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Edewor TI
Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Akintola AO
Department of Science Laboratory Technology, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Ogundola AF
Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Ibikunle GJ
Department of Science Laboratory Technology, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Adepoju AJ
Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Mmuo AJ
Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Owa SO
Department of Biological Sciences, Landmark University, Omuaran, Kwara, Nigeria

Corresponding Author:
Akintola AO
Department of Science Laboratory Technology, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Ogundola AF
Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo, Nigeria

Phytochemical constituents, total flavonoid and phenolic contents and antioxidant activity of leaves of *Syzygium guineense*

Edewor TI, Akintola AO, Ogundola AF, Ibikunle GJ, Adepoju AJ, Mmuo AJ and Owa SO

Abstract
We evaluated the phytochemical constituents and the antioxidant activity of methanolic leaf extract of Syzygium *guineense*. The class of phytochemicals was determined using Harborne's method. The phytochemicals present in the extract were identified using GC-MS. Twenty-seven compounds were separated and twenty were identified. The most abundant compound was carbamic acid phenyl ester with a percentage composition of 13.67%. Spectrophotometric assay was used to determine the total flavonoid and phenolic contents. The assay indicates that the methanolic leaf extract is rich in polyphenols and flavonoids. At the highest concentration of the extract (500ug/ml), the total phenolic and total flavonoid contents of the extract were 274.92mg gallic acid equivalent/g and 8.42 mg quercetin equivalent/g respectively compared to gallic acid and quercetin standard with total phenolic and total flavonoids contents of 385.12 and 12.46 respectively at the same concentration. The antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing power assays. DPPH radical scavenging activity and ferric reducing power of the extract increased in a dose dependent manner and these activities were compared with ascorbic acid, a standard antioxidant. The results indicate that the leaf extract possess significant antioxidant activity as evident in their DPPH radical scavenging activity and ferric reducing power and could serve as source of natural antioxidant.

Keywords: Flavonoids, phenolics, antioxidant, leaves, Syzygium *guineense*, GC-MS

1. Introduction
Humans are constantly exposed to exogenous substances such as environmental pollutants, radiations, chemicals, toxins, deep fried and spicy foods as well as physical stress and endogenous metabolic processes. These processes bring about the generation of reactive oxygen species such as superoxide, hydroxyl, peroxide radicals and hydrogen peroxide which are responsible for cellular damage as a result of oxidative stress. Researchers have shown that these reactive oxygen species are responsible for most deadly diseases which include cancer, cardiovascular, neurodegenerative disorders, atherosclerosis, inflammation, etc. They cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins, initiate lipid peroxidation by abstracting allylic proton from polysaturated fatty acids. [1, 3]. Modern medicine has done a lot in trying to combat these diseases but most of the drugs have side effects that are also detrimental to the patient and expensive to purchase. So, there is the need to find drugs which are more efficient, cheap and with little or no side effects. Researchers have turned to medicinal plants and are investigating them for potent drugs.
Syzygium guineense is a medicinal plant and a member of Myrtaceae family. It is an ever-green tree that grows to a height of about 35 m. The leaves are narrow at both ends. The young leaves are between 5-10 cm in length and 1-4 cm in width while the older leaves are about 10-18 cm in length and 5-8 cm in width. They are simple, opposite, elliptical and lanceolate. The margins are untoothed. *Syzygium guineense* is widely distributed in savannah and tropical forest zones in Africa. The leaves are used as fodder for livestock. A decoction of twigs and leaves is drunk for treatment of diarrhea, colic and abdominal pain. They are also used as enema. The leaves are applied on wounds and boils, drunk against intestinal parasite and used as an eye drop. This shows that the leaves possess anti-inflammatory, antioxidant [4, 5], antimicrobial [6, 8], analgesic [9] and antidiabetic [10] activities. It also possesses immunological activity [11]. The aim of this research is to determine the antioxidant potential, and quantify the flavonoid and polyphenolic contents of the plant leaf.

2. Materials and Methods

2.1 Chemicals and Equipments

The following chemicals used sodium carbonate, sodium hydroxide, aluminium chloride, sodium nitrite, Folin-Ciocalteau reagent, potassium acetate were obtained from E. Merck (Darmstadt, Germany) while quercetin, gallic acid, ascorbic acid and 2, 2- diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Absorbance measurements were recorded on a Genseys 10S VI. 200 217H311008 spectrophotometer.

2.2 Sample collection and preparation

The plant leaves were collected from a traditional health practitioner in Omuaran, Kwara State, Nigeria. The plant was identified by Prof Ogunkunle A. T at the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria based on its local name which was located in the book ''Vernacular of Nigerian plants'' by Gbile and Soladoye, (2002) [12] and the plant specimen with a voucher no LHO 566 was deposited in the University Herbarium.

The leaves were dried for two weeks, then pulverized into fine powder and stored in clean plastic containers.

2.3 Extraction

500g of the pulverized leaves was extracted by using a soxhlet extractor. The extract was concentrated by distilling off the solvent and evaporating to dryness using a rotary evaporator. The solvents for ethanol

2.4 Phytochemical analysis

2.4.1 Preliminary Phytochemical screening

The extracts were screened for the presence of terpenoids, flavonoids, steroids, saponins, cardiac glycosides, anthraquinones and tannins using the method described by Harborne, 2005 [13].

2.4.2 GC-MS analysis

A gas chromatograph (7890A) from Agilent USA hyphenated to a mass spectrophotometer (5975C) with a triple axis detector equipped with an auto injector (10µl syringe) was used.

This was treated with phenylmethyl siloxane. The specifications for the capillary column used for the chromatographic separations and other GC-MS conditions of operation are given in Table 1. MS solution software provided by the supplier was used to control the operating system and to acquire data.

Table 1: GC-MS Conditions of Operation

Operating conditions	Specifications
Column length	30 m
Column internal diameter	0.2 m
Column thickness	250µm
Ion source temperature (EI)	250ºC
Interface temperature	300ºC
Pressure	16.2 psia
Out time	1.8 min
1 µm injector	Split mode
Split ratio	1:50
Injection temperature	300ºC
Column temperature started at	35ºC
Rate of increase of temperature	20ºC/min
Temperature held for	5 min
Total elution time	47.5 min

2.5 Determination of total phenolic content

Total phenolics content of the extract was estimated using the Folin Ciolcateau assay [13]. About 1mg of the extract was dissolved in 1mL of distilled water and 1 mL of Folin-Ciocalteau reagent was introduced into it and the mixture left standing for 5 mins. Later, 10 mL of sodium carbonate and 13 mL of distilled water were added to the mixture and stirred thoroughly for 5 mins, then left to stand in the dark for 90 mins at room temperature. The absorbance was measured at 765 nm using a UV/visible spectrophotometer. Gallic acid was used as the standard. Different concentrations of gallic acid were prepared and the absorbance measured at 765 nm. This was used to obtain the calibration curve for gallic acid. The total phenolic content obtained was expressed in mg gallic acid equivalent/ g extract.

2.6 Determination of total flavonoid content

Total flavonoids content of the plant extract was determined by the aluminium chloride colorimetric method of Chang *et al.*, (2002) [14]. About 3.5 mL of 30% methanol, 0.15 mL of $NaNO_2$ (0.5M) and 0.15 mL of $AlCl_3.6H_2O$ (0.3M) were added to 0.3 mL of the extract in a 10 mL test tube and mixed properly for 5 mins using a vortex mixer. Later, 1 mL of NaOH (1M) was added to the mixture and mixed thoroughly. Then the absorbance of the mixture was measured at 500 nm against a reagent blank. Quercetin was used as the standard. Different concentrations of quercetin were prepared and their absorbance obtained was used to plot the calibration curve. The total flavonoid content is expressed as mg quercetin equivalent/g extract.

2.7 Determination of antioxidant activity

DPPH scavenging potential of the extract was determined using the method of Mensor *et al.*, (2001) [15]. The sample was dissolved in methanol to give a sample concentration of 1mg/mL. 0.5 ml of DPPH-methanol solution was added to 0.1mL of the sample and incubated for 1 hr at room temperature. The absorbance was measured at 517 nm. The percentage inhibition of DPPH was obtained from the equation

$$\% \text{ inhibition} = \frac{A_c - A_s}{A_c}$$

Where

A_c - Absorbance of control

A_s - Absorbance of sample

2.8 Determination of ferric reducing ability

Different concentrations of the methanol extract (100 – 500 µg/ml) were prepared and separately mixed with 2.5 mL of potassium ferricyanide and 2.5 ml of 200 mmol/l sodium phosphate buffer solution (pH = 6.6). This mixture was incubated for 20 min at 50 ºC. Later, 2.5 mL of 10% trichloroacetic acid was introduced into the mixture and the mixture centrifuged for 10 mins at 10000 rpm. Then 1 mL of 0.1% ferric chloride and 5 mL of distilled water were added to 5 mL of the upper layer of the centrifuged mixture and mixed properly, and then the absorbance measured at 700 nm. Ascorbic acid was used as the standard and the assay was done in triplicate.

3. Results and Discussion

Phytochemical screening of plant extracts for presence of medicinal phytocomponents is very important. This could

lead to the identification of compounds that may be vital to the maintenance of human health, and prevention and treatment of sicknesses and diseases. Phytochemical screening of the crude extracts of *Syzygium guineense* leaves revealed presence of flavonoids, alkaloids and tannins while steroids, saponins, cardiac glycosides and anthraquinones were absent as presented in Table 2. Tadesse and Wubneh, 2017 [16] reported presence of anthraquinones and cardiac glycosides while Ezenyi *et al*., 2016 [17] reported their absence and this is in agreement with the findings from this study. Ior *et al*., 2012 [18] reported absence of alkaloids in their sample while it is present in ours, that of Ezenyi *et al*., 2016 and Tadesse and Wubneh, 2017. This observed variation in the phytochemical constituents of the leaf extract could be as a result of soil, climatic and environmental factors. The report of these researchers are in agreement with the findings from this study concerning the presence of flavonoids, tannins and absence of steroids. Phytochemical screening of the extract using GC-MS revealed presence of nitrogenous, carbonyl, polyphenolic, heterocyclic compounds, esters and fatty acid esters as shown in Table 4. The total ion chromatogram of the methanolic extract is shown in Fig.1 A total of twenty-seven compounds were separated and twenty identified. The compounds were identified by comparing the mass spectra of the separated compounds with standard mass spectra of compounds from National Institute of Standards and Technology (NIST, 2011) library incorporated into the equipment. Carbamic acid phenyl ester was identified as the most abundant compound with a percentage composition of 13.67%. Others with appreciable quantity are Tetrahydro-6-octyl-2H-pyran-2-one (4.01%) and 2, 3-dihydrobenzofuran (4.00%). An unidentified compound had a percentage of 5.18% with retention time of 34.72 min. Some flavonoids were identified but in small proportion. Esters have been shown to be important organic compounds that have quite a number of commercial applications [19]. These compounds are largely used in fragrances, cosmetics, flavoring and pharmaceutical industries. They could also be used as plasticizers, lubricants, biological additives and hydraulic fluids [20].

Table 2: Phytochemical Screening of Leaf Extract of *Syzygium guineense*

Secondary metabolite	Methanol extract
Flavonoids	++
Steroids	-
Alkaloids	+
Tannins	+
Saponins	-
Cardiac glycosides	-
Anthraquinone	-

+ = moderately present, ++ = highly present,- =absent

Table 3: Phytochemicals identified in methanolic leaf extract of *Syzygium guineense*

Peak/N	Retention time (min)	% composition	Name of compound	Molecular formula	Molecular weight
1	13.23	13.67	Carbamic acid, phenyl ester	$C_7H_7NO_2$	137
2	13.47	1.94	Ethanone, 1-(3-methylenecyclopentyl)-	$C_8H_{12}O$	124
3	16.59	0.69	Norbomane, 1-methyl-2-hydroxy-	$C_8H_{14}O$	126
4	16.92	2.26	Phenol, 2-methoxyl-	$C_7H_8O_2$	124
5	21.53	1.06	Catechol	$C_6H_6O_2$	110
6	21.94	4.00	Benzofuran, 2,3-dihydro	C_8H_8O	120
7	23.92	0.81	2h-pyran-2-one, 5,6-dihydro-4-(2-methyl-2propen-3-yl)-	$C_9H_{12}O_2$	152
8	24.99	2.03	2-mehoxy-4-vinylphenol	$C_9H_{10}O_2$	150
9	26.30	0.57	2,6-dimethoxyphenol	$C_8H_{10}O_3$	154
10	34.72	5.18	unidentified	-	-
11	35.09	4.01	Tetrahydro-6-octyl-2H-pyran-2-one	$C_{13}H_{24}O_2$	212
12	36.83	2.03	2,6-dimethoxy-4-(2-propenyl) phenol	$C_{11}H_{14}O_3$	194
13	37.49	2.22	Unidentified	-	-
14	38.57	0.80	Unidentified	-	-
15	38.66	1.34	Unidentified	-	-
16	38.72	1.44	6,10,14-trimethyl-2-pentadecanone	$C_{18}H_{36}O$	268
17	38.87	1.42	Unidentified	-	-
18	38.94	0.61	2,4,7,14-tetramethyl-4-vinyl-tricyclo[5.4.3.0 (1,8)]tetradecan-6-ol	$C_{20}H_{34}O$	290
19	38.99	1.91	9-heptadecanone	$C_{17}H_{34}O$	254
20	39.05	0.81	Unidentified	-	-
21	40.91	1.74	Octadecanoic acid, 2-hydro-1,3-propanediyl ester	$C_{39}H_{40}O_4$	572
22	41.06	0.55	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284
23	41.48	2.19	(2(R), 3(S)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2-(H)-chromene-3,5,7-triol	$C_{15}H_{14}O_7$	306
24	41.63	0.57	3,5,7-trihydroxyphenyl)4H-chromene-4-one	$C_{15}H_{10}O_8$	318
25	42.11	0.63	3,5,7-trihydroxy-2-[4-hydroxy-3-[(2(S), 5(S))-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]chromen-4-one	$C_{21}H_{20}O_{12}$	464
26	43.33	1.44	unidentified	-	-
27	43.74	2.17	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390

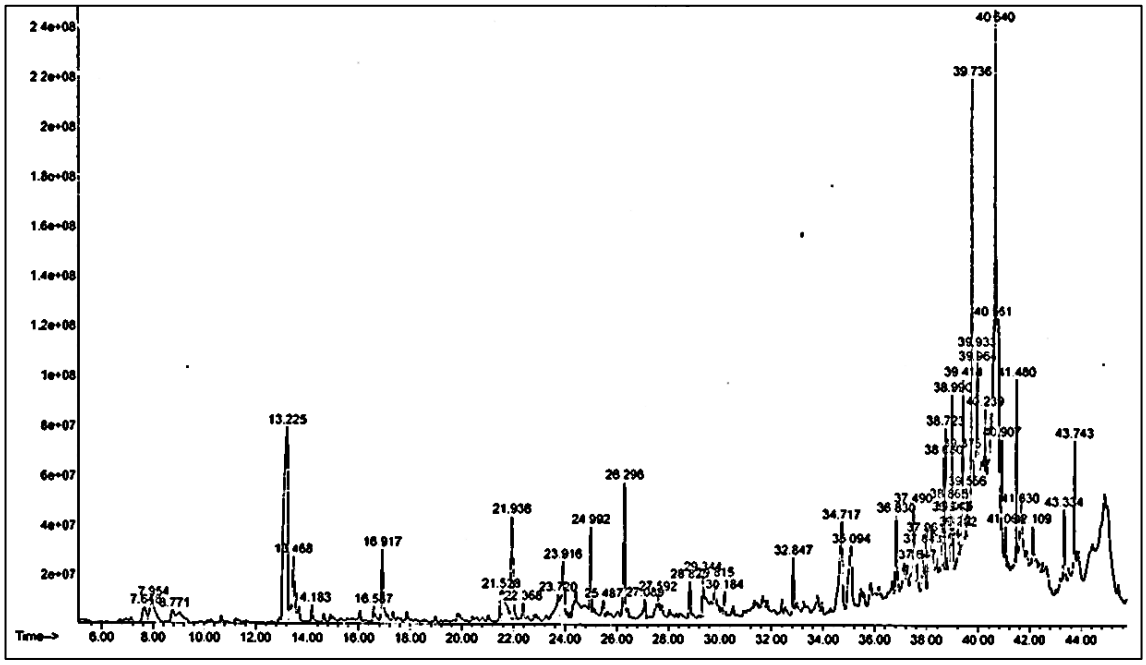

Fig 1: Total ion chromatogram of the methanolic leaf extract of *Syzygium guineense*

Free radicals present in human body are involved in the manifestations of several disorders and diseases while antioxidants inhibit free radicals or protect the body's antioxidant defence mechanism. Natural antioxidants have been shown to be effective in the prevention of destructive processes caused by oxidative stress therefore, there is need to develop antioxidants which are capable of combating these reactive oxygen species and oxidants resulting in the prevention and treatment of diseases and maintenance of human health. The result of the antioxidant activity of the plant extract is presented in Table 4. The extract was able to scavenge DPPH in a concentration dependent manner with scavenging activity of 82.16% at 500ug/ml and this was comparable with ascorbic acid, a standard antioxidant with scavenging activity of 90.85% at the same concentration. DPPH is a free radical, stable at room temperature which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule giving rise to colourless methanol solution. One of the mechanisms involved in antioxidant activity is the ability of a molecule to donate a hydrogen atom to a radical therefore, DPPH radical scavenging activity of the plant extract may be related to its hydrogen donating ability hence DPPH can be used to access the electron donating ability of natural products. Table 5 shows the reductive capability of the plant extract compared with ascorbic acid, a standard antioxidant. The reducing power of the extract increased with increased quantity of the extract with a high absorbance indicating a high reducing ability. In the assay as the concentration of the extract is increasing (100 - 500ug/ml), the absorbance is also increasing (0.36 – 0.84nm), the absorbance values of the extract at different concentrations were found to be lowered than that of ascorbic acid. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. It is associated with the presence of reductants such as antioxidant substance which cause reduction of Fe^{3+}/ferricyanide complex to the ferrous form.

Table 4: DPPH Radical Scavenging Activity of Methanolic Extract of *Syzygium guineense*

Concentration (ug/ml)	Methanolic extract (%)	Ascorbic acid (%)
100	29.09+ 0.25	42.18 + 0.46
200	42.23+ 0.61	70.06+ 0.31
300	61.23+0.38	79.98+ 0.53
400	74.46+0.52	83.16+ 0.32
500	82.16+ 0.47	90.85+0.11

Table 5: Ferric Reducing Ability of Methanolic Extract of *Syzygium guineense*

Concentration (ug/ml)	Methanolic extract (nm)	Ascorbic acid (nm)
100	0.36+ 0.02	0.47.+ 0.06
200	0.42+ 0.04	0.65+ 0.05
300	0.54+0.08	0.86+ 0.04
400	0.66+0.02	1.26+ 0.07
500	0.84+ 0.06	1.94+0.03

Table 6 depicts the result of total phenolic content of the extract. The total phenolic content increased in concentration dependent manner. At the highest concentration of 500ug/ml the total phenolic content of the extract was 274.92 mg Gallic acid equivalent/g compared to gallic acid with total phenolic content of 385.12 at the same concentration. Phenols are a class of organic compounds which are present in most plants. Chemically, they consist of a hydroxyl group (OH) bonded directly to an aromatic hydrocarbon ring. Phenolics retards oxidative degradation of lipid due to the presence of the hydroxyl group. They are regarded as the most important antioxidative components of plants hence total phenolic content can be used as a basis for rapid screening of antioxidant activity of plants [21, 22]. The value indicates that the methanolic extract is rich in polyphenols. Food industries are showing greater interest in plants that possess polyphenols

since they can improve the quality and nutritional value of foods. Table 7 presents the total flavonoid content of the plant extract. The total flavonoids content of the plant increase as the concentration of the plant is increased, at 500ug/m**l** the total flavonoid content of the extract was 8.42mg quercetin equivalent/g compared with quercetin with total flavonoid content of 12.46 at the same concentration. Flavonoids are considered to be important phytochemicals due to their free radical scavenging ability. They suppress reactive oxygen formation, scavenge reactive oxygen species, chelate trace elements involved in free radical production, upregulate and protect antioxidant defenses [23, 25]. Flavonoids and other polyphenolics possess antioxidant activity due to their redox properties and chemical structures especially presence of free hydroxyl groups at position 3. The molecular antioxidant response of polyphenols varies due to differences in their chemical structure. This shows that different phenolic compounds respond differently to Folin-Ciocalteau reagent.

Table 6: Phenolic Content of Methanolic Extract of *Syzygium guineense*

Concentration (ug/ml)	Methanolic extract (Gallic acid equivalent mg/g)	Gallic acid
100	194.29+ 0.28	156.28 + 0.78
200	132.56+ 0.36	195.42+ 0.81
300	186.34+0.48	234.06+ 0.94
400	206.52+0.66	342.14+ 0.66
500	274.92+ 0.74	385.12+0.74

Table 7: Total Flavonoid Content of Methanolic Extract of *Syzygium guineense*

Concentration (ug/ml)	Methanolic extract (Quercetin equivalent mg/g)	Quercetin
100	3.23+ 0.04	7.03+ 0.02
200	3.94+ 0.26	7.24 + 0.04
300	4.78+0.32	8.94+ 0.05
400	6.84+0.26	10.54+ 0.09
500	8.42+ 0.32	12.46+0.07

4. Conclusion

Results from this study show that the methanolic extract is rich in flavonoids and other polyphenolics. This suggest that the plant leaves are a potential source of antioxidants and therefore could be used as natural antioxidants. Further chemical analyses are required to identify those compounds that were unidentified using GC-MS. It will also be needful to isolate the bioactive compounds.

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