2- Materials and Methods

2.1. Materials

2.1.1 Plant material

The fruits of *Vangueria madagascariensis* were collected from Niala, west Sudan in October 2014.Seeds of *Acacia nilotica* subsp. *tomentoza* were collected from Khartoum state Sudan in February-2015. The plants were kindly authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum.

2.1.2 Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer . NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer.Mass spectra were run on a Joel Mass Spectrometer (JMS- AX500).

2.2-Methods

2.2.1 Preperations of reagents for phytochemical screening

Phenolics test reagents

- Aluminium chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1) g of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

Alkaloid test reagents

Maeyer reagent

- Mercuric chloride solution: 1.36 g in 60 ml. distilled water.

- Potassium iodide solution : 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

Wagner reagent

1.27 g iodine and 2 g of potassium iodide in 100 ml distilled water.

2.2.2-Preparation of plant extract for phytochemical screening

(100 g) of powdered shade - dried fruits of *Vangueria madagascariensis* were extracted with 95% ethanol (soxhelt) until exhaustion. This prepared extract(PE) was used for phytochemical screening.

2.2.3- Phytochemical screening

The prepared extract (PE) was used for following tests:

2.2.3.1- Test for unsaturated sterols and for triterpenes

10 ml of the (PE) was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chlorform solution was dehydrated over sodium sulphite anhydrous. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by

two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

2.2.3.2- Test for flavonoids

(20 ml) of the (PE) was evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added. Red colour was observed.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added. A dark yellow colour was formed.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.

2.2.3.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for minutes, then cooled and divided into two portions:

To one portion a few drops of Maeyer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

2.2.3.4- Test for tannins

(10 ml) of (PE) was evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and 10 ml of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution. 5 ml of this

solution was treated with few drops of ferric chloride solution. A dark blue colour was observed.

2.2.4-Extraction of flavonoids

Powder air -dried fruits samples (1 kg) of *Vangueria madagascariensis* were extracted with 95% ethanol ambient temperature for two days. The solvent was removed under reduced pressure giving a brown solid.

2.2.5-Thin –layer chromatography of the crude product

Silica gel and water (1:2) were mixed to form slurry which was spread over clean glass plates. These plates were employed for chromatographic fraction without activation. Part of the crude product (0.1 gram) was dissolved in ethanol (2ml) and applied as concentrated spots on TLC plates. The plate s was developed with different solvents system:

Acetic acid :(5%,10%,20%,30%,40%,50%,60%,) ;acetone and chloroform:(1:1,1:2,1:3,2:1,2:3,3:1,3:2);chloroform:methanol(4:1;3:1;2:3;1:4). However ,the solvent that achieved the optimum fractionation was a mixture of chlroroform:methanol(3:2). Spots were detected under UV Light **.**

2.2.6-Column chromatography

Open column (80 4 cm) was used for fractionation the ethanolic extract . Silica gel with particle size 100-200 mesh from LOBA chemicals was used as stationary phase. The column was packed with slurry of silica gel with chloroform and then allowed to equilibrate for two hours before use.

The column was successively eluted with chloroform: methanol (4:1) , (3:1) and (2:3).The ratio 3:1(chorofrom:methanol) gave a fraction rich in flavonoids and it was further manipulated by TLC. A mobile phase (chlorlform: methanol; 3:2) was used for TLC analysis.

2.2.7- Preparative thin – layer chromatography

(0.5g) of the crude product was dissolved in minimum amount of 95%ethanoland applied on (20x20 cm) silica gel plates as narrow strips. The plates were developed with (chlorlform:methanol;3:2) and the chromatograms were located under UV light .Two bands were scratched and eluted from silica with absolute ethanol .After filtration , the solvent was removed in vacuo to leave compounds I and II

2.2.8 Preparation of shift reagents for UV spectroscopic analysis of flavonoids

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

Sodium methoxide

Freshly cut metallic sodium (2.5g) was added cautiously in small portion to spectroscopic grade methanol (100ml).

Aluminum chloride

Anhydrous reagent grade $AlCl_3$ (5g) was dissolved cautiously in 100ml spectroscopic methanol and filtration was carried out after 24 hours.

Hydrochloric acid

50ml concentrated HCl was mixed with 100ml distilled water. The solution was stored in glass – stoppered bottle.

Sodium acetate

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

Boric acid (H_3BO_3)

Anhydrous powdered reagent grade H_3BO_3 was used.

2.2.9- Stepwise procedure for the use of shift reagents

- Methanolic solution of the compound was first recorded.
- 3 drops of NaOMe were added to the cuvette and after mixing, the NaOMe spectrum was recorded.
- 6 drops of $AlCl_3$ reagent were added to the methanolic solution of the flavonoid, and the $AlCl_3$ spectrum was measured.
- 3 drops of HCl were then added and after mixing, the $AlCl_3$/HCl spectrum was measured.
- Powdered NaOAc was then added to fresh flavonoid stock solution in the cuvette, the mixture was shaken and the NaOAc spectrum was recorded.

- $NaOAc/H_3BO_3$ spectrum was then measured after adding H_3BO_3

2.2.12-Antimicrobial assay

2.2.12.1-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10^8-10^9colony forming units per ml.The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism 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 in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

2.2.12.2-Preparation of fungal suspensions

Fungal cultures were maintained on sabouraud dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

2.2.12.3-Testing for antibacterial activity

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity. (2ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for one of the compounds. Separate Petri dishes were designed for standard antibacterial chemotherapeutic, (ampicillin and gentamycin).

The agar discs were removed; alternate cup were filled with 0.1 ml samples of each compound using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the test compounds and the standard antibacterial

chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in triplicates and averaged.