2.Materials and Methods

2.1- Materials

2.1.1- Plant material

Acacia nilotica, Eucalyptus camaldulensis and *Cassia fistula* were collected from Khartoum (Sudan) . The plants were identified and authenticated by The Institute of Medicinal and Aromatic Plants, Khartoum, Sudan.

2.1.2- Solvents

All solvents used are of analytical grade. Methanol HPLC grade is used for spectroscopic purposes (BDH , England).

2.1.3- Chromatographic materials

- Silica gel G was used for TLC experiments.
- Glass jars, 10x20x24 cm, fitted with covers, were used for chromatographic fractionation.

2.1.4- Equipments

In visualizing TLC plates a multiband UV λmax (254 / 365 nm) portable ultaviolet, a product of Hanovia lamps (6 watt S/Y and L/W) was used. Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV -Visible Spectrophotometer(Shimadzu).

The electron impact ionization (EIMS) mass spectra were obtained on a solid probe using Shimadzu QP-class-500. ^{1}HNMR spectra were obtained on a Bruker AM 500

spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d_6. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal standard and the coupling constants (J) are expressed in Hertz (Hz).

2.1.3-Test organisms

oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in table(1).

Table 1: Test organisms

Ser. No	Micro organism	Type
1	*Bacillus subtilis*	G+ve
2	*Staphylococcus aureus*	G+ve
3	*Pseudomonas aeroginosa*	G-ve
4	*Escherichia coli*	G-ve
6	*Candida albicans*	fungi

2.2- Methods

2.2.1- Preparation of reagents for phytochemical screening

- Flavonoids test reagents

a- Aluminium chloride solution

(1g) of $AlCl_3$ was dissolved in 100 ml methanol.

b- Potassium hydroxide solution

(1g) of KOH was dissolved in 100 ml water.

c- Ferric chloride solution

(1g) of $FeCl_3$ was dissolved in 100 ml methanol.

- Alkaloids test reagents

-Mayer's reagent

(1.358 g) of $HgCl_2$ was dissolved in 60 ml of water and poured into a solution of (5 g) of KI in (10 ml) of H_2O,then sufficient water was added to 100 ml.

- Wagner's reagent

(2 g) of iodine and (6 g) of KI were dissolved in 100 ml of water.

-Glycosides test reagents

-Molisch reagent

(2 g) α-naphthol dissolved in 20 ml EtOH 96%

2.2.2- Shift Reagents

The diagnostic reagents used for the UV spectral measurements of the isolated flavonoids were prepared as follows:

-Sodium methoxide (NaOMe)

Freshly cut metallic sodium(2.5 gm.) was dissolved in100 ml spectroscopic methanol.

- Aluminum chloride

Anhydrous $AlCl_3$(5 gm) was cautiously dissolved in100 ml spectroscopic methanol and filtration was carried out after about 24hours.

-Hydrochloric acid

Fifty ml. concentrated HC1 were mixed with 100 ml. distilled water.

- Sodium acetate

(NaOAc) Anhydrous 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 H_3BO_3 was used.

2.2.3- Stepwise procedure for use of shift reagents for UV

- The UV spectrum of the compound in methanol was first recorded.

- 3 drops of NaOMe reagent were added to the sample and the NaOMe spectrum was recorded, and after 8 minutes the NaOMe spectrum was re-recorded.

- 6 drops of $AlCl_3$ reagent were added to the fresh sample and the $AlCl_3$ spectrum was recorded, 3 drops of HCl were added and after mixing , the $AlCl_3$/ HCl spectrum was recorded.

- Powdered NaOAC was then added to the fresh sample, the mixture was shaked and the NaOAC spectrum was recorded. NaOAC/ H_3BO_3 spectrum was then recorded after adding H_3BO_3.

2.2.4- Preparation of plant extract for phytochemical screening

(100g) of powdered shade-dried roots of *Acacia nilotica* were extracted with 80% methanol (soxhlet) for 6 hours. The cooled solution was filtered and evaporated to dryness. This prepared extract(PE) was subjected to preliminary phytochemical screening for the presence of flavonoids, saponins, tannins,

alkaloids, carbohydrates and/or glycoside, and triterpenes and/or sterols.

-Test for saponins (Froth test)

The dried extract was dissolved in water, transferred into a test tube and shaked vigorously, then it was left to stand for 10 minutes, when a thick persistent froth appears and persists for one hour saponins are present.

-Test for alkaloids

(50 mg) of extract was dissolved in (50 ml) of methanol in a water bath for 20 minutes, the extract was then filtered off and allowed to cool. Two ml of the extract was poured into test tubes. Dragendorff's or Mayer's reagent was added to the tube and the presence or absence of colours or any precipitates was noted.

- Test for tannins

(50 mg) of extract was dissolved in (50 ml) of water .The aqueous extract was then treated with a 15% ferric chloride solution. A blue color indicates condensed tannins, a green color indicates hydrolysable tannins.

- Test for glycosides (Molisch's test)

(1 ml) of the extract solution was pipetted into a test tube, 3 drops of Molisch reagent were added . After mixing, (1ml) of concentrated sulfuric acid was added to the wall of test tube. A positive test for carbohydrates is indicated by a violet ring

forming at the interface between the denser sulfuric acid and the less dense test solution above.

-Test for sterols and triterpenes (Liebermann-Burchard test)

(1 ml) of glacial acetic acid was added to (1 ml)chloroform and cooled to 0°C, then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract.

- Test for flavonoids

(50 mg) of extract was defatted with petroleum ether and the residue was dissolved in(30ml) 80% methanol and filtered. The filtrate was used for the following tests:

(1) To (30 ml) of the filtrate few drops of methanolic aluminium chloride were added . A dark yellow colour was observed .

(2) To (30 ml) of the filtrate few drops of potassium hydroxide solution were added . A dark yellow colour was observed .

(3) To (30 ml) of the filtrate few drops of ferric chloride solution were added . A blue coloration was observed .

2.2.5-Isolation of flavonoids

Powdered shade- dried roots of *Acacia nilotica* (1Kg)were macerated with 95% ethanol at room temperature for 48 hours. The crude extract was applied on Whatman paper (No. 3 mm – 46x 57cm) as narrow strips. The bands were irrigated with 15% acetic acid. The developed chromatograms were air-dried and examined under both visible and UV light (ʎ 366,245nm).. The equivalent bands from each paper were then cut out, combined and cut into small strips and slurred with methanol. After

several hours of contact, with occasional shaking, the liquid was evaporated *in vacuo* to dryness. In this way compounds I(R_f 0.70) and II(R_f 0.50) were isolated from *Aristolochia bracteolate* leaves in chromatographically pure form.

2.2.4-Extraction of oil from seeds of *Ruta graveolens*

Powdered seeds of *Ruta graveolens* (500g) were exhaustively extracted with n-hexane (soxhlet).The solvent was removed under reduced pressure and the oil was kept in the fridge at 4^{o}C for further manipulation.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added, then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes .The

hexane layer was then separated.(5µl) of the hexane extract were mixed with 5ml diethyl ether . The solution was filtered and the filtrate(1µl) was injected in the GC-MS vial.

2.2.5- GC-MS analysis

The oils of *Cassia fistula* and *Eucalyptus camaldulensis* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 µm,

thickness)was used.Helium (purity; 99.99 %) was used as carrier gas.Oven temperature program is given in Table 2, while other chromatographic conditions are depicted in Table 3.

Table 2: Oven temperature program

Rate	Temperature(^{o}C)	Hold Time (min.$^{-1}$)
-	150.0	1.00
4.00	300.0	0.00

Table 3: Chromatographic conditions

Column oven temperature	150.0^{o}C
Injection temperature	300.0^{o}C
Injection mode	Split
Flow control mode	Linear velocity
Pressure	139.3KPa
Total flow	50.0ml/ min
Column flow	1.54ml/sec.
Linear velocity	47.2cm/sec.
Purge flow	3.0ml/min.
Spilt ratio	- 1.0

Different fractions of *Acacia nilotica* roots and the fixed oils from *Cassia fistula* and *Eucalyptus camaldulensis* were screened for their antimicrobial activity against five human pathogenic bacterial strains: Gram-positive (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungal species **:** *Candida albicans*. The cup plate agar diffusion bioassay was used.

2.2.6.1- Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline solution to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average

number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.2.6.2- Preparation of fungal suspension

The fungal cultures were maintained on dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.6.3- Testing of antibacterial susceptibility

The cup plate agar diffusion assay was used to screen the antibacterial activity of the sample and performed by using Mueller Hinton agar (MHA).

(2ml) of the standardized bacterial stock suspension were mixed with (200 ml) of sterile molten nutrient agar which was maintained at 45°C. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle . Each plate was divided into two halves. In each half two cups (10mm in diameter) were cut using sterile cork borer (No 4). Each half was designed for a test solution.

Agar discs were removed, alternate cups were filled with(0.1 ml) samples of each test solution and allowed to diffuse at room temperature for two hours. The plates were then incubated at $37^{o}C$ for 24 hours. After incubation, the diameters of the

resultant growth inhibition zones were measured in duplicates and averaged.

2.2.6.4-Testing of antifungal susceptibility

The above mentioned method was adopted for antifungal activity, but instead of agar , dextrose agar was used. Samples were used here by the same concentrations used above.