2-Materials and methods

2.1. Materials

2.1.1. Plant material

The roots of Sudanese *Albizia Amara* were collected from the Fulla forest -Sudan in January, 2013. Barks of *Acacia mellifera* were collected from Niala–western Sudan during October, 2013.The plants were kindly authenticated by Department of Forest, College of Agriculture, University of Bahri, Sudan .The freshly collected plant material was dried under shade at room temperature, cut into small pieces and powdered in a blender. The powdered plant material was stored in sterile containers for further use.

2.1.2. Materials for chromatographic study

i. Whatman filter paper No.1 (1MM) for paper chromatography (Whatman Ltd. Maistone, Kent, England).

ii. Whatman filter paper No (3MM) for preparative paper chromatography (Whatman Ltd. Maistone, Kent, England).

iii. Silica gel for Column chromatography 0.063-0.200mm (Fluka, Sigma-Aldrich, Switzerland).

iv. Pre-coated silica TLC plates 60 F 250 (E. Merck, Darmstadt, Germany).

Ix- Glass jars (rectangular glass tanks 100 x80 x 40cm) for developing PC and TLC chromatograms.

x- Glass column 130 x 3.5 cm, 100 x 1.5 cm, and 50 x 1cm for separation by column chromatography.

Xi- Ultra - Violet lamp λ **(254/365 nm)**(portable ultraviolet, a product of

Hanovia lamps (6 watt S/W and L/W)) for localization of fluorescent spots on chromatograms and bands on columns

2.1.3. Materials for biological screening

i. Bacterial strains

- Gram positive bacteria

Lactobacillus and Streptococcus mutan.

- Gram negative bacteria

Lactobacillus and Streptococcus mutan.

- Standards

i. *Lactobacillus*: antibacterial standard for G+ve bacteria.

ii. *Streptococcus mutan*: antibacterial standard for G-ve bacteria.

iii. Ampcillin.

-Media for G+ve bacteria

Macconkey agar is used as media for G+ ve bacterial growth,

Peptone from casein 17.0g

Peptone from meat 3.0g

Sodium chloride 5.0g

Lactose 10.0g

Bile salt mixture 1.5g

Neutral red 0.03g

Crystal violet 0.001g

Agar 13.5g

-Media for G-ve bacteria

Nutrient Agar (oxoid, England) is used as media for G-ve bacterial

Lab. Lemco powder 1.0g

Yeast extracts 2.0g

Peptone water 5.0g

Agar No.3 15.0g

Distilled water 1000ml

2.1.4. Material for antioxidant activity

DPPH (2,2-diphenyl-1-picryl hydrazyl),

Gallic acid,

TPTZ (2,4,6-tris(2-pyridyl)-s-triazine),Folin –Ciocateu reagent,

Trolox standard solution.

2.1.5. Equipments

1- Ultra - Violet - Visible spectrophotometer (Shimadzu model UV240 and 240PC) for shifting of compounds.

2- Joel- Nuclear Magnetic Resonance (NMR) spectrophotometer (Brucker AC-250) -500 MHz.

3- Mass spectrometer, Varian Mat 711, Finnigan SSQ 7000 and OMM7070E.

4-Melting Point Determination:Melting points were carried out using Thermosystem FP800 Metler with central processor supplied with a mettler FP 81 MBC cell apparatus.

2.1.6. Solvents

Analytical grade solvents (n-hexane, diethyl ether, chloroform, ethyl acetate, ethanol (95%), methanol and butanol) were used. Methanol used for spectrophotometric analysis was supplied by Merck Co. Dramstadt, Germany. DMSO-d6 (for phenolics) was used in NMR spectral analysis using TMS as internal standard.

2.2. Methods

2.2.1. Soxhlet extraction

3.5 K g of powdered *A.Amara* roots (or 2Kg of *Acacia mellifera* barks) were extracted with 95% ethanol for 10 h. at 60 ^{o}C in a Soxhlet apparatus. The extract was filtered and the solvent was removed under reduced pressure. The dried extract was stored at 5^{o}C in an airtight container for further manipulation.

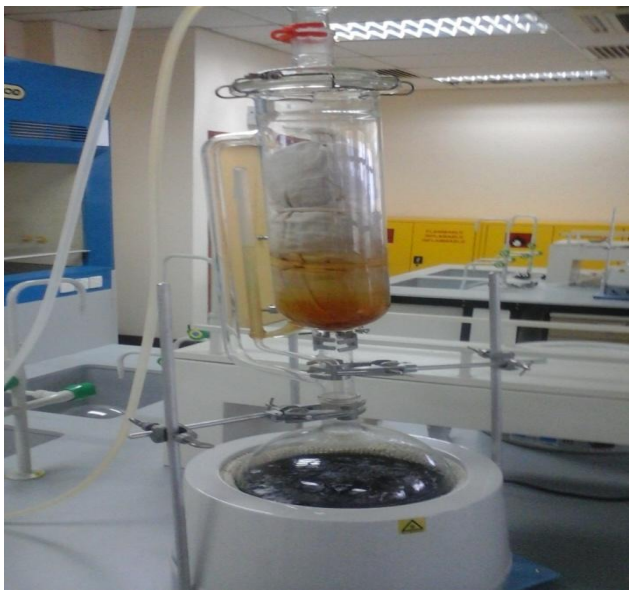
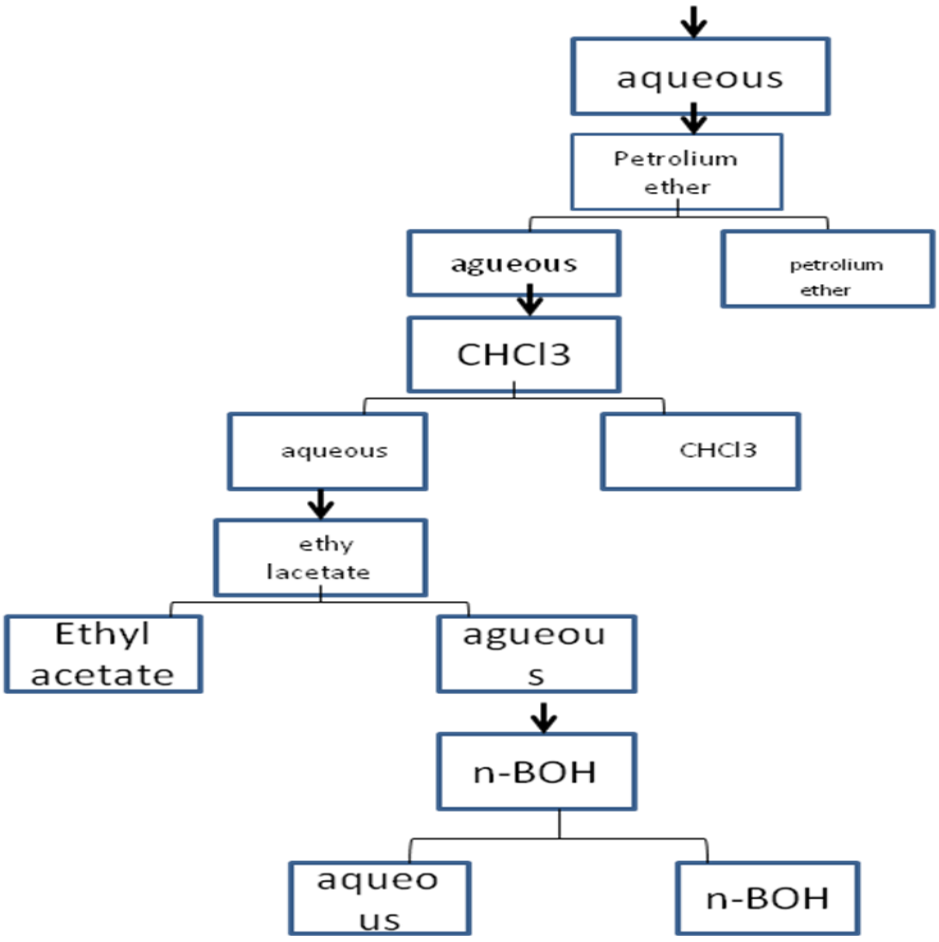


Fig.2.1 (Soxhlet extractor)

2.2.2. Solvent- Solvent Extraction

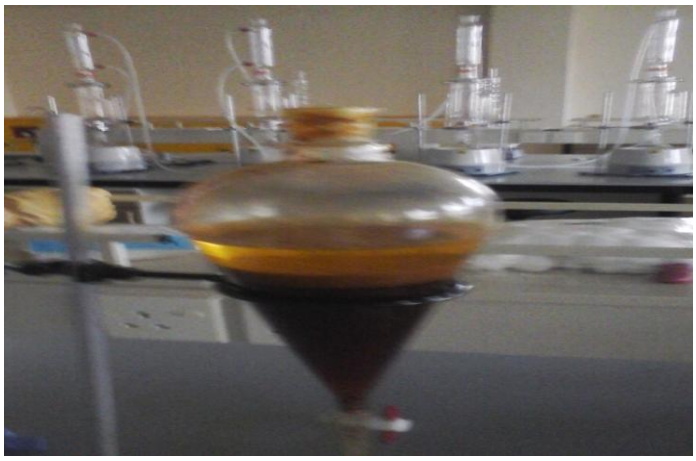
A sequential solvent extraction using a number of solvents of varying polarity was used for the preliminary separation of flavonoids . The alcoholic extract was subjected to separation scheme as sketched below:



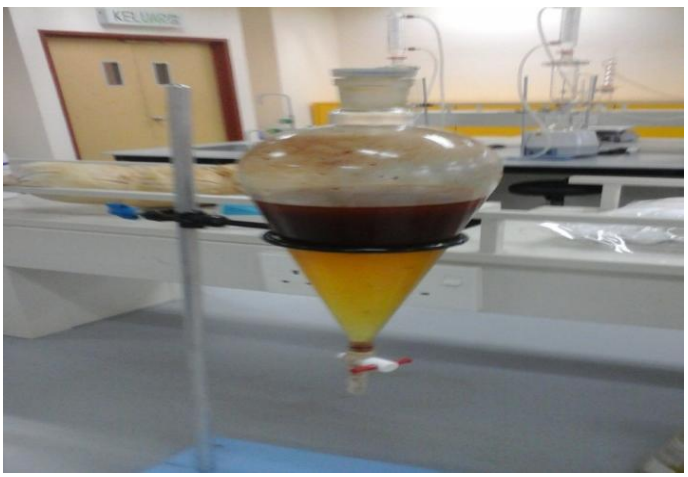
Scheme 2.1

The ethanolic crude extract was placed into beaker and 500 ml of distilled water was added . The mixture was stirred and shaken in order to dissolve maximum amount of extract .The sulrry was filtered at the suction pump. The sticky mass was placed in one liter separating funnel with 500 ml petroleum ether. The content of separating funnel were shaken vigorously and then allowed to settle as two distinct layers .The lower aqueous layer was red in color while upper layer (petroleum ether) was yellow .Then each layer was separated and the process was repeated twice .

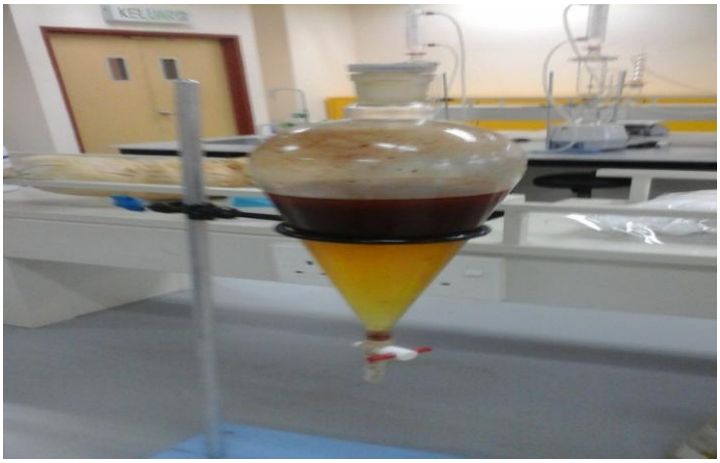
The aqueous layer was further extracted with 700 ml chloroform. The contents of separating funnel were shaken vigorously and the allowed to settle as two distinct layers. The upper aqueous layer was dark in color while lower layer chloroform was yellow .Then both layers were separated. The process was repeated three times. The chloroform layers were combined and concentrated on rotary evaporator, freeze-dried and stored in a small container . The aqueous layer was further extracted as before with 700 ml ethyl acetate and n-butanol successively.



(Fig.2.2) petroluim ether fraction



(Fig 2.3) Chloroform fraction



(Fig.2.4) Ethyl acetate fraction

(Fig.2.5.) n-Butanol fraction

2.2.3. Phytochemical screening

All fractions were screening for the presence of phenolic compounds, flavonoids, tannins, terpenoids, saponins, alkaloids and carbohydrates as follows. [128,129]

a) Test for alkaloids

To the extract, dilute hydrochloric acid was added,then it was shaken well and filtered.For the filtrate, the following tests were performed.

-Mayer's reagent test: To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates the presence of alkaloids.

-Wagner's test: To 2 ml of filtrate, few drops of Wagner's reagent were added. Formation of reddish brown precipitate indicates the presence of alkaloids.

b)Tests for carbohydrates (**Benedict's test):** Equal volumes of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. In presence of sugars the solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution.

c) Test for flavonoids:

i. Alkaline reagent test:

The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid, indicates the presence of flavonoids.

ii. Lead Acetate: The extract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids.

d) Test for tannins i Ferric chloride test: A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of tannins.

ii) Lead acetate test: A small amount of extract was dissolved in distilled water. To this solution few drops of lead acetate solution were added. Formation of white precipitate indicates the presence of phenolic compounds.

e) Test for Saponins (Froth test): The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes . The formation of a persistent slayer of foam indicates the presence of saponins.

2.2.4. Antioxidant, Antibacterial Assay and GC-MS screening:

2.2.4.1. Determination of Volatile Compounds

Volatiles of all extracts (crudes and fractions) were determined via Agilent Technologies 7890A Gas Chromatography (GC) system coupled with Mass Spectrometry (MS) detector. Each sample was prepared at 1000 ppm via dilution in respective solvents and was injected into the system. Blank analysis was also performed. The chromatography settings are; injection source: GC auto sampler and Thermal separation probe (TSP); injection volume: 1 µL (organic sample); injection mode: split less and split ratio 1:5 and oven temperature: initial 35 °C, increase to 180 °C (6 °C/min), held 5 min, increase to 230 °C (1 °C/min) and held 20 min; and initial 35 °C (2 min), increase to 180 °C (2 °C/min), held 5 min, increase to 230 °C (6 °C/min) and held 30 min; for organic and aqueous samples, respectively. Other settings; column: non-polar capillary DB-1 of 100%

dimethyl-polysiloxane (30 m x 0.53 mm id, film thickness 0.25 µm); carrier gas: helium (1 mL/min); ionization energy: 70 eV; front inlet pressure: 6.78 psi, oven equilibrium time: 3 min; maximum oven, post run, front inlet, MS source and MS quad temperature: 350, 290, 250, 230 and 150°C,respectively, for both organic and aqueous samples. The compounds were characterized with the National Institute of Standards and Technology (NIST) Library ChemStation software.

2.2.4.2. Antioxidant techniques:

a)Antioxidant activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)

The determination of antioxidant through DPPH scavenging was carried out according to the standard method[8]. Stock solution was prepared by dissolving 40 mg DPPH in 100 ml methanol and kept at -20 C until used . About 350 ml stock solution was mixed with 350 ml methanol to obtain the absorbance of 0. 7 ± 0.01 unit at wavelength 516 using spectrophotometer (Epoch .Biotek.USA) About 100 µl of sample extracts was added to 1 ml methanolic DPPH and kept overnight for scavenging reaction in the dark. Percentage of DPPH solution activity was determined as follows:

DPPH scavenging activity (%) = [(A_{blank} - A_{sample}) /A_{blank}] × 100.

Where A is absorbance.

b) Ferric reducing antioxidant power (FRAP):

The determination of antioxidant through FRAP was carried out according to the standard method[8].FRAP reagent was prepared freshly using 300 mM acetate buffer , p^H =3.6(3.1g sodium acetate trihydrate,plus16 ml glacial acid made up to 1:1 with distilled water ; 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), in 40 mM HCL ; and 20 mM $FeCl_3$.6H2O in the ratio of 10:1:1 to give working reagent . About 1ml of FRAP reagent was added to 100 µl sample and absorbance was measured at 595 nm wavelength. Calibration curve of Trolox was set up to estimate the activity capacity of sample .The results were express as mg of Trolox equivalent per 100 g of fresh sample (mg TE/100g of FW).

c) Determination of total phenolic content

The determination of antioxidant through TPC was carried out according to the standard method[8]. About 100 µl of extract was added to 0, 5 ml dilute Folin –Ciocateu reagent. The mixture was left for 5 minutes before 1 ml 7.5% sodium carbonate (w/v)was added.The absorbance was measured at 765 nm wavelength with spectrophotometer after 2 hours. Gallic acid was used as the standard phenolic acid to set up the calibration curve.The results were expressed as mg of Gallic acid equivalent per 100 g of fresh sample (mg GA/100g of FW) using the equation obtained from the calibration curve:

$y = 2.683x$

Where: y = absorbance; x = Gallic acid equivalent (µg/g).

d)Cupric reducing antioxidant capacity (CUPRAC):

Cupric reducing antioxidant capacity assay was carried out according to a standard procedure[9]; where 1 m: of 10 mM Cu (II),7.5 mM neocuprine, 1 M ammonium acetate buffer (p^H7) solutions and 0.6 mL water were mixed in a test tube.(0.5)mL of dilute sample (or Trolox standard solution) were added to the initial mixture. The absorbance was measured at 450 nm after 30 minutes. Trolox standard solutions were prepared at concentration ranging: from 40 to 400 µM.

2.2.4.3. Well diffusion method

An inoculum suspension was swabbed uniformly to solidify 20 mL Mueller-Hinton Agar (MHA) for bacteria, and the inoculum was allowed to dry for 5 min. Holes of 6 mm in diameter were made in the seeded agar using glass Pasteur pipettes. Aliquot of 20 µl from each plant crude extract (200 mg/ml) was added into each well on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37^{o}C for 24 h. The resulting inhibition zones were measured in millimeters (mm).The assays were repeated in triplicate and the concurrent values were taken.The activity is expressed as less active, if the zone of inhibition is 9-12 mm, moderate 13-16 mm and high greater than 17 mm.

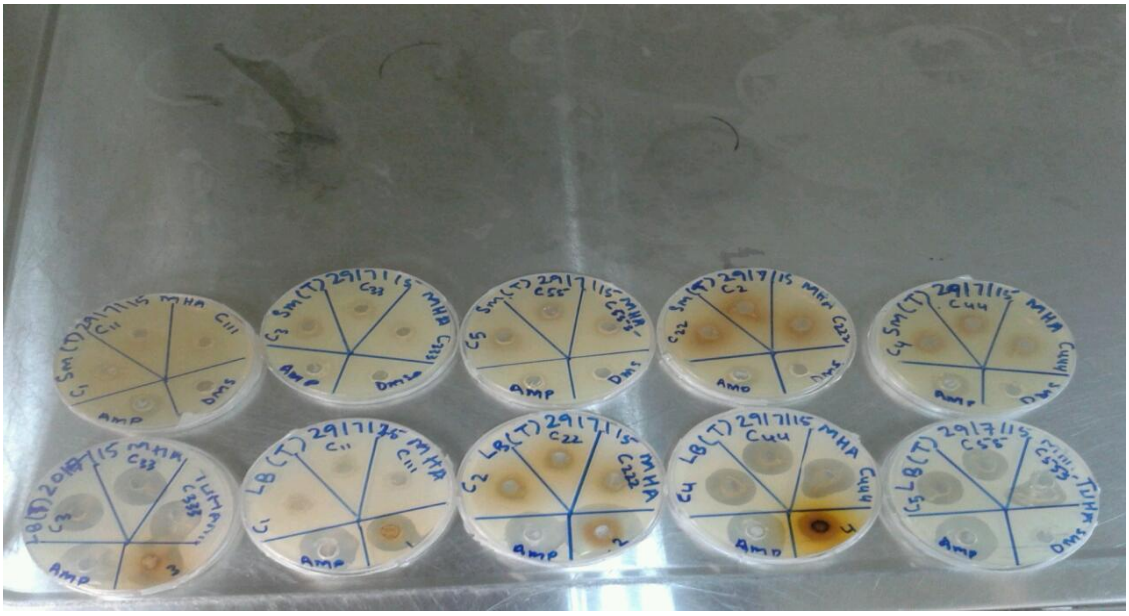


Fig.2.6 Inhibition of bacterial growth by different extracts

2.2.5. Paper Chromatography

Concentrated ethyl acetate extract of *A.amara* roots was applied as a streak on 20 sheets of Whatman 3mm paper (46×57 cm) and run in solvent (BAW:6:1:5;v:v:v) over night. The dried paper were viewed and examined under visible and ultraviolet light, then exposed for 2-3 minutes to ammonia vapors and immediately re-examined to observe changes in color of fluorescence. Chromatograms were located under UV light by pencil, cut off and similar bands were joined and cut into small pieces. The pure flavonoids were eluted from silica by methanol after several hours of contact. Evaporation of the solvent under reduced pressure gave three components: compound II (R_f 0.74); compound III (R_f 0.65) and compound IV (R_f 0.37). The isolates were then screened for their antioxidant activity.

2.2.6. Structural elucidation of flavonoids

Some spectroscopic approaches for identifying flavonoids are considered below:

2.2.6.1. UV-Visible Spectroscopy of Isolated Flavonoids

UV-Visible spectra of isolated flavonoids were recorded on a Varian Cary 100 Scan UV-Visible Spectrophotometer. The spectrophotometer was operated and results were processed using Cary Win UV Scan Application software (version 3.00(182)). Spectra were recorded in a quartz cuvette (1cmx1cmx4.5cm) which did not absorb over the spectral region of interest. A small amount of the isolated flavonoids (ca. 1mg) was dissolved in HPLC grade methanol (10mL) to create a stock solution. The UV-visible spectrum was taken for this solution at a rapid scan rate (600nm/min) to check the optical density. The concentration of the flavonoids solution was then adjusted so that the optical density of the major absorption peak (between 250-400nm) was within 0.6 to 0.8AU. The spectrum of the flavonoids stock solution (2-3mL) was measured at a normal scan speed (50nm/min) over the 200-500nm region. Additional spectra were measured over the regions of peak maxima at a reduced scan rate (10nm/min) to determine the wavelength of maxima accurately. Sodium methoxide solution (3 drops) was added to the cuvette of flavonoids in methanol. The spectrum was then recorded immediately at a normal scan rate. After five minutes the spectrum was re-recorded to check for

decomposition of the flavonoids. The solution in the cuvette was then discarded. Aluminum chloride solution (6 drops) was then added to fresh flavonoids stock solution (2-3mL) in the cuvette. The spectrum was then recorded at a normal scan rate. Hydrochloric acid (3 drops) was then added to the cuvette containing aluminum chloride and flavonoids in methanol. The spectrum was recorded again and the solution discarded. An excess of powdered anhydrous sodium acetate was added to fresh flavonoids stock solution (2-3mL) in the cuvette and shaken. The powdered sodium acetate formed a layer (1-2mm) at the bottom of the cuvette. The spectrum was recorded immediately and again after ten minutes to check for decomposition. Finally, an excess of powdered anhydrous boric acid was added with shaking to the saturated sodium acetate solution. The spectrum was then recorded. In cases where the flavonoid decomposed in the presence of sodium methoxide, a different method was used to obtain a boric acid/sodium acetate spectrum. Boric acid solution (5 drops) was added to fresh flavonoids stock solution (2-3mL) which was quickly saturated with powdered sodium acetate and the recorded immediately.

2.2.6.1.1. Shift Reagents for UV spectra of flavonoids

-Sodium methoxide solution

Freshly cut 2.5g metallic sodium was dissolved, cautiously, in 100 ml spectroscopic methanol (dry methanol).

-Aluminum chloride solution

(5g) anhydrous aluminum chloride was cautiously dissolved in 100 ml spectroscopic methanol and filtration was carried out after about 24 hours.

- Hydrochloric acid

50 ml concentrated hydrochloric acid was mixed with 100 ml distilled water, then keep in glass bottle.

- Sodium acetate

Anhydrous reagent grade sodium acetate was melted and allowed to stand for about 10 minutes. The material was then powdered and stored in a dry bottle.

- Boric acid

Anhydrous powdered reagent grade boric acid was used.

2.2.6.2. NuclearMagnetic Resonance Spectroscopy of(NMR)

NMR spectra of isolated flavonoids were obtained using a Bruker Advance DRX fitted with a 400MHz Bruker Spectrospin superconducting magnet (52mm). Proton 1D experiments were carried out using a 5mm dual ^{1}H probe head. Samples were thoroughly dried by freeze drying over several days and dissolved in dimethylsulfoxide-*d6* for NMR analysis. Operation

of the NMR and processing of spectra were performed using Topspin software (Bruker).

2.2.6.3. Mass Spectrometry

Mass spectrometry was performed by Jonathon Puddick using a Bruker Daltronics Autoflex II MALDI-TOF mass spectrometer. The spectrometer was operated in reflector mode (acceleration voltage 19kV, reflector voltage 20kV). Isolated flavonoids samples were dissolved in methanol and spotted onto an anchorchip (600μm, Bruker) and the methanol evaporated to leave a neat sample. Calibration of the spectrometer was against 2,5-dihydroxybenzoic acid ions (153.02Da and 307.05Da) laid on a neighboring sample spot. A pulsed ion extraction (60ns) and suppression (to 100Da) were used to obtain mass spectra of the samples in negative ion mode.

2.2.7. Antioxidant, Antibacterial Assay:

The determination antioxidant of the four compounds were isolated through DPPH scavenging and FRAP was carried out according to the standard method[8.] Four compounds (three from *Albizia Amara* and one from *Acacia mellifera*) were tested against the *Streptococcus mutans* (SM) and *Lacto bacillus* (LB) for the Antibacterial activities using well diffusion method was mentioned in (2.2.4.3).