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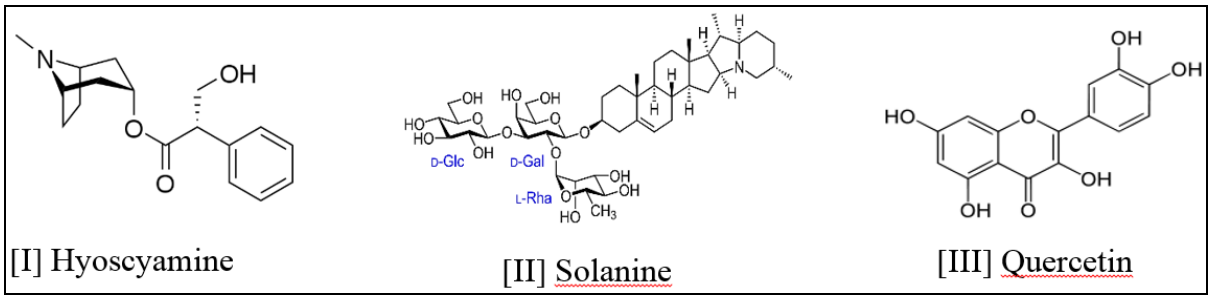
Antimicrobial and phytochemical study of the bioactive fractions of *Guiera senegalensis* from Alasan Tambuwal, Nigeria

Simon Olonkwoh Salihu, Aminu Ahmed Usman

Abstract
The study was carried out to assess the phytochemical constituents of the bioactive fractions of *Guiera senegalensis* leaf extracts. The plant *Guiera senegalensis* is used for traditional medicine among herb users in some parts of Nigeria. The leaves of the plant were extracted by serial exhaustive maceration using petroleum ether (PE), (60^0-80^0), Dichloromethane (DCM) and Methanol (MeOH). Thin layer chromatography of crude extract was carried out on both the crude and bioactive fractions of the extracts. The crude extract of PE prove to be sensitive on *Escherichia coli* and *Pseudomonas aeruginosa* showing significant zone of inhibition of about 14 mm and 21 mm respectively. Dichloromethane crude extract was sensitive only on *Salmonella typhi* while crude MeOH extract showed no activity against all the organisms. Column chromatography was carried out on PE and DCM extracts, using PE/ethyl acetate (4:1 v/v) and n-Hexane/ethyl acetate/DCM (4:1:1 v/v) eluents respectively. 17 fractions from PE extract and 19 fractions from DCM extract were collected. Thin layer chromatography (TLC), of the fractions group the PE and DCM into three each according to their retardation factor (R_f) values. The second fraction of the pet-ether fraction (P_2), shows activity on all the test organisms except salmonella typhi. As for the DCM fractions, the first fraction (D1) was active against *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia,* the second fraction (D2), against all the organisms used, while the third fraction (D3) was active only on *Salmonella typhi* and *Pseudomonas aeruginosa.* Phytochemical screening of the bioactive fractions, which was the aim of this study reveals the present of carbohydrate, steroids, saponins, flavonoids and alkaloids.

Keywords: Antimicrobial, Photochemistry, Bioactive, *Guiera senegalensis* and Alasan

1. Introduction
The use of medicinal plants for the treatment and control of diseases globally and Nigeria in particular has gain popularity and acceptability in different ethnic groups. Many of these indigenous medicinal plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes, Okwu DE [1].
The medicinal value of plants lies on some chemical substance present in them which possess definite physiological action on the human and animal system. The most important classes of these bioactive constituents of plants are alkaloids, flavonoids, tannins, saponins, terpenoids and phenolic compounds, Hill AF [2]. Generally referred to as phytochemicals or metabolites, Okwu DE and Iwu MM *et al.* [1, 3].
Today, intensive research has made it possible to isolate medicinal compounds from a number of botanicals, and their healing properties of such metabolites have been or are being determined. Many of these plant derived compounds are now synthesized in laboratories for use in pharmaceutical preparations, Mohammed F *et al.* and Shyoub Elhassan Mohamed *et al.* [4, 5]. *Guiera senegalensis* is called Sabara" (Hausa), and "Kishishi" (Kanuri), [6].
It is a shrubby and can grow to a height of 3 to 5 m depending on the habitat, Silva O *et al.* [7]. The leaves which are 3 to 5 cm long and 1.5 to 3.0 cm broad are arranged opposite or sub opposite on the stem, Hutchinson J *et al.* [8].
It is widely distributed in the savannah region of west and central Africa Nigeria, Senegal, Gambia, Mali, Niger, Burkina Faso and Ghana, [9-11], in Northern Nigeria it is used extensively for wide range of medicinal purposes, Fiot J *et al.* and Azas OFE *et al.* [12, 13]. For example, it is active against cough, respiratory congestion and fever, Adedapo AA *et al.* and Ali AJ *et al.* [14, 15], and is prescribed as an antitussive, hypertension and hypotension as well as venereal diseases, Adedapo AA *et al.* [13], to ease breathing and to treat lung and bronchial disorders. It is also used against malaria fever, Ancolio C *et al.* [16].



Some findings on the plant elsewhere showed the presence of alkaloids (Hyoscyamine **[I]** and solanine, **[II]**), tannins, terpenoids menthol, coumarins, saponins, flavonoids (quercetin,**[III]**), cardiotonics and cynogenic hetrosides which were assayed in various organs of the plant - leaves, stem bark, fruits and root, Somboro AA *et al.* [17].

The increasing resistance by disease causing organisms to antibiotic has renewed interest for new strategies and incentives towards research on treatment, prevention and development of new drugs against these microbial organisms, Omonigbehin EA *et al.* [18].

Therefore, the present research become necessary for a reason that environmental condition may affect the production of phytochemicals by plants. For example, Shewry, Ward, Zhao, Ravel, Charmet, Lafiandra and Bedő [19] in their research on wheat found that growing wheat in different environment can affect the concentration of phytochemicals such as sterol and various phenols, Alkylresorcinols and Tocols.

In their study on Feverfew, Fonseca, Rushing, Rajapakse, Thomas and Riley [20] found phytochemical contents fluctuate with changes in environmental conditions. Similarly when *Acalypha wilkesiana* was subjected to simulated drought and salinity conditions, lower quantity of alkaloids, flavonoids and tannins where produced while saponins production was increased, Odjegba VJ *et al.* [21].

Another study on the effects of genotype and growing environment on the phenolic contents and antioxidant activities of alcohol-soluble extracts from commercial wheat cultivars indicated there were significant differences among genotypes and environments for total phenolic content, antioxidant activities and concentrations of all the phenolic acids measured, Mpofu A *et al.* [22].

This research was planned to determine the phytochemical constituents in bioactive fractions of *G. senegalensis* leaves by qualitative analysis. The organisms of interest were *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia*. They are responsible for common ailments including typhoid fever, diarrhoea and some gastrointestinal problems. This will add to the database of medicinal plants of Alasan in Tambuwal local government of Sokoto State in particular and Nigeria at large.

2. Materials and methods

2.1 Sample collection and identification

The leaves of *Guiera senegalensis* were collected at Alasan, out sketch of Tambuwal (Fig. 1), along Jega-Sokoto road, in Tambuwal Local Government of Sokoto State, Nigeria in January, 2013. It is located about 12º24′00″N 4º40′00E″, and 279 meters (915 feet) above sea level. The sample was authenticated in the Department of Biological Sciences, Federal University of Technology Minna, Nigeria.

The fresh leaves were rinsed with tap water and air dried for two weeks. The dried leaves were ground with a mortar and pestle and stored in a clean polyethylene bag. The micronized sample was macerated. Serial exhaustive extraction was done using petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH).

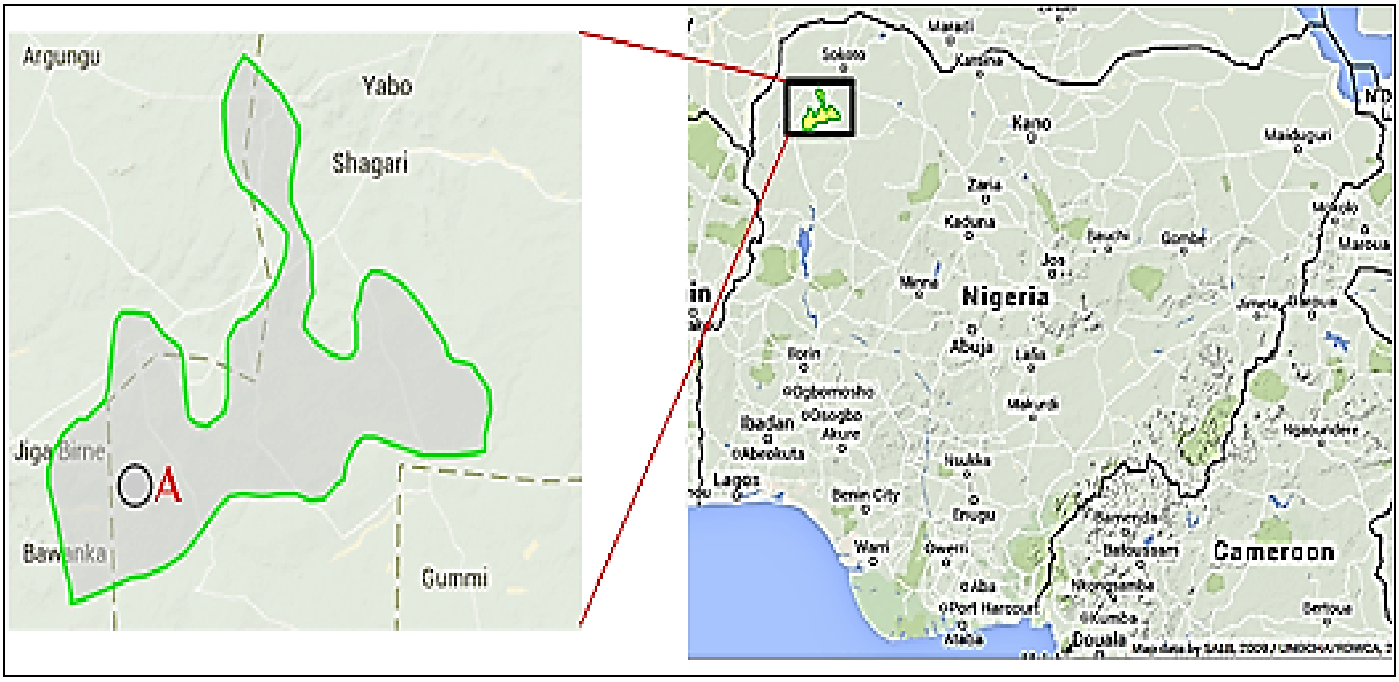


Fig 1: Map of Nigeria showing the sampling area.

2.2 Reagents
The reagents and Thin Layer Chromatography (TLC) precoated plates were purchased from Merck Pharmaceutical and Life Sciences ltd. Ibukun house, 70 Adetokunbo Ademola way, Victoria Island. The reagents were of analytical grade. Nutrient agar was obtained from Bristol Scientific Company Limited Lagos, Nigeria. A supplier for Sigma-Aldrich Company, U.S.A. The incubator was Eurotherm: Model No. 2216E incubator. Ampiclox used as positive control was obtained from Zagbayi Pharmacy Ltd. Opposite Fire Service Office, along Bosso Rd, Minna. Nigeria. It is a product of SmithKline Beecham Pharmaceuticals (Pty) Ltd, 39 Hawkins Avenue, Epping Industria 1. Cape Town 7460.

2.3 Test Organisms
The test organisms for antimicrobial analysis were five bacteria. Pure isolates of these organisms were obtained from the Department of Microbiology, Federal University of Technology Minna, Nigeria. The isolates include *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia*.

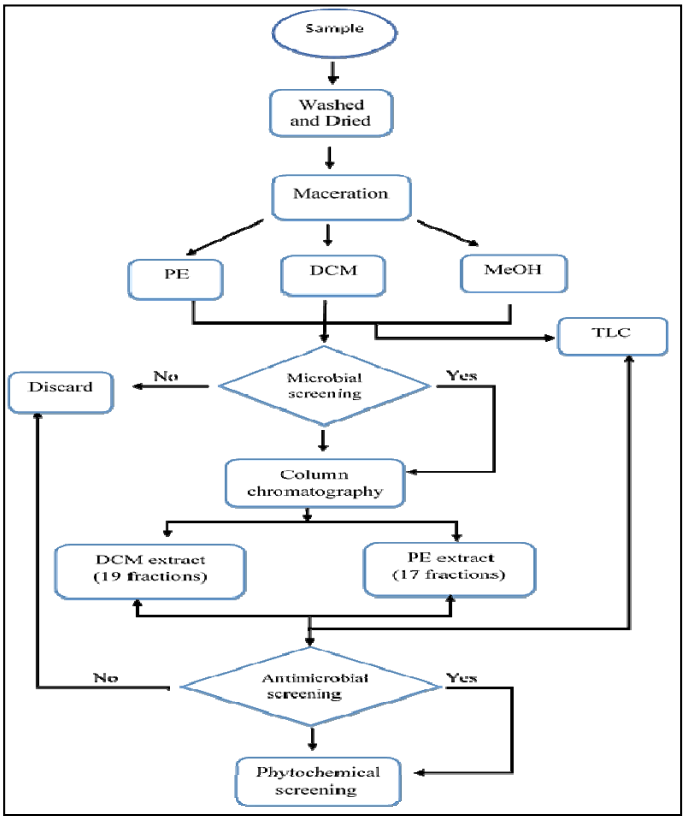


Fig 2: Flow diagram of the experimental procedure. PE = petroleum ether; DCM = dichloromethane; MeOH = methanol; TLC = thin layer chromatography

3. Method
3.1 Extraction
The method of extraction used by Ali, Akanya, Dauda and Ogbadoyi [15] was adopted with some modifications. Briefly, about 150 g of ground sample was soaked in 500 ml PE, (60^0-80^0) in a screw-cap bottle for 48 hours. The extract was decanted and filtered through Whatman Nº. 1 filter paper. The filtrate was concentrated in a water bath at 45 °C after which the extract was transferred into a sample bottle and stored in refrigerator prior to further analysis. The extraction was repeated (after drying the residue) with DCM and finally with MeOH for 48 hours each. The extracts were each concentrated in a water bath at 45 °C and stored in sample bottles pending further analysis.

3.2 Thin layer chromatography of *Guiera senegalensis* leave extracts.
Thin layer chromatography (TLC) of the crude extracts of *Guiera senegalensis* leaves was carried out using TLC pre-coated plate in the following solvents systems.
Petroleum ether extract: PE/ethyl acetate (4:1 v/v); Dichloromethane extract: n-Hexane/ethyl acetate/DCM (4:1:1 v/v/v); Methanol extract: PE/ethyl acetate/MeOH (8:2:1 v/v/v). The R_f (Retardation factor) (**Eq. 1** and **2**), values for each extract were recorded. **Equations 1and 2** were used for tiny and large spots of the samples respectively. The R_f values corresponds to the number of fractions that may be obtained from the sample if eluted through a column.

$$\text{Retardation factor } (R_f) = \frac{D_S}{D_{solv.}} \quad \text{----------Eq. 1}$$

$$\text{Retardation factor } (R_f) = \frac{(D_{SB.} + D_{SF})}{2D_{solv.}} \quad \text{----------Eq. 2}$$

D_S= Distance travelled by sample; D_{SB} = Distance to sample base;

D_{SB} = Distance to sample base;

D_{SF}= Distance to sample front; $D_{solv.}$= Distance travelled by solvent

$D_{solv.}$ = Distance travelled by solvent

3.3 Microbial screening of the crude extract *Guiera senegalensis* leaf extracts.
The agar diffusion method was used. Sterile nutrient agar powder was prepared by dissolving 14 g of the agar powder in 500 ml distilled water, boiled to ensure complete dissolution and sterilised at 121 ºC for 15 minutes and dispensed into labelled petri dishes and allowed to gel. Wells were bored into the nutrient agar using a 4 mm sterile cock borer. 0.2 g of each crude extracts was weighed into sample bottles and dissolve with 1 ml of its solvents, 9 ml sterile distilled water was added to obtain 20 mg/ml of the extract. 0.2 g of control (Ampiclox) antibiotic was weighed and dissolved in 2 ml of distilled water and made up to 10 ml with distilled water. Ampiclox was use for positive control.
0.2 ml, (400 µg/ml) of reconstituted extract or the control, was dispensed into each well and allowed to diffuse for 30 minutes.
The agar was inoculated with the test organisms using a sterile swab stick before incubating at 37 ^{0}C for 24 hours. Zones of inhibition were determined **(Eq. 3)** with the aid of a meter rule. Crude showing no activity against any of the organisms were discarded.

$$\text{Zone of Inhibition } (Z_I) = D_T - D_C \quad \text{---------- Eq. 3}$$

D_T=Total diameer of the zone; D_C=Diameter of the cork borer

3.4 Column chromatography
The column was prepared by wet loading. 26 g of the silica gel was weighed into a 250 ml beaker and mixed with PE -Ethyl acetate, (4:1 v/v), the solvent system which gives best separation from TLC, and stirred to form slurry, the slurry was allowed to stand for 2 minutes to eliminate air bubbles. The

bottom of the column was packed with piece of broken glass followed by a bed of cotton wool. The column was loaded with the slurry by pouring it directly after vigorous stirring. Sides of the column were washed with the solvent mixture to push down stock silica gel from the slurry. The stop tap of the column was open to drain the solvent and ensure good packing of the silica gel. The tap was closed as the solvent front just level with the top of the silica gel. The size of the column was 12 cm (length) and 2 cm (diameter). Glass wool was placed on the on top of the silica gel in the column.

0.5 g of the petroleum ether extract was dissolved in PE/ Ethyl acetate, (4:1 v/v) mixture- the solvent system that gives best separation in TLC. A small amount of the silica gel was added and stirred. The column was loaded with the sample by placing it directly on top of the glass wool in the column. Sides of the glass tube were was to push down the sample gently without disturbing the column. Another piece of glass wool was placed on the loaded sample. The mobile phase (PE/ethyl acetate, 4:1 v/v) was poured gently into the column. The stop tap was then opened and the mixture was allowed to elute. 17 fractions, 5 ml each were collected in test tubes. The test tubes were labelled 1-17 and allowed to evaporate.

The same procedure was repeated for dichloromethane extract using the mixture hexane/ethyl acetate/ DCM (4:1:1 v/v/v) of which 19 fractions, 5 ml each were collected in test tubes. The test tubes were labelled 1-19 and allowed to stand for evaporation.

3.4.1 TLC of the column chromatography fractions.

Precoated TLC plates, 20 cm x 9.8 cm were used. Two lines were drawn using a pencil about 5 mm from two ends (considering the width), of the plate indicating the spotting line and solvent front respectively. For the PE fraction, PE/ethyl acetate (4:1 v/v) was used for TLC analysis. While n-hexane/ethyl acetate/DCM, (8:2:1 v/v/v) solvent system was used to chromatographed the dichloromethane fractions.

The R_f values were calculated and the fractions with similar R_f values were combined. They were named PE_1, PE_2, PE_3 for PE fraction and DCM_1, DCM_2 and DCM_3 for dichloromethane fraction.

3.4.2 Microbial screening of the column chromatography fractions.

The agar diffusion method was used. Sterile nutrient agar powder was prepared and placed in labelled petri dishes and allowed to gel. Wells were bored in to the nutrient agar using a 5 mm sterile cock borer. The dishes were inoculated with each test organism using a sterile swab stick. The sterile swab stick was dipped in to the cultured organism and swabbed on the surface of the media. This was repeated for all organism using different sterile swab stick for each organism, the dishes were labelled.

0.1 ml of each of reconstituted combined fraction, PE_1, PE_2, PE_3,DCM_1, DCM_2, DCM_3 and the eluents used for TLC, (as a control) were each placed into appropriate wells in the prepared agar gel using 5 ml sterile syringes.

The plates containing the nutrient agar gel were incubated at 37^0 C for 24 hours and observed for zones of inhibition. Zones of inhibition for each fraction by each organism were calculated and recorded. Inactive combined fractions were discarded. Active fractions were tested for phytochemicals.

3.4.3 Phytochemical Screening active combined CC fractions of *G senegalensis* leaf extracts.

Phytochemical screening Chemical tests were conducted on active fractions using standard methods as describe by Tijjani, Sallau and Sunusi [11], Trease and Evans [23], Shanti and Amudha [24], with some modifications. The major modification was the use of volumes of the reconstituted fractions instead of grams because the quantities of the fractions were small.

4. Result and discussion

4.1 Thin layer chromatography of *G senegalensis* crude leaf extracts.

The results of TLC as shown in Table 1, revealed the best separation. It was seen that the pet ether extract eluted with pet ether-ethyl acetate (4:1 v/v) gave 5 R_f values. Dichloromethane extract gave 7 R_f values when eluted with hexane-ethyl acetate- dichloromethane (4:1:1 v/v/v) mixture. While methanol extract eluted with hexane-ethyl acetate-methanol (4:1:0.5 v/v/v), mobile phase gave 4 R_f values.

Table 1: R_f values from the TLC of crude *G senegalensis* leaf extracts.

Extract Eluent	Pet. Ether PE–Ethyl acetate (4:1 v/v)	Dichloromethane n-Hexane–Ethyl acetate– DCM (4:1:1 v/v/v)	Methanol PE-Ethyl acetate-MeOH (8:2:1 v/v/v)
Retardation factors	0.98	0.96	0.93
	0.43	0.81	0.68
	0.39	0.69	0.26
	0.36	0.66	0.21
	0.28	0.63	
		0.47	
		0.24	

4.2 Antimicrobial analysis of the *G senegalensis* crude leaf extracts.

The sensitivity test on test organisms using the crude extracts **Table 2**, showed that PE extract was active against *E. coli* and *P. aeruginosa*. Dichloromethane was active against *S. typhi*. Methanol extract was not active against any of the organisms used. While none of the extracts was active against *K. pneumonia* and *S. aureus*. The PE extracts showed higher activity against test organisms.

Table 2: Zones of inhibition (mm) of crude extracts of *G. senegalensis* leaves and control.

	Zones of inhibition						
	Extracts			**Control**			
Test organisms	**PE**	**DCM**	**MeOH**	**PE**	**DCM**	**MeOH**	**Ampiclox**
Salmonella typhi	-	10	-	-	-	-	41
Staphylococcus aureus	-	-	-	-	-	-	43
Escherichia coli	14	-	-	-	-	-	27
Pseudomonas aeruginosa	21	-	-	-	-	-	35
Klebsiella pneumonia	-	-	-	-	-	-	35

Key: - = Absent

4.3 Thin layer chromatography of CC fractions of *G senegalensis* leaf extracts.

The results of TLC R_f values as shown in Table 3 revealed that PE extract eluted with PE/ethyl acetate (4:1 v/v) gave 3 fractions. While the dichloromethane extract eluted with hexane/ethyl acetate/dichloromethane (4:1:1 v/v/v) gave 6 fractions. The R_f values of the fractions were similar to those of the crude extracts.

Table 3: R_f values of fractions from column chromatography of *G. senegalensis* leaf extracts

Extract	Fractions		R_f values
PE	PE_1	1-4	0.94 and 0.96
	PE_2	5-13	0.47 and 0.49
	PE_3	14-17	0.49 and 0.52
DCM	DCM_1	1-13	0.76 and 0.80
	DCM_2	14-15	0.30, 0.66 and 0.68
	DCM_3	16-19	0.46, 0.56, 0.65 and 0.78

4.4 Antimicrobial analysis of the column chromatography fractions of *G senegalensis* leaf extracts.

The sensitivity test (**Table 4**), of the column chromatography fractions was carried out on test organisms and showed that PE_1 was not active against any of the organisms. PE_2, DCM_1, DCM_2 and DCM_3 showed activity against *P. aurigunosa*. PE_2, DCM_1 and DCM_2 showed activity against *K. pneumonia* and *E.coli*. Only DCM_{-2} and $DCM_3$was active against *S.typhi*, and PE_2 and DCM_2 against *S. aureus* of the five fractions DCM_2 showed highest activity against test organisms.

Table 4: Zones of inhibition (mm) of combined column chromatography fractions of *G. senegalensis* leaf extracts and control.

	Zones of inhibition						
Test organisms	**PE_1**	**PE_2**	**DCM_1**	**DCM_2**	**DCM_3**	**C_1**	**C_2**
Salmonella typhi	-	-	-	22	32	-	-
Staphalycoccusaureus	-	16	-	39	-	-	-
Escherichia coli	-	9	8	38	-	-	-
Pseudomonasaeruginosa	-	12	11	30	9	-	-
Klebsiela pneumonia	-	11	10	20	-	-	-

Key: - = absent, PE = pet ether, DCM= dichloromethane, C_1= $Control_1$ (PE/ethylacetate (4:1)), C_2= $Control_2$ (n-Hexane/ethylacetate/dichloromethane (4:1:1)).

4.5 Phytochemical screening of the bioactive fractions of *G senegalensis* leaf extracts.

The results for the phytochemical screening carried out on the bioactive fractions Table 4.5 (PE_1, DCM_1,DCM_2 and DCM_3) of leaves extract of *Guiera senegalensis* showed the presence of carbohydrate in all the fractions, sterols in PE_1 DCM1 and DCM_2,flavanoids and saponins were present only in DCM_2 and DCM_3 and alkaloids were present in DCM_1 and DCM_3.

The phytochemicals were highly present in DCM_2 and DCM_3 and moderately present in DCM_1 and PE_2. Studies conducted on crude extracts of *Guiera senegalensis* elsewhere, showed the presence of alkaloids in roots and leaves of the plant from Mali and Burkina Faso in DCM extracts, Fiot J *et al.* [6], alkaloids, saponins, tannins and antracenes in MeOH extracts of the leaves from Sule-Tankarkar Nigeria, Mohammed F *et al.* [4]. In their research, Somboro, Patel, Diallo, Sidibe, Chalchat, Figueredo, Ducki, Troin and Chalard [17] recorded the distribution of alkaloids, tannins, saponins antracene and flavonoids the leaves roots fruits and bark of *G senegalensis*. While in the gall of the plant from Ouagadougou, Burkina Faso, alkaloids, polyphenols and saponins were detected, Lamien CE *et al.* [25].

Table 5: Phytochemical Screening of the bioactive fractions of the leaves extract of *Guiera senegalensis*

Test	PE_2	DCM_1	DCM_2	DCM_3
Carbohydrate	+++	+++	+	+++
Sterols	+++	++	+	-
Saponins	-	-	++	++
Flavonoids	-	-	+++	-
Alkaloids	-	+	-	++

Key: +++ =Highly present, ++ = moderately present, + fairly present, - = absent. PE= Petroleum ether, DCM= Dichloromethane.

5. Conclusion

It was shown from the antimicrobial analysis that not all crude extracts a fractions thereof were active against the test organisms. The antibiograms indicated different degree of activities which can be interpreted to mean that *Guiera senegalensis* leaves extract or fractions may be used for the treatment of various disease caused by these test organisms.

The phytochemical screening of the bioactive fractions revealed the presence of some metabolites which may be responsible for these activities. From the antimicrobial activity and phytochemical screening of the CC fractions, the active substance in PE extract is probable a steroid while that of the DCM are much likely to be saponins and flavonoids. Again it was observed that it is not suitable to use MeOH for the extraction of bioactive substance for use against the organisms employed in this study.

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