Nigerian Research Journal of Chemical Sciences **(ISSN: 2682-6054)**
Vol. 7, Issue 2, 2019

Phytochemistry, Proximate Analysis and GC – MS Analysis of Essential Oils in Flower of Mistletoe Plant

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ABSTRACT

Essential oils in flower of mistletoe plant were extracted using soxhlet extractor. Qualitative and quantitative analysis of the phytochemicals and nutritive value of the essential oils in mistletoe flower were investigated using standard methods and GC-MS. The qualitative results revealed that tannins, alkaloids, phenol and flavonoids were detected while terpenoid and saponin were not detected in the extract. The quantitative results were tannin 0.84 ± 0.09 mg/100 g, alkaloid 4.47 ± 0.14 mg/100 g, phenol 8.46 ± 0.24 mg/100 mg, and flavonoid 1.71 ± 0.08 mg/100 g. The results showed that the flower have potential to promote good health, reduce diseases risk and can be applied as a source of raw material for the perfume industry.

Key words: Analysis, GC-MS, mistletoe, phytchemicals, proximate

INTRODUCTION

Mistletoe plant is a hemi-parasitic plant that grows on trees such as cocoa, mango, guava, kola nut trees and many more. It is known scientifically for its nutritive content such as carbohydrate, protein, fat, fiber, energy value, and ash. This nutritive content contributes remarkably in animals and human health. Some phytochemical constituents appear to be non-essential to the plant producing them while some serves as defense, colouration and bitter taste, and some of these constituents can be very medicinal. Mistletoe leaves have been known for its use in the treatment of some ailments including hypertension, epilepsy, infertility, arthritis, cancer and diabetes or used as a diuretic agent [1]. Current literature on the flower is scanty or not available to the best of our knowledge. Essential oils are concentrated hydrophobic liquids containing volatile (defined as "the tendency of a substance to vaporize") aroma compounds found in some plants. Mistletoe leaves also contain essential oil which could be a good anti-inflammatory substance

since it contain n-hexadecanoic acid that have been reported to exhibit anti-inflammatory properties [2]. Mistletoe leaf is known to carry out important medicinal roles in human body as a result of the presence of flavonoids in the hexane, chloroform and ethyl acetate extracts from the stem bark. Flavonoids have inherent ability to modify the body's reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobial, antioxidants [3-5]. The aim of this research is to study the phytochemistry, proximate composition, and essential oil content of mistletoe flowers.

MATERIALS AND METHODS

Collection and Preparation

The mistletoe samples used were collected from Cross River University of Technology Staff Quarters in Calabar, Nigeria, from a cocoa tree. The analysis for the phytochemical composition, proximate analysis and oil extraction of the plant were carried out in the Chemistry laboratory of the Chemistry Department, Cross River University of Technology (CRUTECH), Calabar, Cross River State, Nigeria. The plant flower (fresh) obtained was used for oil extraction and moisture content determination while the rest were dried in an electron oven for 3 hours. After drying, the samples were crushed to powdery form using mortar and pestle. The sample in powdered form was stored in bottle until required for analysis

About 15g of the sample was weighed into the soxhlet extractor attached to a round bottom flask containing 250 ml of methanol and a pitch of anti-bumping granules, clamped to a retort stand attached to a heating mantle which was connected to a power source. The essence of adding the anti-bumping granules is to redirect the vapour of the methanol and avoid cracking or breaking of the round bottom flask containing the methanol due to heat. The soxhlet extractor is connected to a condenser with an inlet that allows the flow of water into the system and outlet that allows water to flow out of the system. The inlet and outlet were connected to a water source with rubber tubing. The water flowing into the condenser aids in cooling the system and prevents the escape of the vapour form the system as the methanol is being heated. The vapour dropped back as a result of the cooling effect of the inlet and outlet in the condenser into the round bottom flask through the reflux arm of the soxhlet extractor that was fitted with glass wool.

The extraction was completed when the extract passing through the reflux arm of the soxhlet extractor into the round bottom flask was clear. The methanol was distilled and the plant

extract was left in the round bottom flask. The extracted sample was used for further analyses to determine the phytochemical content

Preliminary Analysis of Phytochemicals

Qualitative analysis of the crude extracts was carried out using standard procedures [6-9]

Test for Alkaloids

1.0 ml of the sample extract was measured into a test tube, 5.0 ml of 2% HCl was added to the test tube on steam bath for 10 mins and filtered with the aid of what man filter paper, 1.0 ml of the filtrates was treated by adding 5 drops of Wegner's reagent and shaken, a reddish brown coloration was observed to be the precipitate for alkaloid.

Test for saponins

Exactly 1.0 ml of the methanolic sample extract was boiled with 5.0 ml of distilled water in test tube for 5 minutes in water bath. It was decanted while still hot. The filtrate was used for the following test:

Frothing test

Exactly 1.0 ml of the filtrate was diluted with 4.0ml of distilled water and shaken vigorously for stable froth on standing. The stable froth was observed for 2 minutes indicating the presence of saponins.

Test for Flavonoids

Exactly 1.0 ml of the methanolic sample extract was measured into a test tube, 1.0 ml of 10% lead acetate was added and shaken for 30 seconds and kept to stand. Formation of yellow precipitate was taken as a positive result for flavonoid.

Test for Tannins

Exactly 1 ml of methanolic sample extract was measured into a test tube and 1ml of 5% bromine water was added and shaken. The formation of greenish to red precipitate was recorded as evidence for the presence of tannin.

Test for Terpenoid

Exactly 5 ml of the methanolic sample extract was measured into a test tube, 2 ml of chloroform was added, and 2 ml of concentrated H_2SO_4 was added carefully by the side of the test tube to

form a layer. No reddish brown colouration at the interface was formed, indicating absence of Terpenoid.

Test for Phenol

Exactly 1 ml of the methanolic sample extract each was measure into a test tube, 1ml of 10% ferric chloride was added and shaken. The formation of a greenish brown colouration was taken as evidence for the phenolics.

Estimation of Phytochemicals

Quantitative determination of the detected secondary metabolites was carried out to know their percentages in the Mistletoe flower following the reported methods [10-13].

Estimation of Saponins

Exactly 5.0 g of dried sample (mistletoe leaf & flower) was measured accurately with an analytical top loading balance into a thimble and was transferred into the soxhlet extractor connected to the condenser and round bottom flask of known weight. 100ml of methanol was used for the extraction for 3 hours to obtain the lipid and the pigment content from the sample first. After the extraction the methanol was distilled off leaving the Saponins after the evaporation the flask and the container was reweighed. The difference between the final and the initial weight of the flask represents the weight of the saponins in the sample.

Calculation for % saponins = $\frac{s-t\ x\ 100\%}{w}$

Estimation of Flavonoid

Exactly1.0 ml of each sample methanolic extracts (mistletoe leaf and flower) was put in separate test tubes. 1 ml each of 5% NaOH was added and shaken for the formation of precipitate. The mixture was decanted to a cuvette for uv-vis spectrophotometer measurement at the wave length of 540nm and result was recorded as absorbance sample.

Calculation for % flavonoid $=\frac{Abs\ sample \times Std\ cone \times 100\%}{Abs\ sample\ standard}$

Estimation of Terpenoid

Exactly 1.0 ml of methanolic extract of sample (mistletoe leaf and flower) was measured into test tube with a stopper, 3.0 ml of acetic anhydride was added gently to the test tube, shaken and cooled in an ice bath for 10 minutes. The colour changed after addition of two drops of

concentrated H_2S0_4 to bluish colouration. 0.1 mg of standard terpenes tablets was weighted and extracted with 5 ml of methanol in separating funnels. 1.0 ml of this standard extract was measured into a test tube and treated as the above sample and the colour was allowed to develop before Uv-Vis spectrophotometer measurement at a wavelength of 520nm and the result was recorded.

Calculation for % Terpenoid $=\frac{Abs\ sample \times std\ con\ c \times 100\%}{Abs\ std}$

Estimation of Tannin

Exactly 1.0 g of the ground mistletoe flower at room temperature was weighed into four different test tubes and dispersed in 10 ml of distilled water and agitated and was left to stand for 30 minutes before centrifuging to obtain supernatant in 5 minutes. The extract obtained was placed into 5 ml volumetric flask with stoppers. The 5th 50 ml flask was fixed with 0.1 of standard tannic acid solution was prepared.

Exactly 1.0 ml of Folin-Denis reagent was measured into each of the five flask containing four samples extract and one Tannin acid extract of the standard followed by 2.5ml of saturated sodium carbonate (Na_2C0_3) solution was diluted to the mark of 50ml flask with distilled water and incubated for 90 minutes at room temperature. The absorbance was measured at 700nm spectrophotometer Jenway model 6405 Uv-Vis spectrophotometer. The reagent blank was measured at zero. The results of the sample and blanks were recorded.

Calculation for % Tannin $= \frac{absobance\ of\ sample \times stabdard\ concentration \times 100\%}{Absobance\ of\ standard}$

Estimation of Phenol

Exactly 0.1 mg of phenol was extracted with methanol in separating funnel. To 1.0 ml of the extract, phenol standard 10% ferric was added and measurement carried out in uv-vis spectrophotometer at the wave length 560nm and result was recorded.

Calculation for % Phenols $= \frac{Absorbance\ sample \times Standard\ Concentration \times 100\%}{Standard}$

Estimation of Alkaloids

1.0 ml of the sample extract was measured into a test tube, 5.0 ml of 2% HCl was added to the test tube on steam bath for 10mins and filtered with the aid of what man filter paper, 1.0 ml of the filtrates was treated by adding 5 drops of Wegner's reagent and shaken, a reddish brown coloration was observed to be the precipitate for alkaloid. The sample was measured with the

Uv-vis spectrophotometer with wave length of 520nm for the percentage content and comparing with standard alkaloid (table) 0.0lg with standard and the result was recorded.

Calculation for % Alkaloid $= \frac{Absorbance\ sample \times standard\ Concentration \times 100\%}{Absorbance\ STancarde}$

Proximate Analysis

The fresh leaves and flower of mistletoe were separated and some were used for moisture content determination while the others were dried and ground into powder and stored in a container for other analysis. The method adopted for the study was the gravimetric method of the Association of Official Analytical Chemist [14].

Determination of moisture content

The moisture content of the whole leaves and flowers of mistletoe were determined by first washing the crucible thoroughly and dried in an oven. Empty weight of the crucible was taken (W_1). 2.0 g of fresh seed of the sample was introduced into the dried crucible and weighed (W_2). The sample was then dried in a ventilated electronically heated oven at 60 % for 24 hours, and then cooled in a desiccator containing magnesium sulphate as drying agent and reweighed.

Moisture (% weight) $= \frac{Loss\ of\ wieight\ on\ drying \times 100\%}{Initial\ samplet\ weight\ (g) \times 1}$

Determination of ash content

To measure the ash content of the sample, two empty crucibles were and 2.0 g of the sample (mistletoe leaves and flowers) was accurately weighed into the crucible and put in a muffle furnace and ashed at 600ºc for 3 hours. At the end of the ashing period, the ashed samples were removed into a desiccator for cooling to room temperature and reweighed.

Calculation for Ash content = (% weight) $\frac{= \text{Weight of fresh sample} \times 100}{Intial\ wt\ of\ sample \times 1}$

Determination of fiber content

The measurement for the fibre content of the sample (mistletoe leaves and flower) was based on two digestions:

Acid digestion: The fat free material stored in the thimble was weighed and transferred into a 400 ml beaker. 50 ml of 1.25% H_2S0_4 was added and the mixture was made up to 200 ml with distilled water. The content of the beaker was filtered through a Buchner funnel with the use of suction pump. The residue was washed with warm distilled water until it was acid free.

Base Digestion: The residue left after acid digestion was transferred into a 400 ml beaker, 50 ml of 1.25% NaOH was added and made up to 200 ml mark with distilled water. The mixture was again heated for 30 minutes with constant stirring. The content of the beaker was filtered using Buchner funnel and washed several times with hot water until it was free from sodium hydroxide. Finally, the residue was washed twice with 95% methanol. This was transferred into a crucible and dried at 100^oC. The weight of oven- dried sample was noted and the residue was ignited in a furnace at 550ºC. The weight of ash left after ignition was also noted. This was done for both the leaves and flower (A & B) and calculation was done using the formula below:

$$\frac{C - A \times 100\%}{A} or \frac{Weight\ of\ sample\ (ash)}{Initial\ wight\ of\ the\ sample} \times \frac{100}{1}$$

Determination of Crude Protein Content

About 5.0 g of the ground sample (mistletoe leaves and flower) was weighed into 250 ml standard kjeldahl flask containing 1 table standard kjeldahl catalyst; some antibumbing chip and 30ml of concentrated $H_2S0_4$was introduced into the flask. The flask was locked into digestion rock and heated gently for 1 hour to prevent vigorous choking and frothing. The flask was then subjected to vigorously heating for 8 hours until a clear bluish colour was obtained. After the digestion, the flask was cooled in tap water and quantitatively transferred into 100 ml standard volumetric flask and made up to mark with distilled water. 10 ml portion of the digested sample was measured into a semi microkjeldahl Mackham distillation apparatus and treated with 30 ml of 40% Na0H into 100 ml beaker containing 10 ml of 2% boric acid plus 2 drops of double indicator (0.1% methyl reagent and 0.1% methyl blue in 100ml of ethanol). The tip of the condensed receiver was immersed in the boric acid, double indicator and the distillation continued until about 3 times the original volume was obtained. The distillate was then titrated with 0.1M HCl solution until a purple-pink end point was reached. The percentage nitrogen content in the sample was obtained with appropriate calculation.

Calculation for % Nitrogen (protein)

$$= \frac{\%\ Nitrogen = {}^{N}/_{10} \times Hcl\ (ml) \times DF \times NF \times 100\%}{Weight\ of\ sample\ in\ milligrame}$$

The protein was determined by multiplying the percentage of nitrogen by the factor 6.25.

Determination of fat content

About 5.0g of sample (leaf & flower of mistletoe) was weighed into a thimble and placed in a soxhlet extractor, and 130 ml of petroleum ether (boiling point 40-60 $^{\circ}$C) was poured into a previously dried weighed round bottom flask. The soxhlet apparatus was set up with condenser connected to the tap inlet and outlet, few chips of antibumbing was placed in the round bottom flask containing ether. The flask was heated on the hot plate for 10 hours, as the oil was extracted the thimble, was then removed and oil dried fat free extract was then used for fibre determination. The petroleum ether in the flask was distilled off and collected and stored for subsequent use. The flask and its content were again dried and weighed accurately. The amount of lipid extracted was obtained as the difference between the weight of the flask before and after.

Fat (%weight) $= \frac{Weight\ of\ fat}{Weight\ of\ sample} \times \frac{1000}{1}$

Determination of total carbohydrate content

The calculation was conveniently done by the differential method total CH=$100 - (\%\ Moisture + \%\ Ash + \%\ Fat + \%\ Protein + \%\ Fiber))$

Determination of total energy value

Energy content: the kilocalories (kcal/100g) value estimation was done by summing the multiplied values for crude protein, crude lipid (excluding crude fibre) and carbohydrate respectively at water factor (4, 9 and 4 kcal) as thus:

Energy value (kcal/100g) = crude protein x 4) + (crude fat x 9) + (total carbohydrate x 4).

Oil Extraction Procedures

Fresh flower of the sample (mistletoe) was fed into a round bottom flask and placed in a soxhlet extraction and 100 ml of distilled water was poured into it. The soxhlet apparatus was setup with condenser connected to the tap inlet and outlet, few chips of antibumbing was placed in the round bottom flask containing the sample with distilled water. The flask was heated on the hot plate for 10 hours. The flask was removed when the clear extract was observed and the extract was turned into a separating funnel and few drops of n-hexane was added and shaken for few minutes and kept for some time. Since the oil is lighter than the water, the oil was observed as the upper layer while the water was the bottom layer. The tap of the separating funnel was opened and the water layer was separated from the oil.

GC-MS Analysis

Using Agilent Technology 7890 GC system coupled with a detector Agilent MS 5975 device. 1 ml of flower was subjected to GC-MS investigation. With a capillary column (HP5) the gas chromatography was prepared, 30m, long, 0.32μm film width and 0.25 μm inner diameter. In a 3-step gradient programmer over temperature was set, initial temperature was set at 40 ^{o}C, till 340 ^{o}C followed by 2°c/minute increase. Flow rate for helium gas was 1 ml per minute. In scanning mode ions or fragments monitored for mass/charge. With the help of mass spectra the identification of components was done by using the mass spectra library.

RESULTS AND DISCUSSION

The results of the qualitative analysis of mistletoe flower are presented in Table 1. Mistletoe flower was found to contain moderate concentrations of tannin, flavonoid and saponin. Alkaloid had low concentration while phenol has higher concentration. Terpenoid and saponins are absent in flowers. The presence of phenol and tannin in the flower qualifies it as an anti-microbial agent. Phenolic and tannin are antioxidant compounds, reducing oxidative stress from cellular metabolism, which decreases the incidence of cardiovascular diseases, certain types of cancer, arthritis and inflammation [15].

Table 1: Result of qualitative analysis phytochemicals of mistotle plant flower.

Phytochemicals	Flowers
Tannins	+
Alkaloids	++++
Phenols	+++
Flavonoids	+
Terpenoid	-
Saponins	-

The quantitative analysis result of mistletoe flower is reported in Table 2. It shows that mistletoe flower contains alkaloids (4.47± 0.14mg/100g), phenol (3.46± 0.24 mg/100g) flavonoid (4.47 ±0.08mg/100g), tannin (0.84 ±0.09). Saponins and Terpenoid were not detected. Hence, the antimicrobial activity of mistletoe flower is due to the abundance of phenol, flavonoids, tannin and saponins. Tannin hastens healing of wounds and inflamed mucus membrane. Also saponins from studies although nontoxic, can generate adverse physiological responses response in animal that consume them.

Table 2: Result of quantitative analysis phytochemicals of mistotle plant of flower

Phytochemicals	Flowers mg/100g
Tannins	0.84 ± 0.09
Alkaloids	4.47 ± 0.14
Phenols	3.46 ± 0.24
Flavonoids	1.71 ± 0.08
Terpenoid	0.00 ± 0.00
Saponins	0.00 ± 0.00

Note: Result is mean of triplicate of samples

The results of the proximate compositions of mistletoe flower as presented in Table 3 indicate that moisture, crude fibre, fat, protein, ash, carbohydrate and energy content are 32.70 % 8.26%, 5.00%, 0.37%, 10.19%, 43 .35% and 219.7 kcal /100g for flower respectively. The fat content was 5.00% for flower. Crude fibre content in the flower is 8.26%. Crude fiber helps in lowering the level of serum cholesterol, lower breast cancer risks as well as risks of coronary heart diseases. The high energy value with good amount of carbohydrate contents constituting 219.7 kcal for flower and a low amount of protein 0.37% for flower show that the value is lower than that reported [16].

Table 3: Result of proximate analysis of mistotle plant of flowers

Parameters	Flowers %
Moisture contain	32.70 ± 0.24
Fiber contain	8.26 ±0.14
Fat contain	5.00 ± 0.05
Protein contain	0.37 ± 0.17
Ash content	10.19 ± 0.15
Carbohydrate	43.35
Energy value	219.7 Kcal/100g

Note: Result is mean of triplicate of sample

The percentage yield and retention time (minutes) of essential oil of mistletoe flower are presented in Table 4. The GC-MS analysis of mistletoe flower revealed that 10 compounds were detected from the oil in the flower. The trend in decreasing order of the percentage content of the compounds detected in flower extract is Eicosyl vinyl ester carbonic acid (19.94%), Heptadecyle isobutyl ester carbonic acid (17.81%), Dimethyl- sitanediol (16.46%), Heptadecyle isobutyl ester carbonic acid (16.02%),9- methyl- Nonadecane (15.58%), 1-bromoeicossanem

(5.11%), Trimethisily fluoride(4.07%),3, 5, 24- trimethyl- tetracontane (1.87%), Crotyl methacrylate (1.74%) and Eicosyl prop- len-zylester carbolic acid (1.44%).

Table 4: GC – MS Analysis of Essential Oil Flower of Mistletoe Plant

S/N	Name and molecular formula	Percent composition	Retention time (min)
1	3, 5, 24- trimethyl- tetracontane. $C_{48}H_{88}$	1.87	3.59
2	Dimethyl- sitanediol $C_2H_8O_2S_i$	16.46	3.62
3	Trimethisily fluoride $C_3H_aFS_i$	4.07	26.73
4	Eicosyl prop- len-zylester carbolic acid $C_{24}H_{46}O_2$	1.44	26.80
5	Crotyl methacrylate $C_8H_{12}O_2$	1.74	27.43
6	Heptadecyle isobutyl ester carbonic acid	16.02	28.24
7	Heptadecyle isobutyl ester carbonic acid 00	17.81	29.08
8	1- bromoei cossane	5.11	29.40
9	Eicosyl vinyl ester carbonic acid	19.94	29.73
10	9- methyl- Nonadecane	15.58	30.29

CONCLUSIONS

The result revealed the presence of tannin, flavonoid and saponin, alkaloid phenol while terpenoid and saponins were not detected in the flowers. The results of the proximate compositions of mistletoe flower showed that the moisture, crude fibre, fat, protein, ash, carbohydrate and energy contents were 32.70 % 8.26%, 5.00%, 0.37%, 10.19%, 43 .35% and 219.7 kcal /100g for flower respectively. The GC-MS analysis of mistletoe flower revealed that 10 compounds were detected from the oil in the flower.

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