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Antimicrobial, Antioxidant, Antidiabetic, Cytotoxic Activities and GC-MS Analysis of Methanolic Extract of *Elaeocarpus sphaericus* Leaves from Nepal

Susan Joshi*[1], Saruna Amatya[2], Ram Darash Pandey[1], Pooza Khadka[1], Jagadeesh Bhattarai[1]

[1]*Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathamandu*

[2]*Tri-chandra Multiple Collage, Ghantaghar, Tribhuvan University, Kathamandu*

Corresponding Authors:** ***Susan Joshi, *Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathamandu*

Abstract: *This research work was focused on bioactivities and Gas Chromatography/Mass Spectrometry analysis of the methanolic extract of Elaeocarpus sphaericus leaves from Nepal. The total phenol and total flavonoid content were found to be 295.41±21.57 mg/g(GAE)and 428.71±56.95mg (QE) mg/g in dry weight using aluminum chloride and Folin-Ciocalteu colorimetric methods respectively. From the GC/MS analysis of hexane and ethyl acetate (1:1) fractions of E. sphaericus leaves extract was identified seventeen different peaks and the major constituents were detected as 2,6,6-trimethyl-Bicyclo[3.1.1] heptane(34.41%),9,12,15-octadecatrienoic acid(11.97%), 1,4-Eicosadiene(10.93%), n-hexadecanoic acid(7.37%), phytol(7.35%) etc. and the methanolic fraction was identified twenty different peaks with the major constituents such as n-hexadecanoic acid (19.58%), 9,12,15-octadecadienoic acid(18.91%), 9,12,15-octadecatrien-1-ol(90%), hexadecanoic acid, methyl ester(6.43),7,8-dimethylbenz[c] acridine(6.07%),4-hydroxy-3-methyl acetop henone (5.27%), 4-[4-fluorophenyl]-6-[trifluoromethyl]-2-pyrimidinamine(4.71%),etc.The methanolic extract revealed effective zone of inhibition towards methicillin- resistant staphylococcus aureus (16mm), S. aureus (15mm), P. vulgaris (14mm) and no activity showed against B. subtilis, K. pneumonia, S. dysentria, E. coli, S. aeruginosa, and S. typhii. by disc diffusion method. The IC_{50} value of antioxidative and anti-diabetic activities of this extract was calculated as 68.98 and 36.90µg/mL respectively which showed strong potentiality for these activities. This extract was found to be highly cytotoxic against brine shrimps (Artemia salina) nauplii due to 61.05µg/ml LC_{50} value.*

Keywords: *phytol, Folin-Ciocalteu, n-hexadecanoic acid, disc diffusion, diffusion*

1. INTRODUCTION

Elaeocarpus sphaericus, a member of Elaeocarpaceae family, the genus *Elaeocarpus* comprises 350 species found throughout Australia, Southeast Asia, Malaysia Japan, New Guinea, Fiji, Hawaii and Madagascar in the west to New Zealand in East [1]. The India subcontinent marks the western limit of *Elaeocarpus* distribution in Asian countries like Nepal, Bangladesh, Bhutan, India, Pakistan and Sri Lanka [2]. Storrs et. al. have recorded 26 species of Elaeocarpus from Himalayan region [3].

E. sphaericus is commonly known as Rudrakshya in Nepal and bead tree in English. It is a tall evergreen broad leaved tree found in tropical and subtropical areas at the altitude ranging from sea coast to 2000m above the sea level. This medicinal plant is about 14.60m-29.20m tall depending on the area and climate with diameter of trunk is up to 1.22 m. The leaves are shinning green on the upper side with a dull leathery on the dorsal side, and ovate with tithed edges. The fruits are globular in shape, 1cm in diameter surrounded with purplish- deep blue or mealy pulp on fully ripening, hence also known as blueberry beads [4]. The bead inside is hard and tubercle. The bead is obtained from seeds of several species of genus of the Elaeocarpus, with *E. sphaericus*, being the major species. According to Hindu mythology, people believe that, anyone who wears rudrakshya beads gets mental and physical powers.

E. sphaericus beads are usually traded from Nepal to neighbor countries and it mostly grows in Central and Eastern Nepal at about 550-1600 m altitude. *E. sphaericus* has been revealed to possess several secondary metabolites such as triterpenes, tannins, flavonoids(qurcetine), alkaloids,

glycosides, steroids, flavonoids, tannins (gallic and ellagic acid), fatty acids (palmitic and linolic), carbohydrates,and protein that have been used for making different medicinal extracts [5-9]. The different parts of this medicinal plant is traditionally use to treat diseases and exhibited pharmacological activities like anti-inflammatory, analgesic, hypoglycemic, antidepressant, antiasthmatic, sedative, antihypertensive, antiulcerogenic, anticonvulsant, and antimicrobial [6,7,10-14]. The leaves of *E. sphaericus* yielded bioactive pytoconstituent such as quercetin, gallic and ellagic acid, elaeocarpidine, elaeocarpineline, isoelaeocarpineline, epiisoelaeocarpiline, epialloelaeocarpiline, alloelaeocarpiline, pseudoepiallo elaeocarpiline and rudrakine [10,13,15-22]. The seeds and leaves were used to cure stress, anxiety, mental problems and also possess anti-inflammatory, antiasthmatic, antioxidant and antimicrobial activities. Plants are the important source of modern pharmaceutical drugs; nearly 25% of the pharmaceutically important drugs prescribed worldwide are derived from plants [23].

Due to large number of infections being caused by microorganisms and the side effects being caused by the antibiotics thus used to treat them, there has been a considerable shift in the demand towards natural herbal medicines [24]. As *Elaeocarpus sphaericus* has been reported to exhibit wide range of medicinal effects such as anti-fungal activity and anti-microbial activity [8,18].The different extracts of fruits of *E. sphaericus* were tested for bacterial strains such as *Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis, Salmonella typhi and paratyphi, Salmonella typhimurium, Vibro cholera, Aeromonas hydrophila, Shigella sp., Klebsilla pneumonia, Enterobacter sp.* and *Pseudomonas sp.* [18]. Some extracts of *E. sphaericus* fruits were found to be effective against bronchial asthma [18] while methanolic extract of this fruits were found to show anti-anxiety properties [8]. According to literature evidences, research works were mostly carried out in biological activities of *E. sphaericus* fruit and barks for pharmacological efficiency [7,8,10,11,16-18,24-27]. Few studies explored the chemical composition and biological activities of *E. sphaericus* leaves despite the richness of bioactive molecules [13,29-32]. However, the chemical composition and biological activities of *E. sphaericus* leaves have not been studied in detail. Therefore, the main aim of this research work were to evaluate the chemical composition and *in vitro* antimicrobial, antidiabetic, antioxidative and cytotoxic activities of methanolic extract of *Elaeocarpus sphaericus* leaves from Kathmandu, Nepal.

2. EXPERIMENTAL TECHNIQUE

2.1. Collection and Identification of Plant Material

The fresh leaves of *Elaeocarpus sphaericus* (Rudraksha) were collected from Kritipur, Kathmandu which is located in 27°40’56” N and 85°17’15” E about 1400m above sea level. The taxonomic identification of the plants was done by comparison with the herbarium species deposited at Central Department of Botany, Tribhuvan University, Kirtipur, Nepal.

2.2. Preparation of Plant Extract

The collected fresh leaves of *E. sphaericus* were cleaned thoroughly with water, shade dried for two weeks at room temperature, pulverized to powder in electrical grinder and stored in clean plastic bag until the further use. The phytochemicals present in the powdered leaves were extracted by cold percolation method using methanol as a solvent. After complete evaporation of the solvent using rota evaporator, crude methanolic extracts of *E. sphaericus* leaves were obtained and stored in sample bottle until the further use.

2.3. Phytochemical Profiling

The phytochemicals present in each extract were subjected to chemical analyses with a specific reagent using a standard protocol [33,34] to detect presence of Secondary metabolites such as alkaloids, terpenoids, steroids, glycosides etc.

2.4. Determination of Total Phenol Content

The total phenol content in the plant extracts was determined by Folin-Ciocalteu colorimetric method based on oxidation-reduction reaction followed by Waterhouse [35]. Gallic acid was used as a reference standard for the construction of a calibration curve. The varying concentrations (10, 25, 50, and 100 µg/mL) of gallic acid solution in methanol were prepared from the stock solution **(**1000

µg/mL)**.** An aliquot of 5 mL of 10% Folin-Ciocalteu reagent (FCR) was mixed with one mL of methanol extract of *A. marmelos* leaves that was shaken for 5 minutes and then added 4 mL of 7% sodium carbonate solution to get a total volume of 10 mL. After that, the mixture was shaken well for 2 minutes and incubated for 30 minutes at 40°C in a water bath. Finally, the absorbance of the mixture solution was measured at 760 nm against the blank (distilled water/ sodium carbonate) solution containing all reagents except gallic acid. All the samples were analyzed in triplicate. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. The total phenolic content of the extracts was expressed as mg gallic acid equivalent (GAE) per gram dry extract (mg/g**).** The average absorbance values obtained at different concentrations of gallic acid were used to plot a calibration curve. The total phenol content of the extract was calculated using the formula; P= CV/M, where, P is the total content of the phenol compounds (mg/gm) in GAE, C is the concentration of gallic acid established from the calibration curve (mg/mL), V is the total volume of extract in assay and M represents the total weight of the plant extract (mg) used in the assay.

2.5. Determination of Total Flavonoid Content

The total flavonoid content of the plant extract was determined by aluminum chloride colorimetric assay [36]. Quercetin was used as a reference standard for the construction of the calibration curve. The stock solution of the extract of concentration 1,000 µg/mL (ppm) was prepared by dissolving 20 mg extract in 2 mL of methanol. Serial dilution was carried out to get 125, 250, 500, and 1000 µg/mL sample solutions. An aliquot of one mL quercetin of each concentration in methanol was poured into a 20 mL test tube containing 4 mL distilled water. Then, at zero time, 0.3 mL of 5% sodium nitrite was added to the test tube. After 5 minutes, 0.3 mL of 10% aluminum chlorides was added, shake well the test tube, and kept it for 6 minutes and 2 mL of 1 M sodium hydroxide were added to the mixture. Immediately the total volume of the mixture was made up to 10 mL by adding distilled water and mixed thoroughly. Finally, the absorbance of the pink color mixture was determined 510 nm wavelength versus blank containing all the reagents except quercetin. The average absorbance values obtained for different concentrations of quercetin were used to plot the calibration curve. All the samples were analyzed in triplicate. The total flavonoid content of the extracts was expressed as mg quercetin equivalent (QE) per gram dry extract (mg/gm). The average absorbance values obtained at different concentrations of quercetin were used to plot the calibration curve. The total flavonoid content of the extract was calculated using same formula as total phenol content.

2.6. Column Chromatography Technique

The crude methanolic extract of *E. sphaericus* leaves was subjected to column chromatography technique using silica gel (mesh 60-120 mesh) as absorbent. This extract was fractionated eluting with different solvent such as hexane, 1:1 hexane and ethyl acetate and methanol to get different fractions. Here in this study, two fractions such as1:1 hexane and ethyl acetate and methanolic fractions were used for identification of chemical component using GC/MS technique**.**

2.7. GC-MS Analysis

GC-MS analysis was performed on the gas chromatography mass spectrometer GC-MS-QP 2010. The phytochemicals in methanolic extract of *E. sphaericus* leaves were analyzed by a gas chromatograph (GCMS-QP 2010) having HP-5MS column (30m X 250µm X 0.25µm) using helium as carrier gas. The sample was injected into the GC inlet under pressure flow control mode maintaining purge flow 3 mL/min after fixing the split ratio at 25:1 and split flow of 25 mL/min. The initial column oven temperature was set at 40ºC and then 15ºC/min to 250ºC for 5 minute with holdup time 1.3789 minute. Diluted sample (2µL) was injected at 250°C temperature and 7.0699 psi pressure, flow rate of 1mL/min, average velocity of 36.262cm/sec. Full scan mass spectra were acquired from 25-500 amu. The total run time was 20 minutes. Identification of the chemical constituents based on the comparison of their retention indices concerning homologous series of n-alkanes and mass spectra. The detected compounds were identified by processing the raw GC-MS data comparing with NIST mass spectral library, Version 2008.

2.8. Antimicrobial Activity

Bacterial inhibitory activity of the extracts is determined to evaluate its antibiotic potential. Inhibition of bacterial growth was tested using agar well disc diffusion method following Dingel et. al. [37].

Antimicrobial activity was screened against bacterial strains, namely methicillin-resistant *staphylococcus aureus* (MRSA), Staphylococcus *aureus* (ATCC 6538p) *Proteus vulgaris* (ATCC6380) *Escherechia coli* (ATCC8739) *Shigella dysenteriae* (Clinical sample) *Pseudomonas aeruginosa* (ATCC 9027) *Saccharomyce scerevisiae* (ATCC18824) *Enterococcus faecalis* (ATCC29212) *Salmonella typhii* (Clinical sample) *Bacillus subtilis* (ATCC6051) and *Klebsiella pueunmoniae* (ATCC 700603). The bacteria strains were grown in a nutrient broth at 37°C, maintained nutrient agar slants at 10°C and standardized to 0.5 Mc Farland (10^6 CFU/ml). The methanolic extract *E. sphaericus* is leaves (100 mg) were dissolved in 1mL dimethyl sulphoxide (DMSO) for this activity. The inoculums containing10^6 CFU/ml bacteria spread on the solid Muller Hinton Agar (MHA) plates with a sterile cotton swabs moistened with bacteria suspension and dried it. The wells were made in the incubated petri plates with the help of sterile cork borer of diameter of 6 mm and were labeled properly and the plant extracts were loaded into the respective wells with the help of micropipette. An antibiotic gentamycin was used as a positive control in the separate well. The petri plates were left for few minutes to diffuse plant extracts in media and incubated overnight at 37°C for 24 hours. The diameter of the resulting zone of inhibition (mm) of growth was measured using measuring scale. All the experiments were carried out in triplicate.

2.9. Minimum Bactericidal Concentration (MBC)

The MBC of methanolic extract of leaves of *E. sphaericus* was evaluated by two fold serial dilution method following standard protocol describe by Baron et. al.[38].

2.10. Brine Shrimp Bioassay

In this work, *Artemia salina* (brine shrimp) is used as the general bioassay tool to perform brine shrimp bioassay as given by Meyer [39]. It determines the LC_{50} values (µg/mL) for the crude extracts. The plant extract or chemical compounds having LC_{50} values less than 1000 ppm (µg/mL) are considered as potentially pharmacologically active. The methanolic extract (20 mg) was weighed out and dissolved in 2 mL methanol to make a stock solution of concentration 1000 µg/mL. From that stock solution, solutions with 1000, 100, and 10 µg/mL concentrations were prepared by serial dilution method. An amount of 2 mL of each solution was transferred to nine different test tubes and 2 mL of methanol was taken in three test tubes (as a blank). After labeling these test tubes, they were kept for 24 hours to evaporate the solvent (methanol). After the complete evaporation of the solvent, 5 mL artificial seawater was added and the solution was mixed thoroughly to suspend the residue. Then ten mature brine shrimp nauplii were transferred into all twelve test tubes. After 24 hours, the number of the total survivors was counted with the help of the disposable pipettes. LC_{50} value is the lethal concentration dose required to kill 50% of organisms used in bioassay. All the experiments were carried out in triplicate. The mortality of *Artemia salina* (brine shrimp) was observed and the LC_{50} value was calculated using Probit analysis.

2.11. Free Radical Scavenging Activity

Free radical scavenging activity of leaves was determined by DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging method followed by Blois [40] with slight modification. The percentage of free radical scavenging activity was calculated using the relation as;

$$\%\ \text{Free Radical Scavenging Activity} = \frac{(\text{Ac}-\text{As})}{\text{Ac}} \times 100$$

Where, Ac = Absorbance of the positive control and As = Absorbance of the sample. IC_{50} value (50% Inhibitory Concentration) is the effective concentration of the sample that is required to scavenge 50% of the DPPH free radical present in the solution. IC_{50} values were calculated from the inhibition curve in the linear range by plotting the extract concentration versus the corresponding % free radical scavenging activity. In brief, 2 mL of different concentrations of plant extract/ standard solution were mixed with 2 mL of 0.2 mM DPPH solution. After mixing, the mixture was incubated for 30 min. in dark at room temperature and the absorbance was measured at 517 nm using a UV spectrophotometer. Ascorbic acid was a positive control as a reference. All the experiments were carried out in triplicate.

2.12. Anti-diabetic Assay

Anti-diabetic activity of plant extracts was determined from α-amylase inhibition assay. It was performed through the modified starch iodine protocol with a slight modification followed by Meyer *et. al.* [39]. Acarbose, a drug used for the treatment of type II diabetes mellitus was used as a standard

reference. The absorbance was measured at 630 nm using an Ultra Violet spectrophotometer. The plant extracts (2 mg) dissolved in 2 mL DMSO (dimethyl sulfoxide) to prepare 1000 µg/mL stock solution. Then extract solutions having 20, 40, 80, 160, 320, and 640µg/mL were prepared by serial dilution from stock solution. In brief, 400 µL of starch solution was pipetted out into a clean and dry test tube and incubated at 37^{O}C for 5 minutes. And 200 µL plant extract/acarbose solution was added in a test tube and again incubated at 37^{O}C for 5 minutes. After 5 minutes, 200 µL α-amylase enzyme solutions was added and again incubated at 37^{O}C for 5 minutes. After 15 minutes, 80 µL HCl solutions was added to quench the reaction and followed by the addition of 1000 µL iodine reagent. The experiments were carried out in triplicate. The % inhibition of α-amylase is calculated by using the expression as;

$$\%\ \alpha - \text{amylase inhibition} = \frac{\text{A1} - \text{A2}}{A0 - A2} X\ 100$$

Where, A1= Absorbance of starch, extract/acarbose, enzyme and iodine, A2= Absorbance of starch, enzyme and iodine) and A0= Absorbance of starch and iodine. From these data, a curve was plotted, and the inhibitory concentration (IC_{50}) value was calculated, which is defined as the concentration of the samples required for a 50% inhibition of enzyme.

2.13. Statistical Analysis

The resulted data were analyzed by using Microsoft Office Excel 2007 for the mean of ($\pm$) standard deviation of three experiments of absorbance for each concentration and from which linear correlation coefficient (R^2) value was calculated. The regression equation is given as,$y = mx + c$.Where, y = Absorbance of the extract, m = Slope from the calibration curve, x = Concentration of the extract and c = Intercept.

3. Result and Discussion

3.1. Phytochemical Screening

The total yield percentage obtained from the methanolic extract of *E. sphearicus*is leaves found to be 17.38%.Phytochemical screening of *E. sphearicus* leaves showed that phytoconstituents like alkaloids, flavonoids, glycosides, saponins, tanins, coumarins, quinones, polyphenols, reducing sugars and terpenoids were presence while volatile oil was absence in methanolic extract of *E. sphaericus* leaves. This result revealed that rich in large numbers of phytochemical constituents justifies the medicinal value of the methanolic extract of *E. sphaericus* leaves. It creates a good scope for further research on it in the field of pharmacological sciences providing a platform for novel drug development for treatment of many chronic diseases and health ailments [19].

3.2. Total Phenolic Content

The total phenolic content of the methanolic extract of *E. sphaericus* leaves was determined by Folin Ciocalteu assay which was conducted using gallic acid as standard that can be quantified at 760 nm using visible light spectrophotometer. The graph of absorption of gallic acid versus concentration was plotted to obtain a calibration curve in Figure 1. The total phenolic content was calculated from the calibration curve using regression equation Y=0.008x+0.193, and R^2=0.993 followed by the formula T=CV/M and expressed as mg gallic acid equivalents (GAE) per gm of extract in dry weight (mg/gm) in Figure 1.The phenolic content of the methanolic extract of *E. sphearicus*is leaves was found to be 295.41 ± 21.57 mg/g(GAE) in dry weight. The phytochemical screening and quantitative estimation of the total phenolic content of the extract studied showed that the leaves were rich in phenolic content and show medicinal activity as well as exhibiting physiological activity.

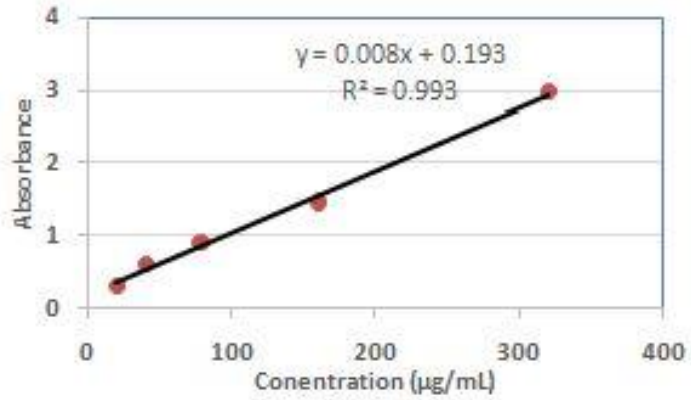

Figure1. *Calibration Plot for Phenolic Determination*

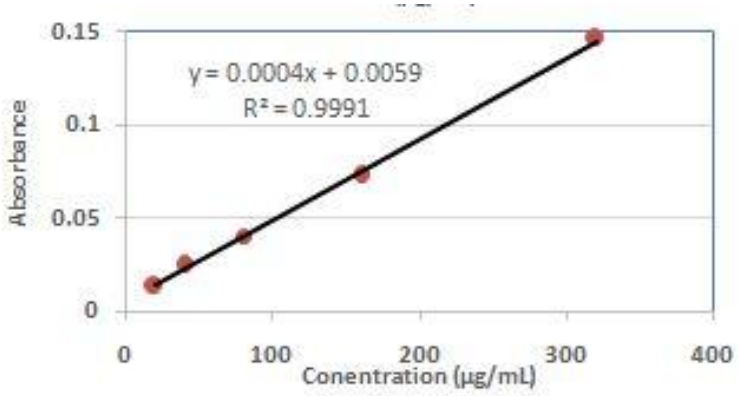

Figure2. *Calibration Plot for Flavonoid Determination*

3.3. Total Flavonoid Content

For the determination of total flavonoid content of the *E. sphaericus* leaves, quercetin was used as standard reference for constructing the calibration curve. The absorption was taken at 510nm by UV-VS spectrophotometer with different concentration 20, 40, 80, 160, 320µg/ml then the graph was plotted absorption versus concentration in Figure 2. The total flavonoid content was calculated from the calibration curve using regression equation Y=0.0004x+0.0059, and R^2=0.993 followed by the formula P=CV/M and expressed as mg gallic acid equivalents (GAE) per gm of extract in dry weight (mg/gm).

The flavonoid content of the methanolic extract of *E. sphaericus* is a leaf was found to be 428.71±56.95mg QE/g in dry weight (mg/gm). The determination of total flavonoid content of the *E. sphaericus* leaves showed that the leaves were rich in flavonoids as secondary metabolites thus, the *E. sphaericus* leaves can be seen as a potential source of useful drugs.

3.4. Column Chromatography Technique

The crude methanolic extract of *E. sphaericus* leaves was subjected to column chromatography using silica gel (mesh60- 120) as absorbent. This extract was fractionated by eluting different solvent such as hexane, 1:1 hexane and ethyl acetate and methanol to get different fractions of leave extracts. Here in this study, two fractions such as1:1 hexane and ethyl acetate and methanolic fractions were used to detect the chemical components present in it using GC/MS technique**.**

3.5. GC-MS Analysis

3.5.1. GC/MS Analysis of Hexane and Ethylacetate (1:1) Fraction of Methanolic Extract

The GC/MS chromatogram of hexane and ethyl acetate (1:1) fraction of methanolic extract of *E. sphaericus* leaves is shown in Figure 3 and Figure 4. In this chromatogram, 17 different peaks were identified, constituting 99.99 % peak area. The chemical constituents identified from GC/MS analysis was tabulated in Table 1. The majority of compounds identified from hexane and ethyl acetate (1:1) fraction of *E. sphaericus* leaves were fatty acids, esters of fatty acids, hydrocarbons and others.

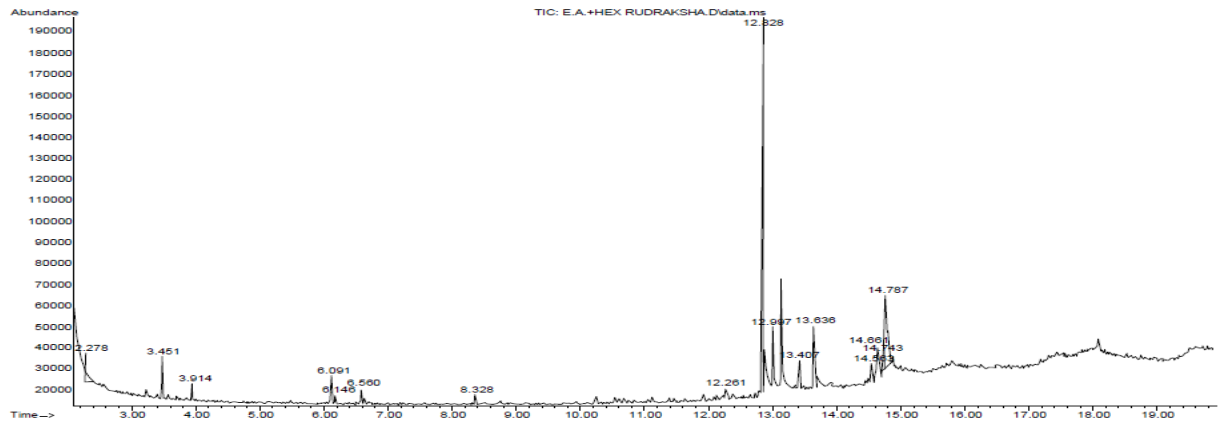

Figure3. *The GC/MS Chromatogram of Hexane and Ethylacetate (1:1) Fraction of E. sphaericus leaves*

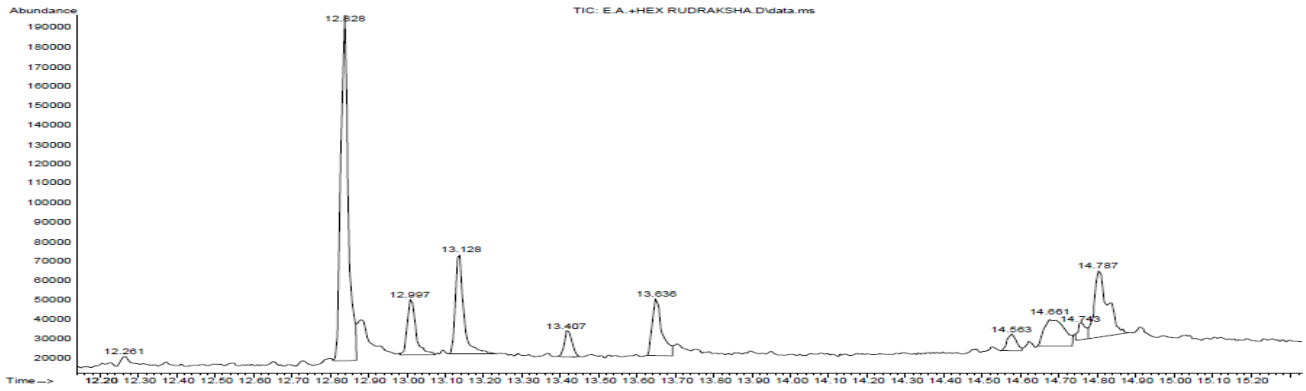

Figure4. *The Expanded GC/MS Chromatogram of Hexane and Ethylacetate (1:1) Fraction of E. sphaericus leaves*

Table1. *Chemical Compounds Identified from Hexane and Ethylacetate (1:1) Fraction of E. sphaericus Leaves*

S.N.	Name of Chemical compounds	Molecular Weight	% Peak Area	Retention Time	Identified Method
1	Trichloro methane	119.38	4.02	2.278	RT and MS
2	2,4-dimethyl- Heptane	128.25	2.91	3.451	RT and MS
3	4-methyl-0ctane	128.26	1.27	3.914	RT and MS
4	4-ethyl-Decane	170.34	2.44	6.091	RT and MS
5	2-Bromo dodecane	249.23	0.91	6.146	RT and MS
6	1-Iodo-2-methyl nonane	268.18	1.31	6.560	RT and MS
7	5-ethyl-2-methyl- Octane	156.31	0.89	8.328	RT and MS
8	Tetradecanoic acid	228.37	1.32	12.261	RT and MS
9	2,6,6-TrimethylBicyclo[3.1.1]heptane	138.25	24.41	12.828	RT and MS
10	3-methyl-Bicyclo[4.1.0]heptane	110.2	6.42	12.997	RT and MS
11	1, 4-Eicosadiene	278.52	10.93	13.128	RT and MS
12	Hexadecanoic acid, methyl ester	270.45	2.57	13.407	RT and MS
13	n-Hexadecanoic acid	256.42	7.37	13.636	RT and MS
14	Cyclobarbital	236.27	2.00	14.563	RT and MS
15	Phytol	296.53	7.35	14.661	RT and MS
16	2-Octyl- Cyclopropaneoctanal	280.48	1.90	14.743	RT and MS
17	9, 12, 15-Octadecatrienoic acid	278.43	11.97	14.787	RT and MS

RT= Retention time, MS =Mass spectrometry

In GC/MS analysis of hexane and ethylacetate (1:1) fraction of ***E. sphaericus*** leave was found to be major constituents as 2,6,6-trimethyl-Bicyclo[3.1.1] heptane (34.41%),9, 12, 15-octadecatrienoic acid (11.97%), 1,4-Eicosadiene(10.93%), n-hexadecanoic acid (7.37%) phytol (7.35%) , 3-methyl-Bicyclo[4.1.0] heptane (6.42%) trichloro methane (4.02%), 2,4-dimethyl- heptane (2.91%), hexadecanoic acid, methyl ester (2.57%), 4-ethyl-decane (2.44%) and cyclobarbital (2.00%), and the

minor major constituents as 2-octyl- cyclopropaneoctanal (1.90%), tetradecanoic acid (1.32%),1-Iodo-2-methyl nonane (1.31%) 4-methyl-0ctane (1.27%), 2-bromo dodecane (0.91%), 5-ethyl-2-methyl-octane (0.89%).

3.5.2. GC/MS Analysis of methanolic Fraction

In GC/MS spectrum, 20 different peaks were identified, constituting 99.99% peak area and tabulated in Table 2. The majority of compounds identified from methanolic fraction of leaves of *E. sphaericus* of were fatty acids, esters of fatty acids, hydrocarbons and others.

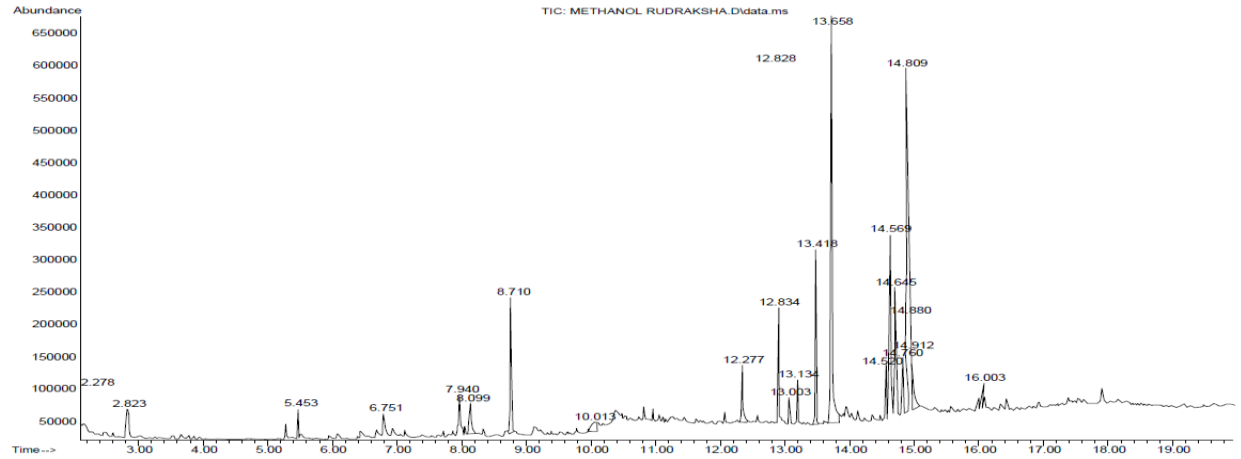

Figure5. *The GC/MS Chromatogram of Methanol Fraction of E. sphaericus Leaves*

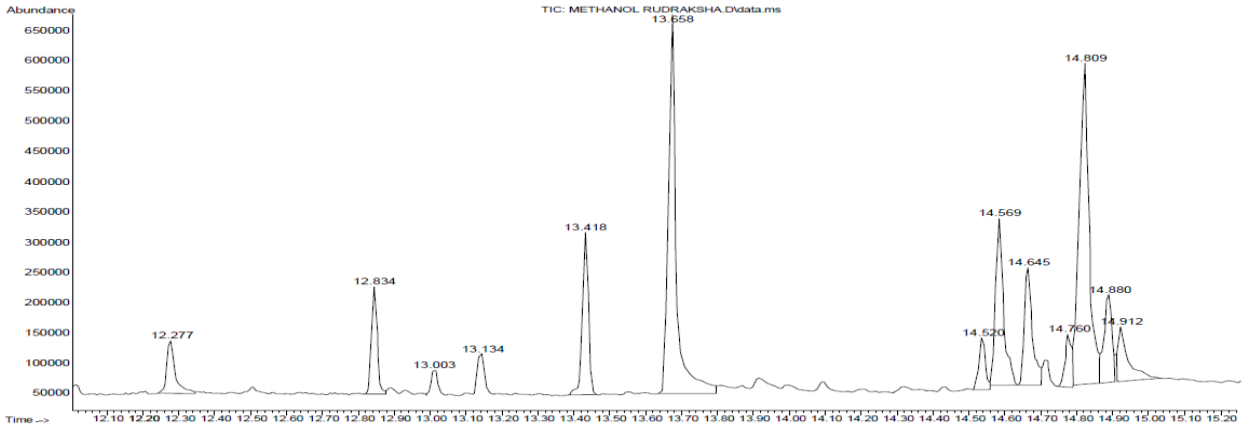

Figure6. *The Expanded GC/MS Chromatogram of Methanol Fraction of E. sphaericus leaves*

Table2. *Compounds Identified from methanol Fraction of Methanolic Extract of E. sphaericus Leaves by GC/MS Analysis*

S.No.	Name of compounds	Molecular Weight	% Peak Area	Retention Time	Identified Method
1	4-chloro-2-nitrotoluene	171.58	2.78	2.823	RT and MS
2	Decane	142.28	1.16	5.453	RT and MS
3	2-Furanmethanol	98.10	1.61	6.751	RT and MS
4	2-Amino-4-methyl but-2-enenitrile	95.16	2.97	7.940	RT and MS
5	2,3-Dimethoxy toluene	152.19	2.09	8.099	RT and MS
6	4-hydroxy-3-methyl acetophenone	150.18	5.27	8.710	RT and MS
7	2R,3S-9-[1,3,4 trihydroxy-2- butoxy methyl] guanine	285.26	1.40	10.013	RT and MS
8	Tetradecanoic acid	229.37	2.67	12.277	RT and MS
9	1-Hexadecyne	222.41	3.92	12.834	RT and MS
10	1-ethynyl-Cyclohexanol	124.18	1.06	13.003	RT and MS
11	3-methyl-Bicyclo[4.1.0]heptane	110.20	1.61	13.134	RT and MS
12	Hexadecanoic acid, methyl ester	270.45	6.43	13.418	RT and MS

13	n-Hexadecanoic acid	256.4241	19.58	13.658	RT and MS
14	[z,z]-9,12-Octadecadienoic acid, methyl ester	294.4721	2.30	14.520	RT and MS
15	[z,z,z]-9,12,15-Octadecatrien-1-ol	264.453	7.90	14.569	RT and MS
16	7,8-dimethyl-Benz[c]acridine	257.28	6.07	14.645	RT and MS
17	[z,z]-9,12-Octadecadienoic acid	264.453	2.63	14.760	RT and MS
18	(z,z,z)-9,12,15-Octadecatrienoic acid	278.4296	18.91	14.809	RT and MS
19	4-[4-fluorophenyl]-6-[trifluoromethyl]-2-pyrimidinamine	351	4.71	14.880	RT and MS
20	Hexadecanedioic acid	286.41	1.39	16.003	RT and MS

RT= Retention time, MS =Mass spectrometry

In GC/MS analysis of methanolic fraction of *E. sphaericus* leaves was found to be major constituents as n-Hexadecanoic acid (19.58%), 9,12,15-octadecadienoic acid (18.91%), 9,12,15-Octadecatrien-1-ol(90%), Hexadecanoicacid,methylester(6.43%),7,8dimethylbenz[c]acridine(6.07%), 4-hydroxy-3-methyl acetophenone (5.27%), 4-[4-fluorophenyl]-6-[trifluoromethyl]-2-pyrimidinamine (4.71%),1-hexadecyne (3.92%), 2-Amino-4-methyl but-2-enenitrile (2.97%), 4-chloro-2-nitrotoluene (2.78%), tetradecanoic acid (2.67%), [z,z]-9,12-octadecadienoic acid (2.63%), [z,z]-9,12-octadecadienoic acid, methyl ester(2.30%),2,3-dimethoxy toluene (2.09%) and the minor constituents as 3-methyl-Bicyclo [4.1.0] heptanes (1.61%), 2-Furanmethanol (1.61%), 2R,3S-9-[1,3,4-trihydroxy-2-butoxy methyl] guanine (1.40%), hexadecanedioic acid (1.39%),decane (1.16%),and1-ethynyl-cyclohexanol (1.06%).

3.6. Antimicrobial Activity

The antimicrobial activity of methanolic extract of *E. sphaericus* leaves was studies against different the gram positive and gram negative bacteria such as methicillin- resistant *staphylococcus aureus* (MRSA), *S. aureus*, *P. vulgaris B. subtilis, K. pneumonia, S. dysentria, E. coli, S. aeruginosa, S. cerevisiae, E. faecalis, S. typhii* by agar well disc diffusion method. The result obtained from antimicrobial activity of methanolic extract of *E. sphaericus* leaves are shown in the Table 3.

Table3. *Antimicrobial Activity of Methanolic Extract of E. sphaericus Leaves*

S.N.	Microorganisms	Zone of inhibition (mm) of Extract	Zone of inhibition (mm) of Gentamycin
1	*Klebsiella pneumonia*	-	10
2	Methicillin- resistant *staphylococcus aureus* (MRSA)	16	14
3	*Shigella dysenteriae*	-	-
4	*Proteus vulgaris*	14	14
5	*Staphylococcus aureus*	15	17
6	*Escherichia coli*	-	14
7	*Pseudomonas aeruginosa*	-	18
8	*Salmonella typhii*	-	17
9	*Bacillus subtilis*	-	15

In order to evaluate the antimicrobial activity of medicinal plant extract, the diameter of zone of inhibition was measured for methanolic extract and reference gentamycin. The methanolic extract revealed effective zone of inhibition(ZOI) towards methicillin-resistant *staphylococcus aureus* (MRSA) (16mm), *S. aureus* (15mm), and *P. vulgaris* (14mm) but no activity showed against *B. subtilis, K. pneumonia, S. dysentria, E. coli, S. aeruginosa and S. typhii.* This indicated that methanolic extract of *E. sphaericus* exhibited the potential antimicrobial activity against both the gram positive and gram negative bacteria.

3.7. Minimum Bactericidal Concentration (MBC)

The MBC of methanolic extract of leaves of *E. sphaericus* was evaluated by two fold serial dilution method following standard protocol. The methanolic extract of *E. sphaericus* leaves revealed the potent MBC value towards *Staphylococcus aureus* 12.5(mg/ml), *Methicillin-resistant staphylococcus aureus* 6.5(mg/ml) and *Proteus vulgaris* 6.5(mg/ml). Thus, the study revealed that the methanolic extract of leaves *E. sphaericucs* leaves is effective against both the gram positive *S. aureus* and

methicillin-resistant *staphylococcus aureus* (*MRSA*) and gram negative *P. vulgaris* bacteria. The methanolic extract of *E. sphaericus* leaves showing large ZOI and small MBC value may contain those compounds which are able to inhibit or kill the microbial population of tested microorganism. The antibacterial activity showed by the *E sphaericus.* leaves extracts could be attributed to the presence of secondary metabolites such as polyphenols, terpenoids, quinones, flavonoids and tannins.

3.8. Brine Shrimp Bioassay

In this study, LC_{50} values (µg/ml) for different concentration (10, 100 and 1000µg/ml) of methanolic extract of *E. sphaericus* leaves were determined in brine shrimp lethality bioassay. The plant extract having less than 1000µg/ml are supposed to be pharmacologically active (toxic in nature). The result of brine shrimp lethality bioassay displayed the toxicity or lethality of methanolic extract of *E. sphaericus* leaves towards *Artemia salina*. The LC_{50} value was calculated as 61.05 (µ g/ml) of methanolic extract of *E. sphaericus* leaves. Thus, the methanolic extract of *E. sphaericus* leaves is highly cytotoxic due to synergic effect of bioactive phytoconstituents which is revealed by GC/MS analysis of hexane and ethylacetate (1:1) and methanolic fraction of *E. sphaericus* leaves as well as secondary metabolites present in this plant extract [41].

3.9. Antioxidant Activity

The antioxidant activity of methanolic extract of *E. sphaericus* leaves was determined by DPPH free radical scavenging method. The scavenging of DPPH free radical determines the free radical scavenging capacity or antioxidant potential of the plant extract which shows its effectiveness, prevention, intersection and repair mechanism against injury in the biological system. In this method percentage of DPPH radical scavenging ability of plant extract and ascorbic acid (standard) were plotted against respective different concentration (10, 30, 50, 70, 90, 110µg/ml) as shown in Figure 7.

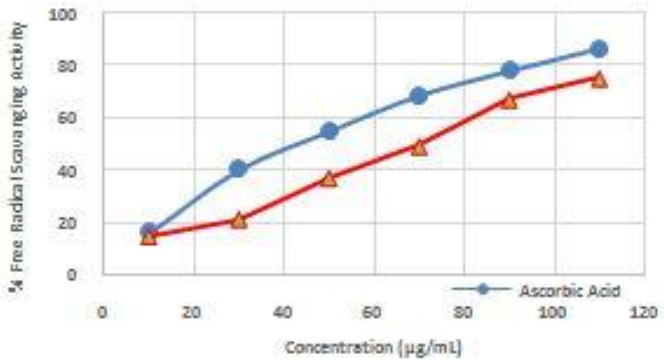

Figure7. *% Inhibition vs Concentration Curve*

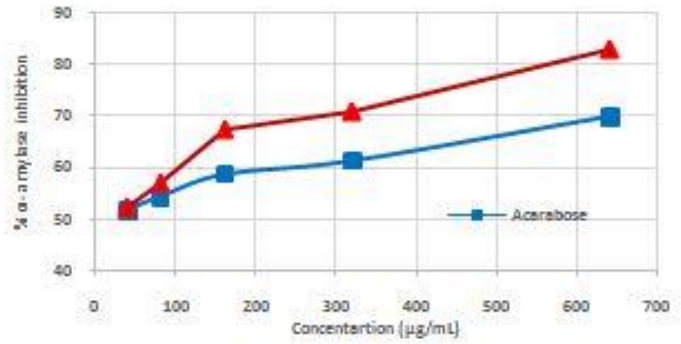

Figure8. *% Inhibition vs Concentration Curve*

The free radical scavenging activity of methanolic extract of *E. sphaericus* was found to be 68.98µg/ml. The standard IC_{50} value of ascorbic acid was found to be 48.93µg/ml. This study revealed that the IC_{50} value of methanolic extract of plant was comparable with standard ascorbic acid. The leaves have superior antioxidant activity may be due to the presence of flavonoids, biflavones and phenols which reveal antioxidant activity [29].

3.10. Anti-diabetic Activity

The anti-diabetic activity of plant extract was evaluated by α amylase inhibitory assay where acarbose is used as a standard. Plant extract decrease the absorption of glucose by inhibiting α-amylase there by indicating the anti-diabetic potential. The % inhibition of plant extract at different concentration (40, 80, 160, 320,640µg/ml) containing starch, α-amylase, iodine and extract/acarbose was calculated. The % inhibition of α- amylase versus concentration curves for acarbose (standard) and plant extract are shown in the Figure 8. The results showed that the methanolic extract of *Elaeocarpus sphaericus* leaves exhibited α-amylase inhibitory activities in *in vitro* assays using starch as substrate. The percentage inhibition of different concentration such as 40, 80,160, 320, 640µg/ml of methanolic extract showed a dose-dependent reduction in percentage inhibition. The IC_{50} value of the methanolic extract of *E. sphaericus* leaves and standard acarbose were found to be 36.90µg/mL and 37.40µg/mL respectively. These values are nearly similar or very close to each others. Thus, according to the experimental results, it was confirmed that the methanolic extract of *E. sphaericus* leaves exhibited strong α-amylase inhibition as compared to acarbose.

4. CONCLUSION

In this present work, we ascertain the presence of the different types of potential bioactive chemical constituents by GC/MS analysis and phytochemical profiling from the methanolic extract of *E. sphaericus* leaves. This extract exhibited strong α-amylase enzyme inhibition with IC_{50} value (36.90µg/mL) less than standard acarbose (37.40µg/mL). Thus it can be utilized as antidiabatic drug with some modification and further research. Plants exhibiting antimicrobial activity can able to help in healing property which is attributed to the secondary metabolites such as alkaloids, flavonoids, steroids, tannins, terpenoids, saponins etc. containing in this plants .The bioactivity analyses revealed that this medicinal plant's leaves is a potential source for antimicrobial, antibiotic, antioxidant, and anti-diabetic medicines due to high content of phenol and flavonoid with presence of different classes of bioactive secondary metabolites. It also revealed the toxic nature towards brine shrimp *naupaulii* due to this evaluation, and this leaves might be good cytotoxic agent with antioxidant or free radical scavenging property in cancer treatment after further more research. Ours results demonstrate that the methanolic extract of *E. sphaericus* leaves might be good resources for further investigation of new potent bioactive chemical compound which caring or healing human related health problem in future.

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