Journal of Bioresources 12 (1): 42–47, July – September 2024

JOURNAL OF BIORESOURCES

journal webpage: https://jbr.rgu.ac.in

ISSN: 2394-4315 (Print)
ISSN: 2582-2276 (Online)

RESEARCH ARTICLE

A report on metabolite content, antioxidant and antibacterial potential of *Gnetum gnemon* L. cone extract

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Article No.: MDJBR98A; Received: 13.07.2024; Peer-reviewed: 20.08.2024; Revised & Accepted: 12.09.2024; Published: 30.09.2024
Doi: https://doi.org/10.5281/zenodo.14800537

Abstract

Plant-derived bioactive compounds have emerged as promising sources of medicinal uses. Herbal medicine which is mainly based on region-specific knowledge and skills has been employed in the Indian subcontinent since ancient times. *Gnetum gnemon*, an important medicinal plant native to Southeast Asia holds a significant therapeutic value in traditional Indian medicine. This research focuses on the less-explored cone extract of *G. gnemon* in Assam (India), shedding light on its metabolite content, anti-oxidant and anti-bacterial properties. The analysis reveals significant phenol and flavonoid content in the *G. gnemon* cone extract demonstrating moderate antimicrobial activity along with DPPH and H_2O_2 radical scavenging effect. Further exploration using GC-MS analysis identifies compounds like Pentatriacontane, Pentacosane, Gamma sitosterol, gamma.-Tocopherol, Vitamin E, Campesterol, and Stigmasterol, known for their anti-corrosive, antioxidant, anticancer, anti-inflammatory and antimicrobial properties. The plant is culturally significant among tribes in Northeast India, where fresh leaves of *G. gnemon* are employed for various medicinal purposes, including malarial treatment. This study addresses the paucity of phytochemical data through investigations into *G. gnemon* cone extracts in Northeast India. This brief report contributes to a deeper understanding of the medicinal potential and paving the way for novel therapeutic applications in traditional and complementary medicine systems and the pharmaceutical industry.

Keywords: *Gnetum gnemon;* Metabolite content; Antioxidant; Antibacterial activities; Assam

1. Introduction

Bioactive compounds of plants present a promising reservoir of medicinal resources that align well with the current requirements for safe and efficient treatment. Indian subcontinent has a long history of treating a number of ailments using herbal medicine. The curative properties of medicinal plants are due to presence of major groups of active compounds like alkaloids, flavonoids, tannins, steroids (Bhat, 2021). Phytochemicals are defined as bioactive nutrient plant chemicals present in leaves, bark, fruits, cones and other plant parts. The study of Phytochemicals has been instrumental in the discovery of new plant natural products which are of commercial values in various industries such as the traditional and complementary medicine systems, pharmaceutical industries (Chukwuebuka et al., 2018). The IUCN status of the *Gnetum gnemon* L. was accessed by Baloch (2011) to be fall under the category of Least Concern (LC) which found distributed in tropical forest of North East India, Indo-Malayan archipelago and North East Australian region. *Gnetum gnemon* L. (Gnetaceae) is a small tree of Gymnosperm group with immense ethnobotanical significance as wild leafy vegetable sources and in traditional medicines for many years mostly in the Asian countries. *'Melinjo'* is the familiar Indonesian name for the plant and has been consumed as safety food for centuries and cultivated in different agroforestry systems. The *G. gnemon* is an evergreen perennial tree; the male and female strobili are used as ingredients in traditional vegetable curry. The plant is extremely popular among few tribes of North east India, mainly in Nagaland, Manipur and Karbi Anglong district of Assam (Kato et al., 2009, 2011). It has been found that *G. gnemon* contains bioactive compounds such as tannins, saponins, flavonoids which are responsible for the antioxidant and anticancer properties (Kato et al., 2011).

In *G. gnemon*, the level of flavonoid varies accordingly with its plant parts. It was reported that, stilbenoids from *G. gnemon* showed moderate antimicrobial activity along with DPPH radical scavenging effect and alpha-amylase inhibition activity (Kato et al., 2009). Other important compounds of the plant that were reported by GC-MS analysis includes - Pentatriacontane, Pentacosane, Gamma sitosterol,phytol acetate, beta-sitosterol, 2-methyltetracosane, tetracontane, heptacosane, nonacosane, hentriacontane, Octadecanoic acid (Vijisaral and Subramanian, 2014). Heptacosane and tetracosane have anti corrosive and antioxidant property (Akpuaka et al., 2013). Stigmasterol have antioxidant, antimicrobial, antiarthritic property (Leopold, 2010).

Figure 1a. Habit of *Gnetum gnemon* L.; b. Cones of *G. gnemon* L.

Table 1. Qualitative phytochemical screening of *G. gnemon* hexane cone extract.

Test		Results
Detection of Glycosides	10%NaOH test	-
	Aqueous NaOH test	+
	Conc. H_2SO_4 test	+
Detection of Proteins and Amino acids	Xanthoproteic test	+
Detection of Flavonoids	Alkaline reagent test	-
	Ammonia test	-
	Conc.H_2SO_4 Test	+
Detection of Phenolic compounds	Iodine test	+
	Gelatin test	-
	Potassium dichromate test	-
Detection of Tannins	10% NaOH test	+
Detection of Alkaloids	Mayer's test	-
	Wagner's test	-
	Picric acid test	-
	Iodine test	-
Detection of Saponins	Foam test	-
	$NaHCO_3$ Test	+
Detection of Phytosterols	Salkowski's test	-
	Hesse's test	+
Detection of Triterpenoids	Salkowski's test	+
Detection of lignins	Labat test	+
Detection of Quinones	Alcoholic KOH test	-
	Conc HCl test	-
Detection of Leucoanthocyanins	Isoamyl Alcohol test	-
Detection of Carboxylic acid	Effervescence test	+
Detection of Gums and Mucilages	Alcohol test	+
Detection of Resins	Turbidity test	-
Detection of Reducing Sugars	Benedict's Test	-
	Fehling's Test	-
Detection of Cardiac glycosides	Keller-Killani Test	-
Detection of Anthocyanins	HCl test	-

The aqueous extract of *G.gnemon* leaves demonstrated a higher antioxidant activity when compared to other extracts such as methanol, ethanol, hexane or chloroform (Wazir et al., 2011). However, some studies reported that the bark ethanolic extract exhibited the highest antioxidant activity (Syahdi et al., 2019).

Various scientific data revealed that *G. gnemon* has antimicrobial, antitoxic, antioxidant, antiquorum sensing and antisenescence properties due to the presence of phyto-constituents like saponins, tannins and stilbenoids (Kato et al., 2009; Kato et al., 2011; Wazir et al., 2011).
Several studies on a *G. gnemon* seed extract reported that the seed has some interesting activities such as antitumor, antioxidant, anti-tyrosinase, anti-diabetes, lipase inhibitor and antimicrobial against pathogenic bacteria (Wazir et al., 2011; Barua et al., 2015).

After extensive literature survey, it was found that most of the earlier works have evaluated phytochemical and biological activities of leaf and bark extract of *G. gnemon* but report on cone extract of *G. gnemon* is still scarce. Hence, present study evaluated metabolite content, anti-oxidant and antibacterial activities of the cone extract of *G. gnemon* collected from Assam.

2. Materials and methods

2.1 Collection and preparation of plant samples

The cones of *Gnetum gnemon* L. was collected from Gauhati University Botanical Garden at a regular interval of time from January 2023 to March 2023. Voucher specimen was prepared by following the standard herbarium techniques (Jain and Rao, 1976). The specimen was identified and authenticated at GUBH, ASSAM and accepted name was verified in standard website (POWO, 2024). Voucher specimen was submitted to the Herbarium of Gauhati University (GUBH) (Accession No. 20506) for future reference.

2.2. Preparation of plant extract

The collected cones were washed and dried in shed for two weeks under controlled environment. The dried cones were subjected to sequential extraction following polarity index of the solvents. Hexane extract was evaporated using Rotary evaporator at 40 °C under 200 mbar pressure. The extracts *Gnetum gnemon* hexane extract (GNHx) were stored in Borosil screw cap glass vials at 4 °C for further analysis.

2.3. Qualitative phytochemical screening

The qualitative test for preliminary screening of phytochemical in the plant extracts was done by standard protocols (Shaikh and Patil, 2020).

2.4. Quantification of total phenol content

The estimation of total phenol was determined by following the standard protocol of Singleton and Rossi (1965). Gallic acid was used as a standard. The total phenol content was determined by using a calibration curve generated from the concentration of gallic acid solution (mg/ml) and expressed in mg gallic acid equivalent/g extract.

2.5. Quantification of total flavonoid content

The estimation of total flavonoid was determined by following the standard protocol of Jia et al (1999). Quercetin was used as a standard. The total flavonoid content was determined using a calibration curve generated from the concentration of quercetin solution (mg/ml) and expressed in mg quercetin equivalent/g extract.

2.6. DPPH scavenging assay

The free radical scavenging activity was measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method (Burits and Bucar 2000; Cuendetet al. 1997) with slight modifications. The absorbance was measured at 519 nm. Ascorbic acid was used as positive control. The percentage of DPPH radical scavenging was calculated according to the formula-

Scavenging activity (%) = $(A_o-A_1)/A_o\times100\%$
Where, A_o is the absorbance of the control and A_i is the absorbance of the sample.

2.7. H_2O_2 scavenging assay

The hydrogen peroxide scavenging assay was determined by using Ruch et al (1989) and Fernando and Soysa (2015) method with slight modifications. The absorbance was measured at 230 nm on the microplate reader against a blank containing only phosphate buffer without hydrogen peroxide. Butylated hydroxytoluene (BHT) acid was used as standard. The percentage scavenging of hydrogen peroxide was calculated using the equation.

Scavenging activity (%) = $(A_o- A_1)/ A_o\times 100$
Where, A_o is the absorbance of the control and A_i is the absorbance of the sample.

2.8. Metabolite profiling using Gas Chromatography - Mass Spectrometry (GC-MS) analysis

The gas Chromatography-Mass Spectrometry (GC-MS) was analyzed using instrument GC-MS DALS. The carrier gas, helium was used at a 1 ml per min flow rate in split mode (10:1) v/v. The extract was injected into the column (60 ×250 µM) at 280°C injector

Table 2. Estimation of Total Phenolic Content

Sample	Total Phenolic Content (mg GAE/gm)
GNHx	39.85±0.794

The values obtained are Mean±SD; where n=3 independent replicates
GAE- Gallic Acid Equivalent

Table 3. Estimation of Total Flavonoid Content

Sample	Total Flavonoid Content (mg QE/gm)
GNHx	290.53 ±7.08

The values obtained are Mean±SD; where n=3 independent replicates
QE -Quercetin Equivalent.

Table 4. IC_{50} of ascorbic acid and hexane cone extract against DPPH

IC_{50} of Ascorbic Acid against DPPH(µg/ml)	9.3 ± 0.823
IC_{50} of Hexane cone extract against DPPH(µg/ml)	168.5 ± 3.12

The values obtained are Mean ± SD; where n=3 independent replicates.
Ascorbic acid acts as standard.

Table 5. IC_{50} of BHT and hexane cone extract against H2O2

IC_{50} of BHT against H_2O_2(µg/ml)	45.6 ± 0.33
IC_{50} of Hexane cone extract against H_2O_2(µg/ml)	141.54 ± 2.57

The values obtained are Mean ± SD; where n=3 independent replicates.
BHT acts as standard

temperature. The initial temperature of the oven started at 60 °C and was held for 1min. Then, it was gradually raised at a rate of 10 °C to 300 °C. The ion sources temperature was maintained at 180 °C. The source and detector temperature were maintained at 150 °C. The detector operates in scan mode ranging from 50-600 atomic mass units. Data acquisition was done using Mass Hunter GC-MS acquisition 2017.

2.9. Evaluation of antibacterial activity using disk diffusion method

The antimicrobial activity of the cone extract was screened by the agar well diffusion method. The bacterial inoculum was uniformly spread using sterile cotton swab on MHA (Muller Hinton agar) plates. Two gram-positive bacterial strains- *Staphylococcus aureus, S. epidermidis* and one gram-negative bacterial strain- *Klebsiella pneumonia* was used as bacterial inoculum. A sterile cork borer was used to create four wells, each measuring 6 mm, in the inoculated media. The wells were loaded with 30 µl of the extract. The systems were placed in an incubator for 24 hours at a temperature of 36°C ± 1°C, in aerobic conditions. Upon completion of the incubation, around the wells the zones of inhibition (ZOI) were observed and were measured. For reference, plates treated with 0.1% DMSO were used as negative controls and ciprofloxacin (5µg/well) were used as positive controls.

3. Result and discussion

3.1. Metabolite content in GNHx

The qualitative phytochemical screening test of the *G. gnemon* hexane cone extract has revealed rich content of phytoconstituents such as steroids, essential oil and terpenoid, flavonoids and polyphenolic compounds of pharmaceutical interests (Table 1). Quantitative estimation has revealed total phenol content of 39.85±0.794 mg GAE/gm and total flavonoid content of 290.53 ±7.08 mg QE/gm in hexane cone extract of *G. gnemon* which is presented in Table 2 and 3. GC-MS metabolite profile (Table 6, Figure 4) has revealed some interesting compounds such as gamma.-Sitosterol was detected in higher concentration (85.72%) which is followed by Vitamin E (17.17%), Campesterol (14.19%), least concentration was reported for gamma.-Tocopherol (10.3%) which is also reported as anti-cancer, anti-oxidant and anti-inflammatory properties by previous workers (Wazir et al., 2011; Barua et al., 2015). These phytocompounds were identified and biological activities were also confirmed through using various scientific literatures as well as NIST chemistry webbook, PubChem which revealed the pharmaceutical importance of these important metabolites.

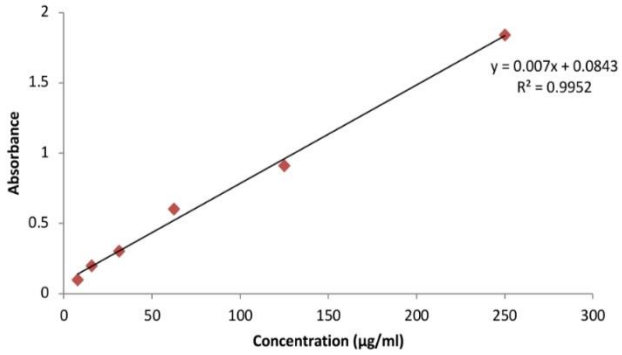

Figure 2. Gallic acid standard curve graph.

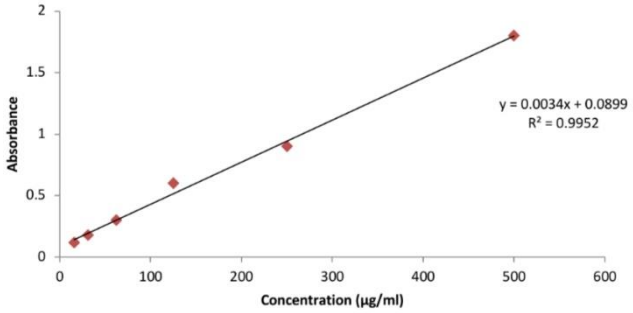

Figure 3. Quercetin standard curve graph**.**

3.2. Anti-oxidant activity

DPPH radical scavenging assay of *G. gnemon* hexane cone extract (GNHx) has revealed significant anti-oxidant activities with IC_{50} value of 168.5 ± 3.124 µg/ml when compared with IC_{50} value of Ascorbic Acid (9.3 ± 0.823 µg/ml) (control) which is present in Table 4 (Figure 2).
H_2O_2 radical scavenging assay of GNHx also revealed significant anti-oxidant activities with IC_{50} value of 141.54 ± 2.57 µg/ml when compared with IC_{50} of BHT (45.6 ± 0.33 µg/ml) which is presented in Table 5 (Figure 3).

3.3. Antibacterial activity

The cone extracts of *G. gnemon* exhibited antibacterial activity with a ZOI of 8.96mm and 6.9mm against gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* respectively. For gram-negative bacteria *Klebsiella pneumonia* the ZOI was observed as 7.06mm. The absence of ZOI was interpreted as the absence of activity (Table 7, Figure 5).

Qualitative and quantitative studies revealed important metabolites in the *G. gnemon* hexane cone extract such as flavonoids, tannins, proteins and amino acids, phenol, triterpenoids, lignins, phlobatanins, carboxylic acid, Glycosides,phytosterols, gums, mucilages and resins. However, reducing sugar, alkaloids, cardiac glycosides, quinones, Leucoanthocyanins and anthocyanins were found absent. It can be concluded that the hexane extract of *G. gnemon* exhibited a notably high total phenol and flavonoid content. Both flavonoid and phenols have been reported to be a rich source of antioxidant (Jia et al., 1999; Wazir et al., 2011). Besides its antioxidant activity, phenol has wide range of properties such as anti-inflammatory, antimicrobial, anesthetic, anti-tubercular, anticancer, analgesic, and anti-Parkinson activities that contributes to its significance (Chukwuebuka et al., 2018). Likewise, flavonoids, known for their antioxidant effects, have been shown to inhibit the initiation and promotion of tumor growth. Furthermore, the consumption of flavonoids has been associated with a decreased risk of coronary heart disease (Bhat, 2021).

Table 6. GC-MS profiling and identification of bioactive phytocompounds in the hexane cone extract of *G. gnemon*.

SN	Retention Time	Peak Area (%)	Compound Name	Molecular Formula	Molecular Weight (gm/mol)
1	26.09	9.15	Phthalic acid, hept-4-yl isobutyl ester	$C_{19}H_{28}O_4$	320.4232
2	26.98	39.19	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507
3	28.08	26.46	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.4772
4	29.29	7.01	Methyl 2-octylcyclopropene-1-heptanoate	$C_{19}H_{34}O_2$	294.472
5	29.66	39.03	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.4721
6	29.76	46.64	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	$C_{19}H_{32}O_2$	292.4562
7	30.14	12.1	Methyl stearate	$C_{19}H_{38}O_2$	298.5038
8	30.29	6.86	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308.4986
9	30.68	27.2	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308.4986
10	30.77	30.83	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	$C_{19}H_{32}O_2$	292.4562
11	31.13	9.84	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312.5304
12	32.29	12.6	Methyl 5,11,14-eicosatrienoate	$C_{21}H_{36}O_2$	320.5
13	32.59	9.18	Heptacosane	$C_{27}H_{56}$	380.7335
14	33.19	11.79	Methyl 5,11,14-eicosatrienoate	$C_{21}H_{36}O_2$	320.5
15	33.95	9.68	Tetracosane	$C_{24}H_{50}$	338.7
16	35.28	8.48	Pentacosane	$C_{25}H_{52}$	352.7
17	35.99	8.79	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	$C_{26}H_{42}O_4$	418.6
18	36.54	9.4	Hexacosane	$C_{26}H_{54}$	366.7
19	37.77	6.21	Heptacosane	$C_{27}H_{56}$	380.7
20	38.95	6.49	Ethyl tetracosanoate	$C_{26}H_{52}O_2$	396.7
21	40.1	21.89	Nonacosane	$C_{29}H_{60}$	408.8
22	41.21	8.65	Triacontane	$C_{30}H_{62}$	422.8
23	41.99	10.3	gamma.-Tocopherol	$C_{28}H_{48}O_2$	416.7
24	42.31	42.88	Nonacosan-10-one	$C_{29}H_{58}O$	422.8
25	42.44	100	14-Methyl-hexadecane-1,2-diol, isopropylidene	$C_{20}H_{40}O_2$	312.5
26	42.81	43.05	Z-14-Nonacosene	$C_{29}H_{58}$	406.771
27	42.93	17.17	Vitamin E	$C_{29}H_{50}O_2$	430.7061
28	43.93	14.19	Campesterol	$C_{28}H_{48}O$	400.6801
29	44.33	10.95	2-Methylheptacosane	$C_{28}H_{58}$	394.7601
30	44.47	15.91	Adipic acid, 2-fluorophenyl hexadecyl	$C_{14}H_{18}FNO_3$	267.30
31	44.84	85.72	gamma.-Sitosterol	$C_{29}H_{50}O$	414.7067
32	45.48	10.04	17-Pentatriacontene	$C_{35}H_{70}$	490.9303

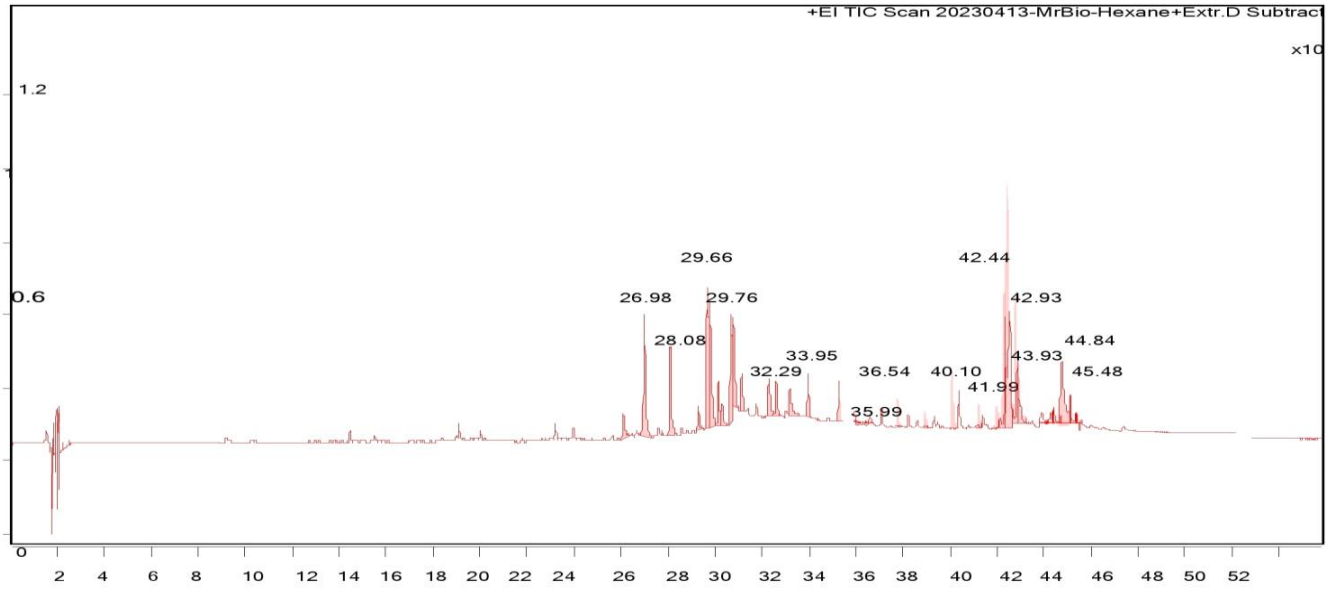

Figure 4. GC-MS chromatogram of the metabolites detected in hexane cone extract of *G. gnemon*

GC-MS profiling of hexane cone extract of *G.gnemon* confirmed presence of compounds such as Phthalic acid, hept-4-yl isobutyl ester, Hexadecanoic acid, methyl ester, Hexadecanoic acid, ethyl ester, Methyl 2-octylcyclopropene-1-heptanoate, 9,12-Octadecadienoic, acid (Z,Z)-, methyl ester, 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z), Methyl stearate, Linoleic acid ethyl ester, Linoleic acidethyl ester, 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z), Octadecanoic acid, ethyl ester, Methyl 5,11,14-eicosatrienoate, Heptacosane, Methyl 5,11,14-eicosatrienoate, Tetracosane, Pentacosane, Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester, Hexacosane, Heptacosane, Ethyl tetracosanoate, Nonacosane, Triacontane, gamma.-Tocopherol, Nonacosan-10-one, 14-Methyl-hexadecane-1,2-diol, isopropylidene, Z-14-Nonacosene, Vitamin E, Campesterol, 2-Methylheptacosane, Adipic acid, 2-fluorophenyl hexadecyl, gamma.-Sitosterol and 17-Pentatriacontene. These compounds have been identified by GC-MS chromatographic technique. Highest peak area was obtained in 14-methyl-hexadecane-1,2-diolisopropylidene (100%) and retention time was 42.44. On the other hand, lowest peak area was obtained in heptacosane (6.21%) and retention time was 37.77. Lowest peak area was also obtained in hexacosane, ethyl tetracosanoate, tetracosane, pentacosane, Linoleic acid ethyl ester. On the other hand, highest peak area was also

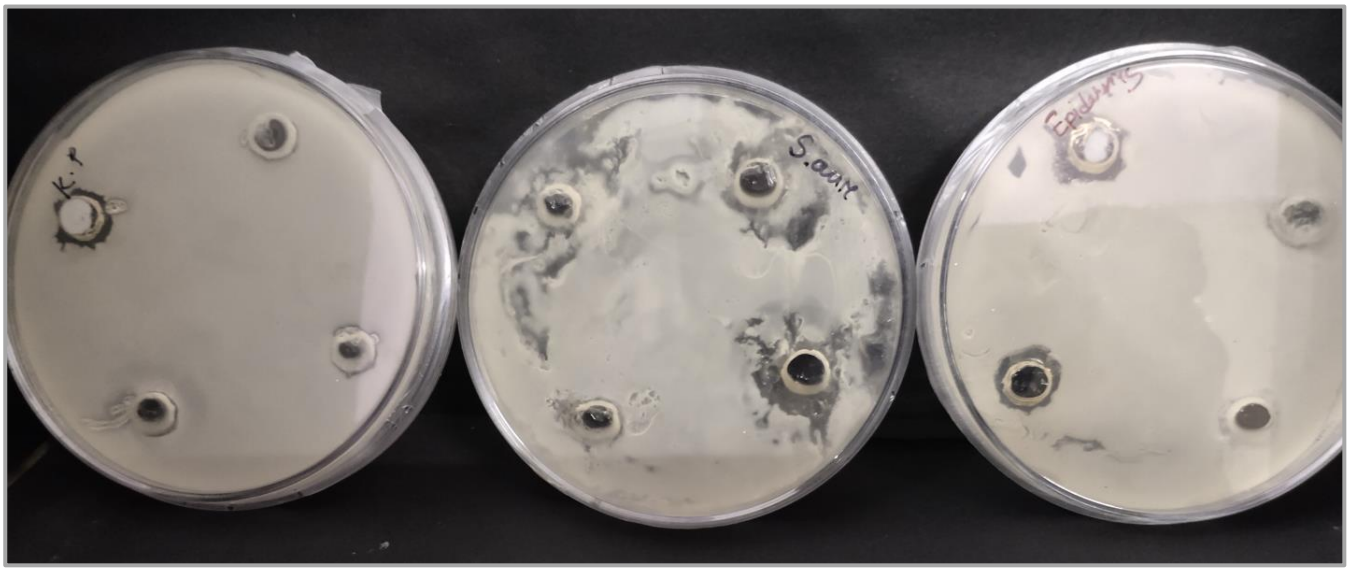

Figure 5. Zone of inhibition against *Klebsiella pneumonia, Staphylococcus aureus* and *Staphylococcus epidermidis* caused by cone extract of *G. gnemon*.

Table 7. Antimicrobial activity *G. gnemon* hexane extract against three bacterial species.

Bacterial strains	Inhibition zone diameter (mm)	Inhibition zone diameter (mm) while using Ciprofloxacin
Staphylococcus aureus	8.96 ± 0.152	17
Staphylococcus epidermidis	6.9 ± 0.1	15
Klebsiella pneumonia	7.06 ± 0.057	15

The values obtained are Mean±SD; where n=3 independent replicates

obtained in gamma sitosterol, 9,12,15-octadecatrienoic acid, methyl ester. The compounds which have antioxidant values (Leopold, 2010) were identified as Gamma-sitosterol, Gamma tocopherol,17-pentatriacontane, Campesterol, Vitamin E, Hexadecanoic acid ethyl ester, Hexadecanoic acid methyl ester, 9,12,15- octadecatrienoic acid methyl ester, Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester and tetracosane.

The antimicrobial effectiveness could be attributed to the active constituents present in the extracts. These findings hold significant practical implications in current times, considering infectious diseases as a leading global cause of death. As the indiscriminate use of synthetic antibiotics is increasing day by day it is posing a serious threat of antibiotic resistance to humankind which can be substituted by using plant-based medicines (Akpuaka et al., 2013). The current study has unveiled the substantial potential of the investigated plant, particularly its cone, in the exploration of antimicrobial compounds that could be applied in addressing microbial infections.

4. Conclusion

This study revealed metabolite profile such as significant content of total phenolic and total flavonoid content, antioxidant and anti-bacterial activities of the cone extract of *G. gnemon*, the important wild vegetable and medicinal sources used by the tribal communities of Assam. Among the important anti-cancer and anti-anti-inflammatory metabolite profiled in GC-MS, gamma.-Sitosterol was detected in higher concentration (85.72%) which is followed by Vitamin E (17.17%), Campesterol (14.19%), least concentration was reported for gamma.-Tocopherol (10.3%) which might be responsible anti-oxidant activities demonstrated in present studies and proven as potential source of bioactive compounds which could be explored further for treatment cancer and inflammation related ailments. Other important volatile metabolites of terpenoid origin detected in higher concentration are Nonacosan-10-one (42.88%), Nonacosane (21.89%), Tetracosane (9.68%) and other volatile essential oil content which might be responsible for the anti-bacterial activities demonstrated against four bacterial strains in present studies.

Acknowledgement

Authors are thankful to Department of Botany and Department of Biotechnology, Gauhati University for their kind support and help. We are also thankful for DST-FIST (Department of Science & Technology - Fund for Improvement of S&T Infrastructure) programme for providing best laboratory equipment in the Gauhati University.

Authors' contributions

PJS, MN, MD, BD and NN constructed the manuscript. NN, MN, RS, DB and BD conceptualized the idea and equally contributed in literature review. While PJS, MN, HK, MH, MB, and MD performed all the experiments. Before submitting the manuscript, all authors thoroughly reviewed and gave their approval.

Declaration of conflict of interests

Authors have no conflict of interests.

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