CHAPTER TWO

MATERIAL AND METHODS

2-Materials and Methods

2.1.1- Materials

Seeds of *Pithecellobium dulce* were purchased from the local market-Khartoum and kindly authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum-Sudan.

2.1.2- Instruments

A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μm, thickness)was used**.**

2.1.3-Test organisms

Pithecellobium dulce oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in table (2.1).

Table 2.1: 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
5	*Aspergillusniger*	fungi
6	*Candida albicans*	fungi

2.2- Methods

2.2.1- Preperations of reagents for phytochemical screening.

i) Flavonoid 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 water.

-Ferric chloride solution

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

ii) Alkaloid test reagents

Maeyer reagent

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

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

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

-Wagner reagent

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

2.2.2- Preparation of plant extract for phytochemical screening

(100 g) Of powdered air- dried fruit pulp of *Pithecellobium dulce* were extracted with 95% ethanol (soxhlet) until exhaustion. This prepared extract(PE) was used for phytochemical screening.

2.2.3- Phytochemical screening

The prepared extract of the plant was screened for major secondary constituents.

i) 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 anhydrous sodium sulphite . (5 ml) portion of the solution was mixed with(0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

ii) Test for flavonoids

(20 ml) of the (PE) were 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.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

iii) Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Maeyer reagent were added., to the other portion few drops of Wagner reagent were added.

iv) Test for tannins

(10 ml) of (PE) were 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 were treated with few drops of ferric chloride solution.

v) Test for saponins

(1g) of dried powdered plant material 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, and allowed to stand.

2.2.4-Extraction of oil from seeds of *Pithecellobium dulce*

Powdered seeds of *Pithecellobium dulce* (500g) were exhaustively extracted with n-hexane (soxhlet).The solvent was removed under reduced pressure and the oil was kept in the fridge at 4ºC for further manipulation.

2.2.4.1--Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

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 oil of the seeds of *Pithecellobium dulce* was analysed 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 2.2, while other chromatographic conditions are depicted in Table 2.3.

Table 2.2: Oven temperature program

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

Table2.2: Chromatographic conditions

Column oven temperature	150.0°C
Injection temperature	300.0°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

2.2.6-Antimicrobial assay

2.2.6.1-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.

2.2.6.2-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.

2.2.6.3-Testing for antibacterial activity

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity of Schiff bases and their Mannich bases. (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.