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LC-MS/MS and tandem MS/MS identification of chemical compounds of the stem bark of *Acacia macrostachya* (Mimosaceae)

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Abstract
The main objective of this work was to identify chemical compounds of biological interest in the methanol extract of *Acacia macrostachya* stem bark using High-Performance Liquid Chromatography coupled with tandem Mass Spectrometry (HPLC-MS/MS) and Global Natural Product Social Molecular Networking (GNPS) databases. A total of seven (07) molecules from four families of secondary metabolites such as Kaempferol-3-O-rutinoside, kaempferol, epicatechin, epicatechin-3-O-gallate, bergaptol-O-β-D-glucopyranoside, 3-hydroxyurs-12-en-28-oic acid and kaempferid were identified. The presence of these compounds in the extract could be responsible of the anti-diabetic and anti-cancer properties of this extract which were reported in our previous investigations.

Keywords: *Acacia macrostachya*, HPLC-ESI-MS/MS, flavonoids, tannins, terpenoids

1. Introduction
Acacia macrostachya (Mimosaceae), a medicinal plant traditionally found in endemic degraded areas, is used by phytotherapists to treat several diseases [1]. In Burkina Faso, given its adaptation to drought conditions, vegetable species is widely distributed throughout the country [2] and possessing various pharmacological potentialities [3, 4]. Indeed, it has been reported in the literature that the various organs of this plant are used against inflammation and oxidative stress [5, 6, 7]. Methanolic extracts of its roots significantly inhibit the proliferation of the KB cancer cell line [3]. Moreover, we reported in previous studies that *A. macrostachya* exhibit very interesting antioxidant, anti-diabetic and anti-cancer properties in two cell line [6, 8]. Indeed, we demonstrated that the methanol extract of the stem bark of this plant species significantly inhibited the enzymatic activity of α-glucosidase, a key digestive enzyme that converts carbohydrates into glucose [8] one part and in second part that the same extract showed significant antiproliferative and apoptotic activities on the U937 cancer line [6]. In addition, other authors have reported that extracts of the root bark of the aforementioned species contain molecules with relevant anti-inflammatory and analgesic properties [5, 9]. Previous work on extracts of the leaves and roots of this plant highlighted the presence of saponins and alkaloids [3, 4, 10]. Phytochemical screening of the same extract revealed the presence of flavonoids, tannins, and terpenoids/steroids. The presence of these families of compounds in a plant extract could be responsible for its biological properties [11, 12]. The aim of the present study is to identify the chemical compounds in the methanolic extract of *A. macrostachya* by using High-Performance Liquid Chromatography coupled with Mass Spectrometry.

2. Material and Methods
2.1 Plant material and extraction
The stem barks of *A. macrostachya*, identified by the botanist Professor Amadé Ouedraogo and referenced with 17252 number at the herbarium of the Joseph KI-ZERBO University were collected in February 2018 in Laongo, a locality located some forty kilometers north of Ouagadougou, Burkina Faso, at GPS coordinates 12°31'50.52 "N; 01°17'2.7 "W. The stem barks were then carefully washed and dried at room temperature away from the sun for two (02) weeks under ventilation.
Dried stem bark was turned into powder using an electric grinder. 100 g of the dry powder was macerated in 500 mL of methanol for 24 hours stirring and at laboratory temperature.

The macerate was then separated from the pomace by filtration through Wattman paper No. 3 filter. The filtrate thus obtained was evaporated to dryness using a rotary evaporator (BÜCHI Labortechnik GmbH, Hendrik-Ido-Ambacht, the Netherlands) and kept at 4 °C for further work.

2.2 Sample preparation
For chromatographic analysis, a 10 mg/mL stock solution was prepared, and kept at 5 °C for use. To record the chromatograms, 1 mg/mL of the solution was used by diluting the stock solution 10 times. The elution solvents were ultra-pure water, methanol (99.9%), formic acid (98%). HPLC grade were purchased from Sigma-Aldrich (Paris, France) and are using without prior purification.

2.3 Liquid Chromatography/Mass Spectrometry analysis (LC/MS)
The compounds were identified by using an Agilent 1260 Infinity chromatograph coupled to an Accurate-Mass-Q-TOF LC/MS 6530 mass spectrometer with an electrospray ionization (ESI) source. Chromatographic separation was carried out using a stationary phase consisting of a Sunfire C_{18} reversed-phase column (150 x 2.1 mm; 3.5 µm, Waters) and a mobile phase consisting of a mixture of acidified distilled water (0.1% HCOOH) (eluent A) and methanol (eluent B) according to the following gradient: from 0 to 30 min 0% A and 100% B; from 35 min to 50 min 98% A and 2% B; from 50 min to 55 min 100% A and 0% B to rebalance the column. The volume injected was 0.25 mL. In MS, the sample is injected at a flow rate of 0.25 mL/min using nitrogen gaze (N_2) at a flow rate of 10 L/min to facilitate sample vaporization. Different collision energies and fragmentation voltages were used to generate MS/MS data. Purine ($C_5H_4N_4$, m/z 121.050873 and HP-0921) and phosphazene ($C_{18}H_{18}F_{24}N_3O_6P_3$, m/z 922.009798) were used as internal locking masses. Complete analyses were acquired in the range of 10,000 (m/z 922) to 4,000 (m/z 121). An MS/MS exclusion list criterion was established to avoid oversampling of the internal calibrant. If the capillary potential is positively charged, the positive fragment ions generated will be analyzed. Once inside, the ions generated are attracted to the extraction cone at a potential of 175 V, where they undergo fragmentation, which continues in the collision cell during tandem mass spectrometry analysis. For ESI-MS spectra recording, the quadrupole is set to allow only ions with m/z between 100 and 1,000 to pass through a conductive capillary heated to 320 °C with a source voltage of 3.5 kV. For the Collision Induced Dissociation (CID) experiments, the mass ions of interest are selected by the quadrupole and subjected to collision by nitrogen gaze in the collision cell at a pressure of 600 bar. All these ions are transmitted to the time-of-flight analyzer (TOF) for separation. The recorded chromatograms were processed using Agilent MassHunter Qualitative Analysis software version B.07.00.

2.4 GNPS database
The GNPS database of molecules and their various fragments was used to identify and confirm the different molecular structures. GNPS facilitates the identification of molecules based on their raw fragmentation mass spectrometry (LC-MS) and tandem MS/MS data. GNPS is a database with over one hundred million MS/MS spectra available to users in over 100 countries worldwide.

3. Results and Discussion
Qualitative analysis of the secondary metabolites of the methanolic extract of *A. macrostachya* stem bark was made by using the total ion chromatogram of the compounds. MassHunter Qualitative analysis software was used to generate the precursor ions of all the compounds recorded, and subsequently the ESI-MS/MS spectra. The identification of the various compounds has been based on the measurement of the molecular mass, the m/z ratio, and the analysis of the characteristic fragment ions obtained and the GNPS database. HPLC analysis of the extract generated the total ion chromatogram (Figure 1)

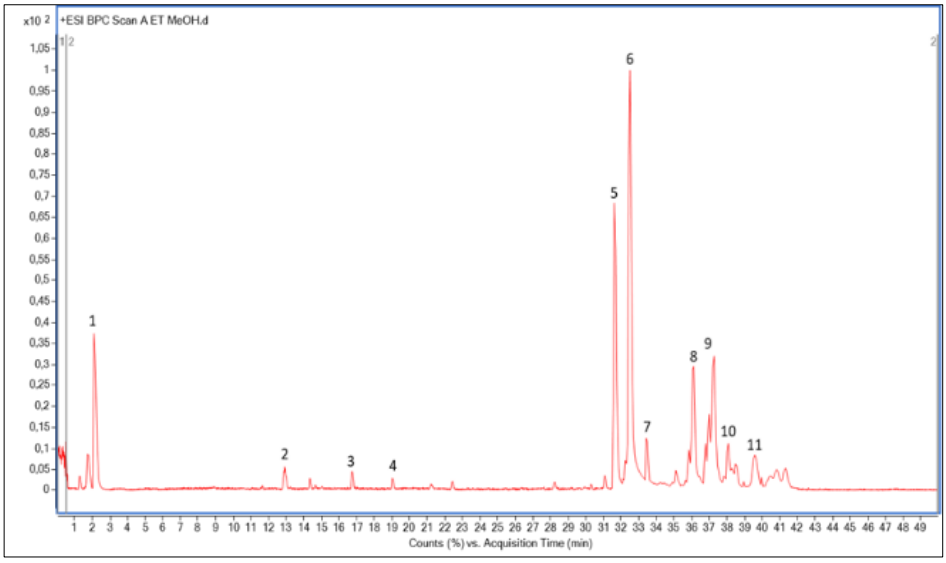


Fig 1: Total ion chromatogram of the methanol extract of *A. macrostachya* stem bark

Figure 1 exhibits clearly the presence of eleven (11) defined peaks at different retention times with different relative intensities. The presence of these peaks probably indicates the presence of at least eleven (11) compounds and agrees with previous reported data.

To identify them, each peak was subjected to LC-MS and LC-MS/MS analysis in positive ionization mode (ESI+). Figure 2 shows the LC-MS and tandem MS/MS positive ionization spectrum of peak 1.

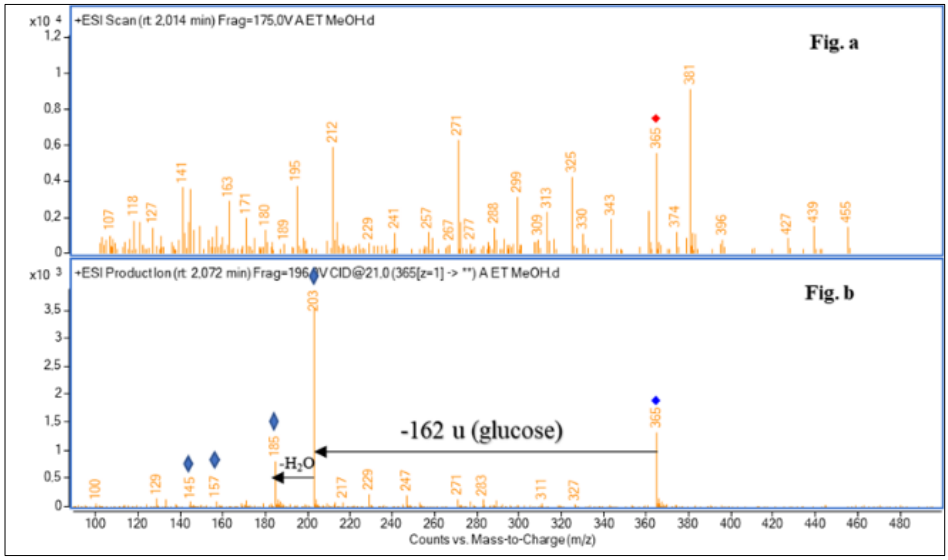
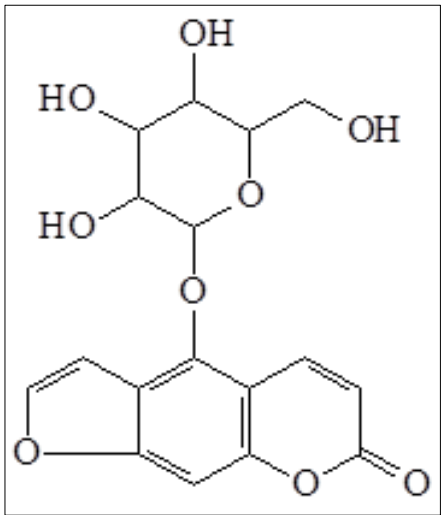


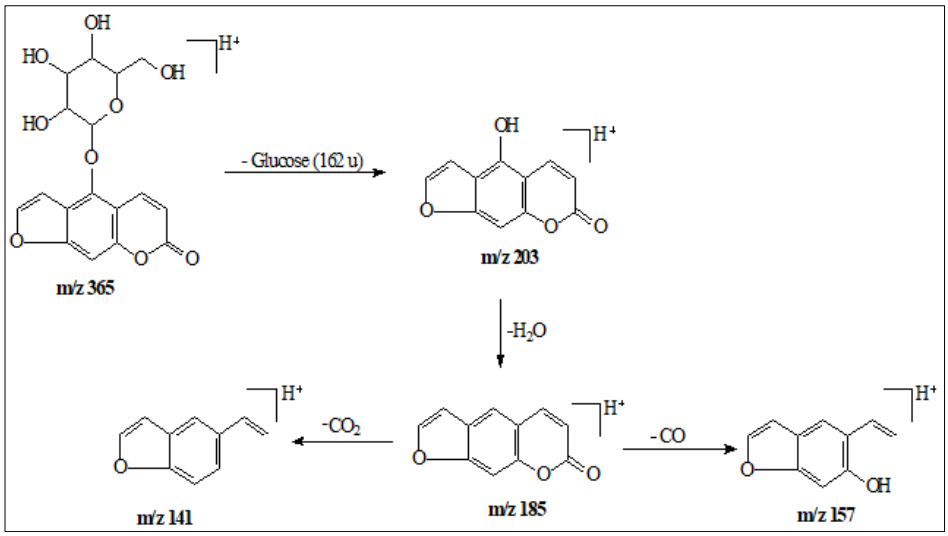
Fig 2: LC-MS and MS/MS spectra of peak 1 in positive ionization mode (ESI+); CE = 21 eV

In this figure, one can see a parent ion at m/z 365 $[M+H]^+$ (Figure 2a) indicating a molecular weight of 364 u. Tandem MS/MS spectral analysis of the ion gives a base peak at m/z 203 ($[M+H-162]^+$) corresponding to the loss of a glucose molecule with a mass of 162 u. Analysis of these data in comparison with those of the literature [13] shows that the base peak at m/z 203 could correspond to the fragment with the formula $C_{11}H_6O_4$; which would imply a molecular peak with the formula $C_{17}H_{16}O_9$.

From the results obtained from the GNPS database and the above, we can suggest that the peak 1 would correspond to the chemical structure of bergaptol-O-β-D-glucopyranoside illustrated in scheme 1. Based on this structure and the presence of the two characteristic fragment ions at m/z 185 corresponding to a loss of a water molecule (-18 u) and at m/z 157 corresponding to loss of a CO molecule (-28 u) in the Figure 2b, the following mechanism of the fragmentation of the base peak at m/z 203 show in Scheme 2 confirm the coumarin structure of the molecule.



Scheme 1: Bergaptol-O-beta-D-glucopyranoside



Scheme 2: Mechanism of bergaptol-O-beta-D-glucopyranoside fragmentation by ESI+

This result is in agreement with the data reported in the literature [13] and confirm the presence of compound in the extract. The ESI+ LC-MS mass spectrum of peak 2 in Figure 3 shows an ion at m/z 595 (Figure 3a) corresponding to a compound with a molecular mass of 594 u in agreement with the molecular formula $C_{27}H_{29}O_{15}$.

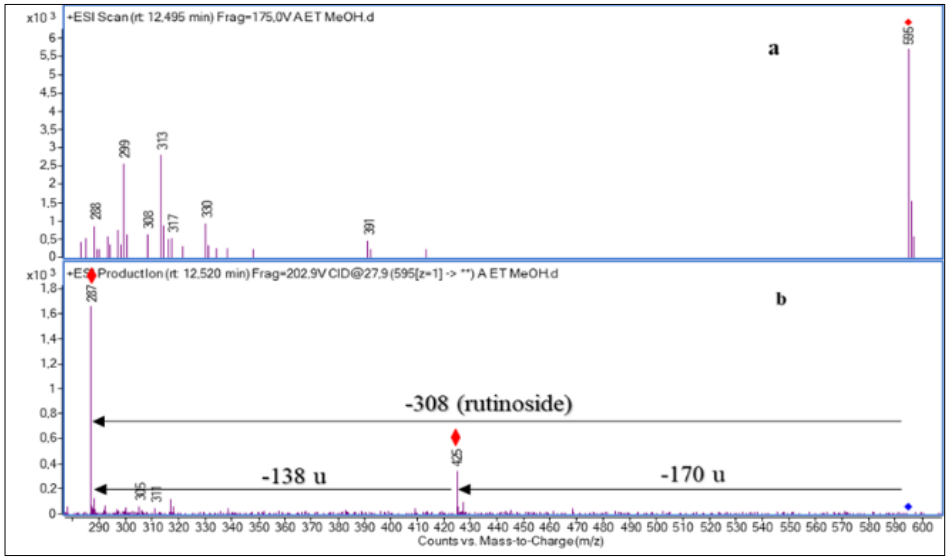


Fig 3: LC-MS spectrum of peak 2 (a) and tandem MS/MS spectrum of the parent ion at m/z 595 (b) by ESI+; CE = 27.9 eV

In the positive ionization chromatogram in Figure 3a, a precursor ion is observed at 595 with a retention time of 12.495 min, corresponding to compound 2. Tandem MS/MS analysis of this parent ion leads to a peak at m/z 287 corresponding to the base peak obtained following a loss of 308 u (Figure 3b). The presence of this peak corresponds to the simultaneous departure of a rhamnose unit (146 u) and a glucose unit (162 u). The spectrum also shows the absence of two characteristic fragment ion peaks at m/z 449 $[M+H-146]^+$ and 433 $[M+H-162]^+$ corresponding the departure of one rhamnose unit followed by the loss of one glucose unit respectively. In addition, the spectrum shows characteristic neutral losses of 170 u and 138 u, confirming glycosylation of the molecule. Taken together, these results suggest that the two sugars are linked and attached to a single glycosylation site.

Furthermore, according to data reported in the literature [14], the peak at 287 corresponds to the peak aglycone, which could be either kaempferol or luteolin. To determine the exact nature of the aglycone, the tandem spectrum was performed and shown in Figure 4.

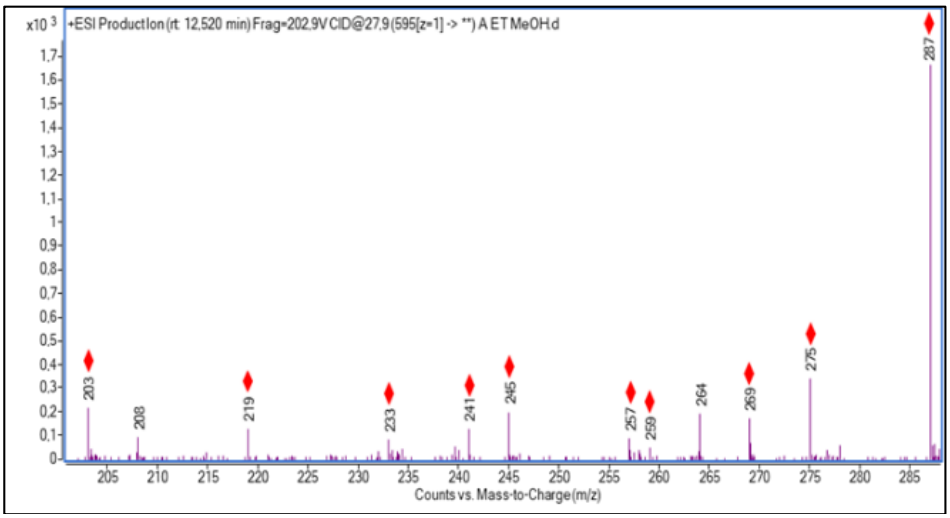
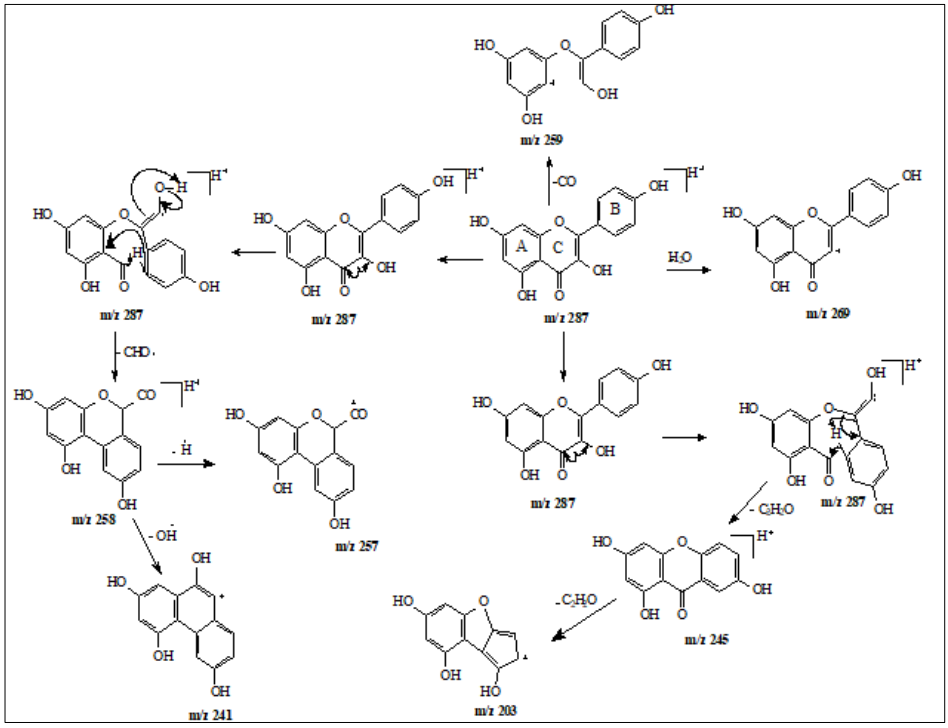


Fig 4: Tandem MS/MS spectrum of the ion at m/z 287 positive mode (ESI+); CE = 27.9 eV

It appears in this figure the presence of ions at m/z 275; 269; 259; 257; 245; 241; 233; 219 and 203, which are consistent with the data reported in the literature [14, 15, 16] for kaempferol aglycone. These different ions could be explained in the mechanism proposed in Scheme 3.



Scheme 3: Mechanism of kaempferol fragmentation by ESI+

Glycosylation can also take place in the 3-O or 7-O position [17]. To determine the exact position of the glycoside, the UV-DAD absorption spectrum of the compound at m/z 595 was recorded. This spectrum shows two characteristic absorption wavelengths at 245 nm and 275 nm (Figure 5).

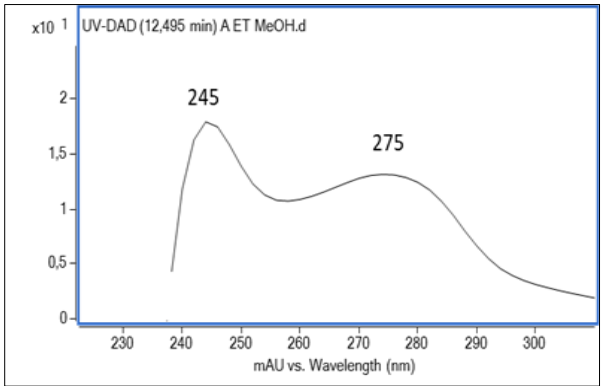
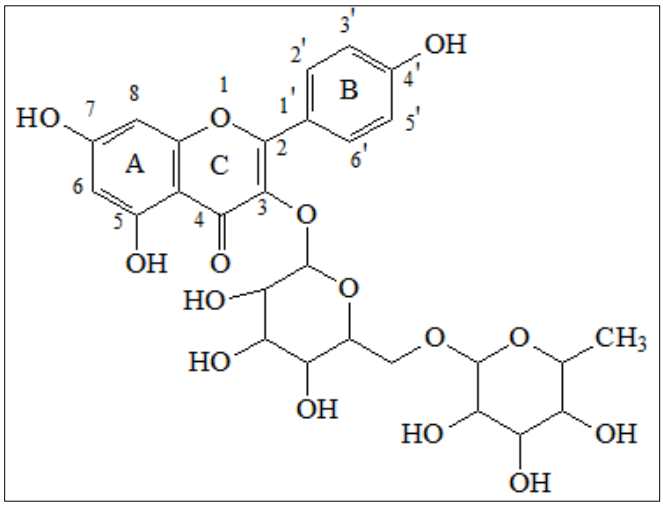


Fig 5: UV-DAD spectra of peak 3

The maximum absorption bands at 245 nm and 275 nm are characteristic of the aromatic rings of flavonoids. The band at 275 nm (band I) is associated with the absorbance of the A ring of flavones and the band at 245 nm (band II) corresponds to the absorbance of the B ring. It is reported in the literature that the UV-visible spectra of most flavones and flavonols show two absorption bands: a band that appears between 290 and 385 nm (band I) and a band around 240-285 nm (band II). Moreover, glycosylation in the 3-O position affects the position of band II and tends to produce a hypsochromic effect (towards the shortest wavelengths). A comparison of these wavelengths with those reported in the literature [16, 18, 19], the glycoside would occupy the 3-O position. Based on all these results, we can identify compound 2 as kaempferol-3-O-rutinoside with the following structure (scheme 4).



Scheme 4: Kaempferol-3-O-rutinoside chemical structure

This compound belongs to the diglycosylated flavonoid family and its presence in methanolic plant extracts has already been reported by Yeqing et collaborators [20].

LC-MS analysis assigned peak 6 to a molecular ion at 301 with retention time of 32.858 min (Figure 6a), suggesting a compound with a molecular weight of 300 u and the molecular formula $C_{16}H_{11}O_6$. The tandem fragmentation of compound 6 shows characteristic fragment ions, which can be seen in Figure 6b.

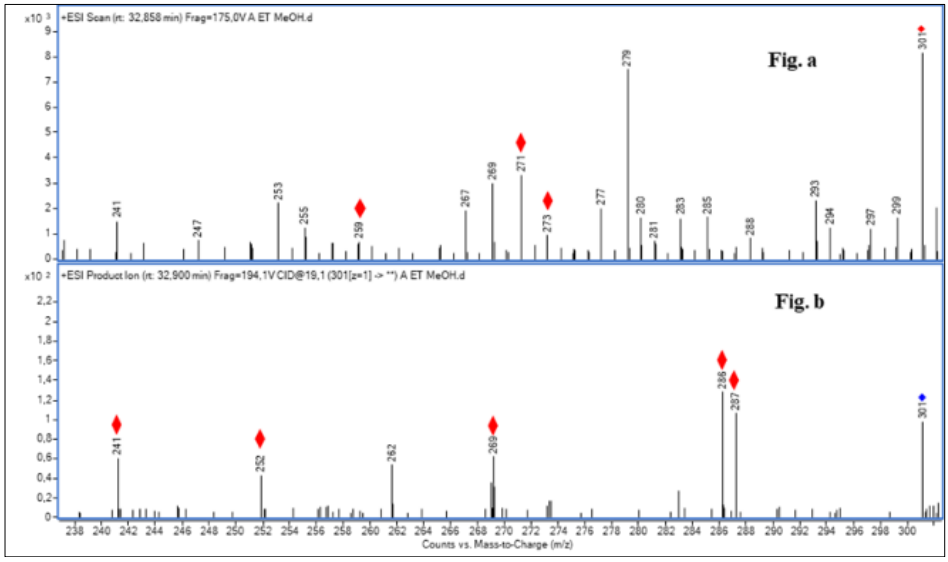
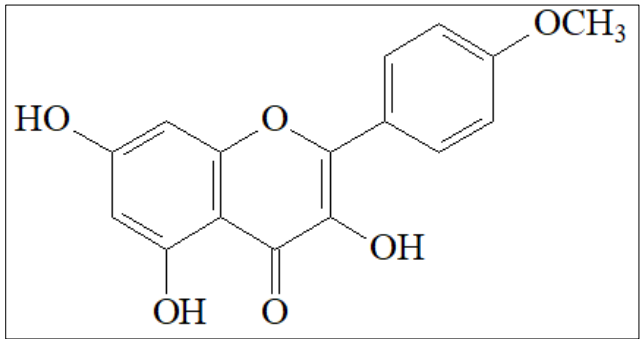
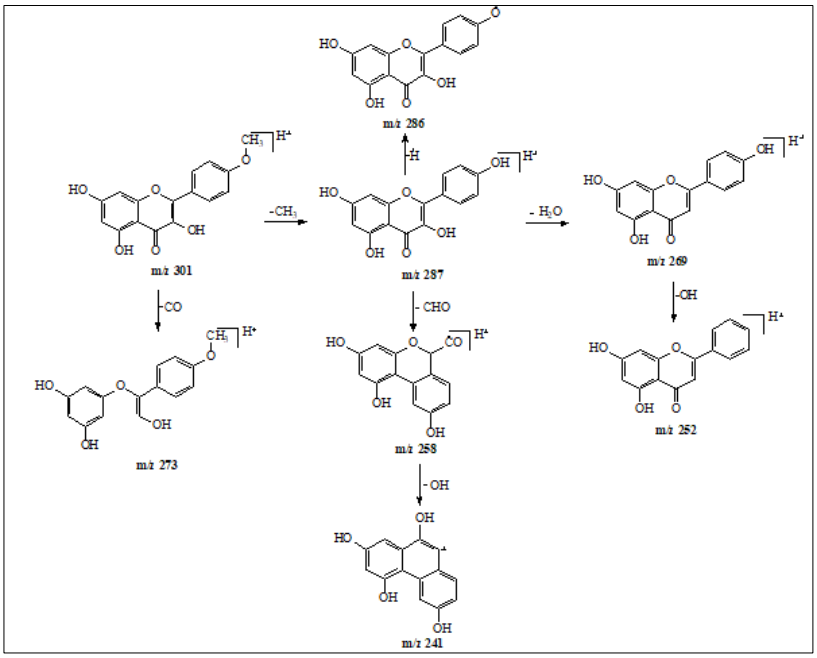


Fig 6: LC-MS and MS/MS spectra of peak 6 in positive ionization mode (ESI+); CE = 19.1 eV

Tandem at m/z 301 revealed significant peaks corresponding to ions at m/z 287; 286; 271; 269; 259; 252 and 241 (Figure 6b). After analysis of the data by GNPS, the molecular peak at m/z 301 corresponded to kaempferid. This result is confirmed by the presence of the fragment ion at m/z 287 (Figure 6b) which indicates, in agreement with results above, the formation of the kaempferol aglycone following the loss of a CH_3 (-15 u) methyl group. The similarity with the fragments obtained from the tandem MS/MS spectra of compound 2 confirms the presence of the kaempferol motif. From the above, especially with the loss of the CH_3 fragment and by data reported in the literature [21], it can be concluded that compound 6 could be attributed to kaempferid with the following chemical structure (scheme 5). This result is in agreement with work reported in the literature which indicated the presence of this molecule in dichloromethane extracts of plants, in particular *Chromolaena odorata* [21]. The formation of the different fragments shown in Figure 6b could be explained by the fragmentation mechanism illustrated in Scheme 6.



Scheme 5: Kaempferid chemical structure



Scheme 6: Mechanism of kaempferid fragmentation by ESI+

In conclusion and taking into account the information obtained from the LC-MS and tandem spectra (MS/MS), seven (07) compounds were fully identified, corresponding to peaks 1; 2; 3; 4; 5; 6 and 7 in methanol extract of *A. macrostachya* stem bark. These results are summarized in Table 1.

Table 1: Summary of compounds identified in the methanol extract of *A. macrostachya* stem bark analyzed by the LC-ESI-MS/MS method in positive mode.

Peak No.	Retention time (min)	Formulas	$[M+H]^+$ (g/mol)	Characteristic fragment ions (m/z)	Identified compounds
1	2.014	$C_{17}H_{16}O_9$	365.30	365 (37.37); 203 (100); 185 (22.78); 145 (2.30)	Bergaptol-O-β-D-glucopyranoside
2	12.495	$C_{27}H_{30}O_{15}$	595.52	287 (100); 275 (20.52); 271; 269 (10.28); 259 (2.93); 245 (11.84); 241 (7.65); 203 (13.03)	Kaempferol-3-O-rutinoside
3	16.676	$C_{15}H_{14}O_6$	291.26	291 (2.07); 273 (3.75); 255 (4.52); 139 (43.22); 123 (100)	(-)-Epicatechin
4	18.221	$C_{22}H_{18}O_{10}$	443.4	291 (2.07); 273 (3.75); 255 (4.52); 139 (43.22); 123 (100)	(-)-Epicatechin-3-O-gallate
5	31.696	$C_{30}H_{48}O_3$	457.3603	443 (0.5); 424 (0.62); 369 (0.83); 313 (2.19); 259 (0.66); 195 (0.56)	3-Hydroxyurs-12-en-28-oic acid
6	32.858	$C_{16}H_{12}O_6$	301.26	301 (15.31); 286 (27.35); 285 (9.50); 241 (15.66)	Kaempferid
7	33.343	$C_{15}H_{10}O_6$	287.23	271 (24.23); 203 (7.58); 153 (12.3); 151 (5.75); 69 (100)	Kaempferol

However, the peaks 8, 9, 10, and 11 could not be formally identified based on the results of LC-MSMS and tandem MS-MS spectrometry.

In summary, all the identified compounds belong to four families of phenolic compounds such as flavonoids for kaempferol-3-O-rutinoside, kaempferid and kaempferol; tannins for (-)-Epicatechin and (-)-Epicatechin-3-O-gallate; terpenoid for 3-hydroxyurs-12-en-28-oic acid and coumarin for bergaptol-O-β-D-glucopyranoside.

The presence of these compounds in the methanolic extract of *Acacia macrostachya* could justify its pharmacological properties, which we have highlighted in our previous work [8, 6]. The therapeutic potential of these molecules, as reported in several scientific studies, has focused on antioxidant, anti-diabetic, and anti-cancer activities [11, 12, 22, 23, 24]. Indeed, Lekshmi *et al.,* showed in 2015 that kaempferid induces the death of colon cancer cells (HL60) by apoptosis and was shown to be non-toxic on normal human fibroblasts [12]. Epicatechin is also a powerful antioxidant and has been implicated in the prevention of chronic non-communicable diseases such as cancer and diabetes. Perveen and al showed in 2017 that di-2-ethylhexyl phthalate (a terpene) significantly inhibits the growth of two skin cancer cell lines, namely SK-MEL-28 and A375P [11]. In summary, it has been shown in the literature that all the compounds identified in this study are potentially cytotoxic on different cancer cell lines, including U937 and K562 cancer cells.

4. Conclusion

The present work focused on the identification of bioactive compounds. Positive-mode LC-ESI-MS/MS analysis of the methanolic extract of *Acacia macrostachya* stem bark enabled us to identify seven (07) compounds, including three (03) flavonoids, and their derivatives, two (02) tannins, one (01) terpenoid, and one (01) coumarin. All seven compounds were identified for the first time in the *A. macrostachya* species. They are kaempferid, kaemperol-3-O-rutinoside, kaempferol, epicatechin, epicatechin-3-O-gallate, bergaptol-O-β-D-glucopyranoside and 3-hydroxyurs-12-en-28-oic acid. All these molecules are known for their well-known anti-diabetic and anti-cancer properties. Following on from this work, we intend to isolate molecules of interest by bio-guidance.

5. Acknowledgments

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6. Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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