2-Materials and methods

2.1 Materials

2.1.1 Apparatus

- Soxhlet apparatus and percolators.
- Rectangular glass tanks 100×80×40 cm for paper chromatography PC.

2.1.2 Instruments

- Ultra violet lamp (G. Switzerland)
- UV –Visible spectrophotometer (Shimadzu UV 2401 PC)
- IR spectrophotometer (Shimadzu FTIR.84005)
- Joel ECA 500 NMR spectrophotometer
- JEOL-Mass spectrophotometer (JMS A×500)

2.1.3 Plant material

Leaves of (Gud –gad) *Geigeria alata* **(**DC) were maintained from a local market at Omdurman city. Plant sample was authenticated by a taxonomist at the herbarium of Medicinal and Aromatic Plants and Traditional Medicine Research Institute. The plant material was used for the phytochemical screening and for the extraction, isolation and characterization of its flavonoids contents.

2.2 -Methods

2.2.1- Preparation of reagents for phytochemical screening

a) Flavonoids test reagents

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

Potassium hydroxide solution: (1g) of potassium hydroxide was dissolved in 100ml water

Ferric chloride solution: (1g) of ferric chloride was dissolved in 100 ml of methanol

B) Alkaloid reagents

i) Mayer reagent

Mercuric chloride	1.35g
Potassium iodide	5g
Distill water up to	100ml

ii) Wagner reagent

Iodine	5g
Potassium iodide	10g
Distilled water up to	100 ml

iii) Modified Dragendorffs reagent

Stock solution A:

(0.85g) of bismuth nitrate was dissolved in (10 ml) acetic acid and (40 ml) of water was added.

Stock solution B:

(8g) of potassium iodide was dissolved in (20 ml) of water.

When testing for alkaloids, (5 ml) of stock solution (A) was mixed with (5ml) stock solution (B). (20 ml) of acetic acid and (100 ml) of water were added.

2.2.2- Preparation of UV shift reagents

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

a) Sodium methoxide

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

b) Aluminum chloride

Anhydrous reagent grade $ALCL_3$ (5g) was dissolved cautiously in (100 ml) spectroscopic methanol and filtration was carried out after 24 hours.

c) Hydrochloric acid

(50 ml) concentrated hydrochloric acid was mixed with (100 ml) distilled water . The solution was stored in glass- Stoppard bottle.

d) Sodium acetate

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

e) Boric acid

Anhydrous powdered reagent grade H_3BO_3 was used

2.2.3- Stepwise procedure for the use of shift reagents

- The UV spectrum of the compound in methanol was first recorded
- 3 drops of NaOMe were added to the cuvette and after mixing the Na OMe spectrum was recorded
- 6 drops of $AlCl_3$ reagent were added to the flavonoid solution, and the $AlCl_3$ spectrum was measured. 3 drops of HCl were added and after mixing the $AlCl_3$/HCl spectrum was measured.
- Powdered NaOAc was then added to the 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.

2.2.4- Preparation of plant extract for phytochemical screening

(100g) of powdered air- dried leaves of *Gegeria alata* were extracted with 80% methanol (soxhlet) for 5 hours. The cooled solution was filtered and the volume was adjusted to (100 ml) by addition of enough 80% methanol. This prepared extract (PE) was used for the following tests.

2.2.4.1 Test for sterols and triterepenes

(20 ml) aliquot of the prepared extract of *Gegeria alata* leaves was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring matters. The residue was extracted (20 ml) chloroform. The chloroform solution was mixed with (0.5 ml) acetic anhydride, followed by two drops of concentrated sulphuric acid. No change in color was observed.

2.2.4.2- Test for alkaloids

(20 ml) of the aliquot of the prepared extract of *Gegeria alata* leaves was evaporated to dryness on a water bath. (5 ml) of 2N hydrochloric acid were

added and the solution was heated with stirring in a water bath for 10 minutes. The mixture was cooled and filtered. To apportion (2 ml) of the filtrate, few drops of Mayer reagent were added. Pale yellow precipitate was observed. Also red precipitate was observed when adding Dragendorff's reagent to the extract.

2.2.4.3- Test for flavonoids

(20 ml) aliquot of the prepared extract of the leaves of *Gegeria alata* were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and the residue was dissolved in (30 ml) 80% methanol and filtered. The filtrate was used for the following tests.

a. To (3 ml) of the filtrate few drops methanolic aluminum chloride were added. A dark yellow color was observed
b. To (3 ml) of the filtrate few drops of potassium hydroxide were added. A dark yellow color was observed.
c. To (3 ml) of filtrate few drops of ferric chloride solution were added. A blue coloration was observed.

2.2.4.4- Test for tannins

(20 ml) aliquot solution of the prepared extract was evaporated to dryness on a water bath and the residue was extracted with n- hexane. The hexane insoluble portion was stirred with (10 ml) of hot saline solution (0.9%wlv of sodium chloride and freshly prepared distilled water). The mixture was cooled and filtered and the volume was adjusted to (10 ml) with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution, a blue coloration was formed.

2.2.4.5- Test for saponins

(1g) of the powdered air dried leaves of *Gegeria alata* was placed in a clean test tube. (10 ml) of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds. Honey comb was formed.

2.2.5- Extraction of flavonoids

Powdered air - dried leaves (1kg) of *Gegeria alata* were macerated with 80% ethanol (5L) at ambient temperature for 48 hours. The solvent was removed in vacuo to give a crude product.

2.2.6- Fractionation of the crude product

The crude product obtained from Gegeria *alata* leaves was dissolved in water (100ml) and successively partitioned by n-hexane, chloroform ethyl acetate and n-butanol. The ethyl acetate, n-butanol and chloroform fractions were checked for the presence of flavonoids and then the flavonoid-bearing fractions (ethyl acetate and n-butanol) were studied by paper chromatography.

Sheets of Watman paper (NO.1 and 3mm . 46×57cm) from Watman Ltd. Maidstone, Kent, England were used for two dimensional ,comparative and preparative separation .Several solvent system were applied to both ethyl acetate and n-butanol fractions. However, the solvent that gave optimum fractionation for both fractions was butanol:acetic acid:water(4:1:5;v:v:v).Chromatograms were located under UV light. Paper chromatography allowed the isolation of compounds I (R_f 0.32) and II (R_f 0.66) from ethyl acetate fraction and compound III (R_f 0.70) from n-butanol fraction.