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Bioassay-Directed Isolation and Structural Characterization of Major Bioactive Compounds against E.coli from *Cordia sinensis* Lam.stem Bark Extract

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Abstract: ***Recently, chemists worldwide have paid attention to the potential of medicinal plants as alternative sources for the isolation of novel metabolites with interesting biological and pharmaceutical properties . The genus Cordia belongs to the family Boraginaceae, with some 300 species distributed worldwide, mostly in the warmer regions of the World. According to literature survey, several uses in traditional medicine have been reported for different Cordia species. Cordia sinensis, which is a medicinal plant found widespread in the drier parts of Sudan, The bark of C. sinensis is used for stomach disorders, menstruation, bladder diseases, gastric ulcers and malaria in western Sudan. The goals of this study was to isolate and identify the structures of the most biologically active components against E.coli from the ethanol extract of C. sinensis bark. Bio-autographic technique was used to detect anti-bacterial components of the extracts and a bioassay-guided isolation approach was adopted wherein a combination of solvent–solvent partitioning and gravity column chromatography was used. Collected fractions were continuously screened in vitro for their ability to inhibit bacterial growth Three compounds considered as the most active component against E.coli were isolated. Chemical structures of the isolated compounds were elucidated using UV, IR, Ms and NMR data analysis and were identified as6-[3, 3-Dmethylallyl]-2,3-dihydrokaempfereol (Aromadendrin), 3, 5, 7, 4'-teterahydroxyflavanone (2,3-di-hydroxykaempfereol) and a steroidal saponin (ß-sitosterol-3-O-D-glucoside***

Keywords: bioactive compounds; bioassay-directed isolation; natural products; extraction; fractionation

1. Introduction

The genus *Cordia* belongs to the family Boraginaceae, with some 300 species distributed worldwide, mostly in the warmer regions of the World (Thirupathi et al; 2008)According to a literature survey, several uses in traditional medicine have been reported for different *Cordia* species (Jamkhande *et al*., 2013).The ethnopharmacological and chemotaxonomic importance of the genus *Cordia* led us to investigate the chemical constituents of one of its species, namely *Cordia sinensis*, which is a medicinal plant found widespread in the drier parts of Sudan, Africa and India (**Shagufta** et al; 2011) The bark of *C. sinensis* is used for stomach disorders, menstruation, bladder diseases, gastric ulcers and malaria(**Mothana *et al*; 2012).** A literature survey revealed that very little phytochemical work has so far been carried out on *C. sinensis*. A methanolic extract of this plant showed strong toxicity in the brine shrimp lethality test and on subsequent fractionation, the major toxicity was observed in the ethyl acetate soluble sub-fraction. Further pharmacological screening of this fraction revealed potent antioxidant activity (**Alhadi *et al.2015***) .

Recently, chemists worldwide have paid attention to the potential of medicinal plants as alternative sources for the isolation of novel metabolites with interesting biological and pharmaceutical properties (Neman and Cragg, 2007). Our previous study showed that ***Cordia sinensis* Lam.** exhibited good antibacterial activity against *E. coli.*

Bio-autography is a useful technique to determine bioactive compound with antimicrobial activity from plant extract.TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Rahalison et al.,1991).

Bioassay-directed fractionations results of crude extracts can help isolation of active compounds **(Ragasa *et al;2015)*** It provides evidence of the possible presence of several active compounds in the crude extracts(**Yeng Chen;et al 2011).** In this study we used a bioautographic technique for the isolation and identification of antibacterial compound(s) against *E. coli* from the methanol crude extract of C. ***sinensis***. The structures of the isolated compounds were determined by means of spectroscopic analyses including UV, I.R, MS, ^{1}D- and ^{2}D-NMR techniques.

2. Material and Methods

2.1 Plant Collection

C. sinensis leaves and bark, collected in December 2019,from the forest of faculty of veterinary science

University of Nyala were identified by a botanist, Department of Botany, faculty of Science and Information technology, University of Nyala, - western Sudan. The plant has been deposited in the herbarium of the same department.

2.2 Solvent Extraction

The shade dried plant material (500g) was extracted with methanol (6.0 L, thrice) at room temperature. The combined methanol extract was evaporated under reduced pressure to give a thick gummy mass (50 g) that was suspended in water and successively extracted with *n*-hexane, ethyl acetate and *n*-butanol to afford the corresponding sub-fractions.

2.3 Column Chromatography

The *C.sinensis bark* extract were further subjected to column chromatography. An open glass column (150 by 200 mm) was packed with silica gel (Merck, Darmstadt, Germany, 0.063 to 0.200 mm). The column was eluted with 150 mL methanol-chloroform (10:90, v/v). The fractions were collected in 5 to 10 mL portions depending on the visible changes in the colorful bands running out of the column. Based on TLC profile of fraction the second column chromatography was performed to further dissociate a few fractions. Then each fraction again re-dissolved in the methanol and test for antibacterial activity against *E. coli* using the disc diffusion method and bio-autographic technique **(Edinardo,*et al.*,2013)**

2.4 Microorganism preparation and antibacterial test

Two *ml* of the standardized bacterial stock suspension were thoroughly mixed with 250 *ml* of a sterile melted nutrient agar and maintained at 40°C. Twenty *ml* aliquots of the inoculated media were poured into a sterile Petri dish (9 *cm* in diameter). The agar was left to set, and in each of these plates, four holes (10 *mm* in diameter) were made using a sterile cork- borer and the agar discs were removed. Alternate holes (wells) were filled with 0.1 *ml* (100 μl) of 100 *mg/ml* samples of each of the extracts and allowed to diffuse at room temperature for two hours. The plates were then incubated, in the upright position, at 37°C for 18 hours. Three replicates were carried out for each extract against the test organism simultaneously; controls involving the addition of the respective solvents instead of the extracts were carried out Fig.2.

2.5 Bioassay guided fractionation of *Cordia sinensis* ethyl acetate fraction, isolation and purification of antibacterial biological active compounds

To isolate and purify the most biologically active compounds from *C. sinensis*, gravity column chromatography was used. The dried ethyl acetate fraction produced from the solvent– solvent partitioning step (and shown in vitro using bioautography assay to the most potent constituent against *E.coli*) was fractionated by primary column chromatography designated column 1, eluted with CHCl3, CHCl3-MeOH and MeOH in increasing order of polarity to obtain five fractions I–V. Fraction I obtained from CHCl3-MeOH (9.9:0.1) was further purified by column chromatography eluting with CHCl3-MeOH (9.7:0.3) to obtain two components 1(10 mg) and **2** (25 mg) from the top and the tail fractions, respectively. Fraction II obtained from CHCl3-MeOH (9.8:0.2) was a mixture of two components, which were separated by column chromatography using the solvent system CHCl3-MeOH (9.5:0.5). Upon TLC chromatography test it was obvious that one of the two components in the mixture of fraction II is identical to component 2 obtained from fraction I**.** To monitor separation of compounds during fractionation, thin layer chromatography (TLC) was employed. all solvents used were technical grade, All column chromatography fractionation steps were carried out using silica gel 60 F254 (0.063– 0.2 mm particle size, Merck (Germany).

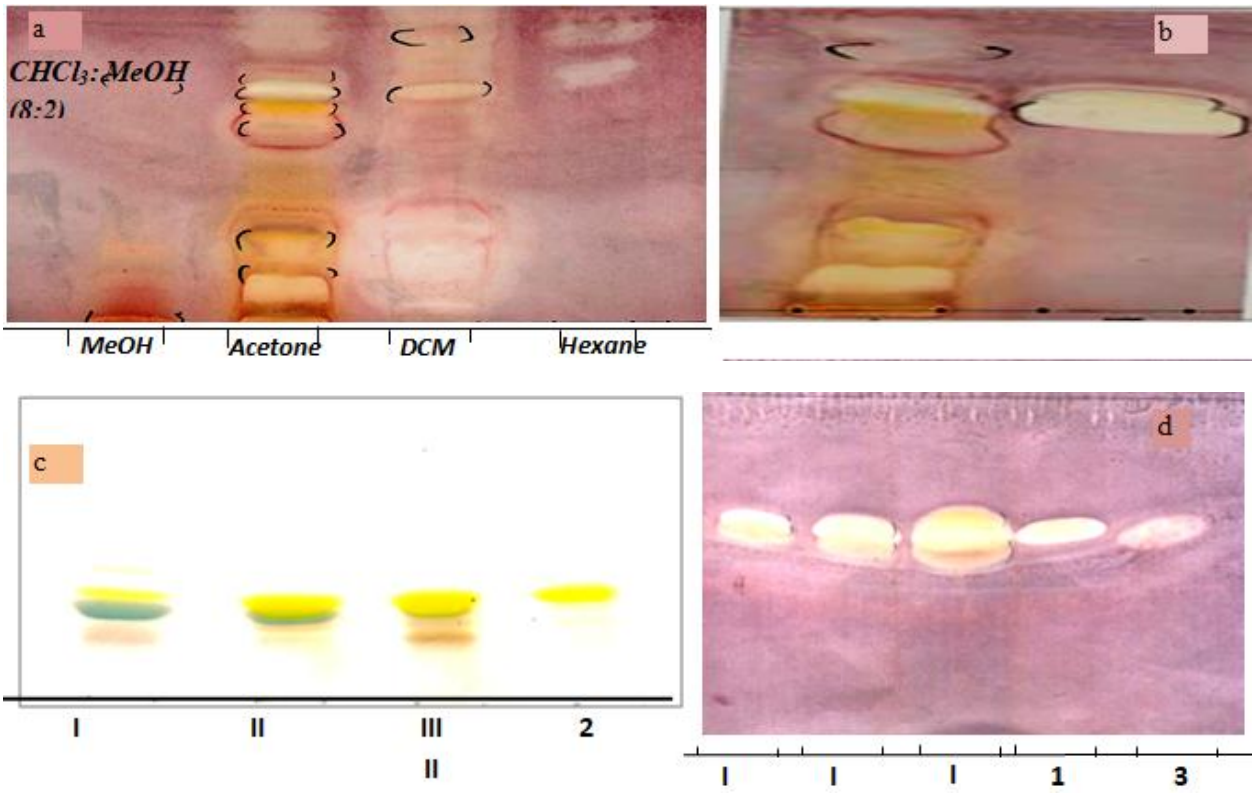
2.6 Sructural identification tools.

dentification of compounds involved a combination of various techniques including nuclear magnetic resonance (NMR), mass spectrometry(MS),ultraviolet(UV) and infrared(IR) spectrometry. NMR both 1H and 13C (one-dimensional spectroscopy) spectra for the isolated compounds were determined on Mercury-200BB "plantkmr-up-ac-za". IR spectra of the isolated compounds were recorded in KBr discs using Fourier Transform Infrared spectrophotometer (FTIR-8400S).

3. Results and Discussion

3.1 Results

From an initial 500 g of dried bark of *C. sinensis*, 41.7 g of crude acetone extract was obtained. Subsequent solvent–solvent partitioning of crude extract (41.7 g) yielded four fractions, namely hexane (8.2 g), acetone (14.2 g), DCM (10.1 g), and methanol (9.2 g). Following the solvent–solvent partitioning procedure the next step was utilized to identify the most potent fraction, as observed by inhibition of *E coli* growth using TLC- autobiographic procedure



Extracts (Fig.1a)

Figure 1: Bioassay guide fractionation and isolation of the most biologically active compounds against *E.coli* from crude extracts and acetone fraction of *C. sinensis* bark (a) Bioautogram of serial exhaustive extraction extracts against *E.coli* (b) indicated that compound 1 was the major component of the active acetone extract. (c) TLC of supernatant solution of fractions I, II and III with compound 2 d) Bioautoghraphy of Supernatant solution of Fractions I, II and III with compounds 1 and cpd3 the bioautographic screening, the chromatogram was developed with $CHCl_3$:

Bioautography

MeOH (8:2). The bacterium *E.coli* was used as the indicator organism for screening of the four crude as shown in Fig 1 above.

Column Chromatography Fractionation of acetone extract

Acetone extract (14 *g*) was packed onto a silica gel column and eluted with a mixture of solvents (hexane, ethyl acetate and methanol). The first samples collected were eluted with hexane: EtOAc (9:1) followed by the ratios: (8: 2), (7: 3), (6: 4), (4: 6), (2: 8), 100 % EtOAc.Atotal of 30 fractions were collected and recombined based on their TLC profiles which were regrouped into five major fractions.

Isolation of Bioactive Compounds

Compound **1** was collected from fraction (**FI)** as crystals and washed with hexane /EtOAc (7:3) to give 20 *mg*. and 10*mg* The bioautogram (Fig.1.b) indicated that compound **1** was the major component of the active acetone extract. Sediment that appeared to be a precipitated compounds, were filtered out from fractions **II** and **III**, and washed with 100% hexane and a combination of hexane and EtOAc gave 80 *mg* of a yellow powdered compound coded as compound **2** and 85 *mg* of a white powdered compound identified as compound **3**.

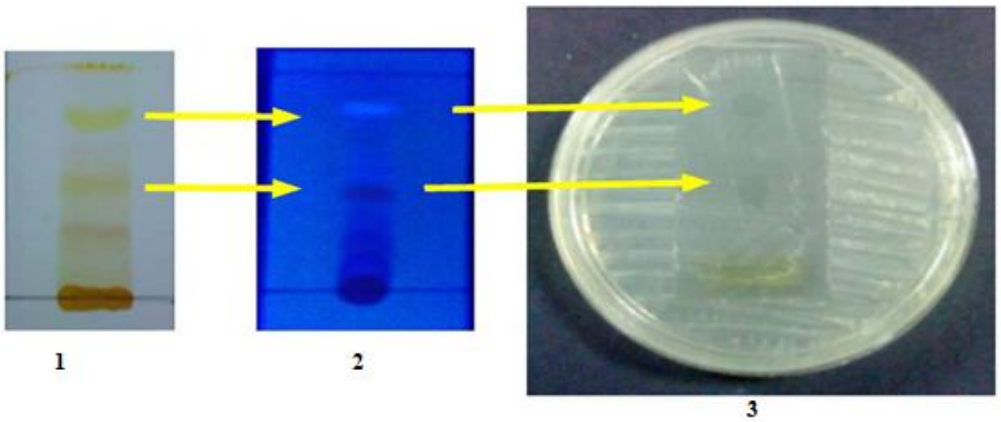


Figure 2: TLC bioautogram showed the spots with the most antibacterial activity from acetone fraction of *C.sinensis* stem bark . 1- TLC chromatogram obtained from acetone fraction before subject to column fractionation 2- TLC chromatogram in 1 visualized under UV lamp (365nm) 3- Inhibition zones of the bacterial growth by agar diffusion or contact bioautography

The spectral data of the isolated compounds:

Compound **1** was crystallized from fraction **FI** of the first silica gel column and from column III as colourless crystals and showed characteristic flavonoid colour reaction on TLC.

It has a melting point of 200 - 202℃. Its UV data showed one major peak at 295 *nm* (Band II) with a shoulder corresponding to 2, 3-dihydroflavonol (flavanonol) skeleton (Mabry *et al*., 1970). IR of its spectrum showed absorption bands at 3552 cm^{-1} (OH), 1616 cm^{-1} (C=O) and at 1521 cm^{-1} for aromatic structure. Molecular formula deduced was $C_{20}H_{20}O_6$ based on Molecular ion peak at *m/z* 356.14 from LREIMS ^{1}H-NMR spectral data showed signals of 1,4-disubstituted ring B, of 2'/6' and 3'/5' protons at δ 7.40 and δ 6.89 (2H each m) respectively. The singlet signal at δ6.01 was attributed to H-8 which suggested the presence of a substituent at C-6, and signals at δ 4.64, 5.06 (d each, J = 11.6 *Hz*) were attributed to H-3 and H-2 respectively and indicated the dihydroflavonol (Mabry *et al.*, 1970). The coupling constant (J = 11.6 *Hz*) indicated *trans* configuration of the two protons. ^{1}H-NMR also showed signals at 5.23(1H, m), 3.26 (2H, d, 6.8 *Hz*) and two methyl signals at 1.75 and 1.64 (s each). The foregoing data indicated the presence of 2, 3-dihydrokaempferol nucleus with 3, 3-dimethylallyl group. The 3,3-dimethylallyl group was located at C-6, based on ^{13}C-NMR spectra by the fact that the resonance of C-6 was shifted down field to 108.71 ppm relative to the corresponding carbon of compound

The ^{13}C-NMR spectra confirmed the presence of dihydro-kaempferol skeleton (Agrawal, 1989) and showed carbons at δ_c 83.7 (C-2); 72.5 (C-3); 161.3 (C-5); 108.7 (C-6); 164.6 (C-7); 94.8 (C-8); 158.2 (C-9); 130.8 (C-2'/6'); 115.2 (C-3'/5'), along with two methyl carbons at δc 17.2 and 25.2 and signals for (CH) at 122.7, (CH_2) at 20.9 and C at 130.7 ppmThe spectral data of ^{1}HNMR and ^{13}CNMR are displayed on table1&2.Compound1 is a flavanol which was identified as 6-[3,3-Dimethylallyl]-2,3-dihydrokaempfereol .(Fig.3,1.) represent the proposed structure of compound 1

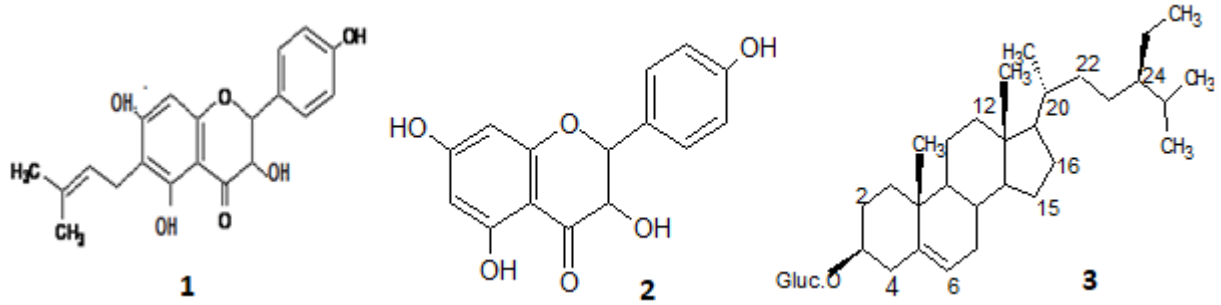


Figure 3: The proposed structures of three isolated compound from the acetone fraction of ***C. sinensis*** **stem bark. Compound1** as 6-[3,3-Dimethylallyl]-2,3-dihydrokaempfereol.**compound2** as2,3-dihydrokaempferol commonly known as (aromdendrin) **compound 3 as** β-sitosterol-3-O- β-D-glucoside

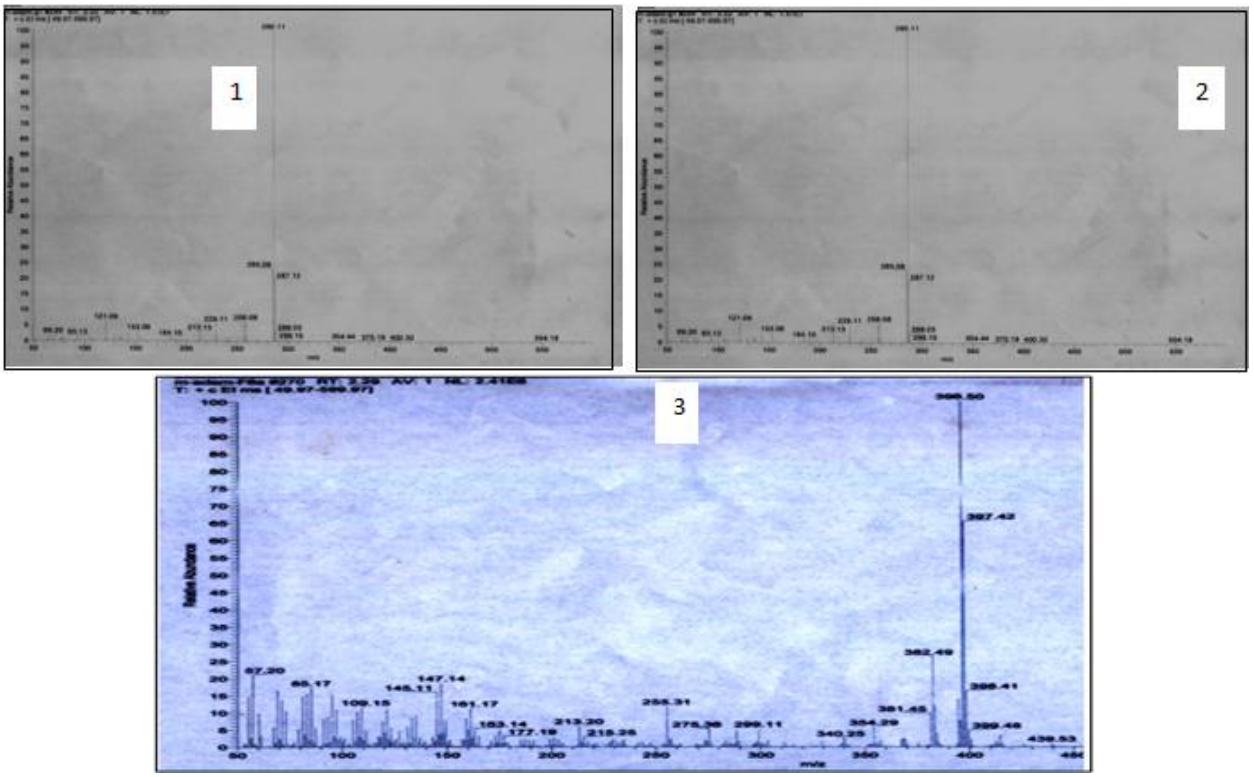


Figure 4: LREIMS spectrum of the isolated compounds 1, 2 & 3

Characterization of Compound 2

Compound2 was isolated from columns I and II as a colourless solid, mp. 218 - 220°C. It gave a deep purple fluorescence under UV-light at 366 *nm* and showed deep red colour on a TLC plate sprayed with vanillin /H_2SO_4. Its UV-spectrum showed absorption maxima at 290 *nm* (Band II with a shoulder as Band I) corresponding to 2, 3-dihydroflavonol (Mabry *et al.*, 1970). The IR spectrum of compound 2 showed absorption bands at 3442 cm^{-1} (OH), 1637cm^{-1} (C=O) and at 1521 cm^{-1} for aromatic structure. ^{1}H-NMR data (Table**1**) and spectra showed two aromatic proton signals at δ 7.42 and 6.90 (two protons, each m, H-2'/6' and H-3'/5'); two signals *meta*-coupled at δ 5.94 and 5.99 (each d, 2.2*Hz*) corresponding to H-6 and H-8 respectively, and two vicinal signals coupled at δ 5.08 and 4.66 (each d, 11.8 Hz) corresponding to H-2 and H-3 respectively. The coupling constant value between H-2 and H-3 (J = 11.8 Hz) indicated *trans* configuration of both protons (Mabry *et al.*, 1970).

The ^{13}C-NMR spectral data (Table) indicated the dihydrokaempferol structure for compound **2** (Agrawal, 1989). This was confirmed with LREIMS analytical data which gave a molecular formula of $C_{15}H_{12}O_6$ (m/z 288.12)

The characterization of this compound was further confirmed by comparison of its UV-, ^{1}H- and ^{13}C-NMR with literature data (Shen and Theander, 1985 and El-Sohly *et al*., 1999) and was identified as 3,5,7,4'-tetrahydroxyflavanone or 2,3-dihydrokaempferol commonly known as(aromdendrin). It is reported for the first time in *C. sinensis*.

Characterization of Compound 3

A steroidal saponin was isolated and indentified as β -sitosterol-3-O-β-D-glucoside. Its structure was presented in Fig1,3. Compound **3** was isolated as a colourless solid precipitate from fraction **F**III. It had a melting point of 278-280°C [(Lit. 284-286), (Shen *et al*., 2008)].

The IR- spectrum showed absorptions at 3415 cm^{-1} (OH), 2988 cm^{-1} (CH_2), 1647 cm^{-1} (C=C) and 1255 cm^{-1} (C-O). steroidal skeleton was easily deduced from the ^{1}H-NMR spectral data (Table **3)** by the appearance of vinylic (R_2C=CHR, δ_H5.31) and hydroxylated methane (RCHOH, δ_H3.03). There was also a crowded signals between δ_H0.5 and δ_H2.0 ppm indicating the presence of -CH_3, -CH_2 and -CH protons included the singlet signal at δ_H0.63 for (CH_3-18) protons and five multiplet for the five methyl protons..

The ^{13}C-NMR (Table3) and DEPT spectra confirmed the presence of 29 carbons: six methyls, eleven methylenes, nine methines and three quaternary carbons one of which (C-5) was olefinic (δ_C140.14) and two at 36.18 (C-10) and at 41.82 ppm for C-13. Confirmation of structure was done by LREIMS (Fig.), which showed the base peak and molecular ion at *m/z* 396 [M^+-sugar moiety] coesponding to $C_{29}H_{48}O_6$.

Final attribution of ^{1}H-, ^{13}C-, DEPT-135 and LREIMS of compound3 were in complete agreement with literature data (Kojima *et al.*, 1990 and Shen *et al.*, 2008). Therefore, compound **3**was elucidated as β-sitosterol-3-O-β-D-glucoside, and it was the first report of its isolation from *C. sinensis.*

Table 1: ^{1}H-NMR (200 MHz) spectral data of compounds **1**, **2.**

Positions	Compounds	
	1 ^{1}Hδ(JinH$_z$)	2 ^{1}Hδ(JinH$_z$)
2	5.06(1H,d,11.6)	5.08 (1H,d,11.8)
3	4.64(1H,d,11.6)	4.66(1H,d,11.8)
6	-	5.94(2H,d,2.2)
8	6.01(1H,s)	5.99(2H,d,2.2)
2'/6'	7.40(2H,m)	7.42(2H,m)
3'/5'	6.89(2H,m)	6.90(2H,m)

Table 2: ^{13}C-NMR Spectral data of Compounds **1**, **2**.

Positions	Compounds	
	1 δC(ppm)	2 δC(ppm)
2	**83.7**	**84.3**
3	**72.5**	**73.1**
4	**198.0**	**198.2**
5	**161.3**	**164.9**
6	**108.7**	**97.04**
7	**164.6**	**167.8**
8	**94.8**	**95.9**
9	**158.2**	**158.8**
10	**97.1**	**101.9**
1'	**129.6**	**129.1**
2'/6'	**130.8**	**130.3**
3'/5'	**115.2**	**115.9**
4'	**158.1**	**158.8**
1"	**…..**	**…..**
2"	**…..**	**…..**
3"	**…..**	**…..**
4"	**…..**	**…..**
5"	**…..**	**…..**
6"	**…..**	**…..**
1"'	**20.9**	**…..**
2"'	**122.7**	**…..**
3"'	**130.7**	**…..**
4"'	**17.2**	**…..**
5"'	**25.2**	**…..**

Table 3: ^{13}C-NMR (50 MHz) and ^{1}H-NMR (200 MHz) spectral data of Compound **3**

Position		**^{13}C(δ_c)**	**^{1}H(δ_H)**
C-1	CH_2	36.18	4.86
C-2	CH_2	31.39	1.43
C-3	CH	67.72	3.03
C-4	CH_2	36.80	2.10
C-5	C	140.41	-
C-6	CH	121.17	5.31
C-7	CH_2	33.32	1.75
C-8	CH	33.32	1.43
C-9	CH	49.57	1.35
C-10	C	36.18	-
C-11	CH_2	20.56	1.40
C-12	CH_2	39.49	1.36
C-13	C	41.82	-
C-14	CH	56.15	1.40
C-15	CH_2	25.40	1.47
C-16	CH_2	28.67	1.48
C-17	CH	55.39	1.47
C-18	CH_3	11.64	0.63
C-19	CH_3	19.06	1.26
C-20	CH	35.45	1.64

C-21	CH_3	18.90	0.93
C-22	CH_2	38.26	1.25
C-23	CH_2	23.84	1.25
C-24	CH	40.75	1.46
C-25	CH	29.23	1.75
C-26	CH_3	19.68	0.89
C-27	CH_3	21.30	0.89
C-28	CH_2	22.57	1.29
C-29	CH_3	11.75	0.89
1’		100.75	
2’		73.44	
3’		76.57	
4’		70.07	
5’		76.72	
6’		61.50	

4. Discussion

Extracts of plants are inherently a complex mixture of different compounds. For the purpose of drug discovery these mixtures of compounds need to be separated and the active compounds be isolated and subsequently identified. Due to the fact that plant extracts usually occur as a combination of various type of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds **(Khan et al; 2021).**

It is a common practice in isolation of the bioactive compounds that different separation techniques such as TLC, column chromatography, flash chromatography Sephadex chromatography and HPLC, should be used to obtain pure compounds, the pure compounds are then used for the determination of structure and biological activity(**Sasidharan et al., 2011**).

While bioassay-guided fractionation can be criticized for being time consuming and resource intensive, the approach compensates for these shortcomings by its effectiveness (Moyo et al 2019). For instance bio-autography allows localizing antimicrobial activities of an extract on the chromatogram, it supports a quick search for new antimicrobial agents through bioassay-guided isolation (Cos et al., 2006). The bioautography agar overlay method is advantageous in that, firstly it uses very little amount of sample when compared to the normal disc diffusion method and hence, it can be used for bioassay-guided isolation of compounds. Secondly, since the crude extract is resolved into its different components, this technique simplifies the process of identification and isolation of the bioactive compounds (Rahalison et al., 1991). The inhibition zones produced on TLC plates by bioautographic technique will be use to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to *Rf* values.

In this study, the bioassay-guided procedure was used to isolate and identify three compounds from *C. sinensis* stem bark. primarily active in vitro against *E. Coli.*

Four solvents were used to extract secondary metabolites from stem bark of the plant under investigation. Acetone and DCM solvents gave higher extractability than hexane and methanol (Fig. 1a).Hexane extract exhibited only a limited activity. The four extracts, except MeOH extract, indicated at least one active compound each in the non-polar region of the chromatogram (Fig.1a). Acetone extract indicated the highest number of active compounds followed by DCM extract. This solvent system gave good separation of compounds against *E. coli* as indicated on the bio-autogram (Fig.1,b).The acetone and DCM extract were the only extracts that indicated promising activity in the bioautography results. Acetone extract was chosen for further investigation because of its high yield (14.2 *g*) and the highest number of active compounds compared with DCM extract. The crude extracts of hexane and MeOH extracts were not promising (at least in this study) because they did not show any marked activity(Fig,1a).

compound1and compound2 were identified as substituted flavanols(**figs.3&4,tables1&2)** ,whereas compound3 identified as a steroidal saponin(**figs.3&4,table3).** Compound1 is the most abundant (*20mg*) and hence the most biologically active against *E.coli,* It showed a broad spectrum antimicrobial activity against the tested bacteria, and polar fraction exhibited better bacterial activity against *E. coli.* Compound2 was the second in order of performance against the inhibition of the tested bacteria growth.

The results of this study are in agreement with several researcher who reported the efficiency of phenols and flavonoids against bacteria and other organisims. Phenolic compounds, including tannins and flavonoids, have demonstrated their therapeutic potential as anti-inflammatory, antifungal, antimicrobial, antioxidant, and wound-healing agents (Phanankosi et al., 2019).The secondary metabolites such as flavonoids and phenols are effective antibacterial substances due to their ability to form complexes with extra cellular and soluble proteins and to form complex with bacterial cell walls leading to the death of the bacteria(Leonor et al; 2017). According to Cushnie and Lamb (2005) the antibacterial activity of flavonoids has been increasingly more documented. Many researchers are a step further, where they have isolated and identified the structures of commercially available flavonoids, such as rutin, quercetin, 3-O-methylquercetin, and various glycosides of quercetin .The activities of these phytochemical components may be responsible for the antibacterial activities observed in the study.

The isolation and identification of these compounds from *C. sinensis* represents a contribution to the phytochemical analysis of the components of this plant and might be useful in further chemotaxonomic studies on the genus *Cordia.*

5. Conclusion

The current study had clearly shown that bioassay-guide strategy was successfully adopted and led to the isolation of two substituted flavanols and a steroidal saponin. The isolated components were all biologically active against *E. coli.* The isolation of biologically active phyto- constituents from plants will help in understanding the mechanism of action and also identification of lead molecules of clinical utility.

6. Authors' Contributions

M.S.K , N.A.E conceived the idea , designed the study carried out the practical and technical work .Z.A.E review the draft manuscript. All authors wrote final version of the manuscript. All authors read and approved the final manuscript

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