PURIFICATION AND CHARACTERIZATION OF EXTRACTS FROM THE STEM BARK OF *SCLEROCARYA BIRREA* (MARULA)

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ABSTRACT

Sclerocarya birrea were used for an array of human ailments in Nigeria and other African countries as an important medicinal plant. 200 g of *Sclerocarya birrea* stem barks were extracted using five different solvents sequentially in order of increasing polarity. The chemical constituents of the extracts from the stem bark of *Sclerocarya birrea* were separated by thin layer chromatography (TLC). The extracts were fractionated by silica gel column and the fractions were collected at regular intervals. Two fractions were collected from the ethyl acetate extract and one from the acetone extract. The ethyl acetate fraction was subjected to structural analysis using Infrared (IR) and Gas Chromatography-Mass Spectrometry (GC-MS) Spectroscopic techniques. The results from IR absorption frequencies showed the presence of OH, benzene ring and t-butyl group and 2, 4-bis (1, 1-dimethyl ethyl) phenol with molecular ion peak at M/Z = 206 and base peak at M/Z = 191 was identified using GC-MS spectroscopy.

Keywords: *Sclerocarya birrea,* Purification, Characterization, Spectroscopy and Phenolic

INTRODUCTION

Medicinal plants contain numerous biologically active compounds such as terpenoids, flavonoids, carotenoids, steroids, simple phenolic, glycosides, tannins, saponins, polyphenols, to mention a few which have shown medicinal activities (Ushie and Adamu, 2010). These medicinal properties that the secondary metabolites possessed may be equal or superior to that found in synthetic combinatorial libraries (Gurib-Fakim *et al*., 2005). The most important of these bioactive constituents of plants are the alkaloids, tannins, flavonoids and phenolic compounds (Edeoga *et al*., 2005). The health benefits of medicinal plants are attributed in part to their unique phytochemical compositions (Donatus and Friday, 2009). Phytochemicals act as anti-oxidant, stimulate the protective enzymes in the liver or block damage to genetic materials, prevent the occurrence of oxidation of chemical species and stimulate anti-oxidant repairing mechanism and scavenging capacity for radicals in the system (Donatus ana Friday, 2009).

Marula *(Sclerocarya Birrea)* is a Nigerian medicinal plants used to cure disease and heal injuries. This plant has various effects on living system. In Nigeria and in some other African countries, the stem bark, roots and leaves *of Sclerocarya birrea* are used for an array of human ailments, including: malaria fever, diarrhea and dysentery, stomach ailments, headache, toothache and body pains etc (Masoko, 2008).

In Nigeria and in some other African countries, the stem bark, roots and leaves *of Sclerocarya birrea* (Marula) are used for an array of human ailments, including: malaria fever, diarrhea and dysentery, stomach ailments, headache, toothache and body pains etc (Masoko, 2008). *Sclerocarya Birrea*, commonly known as Marula in English and Danya in Hausa, is taxonomically derived from the Anacardiaceae plant family. It is an indigenous, fruits bearing tree of sub-saharan Africa (Daniela *et al*., 2013). Daniela *et al*. (2013) used HPLC-MS to investigate the differences in phytochemicals in the roots, bark and leaf of *S. Birrea* for methanol and water extracts that exhibited the best anti-oxidant activities and observed about 36 compounds of which 27 were tentatively phenolic compounds. Extracts of *S. Birrea* have been reported to possess antioxidants, antibacterial, antifungal, astringent anticonvulsant, anti-hyperglycemia, anti-inflammatory and antiatherogenic properties. Several of these properties could be attributed to the high content of polyphenols and its antioxidant activity. Many

potent drugs including anti-malarial, anti-bacterial and anti-diabetic compounds have been purified from medicinal plants (Elujoba *et al.*, 2005). Ndlala *et al* (2007) have reported that the pulp of *S. birrea* possesses high total phenolics, flavonoids and condensed tannins. Mdluli (2005) has also investigated the antioxidant potential of Marula fruits and observed the antioxidant activity of the juice. Moyo *et al.* (2010) also reported that the leaf and young stem extracts of *S. birrea* had high antioxidant activity and possessed high phenolic content, thus indicating a strong relationship between phenolic content and antioxidant activity. Mariod *et al*. (2008) investigated the phenolic content and antioxidant activity of 60 % aqueous methanol extracts from the leaf, bark and root of *S. birrea* and reported that the bark and root extracts had higher phenolic content than the leaf extract. Pharmacological studies by various groups of investigators have shown that *S. birrea* possesses antidiarrhoeal, antidiabetic, anti-inflammatory, antimicrobial, antiplasmodial, antihypertensive, anticonvulsant and antioxidant properties, thus lending pharmacological support to the plant's folkloric, ethnotherapeutic uses in African traditional medicine (Ojewole *et al*, 2010). In line with their high antioxidant activities, these *S. birrea* polar extracts possessed a high total phenolic content (Daniela *et al.*, 2013). Therefore, it is against this background that this study intends to purify and characterized the extracts from the stem bark of *Sclerocarya birrea* (Marula), wild source.

MATERIALS AND METHODS

Equipment and reagents

All chemicals and reagents used in this research work are of analytical grade and were used without further purification. The equipment's used in this research work are: HPLC 1200 model, Shimadzu (Japan), IR Spectrometer by FTIR MB3000 model (USA) and Gas Chromatograph-Mass Spectrometer by Shimadzu (Japan).

Sample Collection and Preparations

Fresh stem bark of *Sclerocarya birrea* were collected in Bauchi. The plants sample was air dried to avoid heat destruction of the active components. The dried sample was ground into fine powder and stored in air tight containers at room temperature.

Extraction

The extraction (200g) was carried out using cold extraction method with the following solvents; n-hexane, chloroform, ethyl acetate, acetone and methanol sequentially in order of increasing polarity. The extract was filtered through Whatman filter paper and then concentrated using rotary evaporator.

Solvent-Fractionation

The crude extracts from the stem bark of *Sclerocarya birrea* were subjected to solvent-fractionation. The extracts were washed three times each with n-hexane, chloroform, ethyl acetate and acetone. The ethyl acetate and acetone extracts were the crude extracts used for this solvent fractionation because they are the most active extracts against the microorganisms tested previously in this study. The crude ethyl acetate (A15) and acetone (A14) extracts from the stem bark of *Sclerocarya birrea* were purified by dissolving the dry extracts in hexane, chloroform, ethyl acetate and acetone sequentially in order of increasing polarity.

Thin Layer Chromatography (TLC)

The chemical constituents of the extracts were analysed by the TLC. For spotting and developing the TLC Plate, few milligrams of the extracts were dissolved in a volatile solvent to make a dilute solution. Drops of the solution to be separated was placed near one edge of the preparative TLC plate (about 1.5cm from the bottom of the plate) using capillary tube and the plate was placed in a container (developing chamber) with enough of the eluting solvents that was filled to a level just below the spot which was made on a pencil line on the plate that served as the origin. The solvent system migrated up the plate, carrying with it the components of the mixture at different rates. The individual components of the sample were detected as separate spots on the plate. Various combinations of polar and non-polar solvents were tested for suitability as mobile phases for the preparative TLC fractionation of the extracts. The ethyl acetate: methanol: water (EMW) solvent system yielded the best results for the crude acetone and ethyl acetate extracts in a ratio of 1:1:1.

The R_f value (retention factor) of each spot was determined as shown in equation (1)

$$\boldsymbol{R_f} = \frac{\boldsymbol{distance\ moved\ by\ component\ of\ the\ solute}}{\boldsymbol{distance\ moved\ by\ solvent}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Column Chromatography

The column was packed with an active solid (Silica gel) and a small liquid sample was applied to the top. The sample was dissolved in a minimum amount of solvent and introduced into the packed column using a pipette. An eluting solvent mixture was then allowed to flow through the column. The ethyl acetate extract was chromatographed over a Silica gel and eluted with the solvent mixture. After development, discrete bands were formed. As the components of the mixture were separated, they begin to form moving bands (or zones) each band (solvent and solute) passed out the bottom of the column and was collected in a test tube. The fractions were collected at regular intervals and each fraction was monitored by TLC analysis to confirm the presence of only one compound in each fraction. The fractions were concentrated using rotary evaporator and dried in Desiccator.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was employed to further purify the active purified components (A15A) of the stem bark of *Sclerocarya birrea*. HPLC conditions were set as described by Sotelo and Serrano (2000) and the UV absorbance detector was set at 280 nm. This was done in Central Advanced Science Laboratory, Usman Danfodio University Sokoto - Nigeria.

Infrared Spectroscopy (IR)

The IR analysis of ethyl acetate fractions was done in Central Advanced Science Laboratory, Usman Danfodio University Sokoto - Nigeria. The IR analysis was employed to identify the functional groups in the sample. The spectrometer model MB3000 with Detector model 114690-131082 was used.

Gas Chromatography – Mass Spectrometry (GC – MS)

The GC/MS analysis of the ethyl acetate fraction was done at Central Advanced Science Laboratory, Usman Danfodio University Sokoto - Nigeria.

RESULTS AND DISCUSSION

The secondary metabolites from the plant were extracted using cold extraction method with n-hexane, chloroform, ethyl acetate, acetone and methanol sequentially

in order of increasing polarity. The results for the percentage yields are presented in Table1 below.

Table 1: Yield and Percentage Recovery from the stem bark of *S. birrea*

Solvent	Yield	% Recovery
n-hexane	1.50	0.75
Chloroform	1.10	0.55
Ethyl acetate	8.20	4.10
Acetone	6.60	3.30
Methanol	9.30	4.65

The thin layer chromatography (TLC) analysis of the acetone and ethyl acetate crude extracts were carried out using ethyl acetate: methanol: water (EMW) solvent system being the suitable solvent mixture. Using the Column Chromatographic technique, A15 fractions were collected at regular intervals and each fraction was monitored by TLC analysis to confirm the presence of only one compound in each fraction. The Fractions are labelled as A15A and A15B respectively. The fractions were concentrated using rotary evaporator and dried in Desiccator.

The number of components observed with their retention factor (R_f values) are presented in Table 2. The HPLC Chromatogram of A15A presented in figure 5 showed a single and purified component.

Table 2: The R_f values from the crude ethyl acetate extract (A15) and crude Acetone Extract (A14) from the stem bark of *S. birrea*

Extract	Number of components	R_f values
A15	2	0.75
		0.68
A14	1	0.56

The thin layer chromatography was used to determine the number of components present in each extract. The ethyl acetate extract gave two spots (A15A and A15B) and the acetone extract gave only one spot indicating that there are two compounds

in ethyl acetate extract and one compound in the acetone extract with R_f values of 0.75, 0.68 and 0.56 respectively.

Therefore, the ethyl acetate purified active component (A15A) was subjected to structural analysis using Gas Chromatography- Mass Spectrometry (GC-MS) and Infrared (IR) spectroscopy.

The IR Spectral data and the GC/MS m/z values for A15A are presented in Tables 3 and 4 below.

Table 3: IR Spectral Data of A15A

Frequency range (cm^{-1})	**Bond type**	**Type of functional group**
3400cm^{-1}	O – H Stretching (broad)	Phenols
2950cm^{-1}	C – H Stretching	Sp^3 carbon, methyl group
1050cm^{-1}	C – O Stretching	Phenols
1600cm^{-1}	C = C Stretching	Aromatic, Benzene
1510cm^{-1}	Carbon-Carbon stretching Vibrations in the aromatic ring	Benzene
1380cm^{-1}	t-butyl	t-butyl group
750cm^{-1}	C – H bending, out of plane ring deformation	Benzene

Figure 1 and 2 Showed the Infrared Spectra of A15A.There is absorption band around 3400 cm^{-1} and this absorption is due to O – H stretching of alcohols and phenols. O – H absorption of carboxylic acids is also around this region but is broader than the O – H absorption of alcohols.

N – H absorption band of amines is also around this region but it is much sharper than this. There is a strong absorption band around 1050 cm^{-1} which is due to C – O stretching of alcohols, phenols and ethers. Even though other functional groups absorb in this region, absence of an absorption in the region suggest the absence of a C – O bond. The stretch is due to C – O bond of alcohols or phenols because its lies towards the lower end of the range. If however, the C – O bond is in carboxylic acid, the stretch will lie at the higher end of the range. The two absorption bands (3400 cm^{-1}

and 1050 cm^{-1}) indicate the presence of an alcohol or phenol. The absorption at approximately 1600 cm^{-1} is due to C = C aromatics. Absorption at 1600 cm^{-1} and 1510 cm^{-1} are due to ring vibrations of the benzene system and are usually present in all derivatives of benzene. Absence of an absorption at 1710cm^{-1} suggest the absence of C = O bond of carbonyl compounds. Therefore, the O – H absorption above is due to alcohols or phenols not carboxylic acids. Absorption around 3000cm^{-1}suggest that hydrogens are attached to Sp^3 or Sp^2 carbon atoms. Absorption below 3000cm^{-1}suggest Sp^3 carbon and above 3000cm^{-1} suggests Sp^2 carbon. In this case, there is absorption around 2950cm^{-1} which suggests the presence of C – H bond of Sp^3 Carbon. Hydrogens are attached to Sp^3 carbons, > 300cm^{-1}, Sp^2 Carbon C – H stretching and < 300cm^{-1}, Sp^3 Carbon C – H stretching. Absorption at 1380 cm^{-1} indicates the presence of methyl, ethyl, Isopropyl and t-butyl groups. In this case it suggests the presence of t-butyl groups. An absorption around 750cm^{-1} indicates the C – H bending (out of plane) of the benzene ring. Therefore, from the above analysis, the compound contains OH group (3400cm^{-1}), C – O (1050cm^{-1}), benzene ring (1600cm^{-}, and 1510cm^{-1}), t-butyl groups (1380cm^{-1}) and the presence of C- H bond of Sp^3 carbon atom.

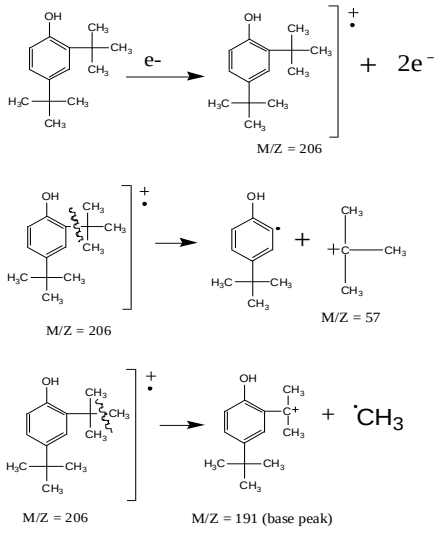
Gas Chromatography – Mass Spectrometric (GC-MS) data analysis revealed the present of 2, 4-bis (1, 1 – dimethyl ethyl) phenol in the ethyl acetate purified active component (A15A) with molecular ion peak at m/z 206 and a base peak at m/z 191 as shown in figure 5 below.

Table 5: GC/MS Fragments with their m/z Values for A15A

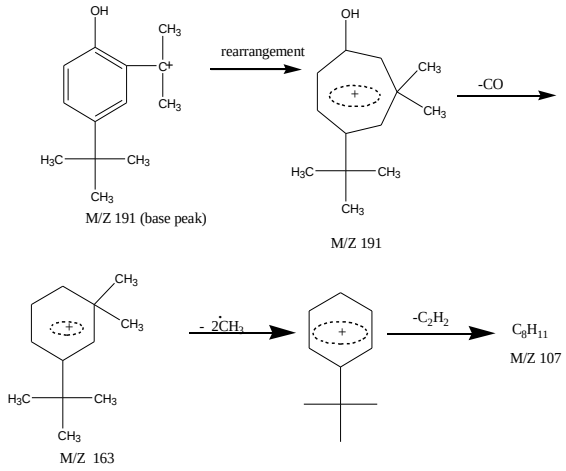
M/Z Value	Fraction
206	$C_{14}H_{22}O^{\cdot+}$
57	$C_4H_9^+$
191	$C_{13}H_{19}O^+$ Base Peak
163	$C_{12}H_{19}^+$
107	$C_8H_{11}^+$

The Fragmentation pattern for compound obtained is given below. Scheme 1 shows how the compound 2, 4-bis (1,1-dimethyl ethyl) phenol, fragments to form the base

peak at M/Z 191 and Scheme 2 shows how the base peak fragments to form other ions. Therefore, from the above analysis, the compound contains OH group (3400cm^{-1}), C – O (1050cm^{-1}), benzene ring (1600cm^{-}, and 1510cm^{-1}), t-butyl groups (1380cm^{-1}) and the presence of C- H bond of Sp^3 carbon atom. Phenols with an alkyl side chains readily fragments at the benzylic carbon, expelling a radical ($\cdot CH_3$) and leaving an ion which rearranges to the hydroxytropylium structure. In this case the compound had a molecular ion peak at m/z 206. It fragments expelling $\cdot CH_3$ radical leaving an ion which appears at m/z 191. This ion at m/z 191 corresponds simply to the loss of CH_3 radical leaving the ion which rearranges to the tert – butyl hydroxyl tropylium ion. Loss of CO from this ion gives another fragment which appears at m/z 163. The ion at m/z 163 loses a neutral molecule (CH_3CH_3, that is $\cdot CH_3$ and $\cdot CH_3$) to give a tert-butyl phenyl cation which loses C_2H_2 to give an ion which appears at m/z 107. From the molecular ion peak, one of the t-butyl group fragments as ion to give a peak which appears at m/z 57. The fragmentation pattern is illustrated in schemes 1 and 2 above. Phenols with alkyl side-chains undergo benzylic fission leaving variants of the hydroxytropylium ion. Favoured modes of fragmentation involve loss of CH_3, CO and C_2H_2.



Scheme 1: Fragmentation Pattern of 2, 4-bis (1, 1 - methyl ethyl) phenol



Scheme 2: Fragmentation 2,4-bis(1,1-dimethyl ethyl) phenol continued

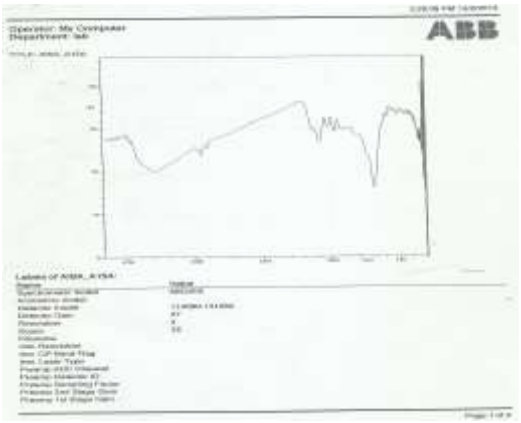


Figure 1: INFRARED SPECTRUM OF A15A

Figure 2: SECOND PAGE OF INFRARED SPECTRUM OF A15A

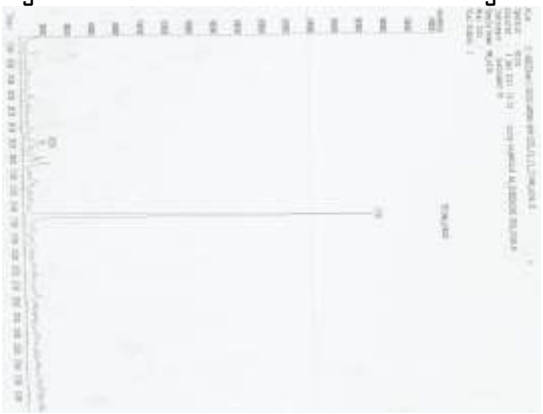


Figure 3: GC SPECTRUM OF A15A

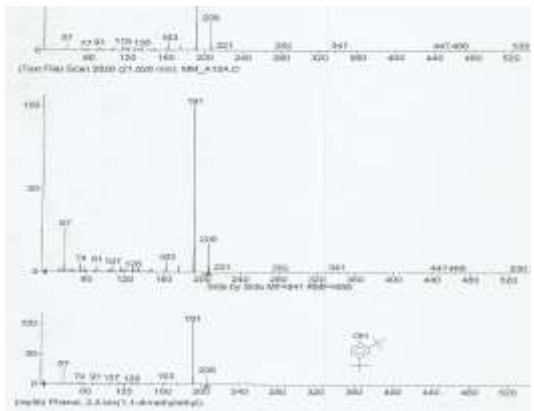


Figure 4: MASS SPECTRA OF A15A

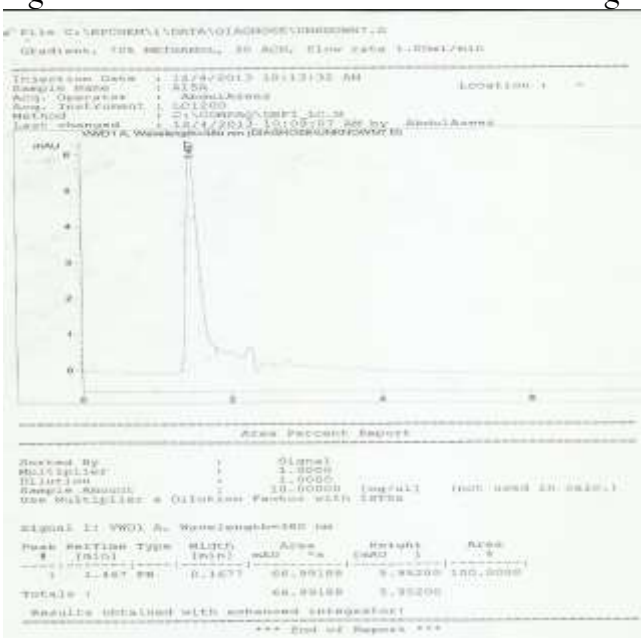


Figure 5: HPLC Chromatogram of A15A

CONCLUTION

Ethyl acetate and acetone crude extracts from the stem bark of *Sclerocarya birrea* were subjected to thin layer chromatographic technique for identification of the number of compounds present in each extract and to column chromatography technique for isolation and purification of the compounds. Two compounds (A15A and A15B) were observed in ethyl acetate extract and one compound (A14) was observed in acetone extract. The ethyl acetate extract gave two spots and the acetone extract gave only one spot indicating that there are two compounds in ethyl acetate extract and one compound in the acetone extract with R_f values of 0.75, 0.68 and 0.56 respectively. The ethyl acetate purified active component (A15A) was subjected to GC-MS and IR spectroscopic technique. The IR spectrum shows the presence of OH, C – O, benzene ring and t-butyl group while using the GC-MS, 2, 4 – bis (1, 1 – dimethyl ethyl) phenol was identified.

RECOMMENDATIONS

Further purification and characterization of the various solvents extract should be employed using other purification methods.

ACKNOWLEDMENT

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