory mechanism	[143]
20	Mome inositol	$C_7H_{14}O_6$	194	27.066	*12.03*	Antioxidant activity	[144, 145]
21	Methyl palmitate	$C_{17}H_{34}O_2$	270.5	27.313	3.63	Antiradical activity, Anticancer activity	[95, 96]
22	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, 1-[2-[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-1-oxopropoxy]ethyl]-2,2,6,6-tetramethyl-4-piperidinyl ester	$C_{45}H_{71}NO_6$	722	27.440	0.66	–	–
23	Palmitic acid	$C_{16}H_{32}O_2$	256.42	28.267	*23.53*	Anti-inflammatory activity, Anti-cancer activity	[97, 98]
24	1-Hexadecanethiol	$C_{16}H_{34}S$	258.5	28.483	1.23	–	–
25	Methyl linoleate	$C_{19}H_{34}O_2$	294.5	30.146	1.92	Antioxidant activity	[100, 101]
26	Methyl elaidate	$C_{16}H_{36}O_2$	296.5	30.253	3.74	Cytotoxic property	[102]

Table 5 (continued)

S.no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
27	Methyl stearate	$C_{19}H_{38}O_2$	298.5	30.659	1.12	Nematocidal activity in plants	[146]
28	Oleic acid	$C_{18}H_{34}O_2$	282.5	31.150	*10.41*	Antischistosomal activity, anti-inflamatory	[105, 106]
29	Stearic acid	$C_{18}H_{36}O_2$	284.5	31.433	6.65	Antimicrobial activity and antidepressant activity in mice	[147]
30	Alcohols, C20-28, ethoxylated	$C_{22}H_{46}O$	326.6	31.727	1.22	Surfactant	[148]

–Not available

Higher peak area % compounds were given in italic

phenol in the methanolic extract suggests that the GNS could be useful as a source of dietary antioxidants and protect living systems against oxidative damage [194].

Total Tannin Content

From this study, the tannin content found in the extracts of GNS is shown in Fig. 4D, and its standard Tannic acid equivalents (TAE) in Fig. 4C. Methanol has the higher tannin content followed by ethyl acetate, ethanol, petroleum ether, hexane, and acetone. The regression equation ($y = 0.0019x + 1.0175$, $r^2 = 0.9711$) was used to calculate the outcomes of our investigation.The range obtained in this study is shown in Table 9. Tannins contribute considerably to the creation of a variety of nutraceuticals with distinct flavors [195]. Mostly tannins are present in the bark of the coniferous trees. Tannins are rich in polyphenols and promising source of antibacterial and antioxidant properties [196]. Tannin extraction makes it easier when the particle size is smaller because solvents may easily permeate the particle, reducing the extraction time [197]. By suppressing gastrointestinal pathogens, tannins aid in the use of protein in the animal body and advantageous to animal health [198]. Tannins are essential compounds that have an anti-phlogistic effect as well as being potent cyclooxygenase-1 inhibitors. The anti-inflammatory properties of tannins could be linked to their anti-phlogistic properties [199].

Assesment of Antibacterial Activity

Agar Well Diffusion Method

The results of the antibacterial potential with respect to the solvents were studied. Various solvent extracts of the GNS were evaluated against *Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Klebsiella pneumoniae*. Overall, the methanolic extracts possess higher zone of inhibition compared to other extracts. Our results revealed that the bioactive chemicals recovered with methanolic extracts were found most effective antibacterial agents. In comparison to other solvent extracts, methanol demonstrated a significantly greater zone of inhibition, whereas the other solvent extracts showed the smallest zones in a concentration-dependent manner due to differences in the polarity (Tables 10, 11, 12, 13). As the polarity of the solvents increases, antimicrobial activity increases [200]. *Aeromonas hydrophila, Pseudomonas aeruginosa,* and *Staphylococcus aureus* were the most sensitive, while *Klebsiella pneumoniae* was the most resistant to all plant components extracts examined in this study. The variation in susceptibility between gram-positive and gram-negative bacteria is due to morphological characteristics within those bacteria, specifically in membrane permeability [201]. The Gram-positive bacteria cell's several layers of peptidoglycan create a resistant structure that prevents the bioactive compounds in the extracts from penetrating but Gram-negative cell walls are made up of single or double layers of peptidoglycan, making them more susceptible to extract secondary metabolites [44, 202]. Methanol extract shows the highest antibacterial activity against all the pathogens tested, which could be due to extracting more soluble metabolites [63]. Further to confirm this study, spectroscopic approaches will be required to detect a variety of microorganisms and its membrane permeability by the extracts [203]. The antibacterial therapeutic impact of phenolic compounds can manifest by inhibiting various microbial pathogenicity factors (e.g., biofilm development, host ligand adherence, and neutralisation of bacterial toxins), diminish membrane fluidity, inhibit nucleic acid and cell wall synthesis, and inhibit energy metabolism [204]. Furthermore, variations in the hydroxyl group's location could have a significant impact on antibacterial action [205]. Importantly, phenols are effective iron scavengers in the bacterial cell, and it inhibits pathogenic microbial growth by reducing the ribonucleotide precursor of DNA [206].

Table 6 GC–MS analysis of GNS Ethyl acetate extract

S. no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
1	Cyclohexanone	$C_6H_{10}O$	98.14	4.630	*10.96*	Antiparasitic activity	[149]
2	Mesitylene	C_9H_{12}	120.19	6.657	0.93	Neurotoxic effect	[150]
3	Butyronitrile	C_4H_7N	69.11	9.471	0.20	–	–
4	Butyronitrile	C_4H_7N	69.11	9.483	0.17	–	–
5	Butyronitrile	C_4H_7N	69.11	9.758	1.22	–	–
6	Butyronitrile	C_4H_7N	69.11	9.857	1.16	–	–
7	3-Decylsulfinyltetrahydrothiophene-4-ol 1,1-dioxide	$C_{14}H_{28}O_4S_2$	324.5	9.923	0.50	–	–
8	3-Decylsulfinyltetrahydrothiophene-4-ol 1,1-dioxide	$C_{14}H_{28}O_4S_2$	324.5	10.105	0.50	–	–
9	3-Decylsulfinyltetrahydrothiophene-4-ol 1,1-dioxide	$C_{14}H_{28}O_4S_2$	324.5	10.130	0.10	–	–
10	Cyanoacetic acid	$C_3H_3NO_2$	85.06	10.236	0.37	Antitumour activity	[151]
11	3-Decylsulfinyltetrahydrothiophene-4-ol 1,1-dioxide	$C_{14}H_{28}O_4S_2$	324.5	10.332	0.98	–	–
12	2,2-Difluorocycloheptan-1-one	$C_7H_{10}F_2O$	148.15	10.601	0.21	–	–
13	Oxalic acid, allyl ethyl ester	$C_7H_{10}O_4$	158.15	10.777	0.18	Bactericidal activity against human and fish pathogen	[152]
14	Bromoazidomethane	CH_2BrN_3	135.95	10.843	0.13	–	–
15	3-Decylsulfinyltetrahydrothiophene-4-ol 1,1-dioxide	$C_{14}H_{28}O_4S_2$	324.5	10.930	0.25	–	–
16	Diundecyl phthalate	$C_{30}H_{50}O_4$	474.7	20.918	1.08	Plasticizer	[153]
17	1-Nonadecene	$C_{19}H_{38}$	266.5	24.838	0.37	Antioxidant, Antibacterial	[138, 139]
18	Isopropyl myristate	$C_{17}H_{34}O_2$	270.5	25.408	1.50	Anticancer activity	[154]
19	1-Nonadecene	$C_{19}H_{38}$	266.5	28.393	0.43	Antioxidant, Antibacterial	[138, 139]
20	Ethyl arachidate	$C_{22}H_{44}O_2$	340.6	31.235	3.30	Antibacterial activity	[155]
21	Hexatriacontane	$C_{36}H_{74}$	507.0		0.74	Antimalarial/larvicidal activity	[81]
22	1-Nonadecanol	$C_{19}H_{40}O$	284.5	31.634	0.31	Antifeedant activity in plants, antimicrobial activity	[156, 157]
23	2,4,6-Cycloheptatrien-1-one, 3-acetyl-2-hydroxy-5-(1-methylethyl)-	$C_{12}H_{14}O_3$	206.24	31.457	0.66	–	–
24	Dioctyl adipate	$C_{22}H_{42}O_4$	370.6	34.556	*35.02*	Plasticizer	[158]
25	1,25-Dihydroxyvitamin D3, TMS derivative	$C_{30}H_{52}O_3Si$	488.8	35.417	2.76	Involved in Metabolic activities of animals	[159]
26	Lupeol	$C_{30}H_{50}O$	426.7	35.674	*21.44*	Antioxidant, anti-inflammatory and antitumour activity	[160]
27	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	36.792	3.04	Antimutagenic activity	[111]
28	Withaferin-A Diacetate	$C_{32}H_{42}O_8$	554.7	37.543	1.38	Antitumour activity	[161]
29	Cholesta-4,6-dien-3-ol, (3beta)-	$C_{27}H_{44}O$	384.6	38.377	4.14	Anti-Alzheimer property	[162]
30	3beta-Acetoxystigmasta-4,6,22-triene	$C_{31}H_{48}O_2$	452.7	39.628	5.98	Antioxidant, anti-inflammatory, and anticancer activity	[163]

–Not available

Higher peak area % compounds were given in italic

Table 7 GC–MS analysis of GNS Ethanol extract

S.no	Name of the compound	Molecular formula	Molecular weight	Retention time	Peak area (%)	Biological applications	References
1	Furfuryl alcohol	$C_5H_6O_2$	98.10	4.034	0.71	Bioinsecticide and antimicrobial agent	[164]
2	4-Cyclopentene-1,3-dione	$C_5H_4O_2$	96.08	4.493	0.62	Antiangiogenic activity	[165]
3	1,2-Cyclopentanedione	$C_5H_6O_2$	98.10	5.374	0.51	Anti-inflammation property, Antimicrobial and antioxidant potential	[127, 128]
4	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.12	10.631	0.86	Antioxidant, anti-inflammatory activity,	[130]
5	Azulene	$C_{10}H_8$	128.17	11.576	1.29	Chemical used in cosmetic	[91]
6	Heptadecane	$C_{17}H_{36}$	240.5	21.059	1.85	Antinflammation, Antibactericidal	[136, 137]
7	Myristic acid	$C_{14}H_{28}O_2$	228.37	24.342	1.34	Antimicrobial activity	[134]
8	Heneicosane	$C_{21}H_{44}$	296.6	24.957	2.08	Antimicrobial activity	[113]
9	Bis(2-methylpropyl) 4,5-dimethylbenzene-1,2-dicarboxylate	$C_{18}H_{26}O_4$	306.4	26.109	1.00	–	–
10	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.40	26.192	1.72	Anticancer activity	[140]
11	Esculetin	$C_9H_6O_4$	178.14	26.298	3.70	Antitumor activity	[166]
12	7,9-Di-tert -butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276.4	26.971	1.03	Anti-Alzheimer property	[167]
13	Mome inositol	$C_7H_{14}O_6$	194	27.202	2.54	Antioxidant activity	[144, 145]
14	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	27.790	1.85	Plasticizer	[153]
15	Palmitic acid	$C_{16}H_{32}O_2$	256.42	28.305	*18.97*	Anti-inflammatory activity, Anti-cancer activity	[97, 98]
16	Ethyl palmitate	$C_{18}H_{36}O_2$	284.5	28.419	*14.35*	Larvicidal and insecticidal agent	[99]
17	Heneicosane	$C_{21}H_{44}$	296.6	28.506	1.82	Antimicrobial activity	[113]
18	Ethyl stearate	$C_{20}H_{40}O_2$	312.5	30.047	1.28	Antioxidant activity	[168]
19	Methyl elaidate	$C_{19}H_{36}O_2$	296.5	30.160	1.41	Cytotoxic property	[102]
20	Ethyl linoleate	$C_{20}H_{36}O_2$	308.5	31.137	8.48	Cosmetic chemical, anti-inflammatory	[103]
21	Ethyl oleate	$C_{20}H_{38}O_2$	310.5	31.250	*14.54*	Cosmetic oil, emulsifier	[104]
22	9-Octadecenoic acid, ethyl ester	$C_{20}H_{38}O_2$	310.5	31.332	0.63	Antioxidant activity	[169]
23	Ethyl stearate	$C_{20}H_{40}O_2$	312.5	31.653	3.44	Antioxidant activity	[170]
24	Eicosane	$C_{20}H_{42}$	282.5	31.730	1.04	Leishmanicidal activity, anti-inflammatory, antioxidant, antipyretic activity	[108, 109]
25	(Z)-9-Tricosene	$C_{23}H_{46}$	322.6	32.848	0.74	Pheromone compound	[119]
26	Ethyl arachidate	$C_{22}H_{44}O_2$	340.6	34.602	1.53	Antibacterial activity	[155]
27	Lupeol	$C_{30}H_{50}O$	426.7	35.697	4.05	Antioxidant, anti-inflammatory and antitumour activity	[160]
28	Hexadecanoic acid ((3E,7E)-(1S,2R)-2-hydroxy-1-hydroxymethyl-16-methyl-heptadeca-3,7-dienyl)-amide	$C_{35}H_{67}NO_3$	549.9	36.497	2.67	Antioxidant and antimicrobial activity	[171]
29	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	36.803	1.28	Antimutagenic activity	[111]
30	Ethyl docosanoate	$C_{24}H_{48}O_2$	368.6	37.905	2.66	Antiplasmoidal activity	[172]

–Not available

Higher peak area % compounds were given in italic

Table 8 The IC_{50} for different extracts of Groundnut shell against DPPH, ABTS

IC50 (µg/ml)	Target	Positive control	Methanol extract	Ethanol extract	Acetone extract	Ethyl acetate extract	Hexane extract	Petroleum ether extract
	DPPH	94.65	789.36	1033.77	1051.99	1047.09	1264.60	1586.13
	ABTS	205.65	480.11	506.24	945.63	1175.85	1036.97	1356.98

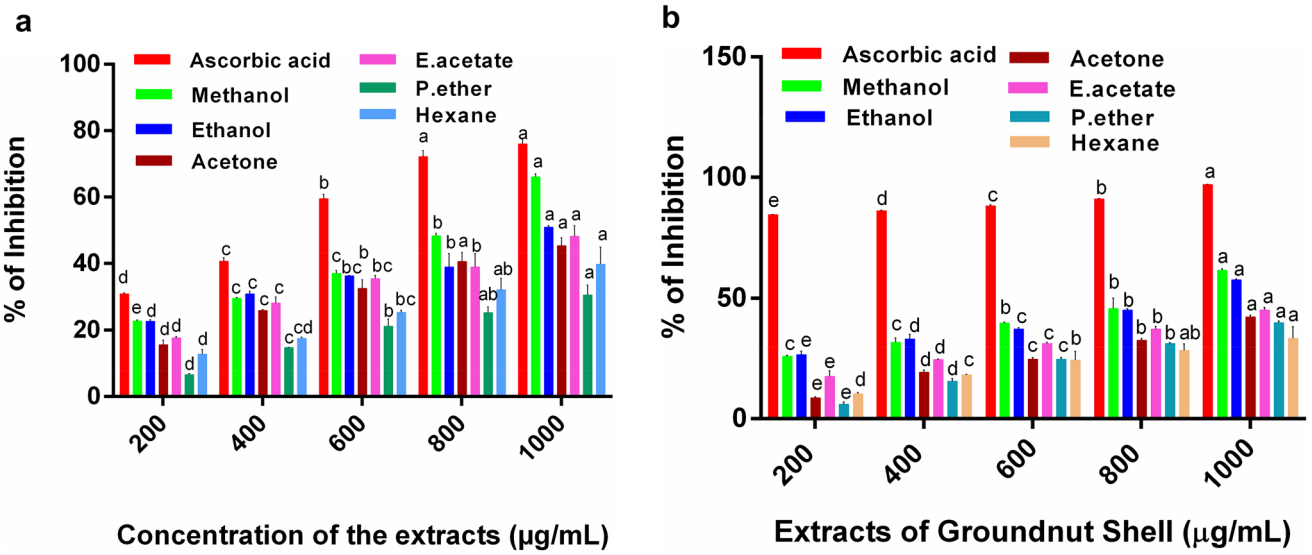


Fig. 3 **a** DPPH Radical scavenging activity of different extracts of Groundnut shell. **b** ABTS Radical scavenging activity of different extracts of Groundnut shell

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration of various GNS extracts using resazurin dye method is demonstrated in Table 14. From the extracts, methanol and ethanol showed potential MIC value against all the tested microorganisms' ranges 250 µg/mL, whereas all other extracts showed MIC at 500 µg/mL. Resazurin reduction could be accomplished by live bacterial cells. Inhibitory potential of the extracts was due to the presence of the long-chain unsaturated fatty acids like oleic acid, palmitic acid, and linolenic acid. These fatty acids hindered the bacterial enoyl-acyl ATP binding reductase involving in the production of bacterial fatty acid synthesis [207]. A similar result was obtained in this study, the presence of unsaturated fatty acids in the GNS extracts confirmed in the GC–MS analysis. The polarization of the solvent is essential since it alters the molecules that enter the organism's membrane, causing growth disruption [208]. Antibacterial action is facilitated by both the hydrophilic and hydrophobic portions of phenolic compounds. The hydrophilic component interacts with the polar section of the bacterial cell membrane, whereas the hydrophobic part interacts with the inner part of the bacterial cell [209]. Suspension of antibacterial compounds move across the membrane, and membrane permeabilization, which disrupts the cell membrane's integrity and causes cell lysis [210]. And it can pass through phospholipid bilayers having a hydrophobic interfacial region, and the permeability increases as the number of hydrophobic solute molecules rises [211]. Bacteria have an outer membrane (OM) that contains lipopolysaccharide (LPS), which gives a hydrophilic surface to the bacterium. Anionic groups in LPS molecules contribute to its stability by electrostatic interactions with divalent cations. Gram-negative bacteria are relatively resistant to hydrophobic drugs and harmful medications because the OM acts as a

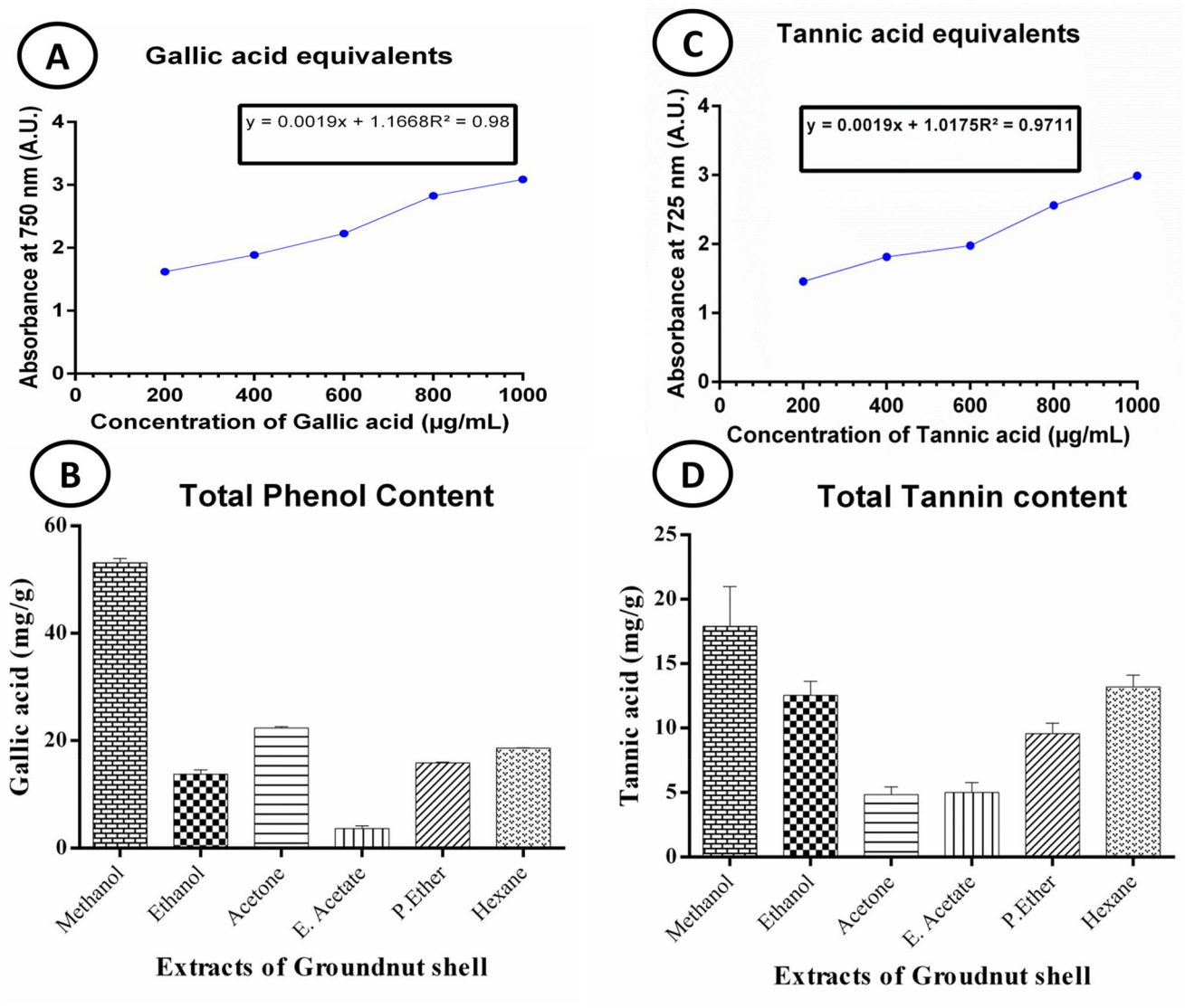


Fig. 4 Standard graph of Gallic acid equivalents (**A**), Quantitative analysis of the total phenolic content (**B**), Standard graph of Tannic acid equivalents (**C**) and Quantitative analysis of the total tannin content (**D**) present in the various extracts of Groundnut shell

Table 9 Total phenol and Total tannin content of the groundnut shell extracts

S. no	Solvent extracts	Total phenol content (mg/GAE g)	Total tannin content (mg/TAE g)
01	Methanol	53.19 ± 1.33	17.91 ± 5.31
02	Ethanol	22.39 ± 0.41	12.56 ± 1.84
03	Acetone	13.75 ± 1.41	4.84 ± 1.05
04	Ethyl Acetate	3.68 ± 0.87	13.19 ± 1.58
05	Hexane	15.85 ± 0.29	5.01 ± 1.32
06	Petroleum Ether	18.61 ± 0.08	9.57 ± 1.39

penetrating barrier against biomolecules and hydrophobic compounds [212]. The antibacterial efficacy is mostly determined by the hydrophilic-hydrophobic balance of hydrophilic compounds, and dimeric amphiphiles give a greater number of positive charges as well as stronger interaction with the lipid bilayer [213].

Acting as the proton donor/acceptor, phenols lead to membrane disrupting activity against the microorganisms [214]. The antibacterial activity of the extracts is due to the presence of phenols in the extract as well as the metabolites extracted by the solvent, because these phenols inhibit

Table 10 Antibacterial activity of the tested extracts against *Aeromonas hydrophila*

S. no	Zone of inhibition (mm)					
	Extracts	Control	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
01	Methanol	30.33 ± 1.25^{a}	7.5 ± 0.5^{e}	11.16 ± 0.76^{d}	13.33 ± 1.04^{c}	18.16 ± 0.76^{b}
02	Ethanol	27.16 ± 1.04^{a}	12.16 ± 0.76^{d}	12.33 ± 0.76^{d}	15.16 ± 1.04^{c}	17.33 ± 1.15^{b}
03	Acetone	26.16 ± 0.76^{a}	–	7.66 ± 0.57^{d}	11.16 ± 0.76^{c}	14.66 ± 0.57^{b}
04	E.acetate	23.66 ± 0.57^{a}	–	8.83 ± 0.28^{d}	11.5 ± 0.5^{c}	13.66 ± 0.57^{b}
05	Hexane	28.33 ± 0.57^{a}	–	–	11.33 ± 0.57^{c}	14.16 ± 0.76^{b}
06	P.ether	29.16 ± 0.76^{a}	–	–	12.66 ± 1.52^{c}	15.33 ± 0.57^{b}

Each result represents the mean ± standard deviation ($n = 3$), and different superscript letters indicate a significant difference between the groups ($p < 0.05$)

Table 11 Antibacterial activity of the tested extracts against *Klebsiella pneumoniae*

S. no	Zone of inhibition (mm)					
	Extracts	Control	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
01	Methanol	25.16 ± 0.76^{a}	–	10.16 ± 0.76^{d}	14.33 ± 1.52^{c}	18.33 ± 0.76^{b}
02	Ethanol	27.33 ± 0.57^{a}	–	8.33 ± 0.57^{d}	11.16 ± 0.76^{c}	13.16 ± 1.04^{b}
03	Acetone	27.16 ± 0.76^{a}	–	7.66 ± 0.57^{d}	11.16 ± 0.76^{c}	15.16 ± 0.76^{b}
04	E.acetate	31.16 ± 0.76^{a}	–	–	12.16 ± 0.76^{c}	15.83 ± 0.28^{b}
05	Hexane	27.16 ± 0.76^{a}	–	–	11.5 ± 0.5^{c}	13.16 ± 0.76^{b}
06	P.ether	29.16 ± 1.04^{a}	–	–	12.16 ± 0.76^{c}	15.16 ± 0.76^{b}

Each result represents the mean ± standard deviation ($n = 3$), and different superscript letters indicate a significant difference between the groups ($p < 0.05$)

Table 12 Antibacterial activity of the tested extracts against *Staphylococcus aureus*

S.no	Zone of inhibition (mm)					
	Extracts	Control	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
01	Methanol	31.16 ± 0.76^{a}	8.16 ± 1.25^{e}	11.33 ± 0.57^{d}	13.66 ± 0.57^{c}	17.16 ± 0.76^{b}
02	Ethanol	30.66 ± 0.57^{a}	–	11.16 ± 0.76^{d}	13.66 ± 0.57^{c}	16.33 ± 0.57^{b}
03	Acetone	26.33 ± 0.57^{a}	–	8.66 ± 0.57^{d}	12.16 ± 0.76^{c}	15.33 ± 1.15^{b}
04	E.acetate	31.66 ± 1.52^{a}	–	8.16 ± 0.76^{d}	11.33 ± 0.57^{c}	13.33 ± 0.57^{b}
05	Hexane	32.66 ± 0.57^{a}	–	10.33 ± 0.57^{d}	13.16 ± 0.76^{c}	16.16 ± 0.76^{b}
06	P.ether	31.66 ± 1.52^{a}	–	–	12.16 ± 0.76^{c}	15.16 ± 0.76^{b}

Each result represents the mean ± standard deviation ($n = 3$), and different superscript letters indicate a significant difference between the groups ($p < 0.05$)

Table 13 Antibacterial activity of the tested extracts against *Pseudomonas aeruginosa*

S.no	Zone of inhibition (mm)					
	Extracts	Control	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
01	Methanol	34.16 ± 0.76^{a}	–	11.33 ± 0.57^{d}	15.33 ± 1.52^{c}	18.33 ± 0.76^{b}
02	Ethanol	31.33 ± 1.52^{a}	–	7.66 ± 0.57^{d}	10.33 ± 0.57^{c}	13.66 ± 0.57^{b}
03	Acetone	32.16 ± 0.76^{a}	–	8.66 ± 0.57^{d}	11.66 ± 0.57^{c}	14.16 ± 0.76^{b}
04	E.acetate	30.16 ± 0.76^{a}	–	11.33 ± 0.57^{d}	13.33 ± 0.57^{c}	14.66 ± 1.52^{b}
05	Hexane	31.5 ± 1.35^{a}	–	7.83 ± 0.76^{d}	10.5 ± 0.86^{c}	13.83 ± 0.28^{b}
06	P.ether	29.33 ± 1.52^{a}	–	–	12.16 ± 0.76^{c}	14.33 ± 0.57^{b}

Each result represents the mean ± standard deviation ($n = 3$), and different superscript letters indicate a significant difference between the groups ($p < 0.05$)

Table 14 Minimum inhibitory concentration (MIC) values of the Groundnut shell extracts against the tested organisms

Solvent used for groundnut shell extracts	Minimum inhibitory concentration (µg/mL)			
	A. hydrophila	*P. aeruginosa*	*S. aureus*	*K. pneumoniae*
Methanol	250	250	250	250
Ethanol	250	250	250	500
Acetone	500	250	250	500
Ethyl acetate	500	500	500	500
Hexane	500	500	500	500
Petroleum ether	500	500	500	500

microbial growth by altering membrane permeability, inhibiting nucleic acid synthesis, and cytoplasmic membrane function, which leads to bacterial cell destruction [215]. The presence of several bioactive elements in the GNS extract and their diverse fractions such as saponins, phenolics, and flavonoids may contribute to the diversity of antibacterial activity [216].

Effects on Growth of the Various GNS Extracts on Microbial Cultures

The obtained results depict the influence of prepared extracts on the growth of *A. hydrophila, P. aeruginosa, S. aureus,* and *K. pneumoniae* (Fig. 5A–D). A modest variation in growth patterns was found in the treated organism as relative to the control species at sub-MIC dosages. However, the growth was completely inhibited at the MIC level. This study confirms that various extracts of the GNS have the potentiality to inhibit the growth of the tested microbial cultures at MIC but not in Sub-MIC level. This inhibitory effect occurred because the hydrophobic molecules in extracts triggered a series of interactions with the bacteria that were being studied [217]. The MIC value determined in this study corresponds to the bacterial growth curve analysis, which revealed inhibition of growth based on polar and non-polar solvent extracts of the GNS [218]. Bacterial growth inhibition was due to the interaction of the bioactive compounds in the microbial cell membrane [219]. These discrepancies in the results may be due to the fact that Gram-positive bacteria have a single-layer cell wall, whereas Gram-negative bacteria have a multilayered cell wall. The structure and composition of both gram-positive and gram-negative bacteria, prevents the medication from penetrating the cytoplasmic membrane. [220]. By breaking the outer membrane, metabolites such Tetracosane, Heneicosane, Myristic acid, Tetradecane, and 1-Hexadecane found in GNS extracts could release lipopolysaccharides [221]. These metabolites have a tendency to attach to the cytoplasmic membrane, causing membrane leakage, membrane integrity loss, and damage to the outer membrane vesicles (OMVs) [222] through ion motive force, [223] in the membrane of the bacteria. The electron density in DNA will be altered, resulting in DNA malfunction and the drug's inhibitory potential. Increased inhibition of the growth is related to the penetration of the compounds into the cell, which cause the cell death. Certain metabolites exerted from the extracts which is comprised of phenols, so that it is disintegrated to the outer membrane and release the lipopolysaccharides by increasing the membrane permeability [224].

Conclusion

From the obtained results, qualitative phytochemical investigation, GNS extracts have therapeutic potential due to the presence of metaboilites such as phenols, tannins, saponins, flavonoids, and alkaloids. FT-IR and GC–MS also identified and confirmed the functional groups and bioactive metabolites. The presence of key components such as octadecane, oleic acid, palmitic acid and mome inositol in the GNS extract suggests that it has a higher antioxidant and antibacterial potential. Methanol surpassed other solvent extracts by preserving and increasing the activity of the metabolites as antioxidant and antibacterial agents against the organisms examined. The study findings show how agricultural waste of GNS can be used to combat pathogens in disease control. Furthermore, this study encourages the use of agro-waste as a potential source in pharmaceutical and feed industries.

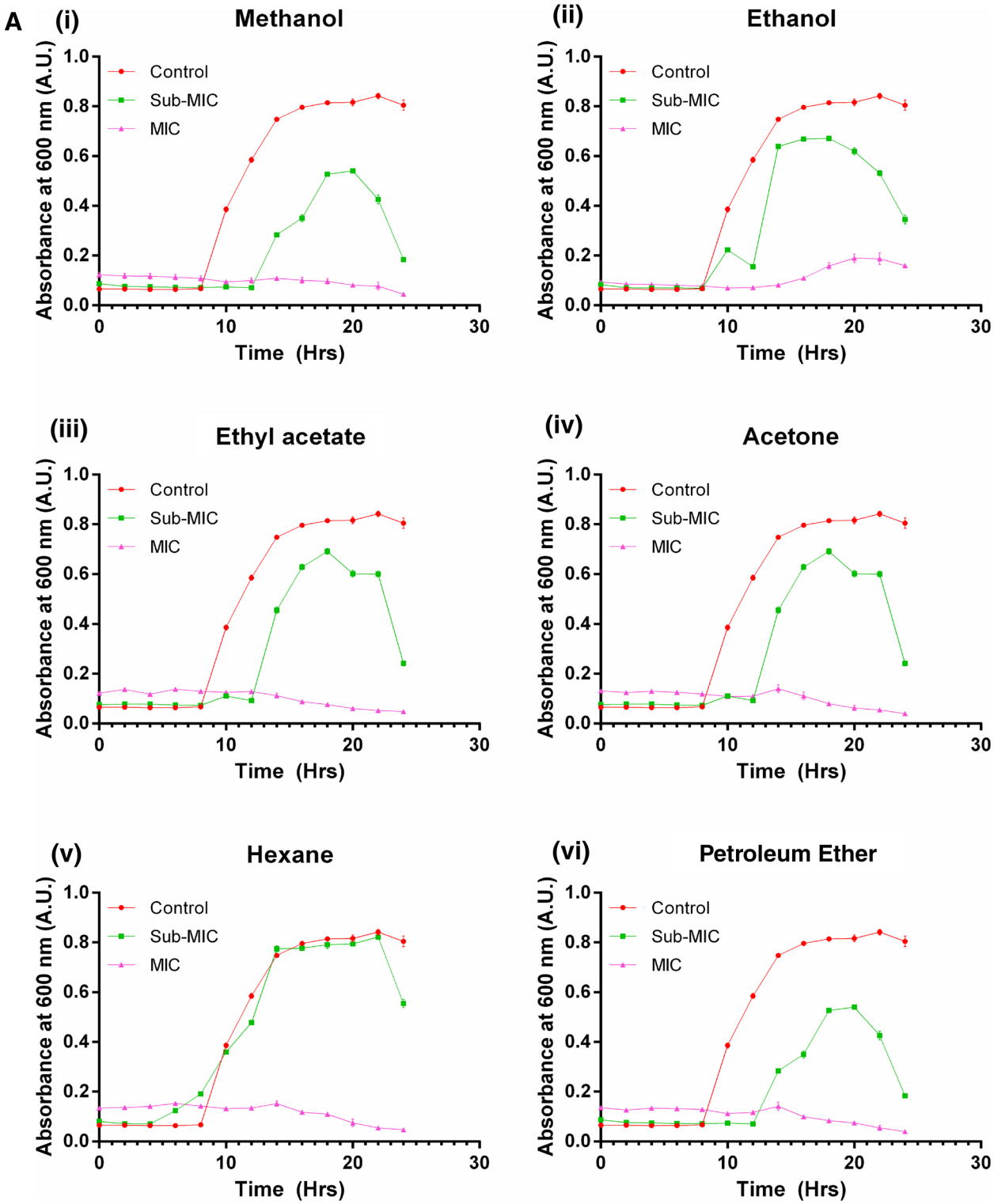
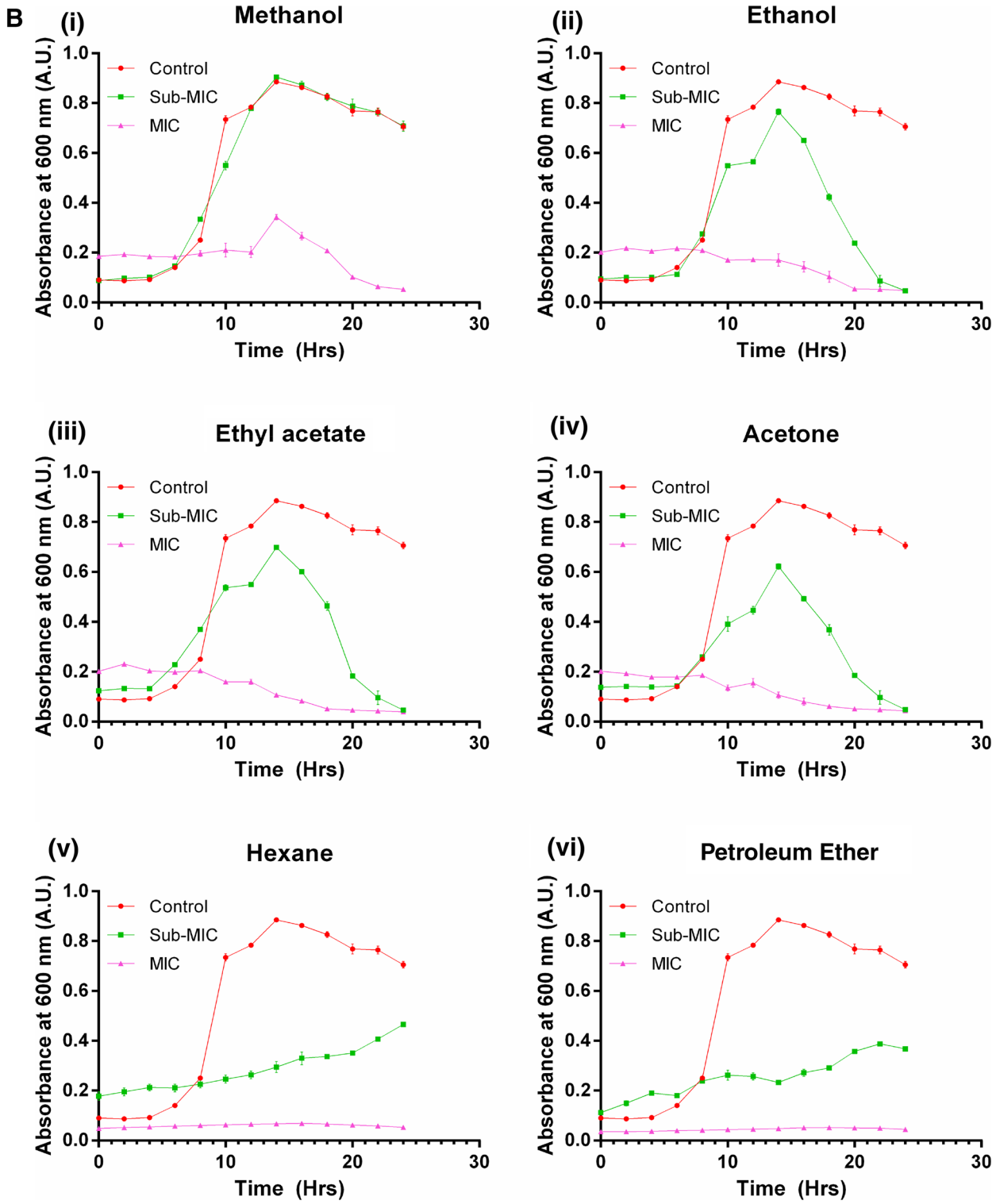


Fig. 5 **A** Growth curves of *Aeromonas hydrophila* under the influence of various extracts of groundnut shell (i) Methanol, ii) Ethanol, iii) Ethyl acetate, iv) Acetone, v) Hexane and vi) Petroleum Ether). **B** Growth curves of *Pseudomonas aeruginosa* under the influence of various extracts of groundnut shell (i) Methanol, ii) Ethanol, iii) Ethyl acetate, iv) Acetone, v) Hexane and vi) Petroleum Ether). **C** Growth curves of *Klebsiella pneumoniae* under the influence of various extracts of groundnut shell (i) Methanol, ii) Ethanol, iii) Ethyl acetate, iv) Acetone, v) Hexane and vi) Petroleum Ether). **D** Growth curves of *Staphylococcus aureus* under the influence of various extracts of groundnut shell (i) Methanol, ii) Ethanol, iii) Ethyl acetate, iv) Acetone, v) Hexane and vi) Petroleum Ether)



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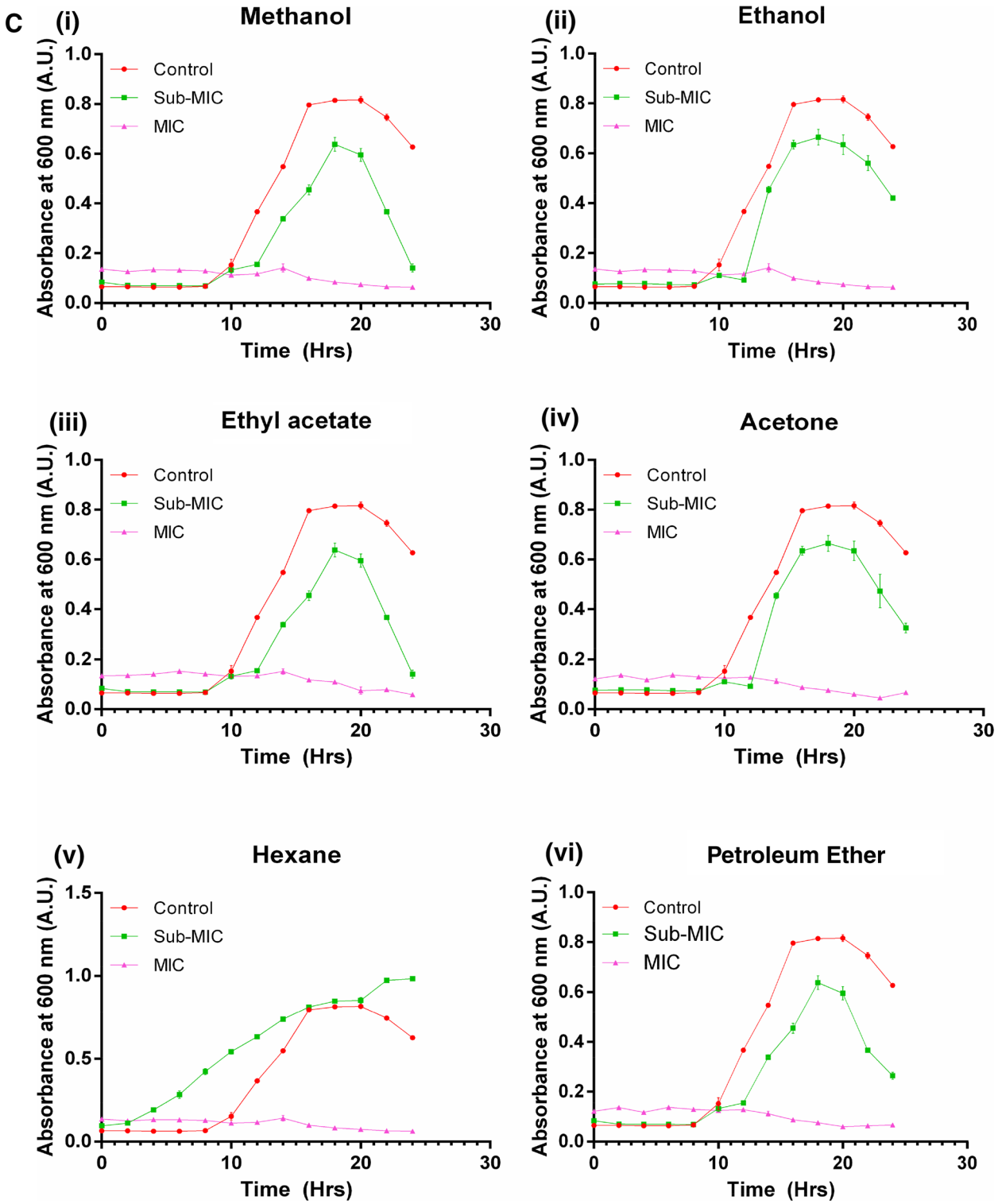

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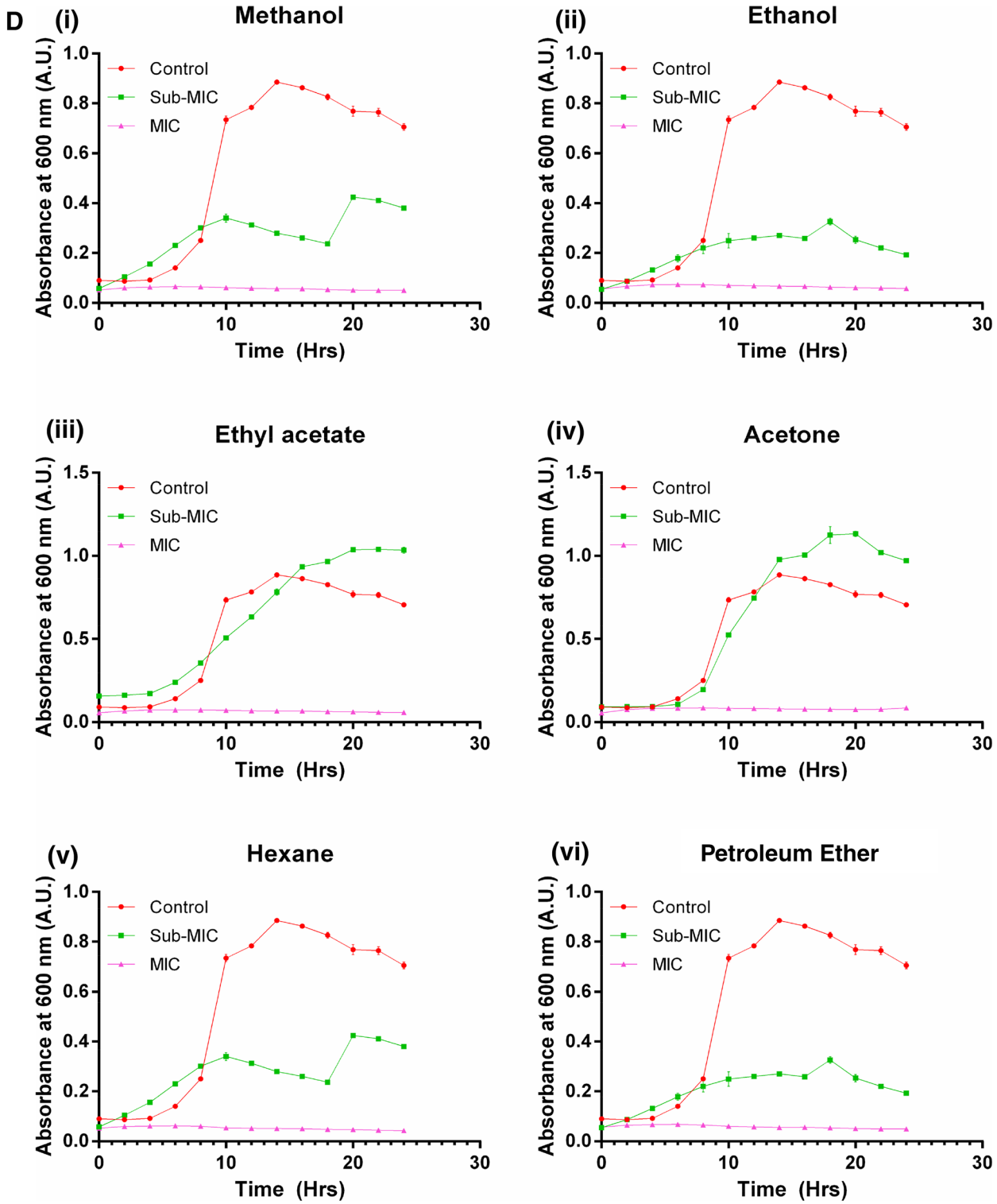

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Data availability All data which were analyzed or generated throughout this study are included in this published article.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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