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EVALUATION OF THE PHYTOCHEMICAL CHARACTERISTICS AND ANTIMICROBIAL ACTIVITY OF *CAJANUS CAJAN* LEAVES

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ASTRACT

Antibiotics resistance has become a serious global issue particularly in developing countries. Therefore and despite of availability of wide range of antibiotics, still there is need for discovery of new antibiotics.[1] In this line of research the antimicrobial activity of different extracts of *Cajanus cajan* leaves was investigated. Different extraction methods including (maceration, percolation and hot continuous extraction using soxhelt) and different solvents including petroleum ether, chloroform, ethyl acetate and methanol were used in this study. Out of the different solvents and extraction methods used, extraction of leaves with methanol /water 80% (v/v) using a hot soxhelt method gave the highest yield % (10%w/w) and the highest antimicrobial and antifungal activity. The antimicrobial activity was confirmed through comparative studies with known antibiotics using standard organisms covering gram+ve and gram-ve species. The specified methanolic extract was subjected to different phytochemical tests including chemical, chromatographic (TLC, HPLC) UV spectroscopy and IR. This study revealed the presence of flavonoids, tannins, alkaloids, sterol compounds and coumarin as major constituents.

KEYWORDS: *Cajanus cajan*, phytochemical, methanolic extract, antimicrobial activity.

INTRODUCTION

There are a lot of kinds of secondary metabolites produced by plants as defense mechanisms against environmental stress or other factors like pest attacks, wounds and injuries.[2, 3] The complex secondary metabolites produced by plants have found various therapeutic uses in medicine.[4-6] Earlier studies have shown that presence of tannins, glycosides, saponins, steroids and various oils have medicinal or antimicrobial value and they tend to be the site for the active medicinal ingredients of such plants.[7] Antibiotics are miracle drugs, they stand against various infectious diseases for decades and saved millions of lives. However, resistance of some micro-organisms to antibiotics is a serious global problem.[8] The recent failure of antibiotics due to the dramatic emergence of multi-drug resistant pathogens and the rapid spread of the new infections, urged the health organizations and pharmaceutical industries all over the world to change their strategy; they stopped going on the slow growing production of more synthetic antibiotics against the fast growing antibiotics-resistant microorganisms; they realized that there are actually considerable alternative sources of natural antimicrobials from plants with different mode of actions; some of them are employed in traditional medicine for centuries and were found to have competitive effects compared to some commercial antibiotics.[9, 10]

Cajanus cajan (Pigeon pea) plant is an erect shrub, grows widely in all parts of the Sudan. It is used as fence, while the green leaves and the tops are used as fodder; the dried fruits of the plant are commonly used as source of protein and the leaves are used traditionally for medicinal purposes. Also it is an important food grain legume in Asia, South America and in southern and eastern Africa. Today, pigeon peas are widely cultivated in more than 25 tropical and subtropical countries, either as a sole crop or intermixed with cereals, such as sorghum (Sorghum bicolor).[11] It is used to treat wounds, malaria, bedsores, against hypoxic-ischemic brain damage and alcohol-induced liver damage and as antioxidant, anticancer and antibacterial.[12, 13] The pigeon pea enriches soil through symbiotic nitrogen fixation. The present study was designed to evaluate extracts of *Cajanus cajan* leaves using different extraction methods and selection of the most effective extract through evaluation of the phytochemical contents and potential antimicrobial activity of *Cajanus cajan* extracts.

MATERIALS AND METHODS

Plant collection

The plant *Cajanus cajan*(*pigeon pea*)leaves were collected from East Nile fields in Khartoum in summer between June and July, then cleaned from dust and dirts. The plant was identified and authenticated by Medicinal and Aromatic Plant Research Institute (MAPRI) Khartoum. The collected leaves were dried under the shade for more than six days. The dried leaves were then grinded into powder with 1 mm size using a Grinder machine. Leaves were stored in well closed container at room temperature.

Preparation of extracts

Hot extraction using soxhelt

Successive extraction using soxhelt and four solvents(Methanol, ethyl acetate, chloroform and petroleum ether) based on their polarity were used for the extraction. A clean and dried leaves were crushed until it became fine to the touch; fifty gm coarse powder of *Cajanus cajan* leaves were weighed and transferred to a soxhelt containing 500mL of petroleum ether, and allowed to extract for 8 hrs. The extract was filtered by cotton wool and evaporated to reduce the solvent volume at room temperature and normal atmospheric pressure. The reduced solvent volume was poured in a petri-dish and left to dry to constant weight. The procedure was repeated using either chloroform, or ethyl acetate or methanol.[14, 15] The obtained crude extracts were weighed and the yield percent calculated, kept in closed container for antimicrobial activity screening and qualitative phytochemical analysis. Methanolic extract gave the higher yield percent and it was used as extracting solvent in different concentrations (100%, 80%, 70% and 50%) v/v.

Cold extraction

Maceration with methanol

The dried powdered leaves of *Cajanus cajan* were weighed (10gm) using a electric balance, and transferred to flasks containing 100mL of either 50% (v/v), 70% (v/v),80% (v/v)or 100%(v/v) of methanol in water. Each flask was covered by aluminum foil and allowed to stand for 24 hrs at room temperature. The extract of each flask was filtered using cotton wool. The extracts were evaporated to reduce the solvent volume, which was then poured into a petri-dish and left to dry to constant weight. The yield percent was then calculated for each methanolic extract.

Percolation

The dried powdered leaves of *Cajanus cajan* were weighed (10gm) using an electric balance, and transferred to 100ml volumetric flask the volume completed to100ml with either methanol/water using 50% (v/v), 70% (v/v), 80% (v/v)or 100%(v/v); each flask content was extracted using a percolator. The extract of each flask was filtered using cotton wool, then treated as mentioned above.

Phytochemical Screening tests

The extract obtained with(Methanol 80% v/v) was subjected to phytochemical screening tests using method described by (Harbone, 1984 and modified by Evans, 1989).[16] The presence of secondary bioactive metabolites such as saponins, alkaloids, glycosides, flavonoids, coumarine, triterpine and deoxy sugar (Keller killani test) and tannins were indicated by visual observation of color change or by precipitate formation.[1]

Preliminary screening tests on plant extracts for antimicrobial activities

Preparation of standard bacterial suspension

One mL aliquots of 24 hours broth cultures of the standard organisms were aseptically distributed onto nutrient agar slopes and incubated at 37ºC for 24 hrs. The bacterial growth was harvested and washed off with sterile normal saline, to produce a suspension containing about 108- 109 colony forming units(cfu) per mL The suspension was stored in refrigerator at 4ºC till used. The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS).[17]

Bacterial suspensions were diluted with sterile physiological solution to 108 cfu/ ml (turbidity = McFarland standard 0.5). One hundred micro liters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculums were allowed to dry for 5 minutes. Sterilized filter paper discs (What man No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extracts. The inoculated plates were incubated at 37°C for 24 hrs in the inverted position. The diameters (mm) of the inhibition zones were measured.

Preparation of standard fungal suspension

The fungal cultures were maintained on Sabouraut dextrose agar, incubated at 25ºC for three days. The fungal growth was harvested and washed with sterile normal saline, finally suspended in 100 mL of sterile normal saline, and the suspension was stored in the refrigerator till used.[18]

Antifungal activity was studied by an agar- diffusion method; each of the inoculate of the tested organisms (1 mL) was poured into sterile petri-dish. A medium (about 45ºC) was poured into each of the Petri dishes (20 mL). The medium was left to stand to allow it to set. Cups were cut into the media with the aid of a sterile cork borer of 10mm diameter and the agar discs were removed. The cups were marked and different concentrations of the plant extracts were placed (0.1 mL) into the cups using sterile syringes. Plates were then incubated at 25ºC for 48 hrs. The sensitivities of the test organisms to the plant extracts were indicated by clear zones of growth inhibition around the cups containing the plant extracts and the diameter of the clear zone was taken as an index of the degree of sensitivity.[18]

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by adopting the techniques as specified by (NCCLS, 1998). A twofold serial dilution of the reconstituted extract was prepared. Each dilution was seeded with bacterial suspension (1 × 106cfu/ml) and incubated for 24 hrs at 37°C. MIC was determined as the least concentration of the extract that inhibits the growth.[17]

Ultra-Violet (UV) screening

From the dried selected methanolic extract, about 0.5mg was weighed accurately and dissolved in 200 ml methanol/water 80%(v/v); the obtained solution was screened between200-350nm versus blank.

Infra-Red (IR) spectra of the extract

From the dried methanolic extract of *Cajanus cajan* leaves, 0.2mg was accurately weighed and thoroughly mixed with potassium bromide powder then compressed with aid of hand compressor to obtain a uniform disc which was placed in clean sample holder before recording the IR spectra of the methanolic extract.

Chromatographic tests

A. Thin-layer Chromatography (TLC)

Spots of the plant extract solution were applied at bottom of the plate with aid of capillary tube and allowed to dry; each plate was then inserted into a jar containing different ratios of methanol and water, ethyl acetate and toluene and ethyl acetate as mobile phases. Silica gel was used as stationary phase. The different TLC systems used were as followed:

Flavonoids: Stationary phase: silica gel; mobile phase: ethyl acetate, methanol and water (100:13.5:10); Spraying reagent: acetic anhydride, concentrated sulphuric acid and ethanol in ratio 1:1:10.

Coumarins: Stationary phase: silica gel, mobile phase: toluene and ethyl acetate (93:7); Spraying reagent: ethanolic KOH in ratio 1:9.

The RF values for each methanolic extract was compared with RF values for two drugs containing sterol ring e.g dexamethasone sodium phosphate injection 4.0mg/ml and Hydrocortisone sodium succinate injection equivalent to 100mg of Hydrocortisone conc. 100mg/5ml.

B. High Performance Liquid Chromatography (HPLC)

The methanol extracts were filtered through membrane filter before injecting into HPLC system (Prostar model 210SDM). The different parameters optimized were: mobile phase, methanol: water at a ratio of (80:20) v/v. Flow rate was 2ml/minute. Injection volume: 20µL; temperature 25±1ºC. The UV detector wavelength was set at 271 nm.

C. Gas chromatograph mass spectrophotometer (GC-MS) analysis

The methanolic extracts of *Cajanus cajan* leaves was dissolved in HPLC gradient methanol and analyzed in gas chromatograph with Mass spectrophotometer (GC-MS) analysis. Analysis was carried out in a GC-MS equipped with a VF 5MS (30 m × 0.25 mm ID, 0.25 m film thickness), coupled to a detector interfaced with Chem station NIST mass spectral library. Column temperature was programmed from 70°C to 200°C. Injection was performed at 250°C. Helium was used as the carrier gas at a flow rate of 1.51 ml/minute. Mass spectra was recorded in the scan mode at 70 eV (50-600amu). Total running time is 35minutes. The data obtained was compared with the mass spectral compounds available in NIST library (Sampath and Rama, 2011).

RESULTS AND DISCUSSION

Solvents methanol, methanol in water(50% v/v,70%v/v or 80%v/v), ethyl acetate, chloroform and petroleum ether were used as preliminary extracting solvents for the *Cajanus cajan* leaves. Different extraction methods including maceration, soxhelt hot method or percolation were used in this study. The percentage yield value of each extract is shown in Table (1). it is clear that solvent methanol (80%v/v) using the hot extraction soxhelt method gave the highest yield % (I0%). This is in agreement with finding reported by Meena Sahu, *et al.,* (2014)[19], who stated that hot extraction using soxhelt is the best method for methanolic extraction for *Cajanus cajan* leaves constituents. First extraction trials using water as extracting solvent resulted in an extract with many limitations and drawbacks. The water extract was found to have high susceptibility to fermentation within one day; this could be attributed to the easily extractable sugars by water, and therefore pure water was excluded as extracting solvent. On the other hand although solvent ethanol gave good extraction yield, but it is excluded as it has antimicrobial property that can enhance antimicrobial activity of the plant extract. The extraction method was found to have impact on the % yield as well as on the extraction of constituents of appreciable activity. This observation was concluded when using extraction methods like hot infusion, decoction and soxhelt methods using petroleum ether, chloroform or ethyl acetate solvents. These extract did not show promising antimicrobial activity in agreement with previous reports by the findings of Nwachukwu, *et al*., 2010, Pal, *et al.,* 2011, Obiorah, *et al*, 2012.[1, 2, 20]

On the other hand the methanol extract using the maceration method gave % yield ranging between 4.55-6.17% w/w depending on the methanol/water %v/v (Table1); the hot soxhelt extraction method using methanol/water (80%v/v) gave the highest yield (10%w/w)(Table1); this methanol/water extract besides showing the highest yield, also showed high antimicrobial activity against strains of *Staphylococcus aureus* and *Bacillus subtilis* (gram+ve*)* and against strain *Escherichia coli* and *Pseudomonas aeruginosa (*gram-ve*)* (Table 3).

Also the extract was active against *Aspergillus niger* and *Candida albicans* fungi(Table 3). (Table 3) shows a comparative study of the antimicrobial activity of the methanolic extract with known gram +ve standard drugs when they were incubated with standard organisms. The possible significant antifungal activity of the extract was deduced from the inhibition zones caused by *Aspergillus niger* and *Candida albicans* (Table 3).

The methanolic extract showed promising significant antimicrobial activity when compared with known antibiotics ciprofloxacin (CIP) gentamycin (G), clindamycin(CD) and amikacin (AK); although the response of the extract was lower than these drugs as shown by the values of the inhibition zones (Table 5) but this could be attributed to the concentrations of the drugs used relative to the active material concentration in the crude extract. On the other hand the extract was found active against all the studied organisms in comparison with (CD) which was active only against *Bacillus subtilis (*Table 5). The result of the study of the evaluation of the minimum inhibitory concentration (MIC) of the crude methanolic extract of the plant is presented in (Table 4). The concentration of 3.15µg/ml was taken as the MIC of the crude extract which reflects a suitable active constituent's concentration (Table 4).

Phytochemical properties of the extract

Plants constituents normally are useful as identification means for the plants species through selection of a plant constituent as a marker. The constituent also serve as guidance for the possible medical uses of the plant.

The general phytochemical tests of Cajanus cajan leaves revealed that the methanolic extract contain high amounts of alkaloids, coumarin, flavonoids, tannins, triterpenes, unsaturated sterols and deoxy sugar (Table 2). Further confirmation of the presence of the for mentioned methanolic extract constituent (Table 2) was carried using spectrophotometry, chromatographic methods (TLC, HPLC and GC-MS).

The IR spectrum of the extract showed a strong broad absorption band around 3400 cm-1, which is indicative of the presence of conjugate (-OH) group (phenolic); this in an addition to various bands around 2950-3030 cm-1 referring to the aliphatic and aromatic (C-H) stretches. This is further confirmed by the existence of strong (C=C) stretches around 1620 cm-1-1530 cm-1 in the fingerprint region of the spectrum. Absorption at 1750 cm-1 possibly indicates the presence of (C=O) bond. The overall picture of the IR could indicate the presence of phenolic and flavonoids component which has common groups (Fig 2).

The UV spectrum of the extract showed absorbance band with λ_{max} at 271nm (Fig.1) with rather characteristic shape, which is generally, but not specifically, attributed to phenolic compounds (tannins) and flavonoids which are known to absorb between the range 230-290nm.[21]

The TLC experiments carried using the three mobile phase systems confirmed the presence of flavonoids, coumarins and sterols through measurement of equivalent retardation factor (Rf) values of spots separated from the extract compared with reference appropriate drugs (Tables 7,8).

HPLC and GC-MS results

The injected extract onto the HPLC column revealed a major peaks at RT 6.573 minutes. together with two small peaks before the major one (RT between 5-6 minutes.) and a shoulder peak at the base of the major peak. Although further suitable HPLC system is required to resolve these peaks and further extract purification could be needed, however the extract can be considered having a relatively high purity.

Also the major peak resolved at RT 6.573minutes. needs identification through injection of reference materials of the different identified constituents in the extract (Fig 3,4).

GC-MS also reveal presence of possibly important components. One of the main component chemical structure is ($C_{15}H_{24}$) 1-(hex-5-enyl)-2, 3, 4, 5 tetra methyl cyclo- penta-1,3-diene (Exact mass: 204), Elemental Analysis: C, 88.16; H, 11.84**).**

Table (1): Yield percentage of *Cajanus cajan* extract:

Extraction method	Yield % (± 0.5% w/w)
Maceration by MeOH 50% (v/v)	4.55
Maceration by MeOH 70% (v/v)	6.04
Maceration by MeOH 80% (v/v)	6.58
Maceration by MeOH 100% (v/v)	6.17
Soxhelt by petroleum ether	4.5
Soxhelt by ethyl acetate	5.0
Soxhelt by chloroform	5.24
Soxhelt by MeOH 80% (v/v)	10.0
Percolation by MeOH80%	4.0

Table (2): General phytochemical tests of *Cajanus cajan* extract

No.	Test	Results
1	Alkaloids	+++ve
2	Anthraquinones	-ve
3	Coumarins	+++ve
4	Cardiac glycoside	-ve
5	Flavonoids	++ve
6	Saponins	+ve
7	Tannins	+++ve
8	Triterpenes and unsaturated sterols	+++ve
9	Deoxy sugar	+++ve

Table (3) Inhibition zones of methanolic extract of *Cajanus cajan* against standard microorganisms

Organism Extract	*E. c*	*P. s*	*B. s1*	*S. a*	*Ca.a*	*As. n*
Methanol	21 - 22	17-18	19-20	16-17	17-18	16-17
Ethanol	14-15	15-14	15-14	13-12	14-15	-
Petroleum ether	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-

Table (4): MIC of methanolic extract of *Cajanus cajan*

Extract conc.	E.C	Ps	Sa	Bs	Ca	An
50mg/ml	16	11	11	11	14	13
25mg/ml	12	11	11	10	11	12
12.5mg/ml	9	10	11	9	9	11
6.25mg/ml	10	9	10	5	11	11
3.15mg/ml	8	8	10	5	10	10

Table (5): Comparison of inhibition zones of reference drugs and the methanolic extracts against standard microorganism

Organism	AK	CD	G	CIP	Extract
E.coli	00	00	26	30	21.5
P.a	16	00	31	30	17.5
S.a	20	00	22	18	16.5
B.s	27	20	30	30	19
Ca.a					17.5
As.n					16.5

G=Gentamicin, CD=Clindamicin, CIP=Ciprofloxacin, AK=Amikacin Methanolic extract 80% v/v.

Table (6): Inhibition zones of the extract with reference drugs against standard microorganisms

Organism	AK+Extract	CD+Extract	G+Extract	CIP+Extract
E.coli	27	40	30	38
P.a	30	40	32	38
S.a	27	18	30	32

Table (7): Retardation factor (RF) of the extract and reference drugs containing sterol ring

Component	RF
Methanolic extract	1
Dexamethasonesodiumphosphate4mg/ml	0.91, 0.76
Hydrocortisonesodium succinate eq to100mg/ml	0.91

Table (8): Retardation factor (RF) of the extract for flavonoids and coumarin

Component	Stationary phase	Mobile phase	Spray reagent	RF
Flavonoids	Silica gel	ethyl acetate, methanol, water 100:13.5:10	Acetic unhydride, H2SO4, ethanol in ratio 1:1:10	0.164, 0.273,0.4 and 0.53
Coumarins	Silica gel	Toluene, ethyl acetate 93:7	Ethanolic KOH 1:9	0.2, 0.3, 0.55, 0.81and 0.90,

1.UV spectrum of methanolic extract of *Cajanus cajan*

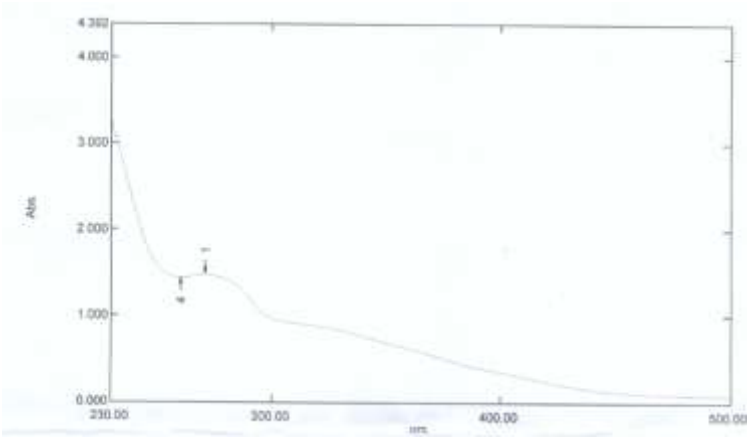

Figure (1): UV spectrum of methanolic extract of *Cajanus cajan*

2.IR spectrum of methanolic extract of *Cajanus cajan*

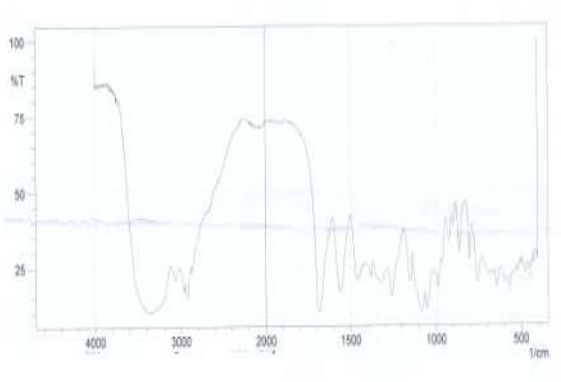

Figure (2): IR spectrum of methanolic extract of *Cajanus cajan*

3. HPLCof methanolic extract of *Cajanus cajan*

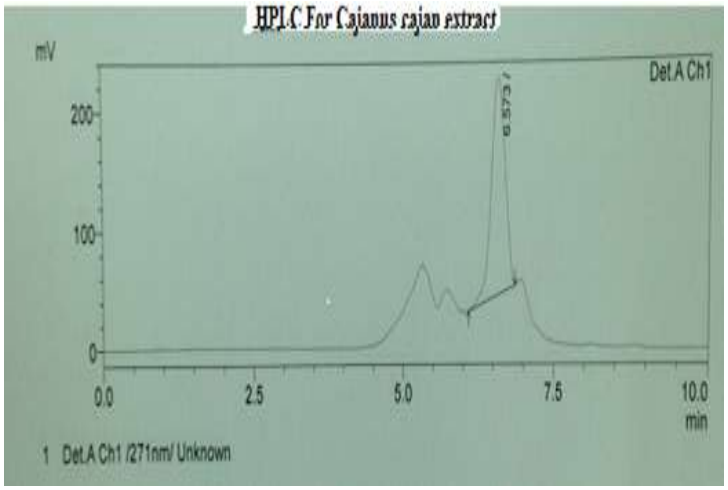

Figure (3):Typical HPLC chromatogram of methanolic extract of *Cajanus cajan*

4. GC-MS of methanolic extract of *Cajanus cajan:*

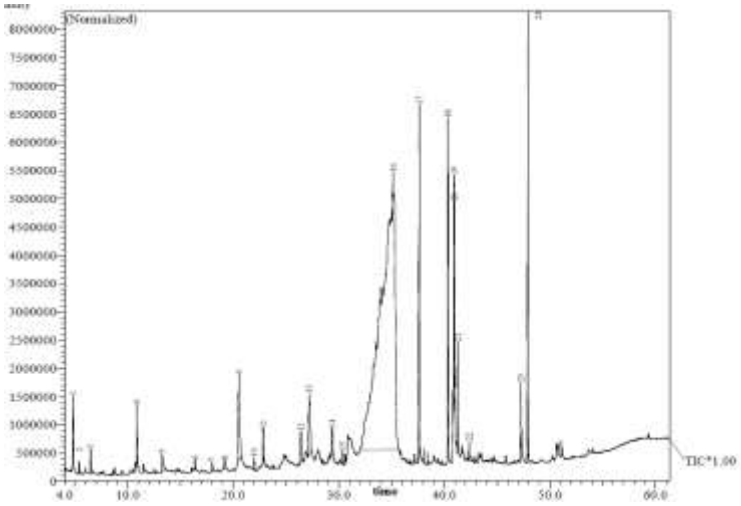

Figure (4):GC-MS of methanolic extract of *Cajanus cajan*

CONCLUSIONS

The specified methanolic extract was subjected to different phytochemical tests including chemical, chromatographic (TLC, HPLC) UV spectroscopy and IR. This study revealed the presence of flavonoids, tannins, alkaloids, sterol compounds and coumarin as major constituents.

This work is considered a preliminary study to exploit the general phytochemical properties and antimicrobial activity of this plant. No doubt further more elaborated investigation to add up and to confirm these findings are the further aims.

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