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Two Flavones from *Acanthospermum hispidum DC* and Their Antibacterial Activity

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Abstract

Two new flavones, namely 5,7,2',5'-tetrahydroxy-3,4'-dimethoxyflavone and 5'-acetoxy-5,7,2'-trihydroxy-3, 4'-dimethoxyflavone were successfuly isolated from the leaves of *Acanthospermum hispidum DC* and identified by UV-Vis, IR, ^{1}H-NMR and EI-MS techniques. Both compounds exhibited antibacterial activity against Salmonella typii, Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Bacillus subtilis, Pseudomonas aeruginosa and Shigella dysenteriae with minimum inhibition concentration (MIC) ranging from 0.001 - 0.20 but was inactive against Escherichia coli, Corybacterium pyogenes and Proteus vulgaris.

Keywords: *Acanthospermum hispidum DC*, Flavonoids, Antibacterial, Spectroscopy

1. Introduction

Although the main use of plants is directed to nutritional purposes; however they are also used at various religious functions, for economic purpose and more importantly for medicinal purposes [1]. Nowadays, pathogenic microorganisms are acquiring multiple resistances to existing antibiotics at an alarming rate globally [2]. All these factors necessitated the search for alternative medicine of natural origin for the treatment of microbial infections and traditional folk medicine is being used as a guide. Many herbs used in folklore for the treatment of diseases in different parts of the world are being screened for antimicrobial activities and the results obtained from these scientific studies so far have rationalized the tradomedical use of many plants and plant parts [3-5].

Acanthospermum hispidum DC is a member of the family of plants called *Asteraceae*. It is found in East, West and Central Africa [6]. In USA, it is found in Georgia, Virginia, Florida, New Jersey and Alabama [7]. Other countries where it is found include Paraguay, Argentina, Bolivia, India, Brazil, Nicaragua, Honduras, Hawaii, Australia and Venezuela [8].

In the specific case of *A. hispidum*, there is a huge volume of literature related to research on numerous chemical components extracted from this plant, as well as chemotaxonomic and phytotherapeutic investigations. The oldest phytochemical studies dated to 1975 when investigations concerning sesquiterpene lactones with potential biological activities were reported [9] and in 1976 further studies on *A. hispidum* were published [10,11]. The sesquiterpene lactones found in *A. hispidum* were chemically distinct from other sesquiterpenoids due to the presence of a α-methylene-γ-lactone system, many of them containing α,β-unsaturated carbonyl and epoxides, which are part of a larger family of compounds with a wide spectrum of biological activities. Investigations of species of *Acanthospermum* have led to the isolation of *cis-cis*-germacranolides and melampolides [8]. Additional compounds isolated from *A. hispidum* are alkaloids [12], polyphenolic constituents (flavones, caffeic acid and phenylpropans, acanthospermol galactoside [13], sesquiterpene hydrocarbons, β-caryophyllene, α-humulene, bicyclogermacrene, germancrene D, α-isabolol, nonanal, carvacrol and methyl carvacrol [8], saponins and lipids [14-20].

Many studies have shown that flavonoids play important pharmacological roles against various human diseases, such as cardiovascular disease, cancer, inflamemation and allergies [21,22]. The aim of the present study consists of the investigation of the flavonoidal constituents of the ethyl acetate fraction of the methanolic leaf extract of *Acanthospermum hispidum DC* that grows in Nigeria. The antibacterial activity of the isolated compounds is also reported.

2. Experimental

2.1. General

Melting points were determined using a Stuart Model SMPI (MG155) instrument and are corrected against the temperatures of certified reference standards. The elemental analyses were carried out using a Carlo Erba 1106 Elemental analysis. The IR spectra were recorded on a Perkins Elmer 257 spectrophotometer. UV-Vis spectra were determined using a Pye Unicam, Hetr spectrophotometer. ^{1}HNMR spectra were recorded in Methanol-d_4 on a Varian Oxford NMR YH 200 instrument operating at 500 MHz. δ values are given in ppm, using TMS as internal standard. EI mass spectra were measured on a Bruker FTMS 4.7 T mass spectrometer. Merck silica gel was used for TLC. Spots were detected on TLC plates by using iodine tank. Column chromatography (CC) was carried out on silica gel 60 (70 - 230 mesh).

2.2. Plant Material

The plant material used in this study was the leaf of *A. hispidum.* This material was taken to Department of Pure and Applied Biology of Ladoke Akintola University of Technology, Ogbomoso, Nigeria for identification.

2.3. Extraction and Isolation

The dried plant leaves (1 Kg) was first extracted with *n*-hexane for 24 h, then with MeOH for 24 h using cold extraction method. The crude extract was subjected to accelerated gradient chromatography (AGC) to afford 100 fractions of 10 ml each. Fractions 74 - 77 were bulked together and coded as EA1 then further separated on open column using EtOAc: MeOH 9:1 v/v as eluent. This afforded 20 fractions coded as EA1(A-M). Fractions EA1(F-H) were bulked together based on similar R_f values and further chromatographed on open column using EtOAc: DCM: MeOH 5:4:1 v/v/v as eluent to yield 10 fractions coded EA1(F-H) (1 - 10). Fractions EA1(F-H)(10) were recrystalized from methanol to yield compound **1** (467mg) as a white amorphous powder (found: C, 58.99; H, 4.06; O, 36.95; Calc. for $C_{17}H_{14}O_8$: C, 58.96; H, 4.05; O, 36.99), m.p: 169˚C - 171˚C; MW: 346; UV λ_{max} MeOH (nm) logε: 259 (3.02), 325 (2.12); IR (KBr) cm^{-1}: 3422, 1645, 1560, 1412, 1162, 1086; ^{1}HNMR (500MHz): 6.20(s), 6.33(s), 6.56(s), 6.91(s), 3.71(s)(3H), 3.88(s)(3H); EI-MS, (70Ev): m/z: 346 $[M]^+$ (38), 329 (42), 315 (100), 303 (30), 194 (20), 174(30), 167 (18), 153 (32), 69 (77).

Fractions EA1(I, J) were combined and further purified on open column using EtOAc: MeOH: $CHCl_3$ 6:1:1 v/v/v as eluent to afford 10 fractions coded EA1 (I, J)(1-10). Fractions EA1(I, J)(5) were recrystalized from methanol to afford compound **2** (449mg) as a white amorphous powder ((found: C, 58.80; H, 4.12; O, 37.08. Calc. for $C_{19}H_{16}O_9$: C, 58.77; H, 4.15; O, 37.08), m.p: 182^{o}C - 184^{o}C; MW: 388; UV λ_{max} MeOH (nm) logε: 255 (3.31), 338 (2.34); IR (KBr) cm^{-1} : 341, 1772, 1646, 1436, 1162, 1086; ^{1}H-NMR (500MHz): 6.20(s), 6.28(s), 6.85(s), 7.12(s), 3.61(s)(2H), 3.90(s)(3H), 2.15(s)(3H); EI-MS, 70Ev, m/z: 388$[M]^+$, (33), 346 (75), 329 (35), 315 (100), 194 (20), 179 (30), 167 (18), 153 (32), 69 (77).

2.4. Organisms

Clinical isolates of *Salmonella typii*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Bacillus subtilis*, *Corynebacterium pyogenes*, *Proteus vulgaris*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from Baptist Medical Center, Ogbomoso, Nigeria.

2.5. Preparation of the Medium

Nutrient agar medium was prepared by dissolving 2.8 g of nutrient agar in 100 ml of distilled water. The medium was sterilized in an autoclave at 121˚C for 15 minutes. It was cooled to 45˚C and poured into sterile Petri dishes to solidify.

2.6. Preparation of Test Samples

10 mg each of the extracts was dissolved in 10 ml each of redistilled solvents (chloroform, ethyl acetate methanol and distilled water). The activity of Streptomycin, a standard antibiotic was also determined and used as the positive control; 10.0 mg of it was dissolved in 10 ml of distilled water. Distilled water and redistilled solvents were used as negative control.

2.7. Disc Diffusion Test

Disc diffusion method was employed [23]. This involved the use of filter paper disc as carrier for the antibacterial agents. Sterilized discs cut from Whatman No. 1 filter paper were impregnated with solutions of the antibacterial agents. The solvent was evaporated and the disc dried properly. The nutrient agar medium was inoculated with the test organism and the impregnated disc placed on the surface of the nutrient agar. The antibacterial agent upon contact with the agar diffused into all directions. The ability of the test organism to grow or not in the presence of the test sample was then determined

within 24 hours by measuring the zones of inhibition. The plates were incubated upside down at 37°C. All tests were done in quadruplicate and the antibacterial activity was expressed as a mean of inhibition diameters (mm) produced by the leaf extracts with streptomycin as the standard antibiotic.

2.8. Minimum Inhibitory Concentration (MIC)

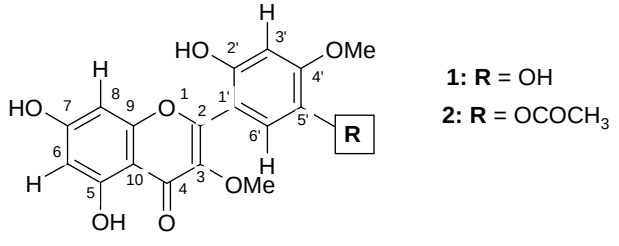
The minimum inhibitory concentration (MIC) of the extracts against the sensitive microorganisms was determined using the disc diffusion method. Serial dilutions of the isolated compounds were prepared (10.0, 5.0, 2.5, 1.25, 0.625, 0.313 and 0.156 mg/ml). Each of the innocula was poured into each Petri-dish and the agar was later poured and allowed to set. Wells were made using the sterile 3 mm cork borer. Serial dilutions of the isolated compounds were added into the marked wells. The plates were incubated at 37˚C for 24 h. The growth was observed to determine the sensitivity of the microorganism using clear zones of no microbial growth. The least concentration of the extracts that had inhibitory effect was taken as the minimum inhibitory concentration (MIC) of that extract against such microorganism.

3. Results and Discussion

3.1 Structural Elucidation

Our flavones were isolated from the ethyl acetate fraction obtained from fractionation of the crude methanolic leaf extract of *A. hispidum*. Compounds **1** and **2** were obtained as white amorphous powder after repeated column chromatography. Their assigned structure is shown in **Scheme 1**.

Thus, the structure of compound **1** was established based on elemental analysis, UV-Vis., IR, ^{1}HNMR and MS data. The EI-MS of compound **1** exhibited a molecular ion peak at *m/z* 346 $[M]^+$ (calc. 346.291) as well as elemental analysis (found: C, 58.99; H, 4.06, O, 36.95; required: C, 58.96; H, 4.05, O, 36.99). Data provided by ^{1}H-NMR (**Table 1**) indicates the presence of four aromatic protons (δH 6.20 (s), 6.33(s), 6.56(s), 6.91(s)) which are typical of protons at C-6, C-8, C-3' and C-6' of a flavone skeleton [24] and two methoxy signals at δH 3.71(s)(3H) and 3.88(s)(3H). Based on the structural pieces of information from ^{1}HNMR, coupled with the use of the 'rule of 13' [25], the molecular formula was established to be $C_{17}H_{14}O_8$.



1: R = OH

2: R = $OCOCH_3$

Scheme 1. Structure of Compounds 1 and 2.

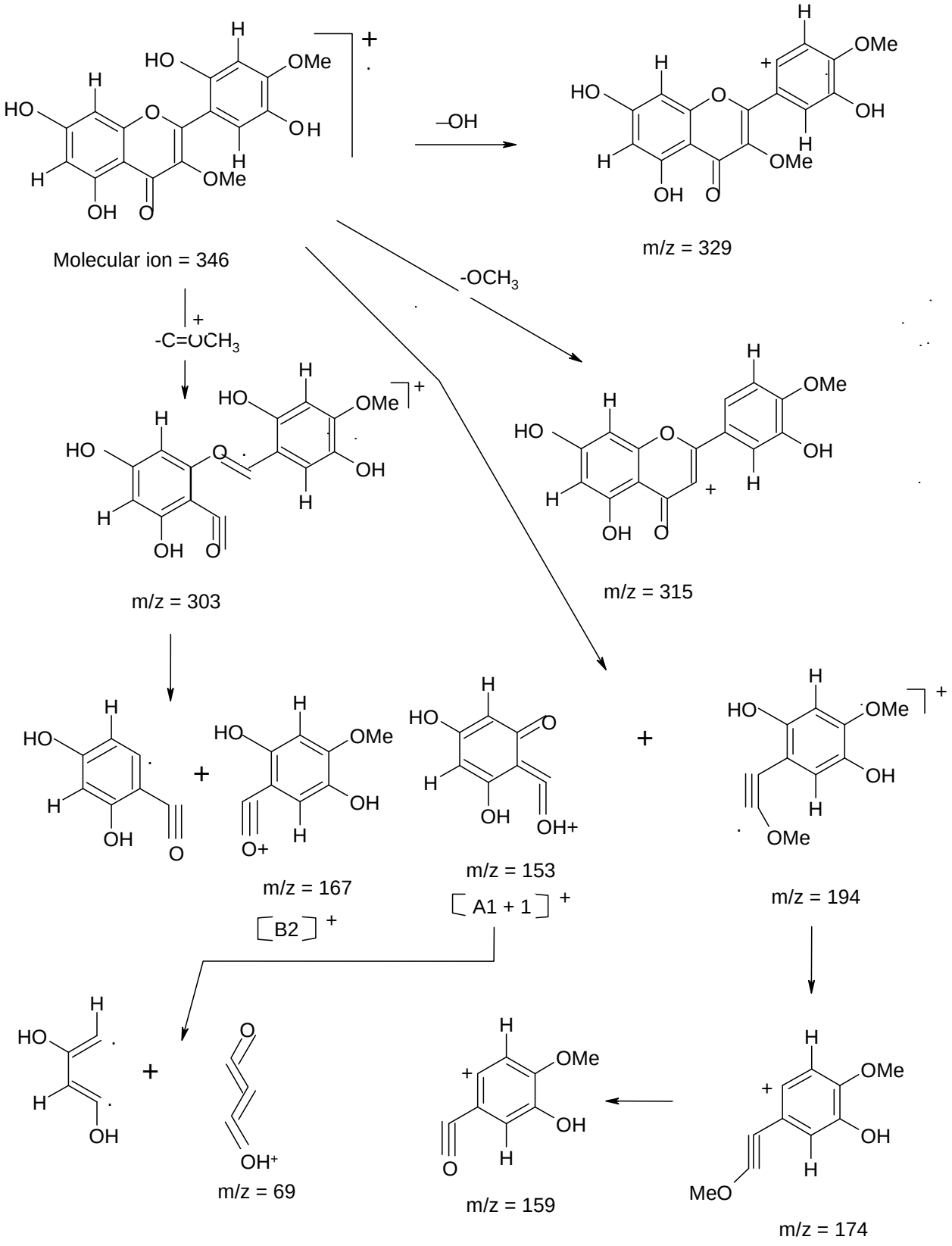
The IR spectrum (KBr) of **1** revealed the presence of hydroxyl (3422 cm^{-1}) and carbonyl (1645 cm^{-1}) groups. Bands in the range 1645 - 1065 cm^{-1} are typical of a flavone skeleton [24].

Next, the UV spectrum of **1** exhibited absorption maxima at 259 nm (band II) and 350 nm (band I) that are characteristic absorption bands of a flavone skeleton [17]. No shift in band I was observed after the addition of $AlCl_3$ and $AlCl_3$/HCl, suggesting a hydroxyl-keto complex formation at the C-5 hydroxyl [17, 26]. The UV spectral data recorded with various shift reagents clearly indicated a free hydroxyl group at C(5) and C(7) with no *ortho*- dihydroxyl groups on rings A and B. These findings were also supported by observation of an EI-MS fragment at *m/z* 153 $[A1 + 1]^+$, which accounts for a ring A fragment with free hydroxyl groups at C(5) and C(7) [27]. Characteristic losses of $[M - 17]^+$ at *m/z* 329 and $[M-31]^+$ at *m/z* 315 were signs of 2'-OH and a 3-OMe system [17]. The second methoxy group was placed in position 4' based on an EI-MS fragment at m/z 167 $[B2]^+$ which accounts for a ring B fragment. Thus, compound **1** was finally identified as 5,7,2',5'-tetrahydroxy-3,4'-dimethoxyflavone. The fragmentation pattern is as shown in **Scheme 2**.

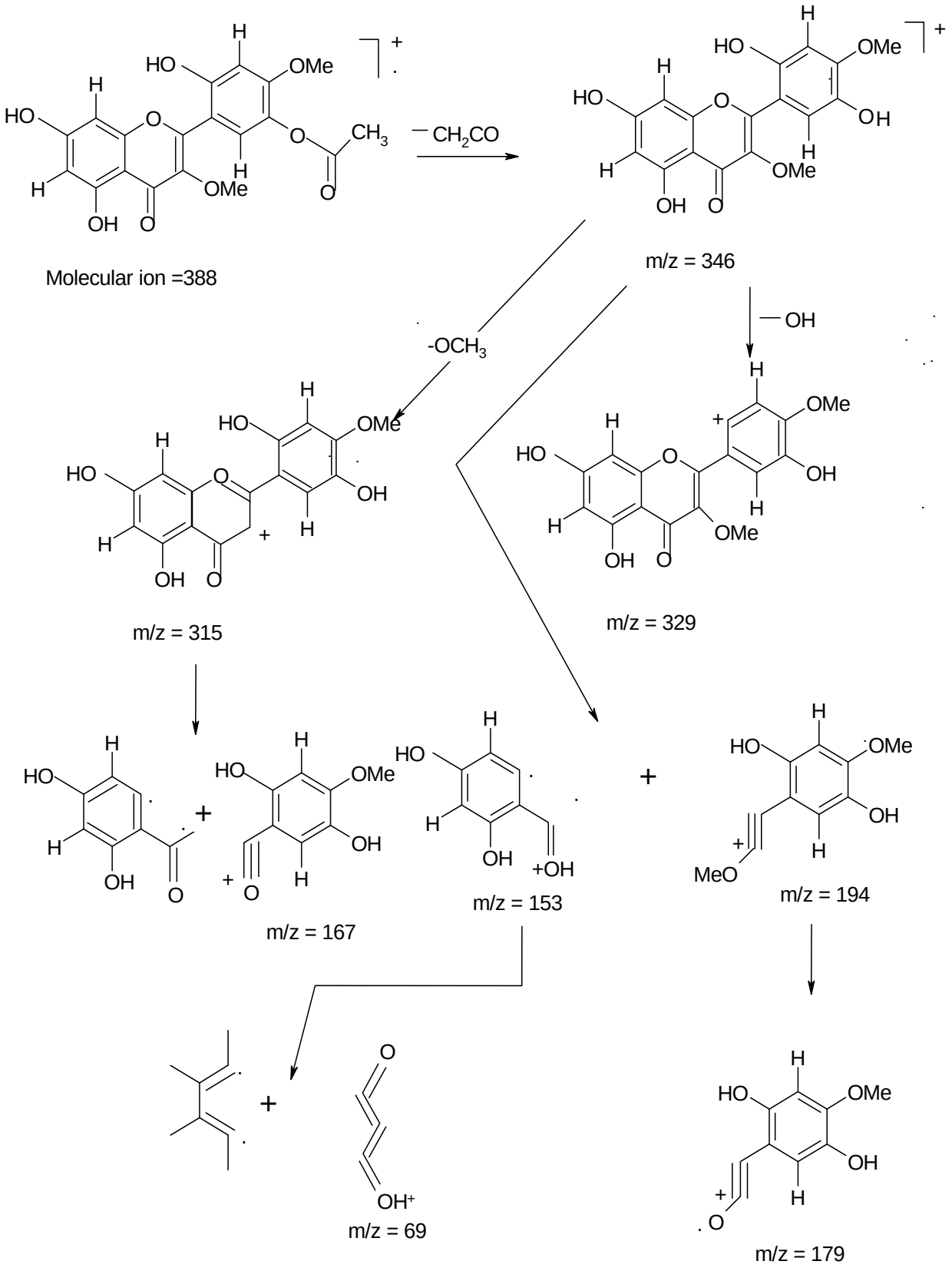
Similarly, the structure of compound **2** was established based on elemental analysis, UV-Vis., IR, ^{1}HNMR and MS data. The EI-MS of compound **2** gave a molecular ion peak at m/z 388 $[M]^+$ (calc: 38.328) as well as the elemental analysis (found: C, 58.80; H, 4.12; O, 37.08; required: C, 58.77, H 4.15; O, 37.08). The ^{1}H-NMR data (**Table 1**) exhibited a flavonoid pattern and showed signals at δ_H (ppm) 6.20(1H, s), 6.28 (1H, s), 6.85(1H, s) and 7.12(1H, s) typical of protons at C-6, C-8, C-3' and C-6' of a flavone skeleton [24]. Chemical shifts at 3.90 ppm and 2.15 ppm suggested that there are substitutions

Table 1. Relevant proton NMR data of compounds 1 and 2 (500 MHz. [D_4] MeOH. r.t).

	Compound **1**	Compound **2**
Carbon position	^{1}H (δ)	^{1}H (δ)
6	6.20(s)	6.20(s)
8	6.33(s)	6.28(s)
3'	6.56(s)	6.85(s)
6'	6.91(s)	7.12(s)
MeO-3	3.71(s)(3H)	3.61(s)(3H)
MeO-4'	3.88(s)3H)	3.90(s)(3H)
AcO-5'	-	2.15(s)(3H)



Scheme 2. Fragmentation pattern for compound 1.



Scheme 3. Fragmentation pattern for compound 2.

in the B ring of the flavonoid ((MeO-4') and (AcO-5')). Based on the structural pieces of information from ^{1}HNMR and mass spectrum coupled with the 'rule of 13'[25], the molecular formula was established to be $C_{19}H_{16}O_9$. The IR spectrum (KBr) indicated the presence of hydroxyl (3418 cm^{-1}) and chromone (1646 cm^{-1}) groups. Bands in the range 1650 - 1065 cm^{-1} are typical for a flavone skeleton [24]. The band at 1772 cm^{-1} indicated that compound **2** had an acetyl group. The UV spectrum exhibited absorption maxima at 255 nm (band II) and 338 nm (band I) that are characteristic absorption bands of a flavone skeleton [17]. No shift in band I was observed after the addition of $AlCl_3$ and $AlCl_3$/HCl, suggesting a hydroxyl-keto complex formation at the C-5 hydroxyl [17,26]. Fragments at m/z 329 $[M\text{-}COCH_2\text{-}17]^+$ and at m/z 315 $[M\text{-}COCH_2\text{-}31]^+$ revealed a OH substitution in C(2') and a OCH_3 group in C(3). UV spectral data supported these findings by confirming free OH groups at C(5) and C(7) and no free OH group at C(4') as well as no *ortho*-dihydroxy substitution in rings A and B. From these results, compound **2** was identified as 5'-acetoxy-5,7,2'-trihydroxy-3,4'-dimeth- oxyflavone. The fragmentation pattern for compound **2** is as shown in **Scheme 3**.

3.2 Antibacterial Screening

The antibacterial screening of the isolated compounds inhibited the activity of the following bacteria: *Salmonella typii*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* with MIC ranging from 0.001 - 0.2 mg/ml but was inactive against *Escherichia coli*, *Corybacterium pyogenes* and *Proteus vulgaris* (**Table 2**).

Thus, compound **2** showed a higher activity against all the screened bacteria except for *S. dysentery*. This activity was more pronounced against Gram-positive than Gram-negative bacteria. This could be as a result of the morphological differences between this micro-organisms. The Gram-positive bacteria have an outer peptidoglycan layer which is not an effective permeability barrier [28] which makes it to be more susceptible to the compounds under investigation while Gram-negative bacteria have an outer phospholipidic membrane that carries the lipopolysaccharide components which makes the cell wall impermeable to lipophilic solutes [29]. Therefore the inactivity against *P. vulgaris* could be related to the high lipid content of the cell wall of *P. vulgaris*, which may have trapped the compounds while its inhibition against *S. aureus* is as a result of the cell wall lacking a lipid layer and therefore could be penetrated by the isolated compounds. The results indicated that the presence of hydroxyl groups at positions C-5 and C-7 was very important for bioactivity.

Now, comparing the inhibitory activity of compounds **1** and **2** against *S. aureus* and *P. vulgaris* showed that most of the activity was related to the presence of hydroxyl groups at positions 3', 4', 5' in ring B and at C-3. However, it was observed that the presence of C-2, C-3 double bond was not crucial for antibacterial activity [23] and the most active antibacterial were reported to be compounds such as Epigallocatechin and dihydrorobinetin with hydroxyl groups at C-5 and C-7 and three substitutions in ring B which is also observed with compounds **1** and **2**.

Table 2. Antibacterial activities of compounds 1 and 2.

TEST ORGANISM	**Zones of MIC inhibition (mm)** (mg/ml)		**Zones of MIC inhibition (mm)** (mg/ml)	
BACTERIA	**Compound 1**		**Compound 2**	
Bacillus subtillis	30.00	0.02	40.50	0.20
Staphylococcus aureus	20.00	0.01	45.00	0.001
Kliebsiella pneumoniae	25.00	0.01	60.00	0.001
Corynebacterium pygenes	0.00	0.0	0.00	0.00
Proteus mirabilis	30.00	0.01	70.00	0.001
Shigella dysenteriae	45.00	0.20	35.00	0.20
Escherichia coli	0.00	0.00	00.00	0.00
Proteus vulgaris	0.00	0.00	0.00	0.00
Pseudomonas aeruginosa	40.00	0.01	50.00	0.01
Salmonella typhii	40.00	0.10	30.00	0.10

Zones of inhibition are mean of quadruplicate determinations.

4. Conclusions

The isolation and spectroscopic characterization of antibacterial flavonoids from the methanolic leaf extract of *A. hispidum* DC was described. The antibacterial flavonoids are flavones and were identified as 5,7,2',5'-tetrahydroxy-3,4'-dimethoxyflavone and 5'-acetoxy-5,7,2'-trihydroxy-3,4'-dimethoxyflavone.

5. References

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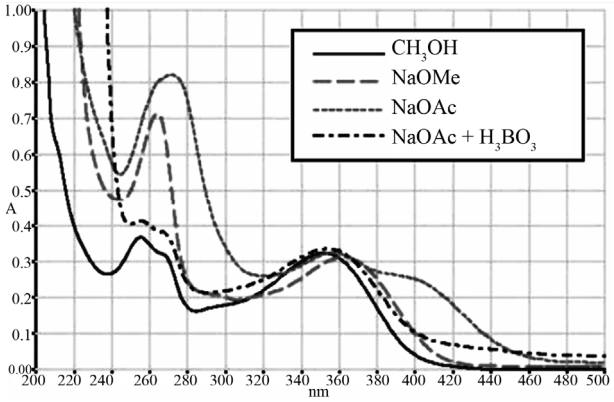
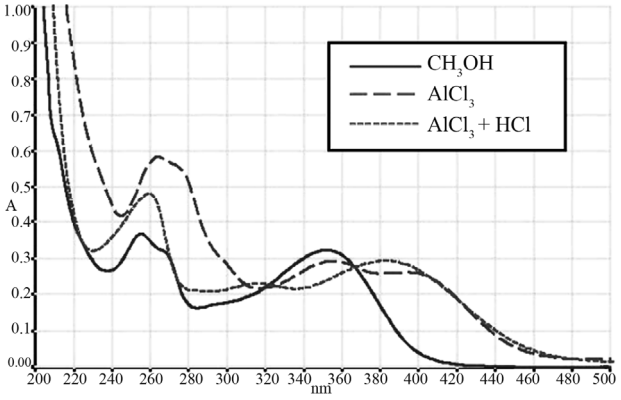
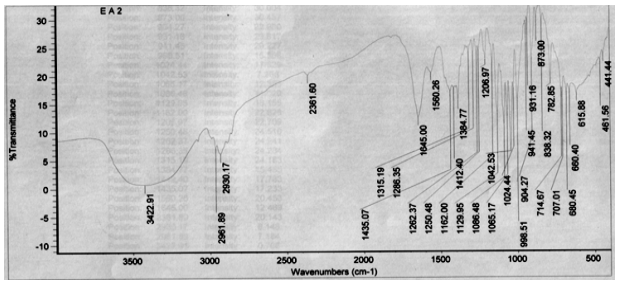
Appendices



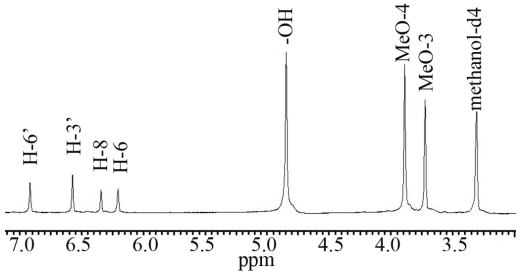
Appendix 1. UV spectra for compound 1 with classical shift regents.



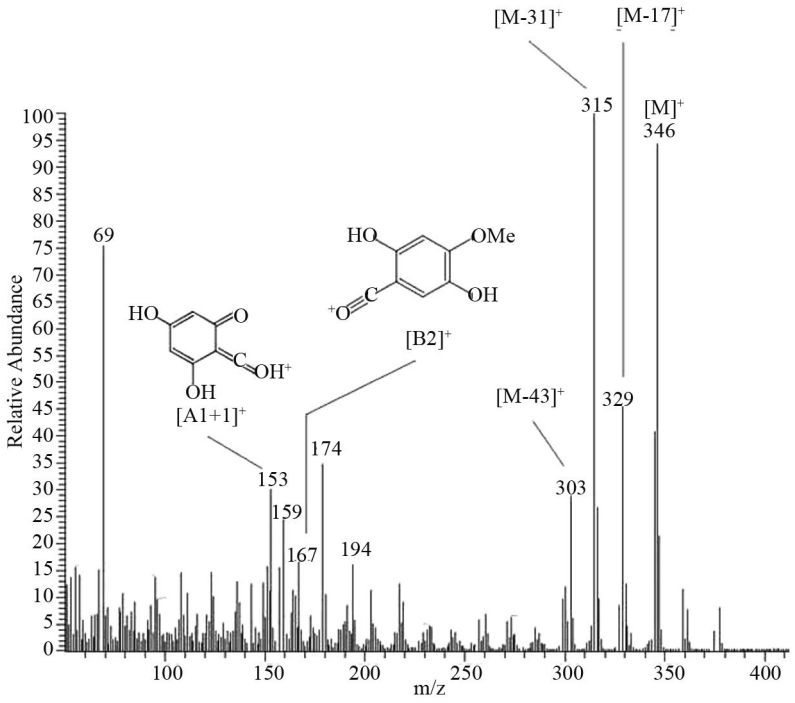
Appendix 2. UV-visible spectra for compound 1 with classical shift regents.



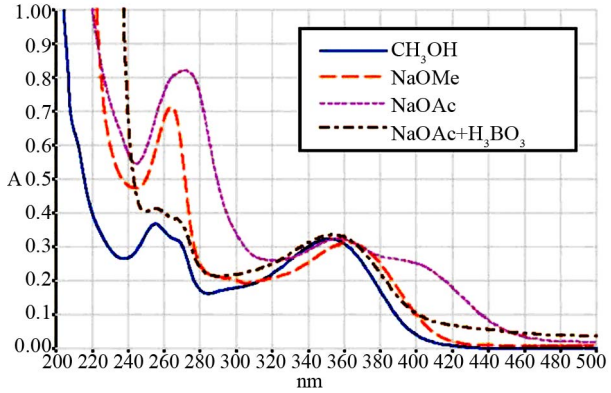
Appendix 3. IR spectrum of compound 1.



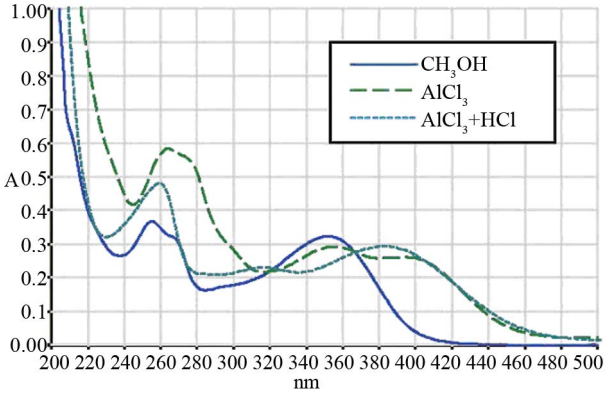
Appendix 4. ^{1}H-NMR spectrum of compound 1 (500 MHz, methanol-*d*4)



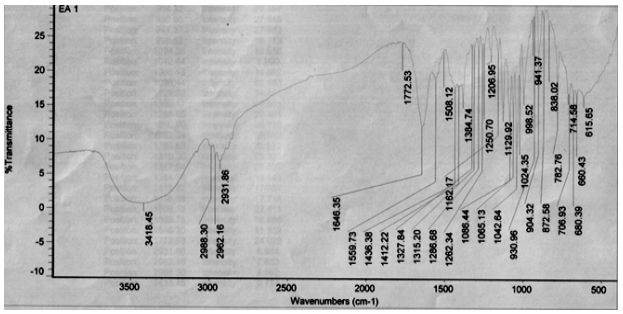
Appendix 5. EI-MS spectrum of compound 1



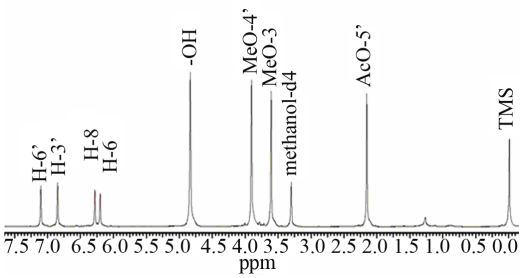
Appendix 6. UV-visible spectrum for compound 2 with classical shift regents



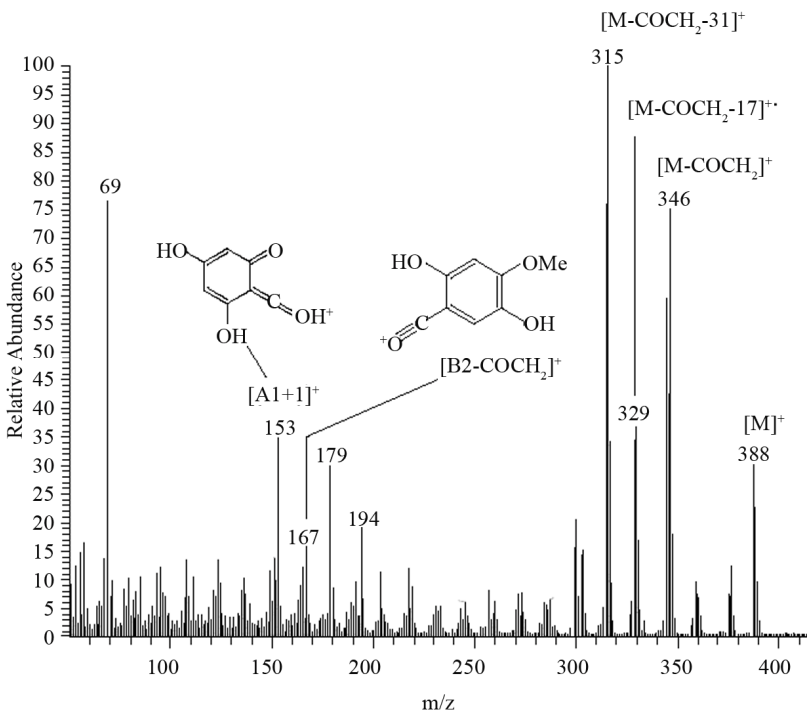
Appendix 7. UV-visible spectrum for compound 2 with classical shift regents.



Appendix 8. IR spectrum of compound 2.



Appendix 9. 1H-NMR spectrum of compound 2 (500 MHz, methanol-d4).



Appendix 10. EI-MS spectrum of compound 2.

