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Cytotoxic, antioxidant and antidiabetic activities versus UPLC-ESI-QTOF-MS chemical-profile analysis of *Ipomoea aquatica* fractions

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Dedicated to Professor Arnold Vlietinck on the occasion of his 80th birthday.

Abstract

Ipomoea aquatica (IA), is a common green leafy vegetable, which has numerous uses in traditional medicine. This study focused on the determination of the cytotoxic, antiradical and antidiabetic properties of various fractions of the *I. aquatica* methanolic extract, as well as on the tentative identification of some bioactive compounds in the same fractions. The cytotoxicity was determined by the brine shrimp lethal test. The antioxidant activities of the *I. aquatica* fractions were investigated through three assays. The antidiabetic activity (*in vitro*) was measured by *α*-glucosidase and *α*-amylase inhibition assays. Phytochemical qualitative analyses demonstrated the presence of alkaloids, terpenoids, phenols and flavonoids in the ethyl acetate-methanol and methanol fractions. The total phenolic and total flavonoid contents were found to be highest in the ethyl acetate-MeOH fractions. The evaluation of the cytotoxicity showed that the hexane-dichloromethane fraction is the most toxic, while the others are moderately toxic. The antioxidant activity assays showed that the ethyl acetate-MeOH fractions are the most potent, while the *α*-glucosidase and *α*-amylase assays revealed that the hexane-dichloromethane fraction might contain a potent antidiabetic agent. Some bioactive substances in the MeOH fractions, such as salicylic acid glucoside, 1-O-sinapoyl-β-D-glucose derivative and dihydroferulic acid derivative were tentatively identified. To the best of our knowledge, this is the first report to detect and identify these compounds in this species. Based on the results of this study, it may be concluded that *I. aquatica* is a potent antioxidant agent and could be a good candidate as natural antioxidant in food and therapeutics.

Keywords: *Ipomoea aquatica*; Convolvulaceae; chemical-profile analysis; cytotoxic activity; antioxidant activity; antidiabetic activity

Introduction

For many centuries, natural products have played a crucial role in human disease treatments. Secondary metabolites of medicinal plants are considered main sources in natural drug discovery. Recently, herbs are more effectively and systematically examined for a variety of pathophysiological cases [1]. The use of phytotherapeuticals, traditional medicines, and dietary supplements, should also be related to safety, efficacy, quality, and consistency.

Ipomoea aquatica Forsskal, belonging to the Convolvulaceae family, has numerous bioactive constituents, such as phenol-, flavonoid-, and alkaloid compounds. According to the Indian indigenous system of medicine (Ayurevda), green leaves of *I. aquatica* are recommended to be taken orally for the treatment of diabetic disorders [5]. Furthermore, in Africa and Sri Lanka, *I. aquatica* is also applied as antidiabetic therapy [6]. Findings of another study demonstrated similar oral hypoglycemic effects of single and multiple doses of the aqueous extract of IA on diabetic rats and on type 2 diabetes mellitus (T2DM) patients as of the drug tolbutamide [7]. Literature study exhibited that *I. aquatica* has potent antioxidant properties and is a good scavenger of the superoxide radical, the hydroxyl radical, and the DPPH radical [8].

Reactive oxygen species (ROS), such as the singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), and hydrogen peroxide (H_2O_2) can attack biomolecules and result in, for instance, protein-, lipid-, and DNA damage; cell ageing, oxidative stress, cardiovascular diseases and neurodegenerative diseases, e.g. Alzheimer, and cancer. Antioxidants have the capacity to scavenge or quench both ROS and reactive nitrogen species (RNS), which are formed as byproducts of the respiration [9].

Diabetes mellitus is a complex disorder disease resulting from the body's inability to produce insulin, causing hyperglycemia. It is considered globally as a significant health problem, which is increasing with the increase of obesity and advancing age in the global population. Currently there are different antidiabetic drugs, for instance, acarbose, miglitol and voglibose, which act by inhibiting the α-glucosidase and α-amylase activities.

However, their continuous use is often associated with undesirable side effects, such as diarrhea, flatulence, abdominal discomfort, adverse gastrointestinal symptoms and occasionally liver toxicity [10, 11]. Therefore, natural α-glucosidase and α-amylase inhibitors, without adverse or unwanted secondary effects are required.

Overall, this work aims investigating the bioactivity of different fractions of an *I. aquatica* methanolic extract, as well as the identification of bioactive substances of the most active fraction(s). This requires performing some qualitative and quantitative analyses of the phytochemical constituents in the *Ipomoea aquatica* fractions, as well as determining some biological properties, including the cytotoxic, antioxidant and antidiabetic activities, in order to determine the most toxic, antioxidant, and antidiabetic fraction(s). This is to our knowledge the first study that focuses on the phytochemical composition and biological activities of African plants of this species. The results of this work may be useful for the food or pharmaceutical industry in the development of natural antioxidants and/or antidiabetic agents from plant sources.

Results and Discussion

The preliminary qualitative screening analysis of the different *Ipomoea aquatica* fractions showed the presence of different types of active constituents, including phenols, saponins, flavonoids, terpenoids, alkaloids and carbohydrates as shown in Table 1.

Our results showed that the ethyl acetate (IA8), ethyl acetate-methanol (IA9-1, 9-2 and 9-3), methanol (IA10-1 and 10-2) and aqueous (IA11) fractions were positive for the presence of alkaloids. The fractions IA2, 3, 4 and 6-1 to 11, which include the hexane-dichloromethane (IA2, 3 and 4), dichloromethane-ethyl acetate (IA6-1, 6-2, 7), ethyl acetate (IA8), ethyl acetate-methanol (IA9-1, 9-2 and 9-3), methanol (IA10-1 and 10-2) and aqueous fractions (IA11) all tested positive for terpenoids. In fractions IA7, 8, 9-1, 9-2, 9-3, 10-1 and 10-2 the presence of phenols was demonstrated. The results also indicated higher amounts in fractions IA9-1 to IA10-2. Flavonoids were found in the ethyl acetate-methanol and methanol fractions (IA9-1 to 10-2). In addition, the fractions of ethyl acetate, ethyl acetate-methanol, methanol and water (IA9-1 to IA11) showed to be also positive for

tannins. Comparison indicated that most bioactive constituents were found in the hydrophilic fractions (IA9-1 to IA11).

The total phenolic content of the *I. aquatica* fractions is expressed as mg GAE/g dry weight sample, and the results are shown in Table 2. The TPC was estimated using a standard curve of gallic acid ($y = 4.71x + 0.03$; $R^2 = 0.985$). The TPC values of the hydrophilic *I. aquatica* extracts ranged from 24.38 to 50.86 mg GAE/g DW. The aqueous fraction (IA11) contained the highest phenolic content, followed by the MeOH fraction (IA10-1) and the ethyl acetate-MeOH fraction (IA9-1) (Table 2). However, when combining the contents found in the three ethyl acetate-MeOH fractions on the one hand and in the two MeOH fractions on the other, the highest TPC is found in the ethyl acetate-MeOH fractions (IA9-1 to 9-3), followed by methanol fractions (IA10-1 & 10-2) and aqueous fraction (IA-11) with 81.35, 66.04 and 50.86 mg GA/g DW, respectively. One thus might expect that the ethyl acetate-MeOH and the MeOH fractions will exhibit a high antioxidant activity.

The total flavonoid content was determined using a standard curve of quercetin ($y = 24.75x + 0.01$; $R^2 = 0.996$). The TFC values ranged from 6.70 to 9.51 mg QE/g DW; the ethyl acetate-methanol fractions (IA9-1 to IA9-3) were found to contain the highest, followed by the methanol (IA10-1 and IA10-2) and aqueous (IA-11) fractions (Table 2). The flavonoid contents in the hydrophilic *I. aquatica* fractions were found to decrease in the following sequence: IA9-2 > IA11 > IA10-1 > IA9-1 > IA10-2 > IA9-3. The TFC values are 23.35, 14.94 and 8.19 mg QE/g DW for the (three) ethyl acetate-MeOH fractions, the (two) MeOH fractions and the water fraction, respectively. The differences between *I. aquatica* fractions (IA9-1 to IA11) for the total phenolic/flavonoid contents were found to be statistically significant (Tukey's multiple range test, $p < 0.05$).

Another study, performed by Datta et al. [12] on the methanolic extract, showed TPC and TFC values of 13.95 mg GAE/dry extract and 10.86 mg rutin equivalent (RE)/g dry extract, respectively. Comparison with the results of our study indicated statistical differences, $p < 0.05$ for TPC (TFC was not compared because of different equivalents used). Indeed, our findings revealed higher total phenolic contents, which may due to the difference in used plant parts and extraction procedure.

Tables 1-2

The results of the brine shrimp lethal test (BSLT) on the *I. aquatica* fractions were expressed as LC_{50} values (Tables 3 and 4). Values ranging between 0 and 500 µg/mL implies toxicity, between 500-1000 µg/mL moderate toxicity and above 1000 µg/ml non-toxicity [13]. The results for group A extracts are summarized in Table 3, while those for group B are given in Table 4. The LC_{50} values of these fractions range from 440 to >1000 µg/mL. Generally, most *I. aquatica* fractions showed a moderate toxicity. However, the LC_{50} values of the lipophilic fractions tends to be slightly lower than those of the hydrophilic. Among of the lipophilic fractions, IA4 (hexane-DCM, 25:75, v/v) is considered the most toxic with LC_{50} = 440 µg/mL. In addition, the hydrophilic fractions revealed not much difference between their LC_{50} values. Fraction IA10-2 is considered a non-toxic fraction.

In a study performed by Octaviani et al. [14] on an ethanolic extract of *I. aquatica*, toxicity was seen with an LC_{50} = 266 µg/mL, while 100% mortality was reached at concentration of 1000 µg/mL. However, another study by Ullah et al. [15] revealed potent toxicity of an *I. aquatica* methanolic extract with LC_{50} = 33 µg/mL. Differences between the toxicity values of both studies are thus observed, and both differ substantially from the values reported in this study since most of our extracts were only found moderately toxic. Possible, the variation between the results originates from differences in the plant location, collection date and/or extraction approaches.

Tables 3-4

The antioxidant activity of the lipophilic and hydrophilic fractions of *I. aquatica* were examined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. No large differences between the different hydrophilic *I. aquatica* fractions were found. IC_{50} values were summarized in Table 5. The IC_{50} values ranged from 0.33 to 1.90 mg/mL, and the activity was compared to trolox (0.01 mg/mL). The two MeOH fractions IA10-1 and 10-2 showed the highest inhibition value against the DPPH radical with IC_{50} values of 0.33 and 0.34 mg/mL, respectively, followed by the ethyl acetate-MeOH fractions, IA9-2 (0.37 mg/mL), IA9-3 (0.48 mg/mL) and IA9-1 (0.49 mg/mL), the ethyl acetate IA8 (0.91 mg/mL) and the

IA7 dichloromethane-ethyl acetate fraction (1.90 mg/mL). The ethyl acetate-MeOH and MeOH fractions contained the highest amounts of phenolic and flavonoid compounds as indicated higher in the qualitative and quantitative analyses. The presence of phenolic and flavonoid compounds may explain the capability of these fractions to expose a higher antioxidant activity than other fractions.

The aqueous fraction and the lipophilic fractions, such as hexane, hexane-dichloromethane, dichloromethane and dichloromethane-ethyl acetate, showed a weak antioxidant activity with inhibition below 50%, for which IC_{50} could not be determined.

On the other hand, the antioxidant activity of *I. aquatica* fractions was performed using the $ABTS^{\bullet+}$ assay, where trolox was used as standard. The various fractions of *I. aquatica* were found to be considerably different in their $ABTS^{\bullet+}$ radical scavenging activities. The activity of the various *I. aquatica* fractions decreased in the following sequence: IA11 (aqueous fraction) > IA9-2 (ethyl acetate-MeOH) > IA9-1 (ethyl acetate-MeOH) > IA9-3 (ethyl acetate-MeOH) > IA10-1 (100% MeOH) > IA10-2 (100% MeOH) > IA8 (100% ethyl acetate) > IA7 (DCM-ethyl acetate, 50:50, v/v) > IA6-1 (DCM-ethyl acetate, 75:25, v/v) > IA6-2 (DCM-ethyl acetate, 75:25, v/v) and IA5 (100% DCM), as shown in Table 5. The IC_{50} values ranged from 0.26 to 0.80 mg/mL. Our findings also indicate that the ethyl acetate-MeOH (IA9-1 to IA9-3) and MeOH (IA10-1 and IA10-2) fractions show higher antioxidant properties. This is found to be similar to the DPPH results. The aqueous fraction had the highest antioxidant activity.

Doka et al., [16] evaluated also the antiradical activity of the ethanolic extract of leaves of this species from Southern West Kordofan (Sudan) using the ABTS approach and found an IC_{50}= 0.39 mg/ml.

Datta et al. [12] determined the antioxidant activities of a methanolic extract of *I. aquatica* (leaves and stems) by the DPPH and ABTS assays, and the percentage of inhibition is found to be higher for ABTS with 33.4 % and 7.8 %, respectively. This is similar to our findings, where the ABTS assay showed a higher inhibition percentage than DPPH. Moreover, in our study, the inhibition percentage in the DPPH approach was

slightly higher for fraction IA9-2 (86 %) than for trolox (84.8 %). In the ABTS assay, theinhibition reached 96.3 %, while for trolox it was 98.2 %.

The findings by Zengin et al., [17] on the antioxidant activity of the leaves extracts of a related species (*I. batatas*) using the DPPH and ABTS assays showed higher antiradical properties in the decoction extract, followed by the Soxhlet and microwave extracts. The differences in results between our and other studies can have different reasons, such as species, the plant part studied (aerial parts versus leaves), the regions of origin, the harvest season and the extraction procedure.

The ABTS assay also succeeded to evaluate some lipophilic fractions for which the DPPH test failed. The main reason for this is that $ABTS^{\bullet+}$ can be solubilized in both aqueous and organic media. Therefore, the antioxidant capacity can be measured for both hydrophilic and lipophilic compounds [18].

Table 5

The ORAC assay is the most widely accepted method for comparing and standardizing the nutritional supplements because of its sensitivity, specificity and relevance to the human body. Because of to the nature of the lipophilic fractions they require a specific sample preparation as well as a specific approach for performing the ORAC assay [19, 20]. Therefore, we focused on performing the ORAC assay only on the hydrophilic *I. aquatica* fractions. The results are also presented in Table 5. The ethyl acetate-MeOH fractions, especially IA9-1 and IA9-2 show the most potent scavenging capacity against $ROO^{\bullet}$ with respectively 1171 and 952 µmol TE/g DW, followed by the aqueous fraction (IA11), the MeOH fraction (IA10-1), IA9-3, and IA10-2. To the best of our knowledge, there is no previous study on the ORAC radical scavenging activity from *I. aquatica* fractions.

The results of this assay confirmed the results of the ABTS assay (Table 5), with the ethyl acetate-MeOH fractions exhibiting the highest antioxidant activity, followed by the aqueous fraction, and the MeOH fractions. However, when combining the results of the three ethyl acetate-MeOH fractions on the one hand and the two MeOH fractions on the other, the findings revealed that the IA9-1 + IA9-2 + IA9-3 (ethyl acetate-MeOH) fractions can be considered as stronger antioxidant scavengers, followed by the IA10-1 + IA10-2

(MeOH) fractions, and the IA11 (aqueous) fraction with 2828, 1349 and 766 µmol TE/g DW, respectively. This result was expected because the ethyl acetate-MeOH fractions (IA9-1 to IA9-3) together were found to contain higher amounts of phenolic and flavonoid compounds than the other fractions (Table 2).

The IC_{50} values of trolox and the different *I. aquatica* fractions from the DPPH and ABTS assays presented statistically significant differences (Dunnett's Multiple Comparison test, Table 5), whereas, the ORAC findings did not (Tukey's Multiple Comparison test). The differences found in the results with the DPPH, ABTS and ORAC assays may result from different probes, reaction conditions/mechanisms and quantification methods [21]. However, in all three tests the hydrophilic fractions showed the strongest antioxidant properties. In view of this, we can conclude that the compounds with the strongest antioxidant activity are clearly present in the hydrophilic fractions.

α-glucosidase inhibitors, which interfere with the enzymatic action, could slow down the liberation of D-glucose from oligo- and disaccharides, resulting in delayed glucose absorption and decreased postprandial plasma glucose levels [22]. The study of plants for their hypoglycemic, antioxidant and hypolipidemic activities may therefore provide new pharmacological approaches in the treatment of diabetes mellitus [23]. Preliminary experiments were carried out to establish the optimal conditions of enzyme-, substrate- and sample concentrations: pNPG was set at 2.5 mM; α-glucosidase at 0.2 U/mL; and inhibitor concentration at 1 mg/mL. Since the ethyl acetate-MeOH, MeOH and aqueous fractions contained the most active compounds (Table 1), the highest phenolic and flavonoid contents (Table 2), and showed antioxidant effects (Table 5), they are considered good candidates to be glucosidase inhibitors. However, these fractions showed a weak or non-inhibitory activity (data not shown) against α-glucosidase. Several factors can, however, interfere with enzyme inhibition. A first may be the presence of phytochemical constituents in the sample which can prevent the active compounds to inhibit α-glucosidase. A second is that the carbohydrates (sugars) present in the samples may interfere with the results. The fact that a decreased inhibition at increased sample concentrations was observed (data not shown) may confirm this hypothesis. Therefore, these fractions first require a pretreatment to remove the sugar compounds.

The lipophilic fractions, such as hexane-dichloromethane, dichloromethane and dichloromethane-ethyl acetate, on the other hand, demonstrated inhibitory activity against α-glucosidase (Table 6). Hexane-dichloromethane (IA4), dichloromethane (IA5) and dichloromethane-ethyl acetate (IA6-1) were the most active fractions, followed by dichloromethane fraction (IA7) with respective IC_{50} values of 0.16, 0.14, 0.17 and 0.37 mg/mL. The difference between the IC_{50} values of these fractions was statistically significant (Tukey's Multiple Comparison test, $p < 0.05$).

A study on three different cultivars of *I. aquatica*, on the other hand, demonstrated that 70% (v/v) methanol extracts as more potent α-glucosidase inhibitor (IC_{50} values 0.21, 0.48 and 0.58 mg/mL) than the aqueous extracts [24]. Meanwhile, 100% methanol extracts exhibited inhibition with IC_{50} = 0.46, 0.60 and 0.64 mg/mL. Our results revealed more potent inhibition for the glucosidase enzyme compared to the latter study.

This may be due to the fractionation of the crude extract, which may increase the concentration of given bioactive compounds in given fractions and thus their opportunity to interact with the α-glucosidase enzyme.

Here also, the ethyl acetate-MeOH, MeOH and aqueous fractions failed to delay the breakdown of carbohydrates and/or to interact with α-amylase. Again, these fractions showed an inverse relation between the inhibition and the inhibitor concentration (data not shown), i.e. a decreased inhibition is seen with increased sample concentrations. Several attempts were made to determine the inhibiting activity of these fractions, but without success. Although for some plants an interesting α-amylase inhibitory activity is reported in addition to the more common α-glucosidase inhibitory effect [25, 26], usually a stronger effect is found on α-glucosidase activity [27-29]. According to Oboh et al. [30], plant extracts with the highest phenolic content do not always demonstrate the strongest inhibitory activity on α-amylase, which indicates the importance of the nature of the molecules and their interactions. In addition, the flavonoid compound luteolin has been examined and reported for its antidiabetic effects [31], while in another study [32], it was concluded that the same flavonoid did not have enough power to delay or inhibit the release of glucose in the gastrointestinal tract. Furthermore, it was seen that, pancreatic α-amylase

inhibitory activity was not only affected by the flavonoids concentration, but also by their specific composition and molecular structure [33].

Only the hexane-dichloromethane fraction (IA4) (Table 6), exhibited a good inhibitory activity against α-amylase with an IC_{50} = 1.08 mg/mL. The IA5, IA6-1, IA7 and IA8 fractions only showed a weak inhibition of pancreatic α-amylase with inhibition values below 50%, preventing the determination of the IC_{50} value.

Table 6

The findings of the phytochemical analysis (Section 3.1) indicated the presence of the most bioactive substances in the ethyl acetate-MeOH and MeOH fractions. The strongest antioxidant fractions were also found in the ethyl acetate-MeOH and MeOH fractions, whereas the most potent toxic and antidiabetic agents were located in *n*-hexane-DCM fractions. In previous work [34], the bioactive substances in the ethyl acetate-MeOH fractions (IA9-1 till IA9-3) were identified using UPLC-ESI-QTOF-MS. In this present study, a tentative identification and comparison of the bioactive compounds is made in both ethyl acetate-MeOH and MeOH fractions. This step is compulsory in order to determine which substance(s) may be responsible for the antioxidant activity in these plant extracts. For this purpose, negative ionization mode was selected, because the sensitivity of this mode is sufficient to detect the most phenolic compounds. The identification of a peak was achieved by comparing retention time and MS spectra with reference standards and literature data. Some bioactive substances, such as dihydroxy benzoic acid pentoside, rutin, nicotiflorin and salicylic acid (**Fig. 1**, peaks 1, 7, 10, 11, respectively) were tentatively identified based on comparing the chemical profiles (MS spectrum) from MeOH fractions with the MS spectrum of EtOAc-MeOH fractions. The intensity of these compounds in the MeOH fractions was found to be less than in the EtOAc-MeOH fractions, which explained and confirmed of the higher antioxidant activity of the ethyl acetate-MeOH fractions compared to MeOH fractions.

Since EtOAc-MeOH fractions, followed by MeOH fractions, revealed the highest phenolic/flavonoid contents and total antiradical activities, these extracts were chosen for the identification of their major bioactive substances. Actually, the major bioactive

compounds in the three EtOAc-MeOH fractions were tentatively identified in a previous study [34]. In the present study, the major bioactive compounds in two MeOH fractions were tentatively identified. As shown in Table 7, the MS data of peak (**2**) revealed a deprotonated molecular ion $[M - H]^-$ at m/z 299.077, with a major fragment ion at m/z 137.024 $[M – H\text{-}162]^-$, suggesting the presence of a hexose group, and corresponds with molecular formula $C_{13}H_{15}O_8$. This compound was tentatively assigned as salicylic acid glucoside (**Fig. 2**) by comparing with the literature [35]. Comparing the MS spectrum of peak (**5**) with the literature [36], this compound was assigned as 1-O-sinapoyl-β-D-glucose derivative with molecular formula $C_{17}H_{21}O_{10}$. The MS/MS spectrum of this compound provided the fragment ion of sinapic acid at m/z 223.061. The MS spectra of peak (**6**) showed an $[M - H]^-$ ion peak at m/z 371.098, producing a daughter ion at m/z 195.066 with UV absorbance at 275 nm and a molecular formula of $C_{16}H_{19}O_{10}$. Therefore, this compound was tentatively identified as dihydroferulic acid derivative, according to [37]. To the best of our knowledge, there are no previous studies reporting the presence of salicylic acid glucoside, 1-O-sinapoyl-β-D-glucose derivative and dihydroferulic acid derivative (**Fig. 2**) in the *I. aquatica* plant. Finally, there are also four unknown peaks (**4**, **8**, **9**, and **12**) that could be phenols according to their UV patterns. However, these peaks are unidentified because of insufficient mass data.

As mentioned above, all main detected compounds in the MeOH and EtOAc fractions are tentatively identified as phenolic acids and flavonoids. These phytochemicals are known for their potential antiradical and antioxidant activity. The antioxidant activity of phenolics/flavonoids originates from their ability to eliminate the free radicals. Consequently, they have the capacity to prevent several ailments. Glycoside flavonoids, such as rutin and nicotiflorin are present in a large number of therapeutic medicinal plants [38]. These compounds receive great interest because of their antioxidant, antiviral and anti-inflammatory activities, as well as their ability to protect nerve cells under stress, e.g. oxidative stress and hypoxia [39]. In addition, the findings of Peyrot et al. [40] showed the antioxidant properties of sinapoyl glucose to be comparable to classical antiradicals, such as trolox or butylated hydroxytoluene (BHT). Hence, the presence of such compounds in the MeOH fractions may contribute to the potency of the antioxidant activities of these fractions.

In conclusion, the presence of some bioactive substances, such as phenols and flavonoids in the ethyl acetate-MeOH and MeOH fractions may explain their potent antioxidant properties. To the best of our knowledge, this is the first study reporting on the phytochemical composition and biological activities of *I. aquatica* in a fractionated methanolic extract. The qualitative analyses displayed the presence of the most active compounds in the ethyl acetate-MeOH and MeOH fractions. In addition, the quantitative analysis indicated that the ethyl acetate-MeOH and MeOH fractions contained the highest amounts of phenolic and flavonoid compounds. Investigation of the cytotoxicity, by the shrimp lethal assay, showed moderate toxicity for all fractions, except for the hexane-dichloromethane (25:75, v/v) fraction, which exhibited a higher toxicity. Results from three antioxidant activity assays (DPPH, ABTS and ORAC) demonstrated the antioxidant potential of the ethyl acetate-MeOH, MeOH and aqueous fractions. These latter fractions were rich in phenolic and flavonoid constituents. Furthermore, a potential antidiabetic activity, estimated by α-glucosidase and α-amylase inhibition assays, was found only in the hexane-dichloromethane fraction. The ESI-QTOF-MS analysis of the two MeOH fractions revealed the presence of bioactive compounds of which some were also found previously in the ethyl acetate-MeOH fractions. Different active compounds, such as salicylic acid glucoside, 1-O-sinapoyl-β-D-glucose derivative and dihydroferulic acid derivative are described for the first time in this plant species. This study might also contribute to the progress of its nutraceutical research in the field of functional foods.

In future studies, the active cytotoxic, antioxidant and antidiabetic compounds in the different fractions have to be identified. This will allow clarifying the relationship between the chemical structure of these compounds and the reported activities.

Materials and methods

Plant material and extraction preparation

Fresh plant material of *Ipomoea aquatica* (aerial parts) was collected from Darfur, West Sudan, Sudan in March 2010. The plant material was authenticated by Dr. El-Taib Ahmed and a voucher specimen (1210KR1) was deposited in the Herbarium of the Department of Botany, Faculty of Science, Khartoum University, Sudan.

Chemicals and reagents

Dimethyl sulfoxide, hydrochloric acid, sodium citrate, copper (II) sulfate pentahydrate, iodine, iron (III) chloride, copper (II) acetate monohydrate, ninhydrin, potassium iodide, sodium potassium tartrate and Folin & Ciocalteu's reagent were purchased from Merck. Aluminium trichloride hexahydrate was obtained from Fagron. Bismuth subnitrate and magnesium were purchased from UCB. Gallic acid, quercetin, Trolox, DPPH, ABTS, fluorescein sodium salt, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), *α*-glucosidase from *Saccharomyces cerevisiae*, *α*-amylase from porcine pancreas, p-nitrophenyl α-D-glucopyranoside (pNPG), and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich, while ultrapure water was produced in house by an Arium® pro ultrapure water system .

Preparation of the extract

The plant extraction was achieved as reported in a previous study [34]. Briefly, the air-dried aerial parts of *I. aquatica* (1 kg) were ground in a mechanical grinder to obtain a powder form. The extraction was executed using maceration with methanol (4 L) for 24 h. The solvent was removed using a rotatory evaporator to get a crude methanolic extract in a gummy form.

Fractionation of the methanolic extract

The methanolic crude extract was subjected to silica gel flash column chromatography (60-120 mm mesh) and eluted with *n*-hexane, dichloromethane (DCM), ethyl acetate (EA), methanol (MeOH) and 100% water (H_2O), gradually increasing the degree of polarity to finally get fifteen fractions eluted with different solvent compositions as described in [34], where, IA1 (100% *n*-hexane); IA2 (hexane:DCM, 75:25, v/v); IA3 (hexane:DCM, 50:50, v/v); IA4 (hexane:DCM, 25:75, v/v); IA5 (100% DCM); IA6-1, IA6-2 (DCM:EA, 75:25, v/v); IA7 (DCM:EA, 50:50, v/v); IA8 (100% EA); IA9-1, IA9-2, IA9-3 (EA:MeOH, 50:50, v/v); IA10-1, IA10-2 (100% MeOH); IA11 (100% H_2O).

Phytochemical screening analyses

Qualitative phytochemical analysis of the lipophilic and hydrophilic *Ipomoea aquatica* fractions to search for the presence of various bioactive constituents, such as alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, steroids, reducing sugars, carbohydrates, cardiac glycosides, proteins, and amino acids were performed using the standard methods in [41-45].

Determination of total phenolic content

The total phenolic content (TPC) of *I. aquatica* fractions was measured in alkaline medium using the Folin & Ciocalteu's reagent [46] and expressed as gallic acid equivalents (GAE) /gram powder on dry weight (DW) basis.

Determination of total flavonoid content

The total flavonoid content (TFC) was measured as described in [47] and expressed as quercetin equivalents (QE) per gram powder on dry weight basis (mg QE/g DW).

Determination of the cytotoxic activity by the brine shrimp lethal test

The brine shrimp lethal test (BSLT) was performed to predict the toxicity of the different *I. aquatica* fractions [48]. The percentage mortality was plotted against the various concentrations of each *I. aquatica* fraction to estimate the lethal concentration (LC_{50}) in µg/mL for each fraction.

Antioxidant activity

DPPH radical-scavenging capacity

The DPPH scavenging activity of various *I. aquatica* fractions was estimated according to the method described in [49]. The DPPH radical-scavenging activity (RSA) of each solution was calculated as the percentage of inhibition (%) by using the following equation:

$$\text{RSA}\ (\%) = \frac{(A_{\text{Blank}} - A_{\text{Sample}})}{A_{\text{Blank}}} \times 100 \qquad (1)$$

ABTS radical-scavenging capacity

The radical scavenging capacity of various *I. aquatica* samples for the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate radical cation ($ABTS^{\bullet+}$) was determined as described in [50]. The free radical scavenging capacities of the samples were expressed as IC_{50} (mg/mL). The ABTS antioxidant capacity of each solution was calculated as the percentage of inhibition (%) according to equation (1).

Oxygen radical absorbance capacity (ORAC)

The ORAC antioxidant capacity of the *I. aquatica* fractions was evaluated by a peroxyl radical ($ROO^{\bullet}$) according to the method described in [51]. This assay was performed using a VICTOR^3 1420 multilabel counter (Perkin-Elmer, Waltham, MA). The ORAC values are expressed as micromoles trolox equivalents (TE) per gram sample on dry weight basis (µmol TE/g DW).

Antidiabetic activity

***α*-glucosidase inhibition assay**

The *α*-glucosidase inhibitory activities were determined according to the method described in [26] using *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as substrate. The *α*-glucosidase inhibitory activity was expressed as the percentage of inhibition (%) and was calculated by the following equation:

$$\text{Inhibition } (\%) = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})}{\text{Abs}_{\text{Control}}} \times 100 \qquad (2)$$

The final results are expressed as IC_{50} values. The IC_{50} was estimated by regression analysis using GraphPad Prism software.

***α*-amylase inhibition assay**

The *α*-amylase inhibitory activity was evaluated using the method reported in [52]. The *α*-amylase inhibitory activity was expressed as percentage inhibition (Eq. 2) and the IC_{50} values were calculated as mentioned above in the *α*-glucosidase assay.

UPLC-ESI-QTOF-MS chemical-profile analysis

Ten mg of dried *I. aquatica* sample was dissolved in 1 ml MeOH (HPLC grade) and then injected into an Acquity UPLC I-Class system (Waters, Milford, MA, USA) chromatograph equipped with a binary pump and PDA detector (Waters), and coupled to a Waters electrospray ionization quadrupole time-of-flight tandem mass spectrometer (ESI–Q–TOF/MS) XevoG2-S. The used conditions were as described in our previous work [34]. Briefly, an Acquity UPLC BEH phenyl 1.7 µm (2.1 × 100 mm) column at 25 ºC with a flow rate (0.3 mL/min) was used. A mobile phase was composed of 0.1% formic acid in Milli-Q water (solvent A) and 0.1% formic acid in MeOH (solvent B) was used with the following gradient conditions: starting with 5% B, ramping to 95% B in 25 min, held at 95% B till 25.5 min, return to 5% B at 26 min and reconditioning at 5% B till 30 min. The injection volume was 1 µl and the PDA detector wavelengths were in the range of 190–400 nm. The data were processed using MassLynx™ 4.1 software with MSE program (Waters). Finally, the interpretation of the observed MS spectra was done based on the comparison with those found in the literature and in other online databases, such as MassBank, ChemSpider, and mzCloud.

Statistical analysis

Analyses were performed in triplicate and the results of the total phenolic content, total flavonoid content are expressed as mean value with standard deviation, while antioxidant and antidiabetic activities (IC_{50}) are expressed as geometric mean value and 95% confidence interval. The IC_{50} values were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's Multiple Comparison tests. Statistical analyses were performed with and all IC_{50} values were calculated by the software GraphPad Prism® Software version 5.00.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Chemical profiles of the methanol fractions (IA10-1 & 10-2) and the ethyl acetate-MeOH fractions (IA9-1, 9-2 & 9-3) using UPLC-DAD-ESI-QTOF-MS. Experimental conditions: see text. Only 9 minutes of analysis are displayed since all peaks eluted before that time.

Fig. 2. Chemical structures of three compounds in the two MeOH fractions (IA10-1 & 10-2) that were tentatively identified using ESI-QTOF-MS.

Table 1

Preliminary phytochemical screening analysis of the lipophilic and hydrophilic *I. aquatica* fractions

Type of Constituents	Fraction														
	IA1	IA2	IA3	IA4	IA5	IA6-1	IA6-2	IA7	IA8	IA9-1	IA9-2	IA9-3	IA10-1	IA10-2	IA11
Proteins															
Biuret test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Amino acids															
Ninhydrine test	–	–	–	–	–	–	–	–	–	+	++	++	+++	+	–
Carbohydrates															
1. Fehling test	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–
2. Benedict's test	–	–	–	–	–	–	–	–	–	++	++	+	+	+	–
3. Barfoed's test	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
4. Iodine test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phenols & Tannins	–	–	–	–	–	–	–	+	+	++	+++	++	++	++	–
Flavonoids															
1. Alkaline reagent	–	–	–	–	–	–	–	–	–	++	++	++	+++	+++	–
2. Shinoda's test	–	–	–	–	–	–	–	–	–	+	++	+	+++	++	–
Saponins															
Froth test	–	–	–	–	–	–	–	–	–	++	++	++	+++	+++	+++
Terpenoids															
Salkowki's test	–	+	+++	+++	–	+	+	+	+	+	+	+	+	+	+
Steroids															
Liebermann Burchard test	++	+++	++	++	+++	+++	+	++	+	–	–	–	–	–	–
Cardiac glycoside															
Keller-Kiliani's test	–	+++	+++	–	–	+++	+++	+++	+++	+	+	+	++	+	+++
Alkaloids															
Dragendorff's test	–	–	+	–	–	–	–	–	+++	+	+	+	+++	++	+++

+++ = highly present, ++ = moderately present, + = limitedly present, – = absent.

Table 2

Total phenolic and total flavonoid contents in the hydrophilic *Ipomoea aquatica* fractions expressed as mg GAE/g and mg QE/g, respectively (mean ± SD (n = 3)).

Fraction	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
IA9-1	31.45 ± 1.29[b]	7.15 ± 0.24[a]
IA9-2	24.38 ± 0.57[a]	9.51 ± 0.43[c]
IA9-3	25.51 ± 0.19[a]	6.70 ± 0.19[a]
IA10-1	37.64 ± 1.45[c]	7.97 ± 0.35[b]
IA10-2	28.40 ± 1.01[b]	6.96 ± 0.39[a]
IA11	50.86 ± 3.96[d]	8.19 ± 0.64[b]

The significance of differences between the fractions was tested using Tukey's Multiple Comparison test (letters a-d indicate significant different groups).

Table 3

Results of the brine shrimp assay for the lipophilic *I. aquatica* fractions **(group A)**

Fraction	Mortality (%) with various concentrations (µg/mL)					LC_{50} (µg/mL)	Activity
	10	100	200	500	750		
IA1	0.00	0.00	3.33	6.67	50.00	750	moderately toxic
IA2	0.00	3.33	3.33	13.33	56.67	712	moderately toxic
IA3	0.00	0.00	3.33	26.67	53.33	719	moderately toxic
IA4	0.00	0.00	10.00	60.00	76.67	440	toxic
IA5	0.00	0.00	10.00	33.3	40.00	>750	ND
IA6-1	0.00	0.00	3.33	33.3	36.67	>750	ND
IA6-2	0.00	0.00	10.00	36.67	66.67	611	moderately toxic
IA7	0.00	0.00	13.33	26.67	56.67	694	moderately toxic
IA8	0.00	0.00	10.00	43.33	46.67	>750	ND

ND = not determined, the percentage of mortality less than 50% at 750 µg/mL.

Table 4

Results of the brine shrimp assay for the hydrophilic *I. aquatica* fractions **(group B)**

Fraction	Mortality (%) at various concentrations (µg/mL)					LC_{50} (µg/mL)	Activity
	10	100	500	750	1000		
IA9-1	0.00	6.67	10.00	13.33	86.67	875	moderately toxic
IA9-2	0.00	0.00	23.33	30.00	80.00	850	moderately toxic
IA9-3	0.00	13.33	23.33	26.67	50.00	1000	moderately toxic
IA10-1	0.00	3.33	20.00	23.33	66.67	903	moderately toxic
IA10-2	0.00	6.67	13.33	20.00	23.33	>1000	non-toxic
IA11	0.00	6.67	6.67	10.00	80.00	893	moderately toxic

Table 5

Antioxidant activity expressed as IC_{50} (mg/mL) and TE/g DW of the *I. aquatica* fractions, determined with the DPPH, ABTS and ORAC assays

Standard or Fraction	DPPH IC_{50} (mg/mL)	CI (95%)	ABTS IC_{50} (mg/mL)	CI (95%)	ORAC (µmol TE/g DW)
Trolox	0.006	0.005-0.007	0.025[a]	0.023-0.028	---
IA1	> 2	---	> 1	---	ND
IA2	> 2	---	> 1	---	ND
IA3	> 2	---	> 1	---	ND
IA4	> 2	---	> 1	---	ND
IA5	> 2	---	0.80***	0.67-0.95	ND
IA6-1	> 2	---	0.56***	0.53-0.60	ND
IA6-2	> 2	---	0.69***	0.59-0.79	ND
IA7	1.90***	1.73-2.09	0.53***	0.49-0.58	ND
IA8	0.91***	0.87-0.96	0.53***	0.50-0.55	ND
IA9-1	0.49***	0.47-0.52	0.31***	0.27-0.35	1172 ± 160[ns]
IA9-2	0.37***	0.32-0.44	0.32***	0.28-0.37	952 ± 374[ns]
IA9-3	0.48***	0.42-0.55	0.34***	0.33-0.36	704 ± 143[ns]
IA10-1	0.33***	0.31-0.36	0.36***	0.34-0.38	713 ± 225[ns]
IA10-2	0.340***	0.338-0.342	0.52***	0.49-0.54	636 ± 180[ns]
IA11	> 2	---	0.256***	0.252-0.260	766 ± 126[ns]

ND = not determined, the percentage of inhibition was less than 50%.

The significance of differences between the IC_{50} of the fractions and trolox as control was determined by ANOVA analysis, followed by Dunnett's Multiple Comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); CI 95% confidence level. For the ORAC assay, the differences between the TE of the fractions were tested using Tukey's Multiple Comparison test (ns= non-significant). The result from three experiments is presented as Mean ± SD.

Table 6

Antidiabetic activity of some *I. aquatica* fractions using α-glucosidase and α-amylase inhibition assays (n= 3) expressed as IC_{50} (concentration at which 50% enzyme inhibition occurs)

Fraction	α-Glucosidase IC_{50} (mg/mL)	CI (95%)	α-Amylase IC_{50} (mg/mL)
IA4	0.16[a]	0.14-0.19	1.08
IA5	0.14[a]	0.07-0.29	> 1
IA6-1	0.17[a]	0.13-0.21	> 1
IA6-2	NS	---	NS
IA7	0.365[b]	0.358-0.373	> 1
IA8	> 1	---	> 1

NS = Insufficient sample quantity.

The differences between the fractions were analyzed using Tukey's Multiple Comparison test ($p < 0.05$). CI: 95% confidence level. The superscript letters a and b indicates significant differences.

Table 7

Phytochemical profile of the two MeOH fractions of *I. aquatica* methanolic extract using the ESI-QTOF-MS approach in negative ion mode

Peak	t_R (min)	UV-vis (nm)	$[M-H]^-$ (m/z)	Fragment ions (m/z)	MF	Tentative name	Fraction	Ref
1	2.91	209, 312	285.062	175.061	$C_{12}H_{13}O_8$	Dihydroxybenzoic acid pentoside	IA9-1, 9-2, -3, 10-1 & 10-2	[34]
2	3.15	203, 270	299.077	211.062, 137.024, 93.035	$C_{13}H_{15}O_8$	Salicylic acid glucoside	IA9-3, 10-1 & 10-2	[35]
3	3.34	211, 312	417.104	293.118, 144.045	$C_{17}H_{21}O_{12}$	Dihyroxybenzoic acid di-pentoside	IA9-1, 9-2, 9-3, 10-1 & 10-2	[34]
4	4.00	217, 271	461.169	215.083, 158.083	$C_{20}H_{29}O_{12}$	Unknown	IA9-1, 9-2, 9-3, 10-1 & 10-2	
5	4.15	217, 286	385.114	258.098, 223.061, 172.098	$C_{17}H_{21}O_{10}$	1-O-sinapoyl-β-D-glucose dervative	IA10-1	[36]
6	4.66	217, 275	371.098	195.066	$C_{16}H_{19}O_{10}$	Dihydroferulic acid derivative	IA10-1	[37]
7	5.24	254, 353	609.146	361.223, 300.027, 271.026	$C_{27}H_{29}O_{16}$	Rutin	IA9-1, 9-2, 9-3, 10-1 & 10-2	[34]
8	5.37	217, 267	449.202	264.087, 246.077, 217.108	$C_{20}H_{33}O_{11}$	Unknown	IA10-1 & 10-2	
9	5.51	218	367.067	173.009	$C_{14}H_3N_{14}$	Unknown	IA10-1 & 10-2	
10	5.80	218, 346	593.150	359.124, 261.134	$C_{27}H_{29}O_{15}$	Nicotiflorin	IA9-1, 9-2, 9-3, 10-1 & 10-2	[34]

11	6.48	219, 300	137.024	116.928, 93.035	$C_7H_5O_3$	Salicylic acid	IA9-1, 9-2, 9-3, 10-1 & 10-2	[34]
12	7.63	221, 273	265.072	231.123, 143.035, 116.928	$C_{13}H_{13}O_6$	Unknown	IA10-1 & 10-2	

Peak numbers correspond to peaks in **Fig. 1.** t_R: retention time, MF: molecular formula.

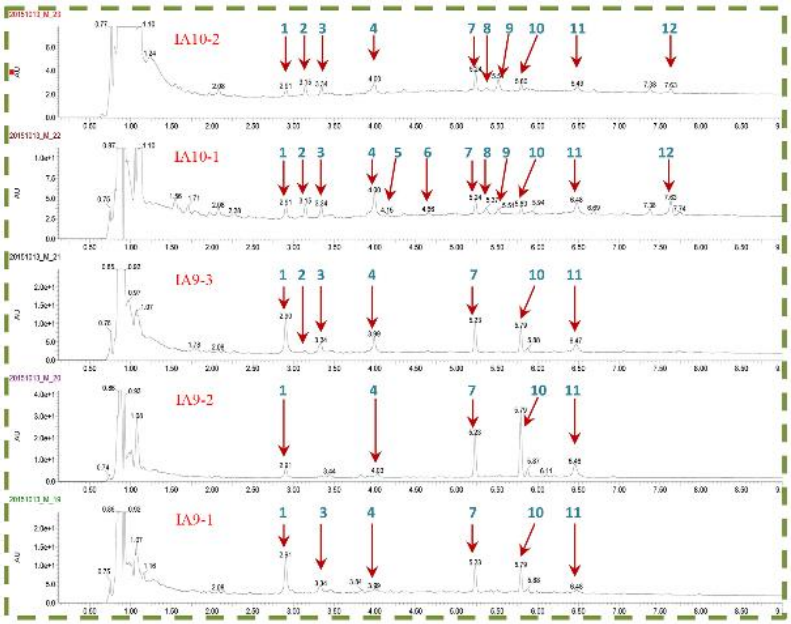
IA10-2
1 2 3 4 7 8 9 10 11 12
IA10-1
1 2 3 4 5 6 7 8 9 10 11 12
IA9-3
1 2 3 4 7 10 11
IA9-2
1 4 7 10 11
IA9-1
1 3 4 7 10 11

Fig. 1.

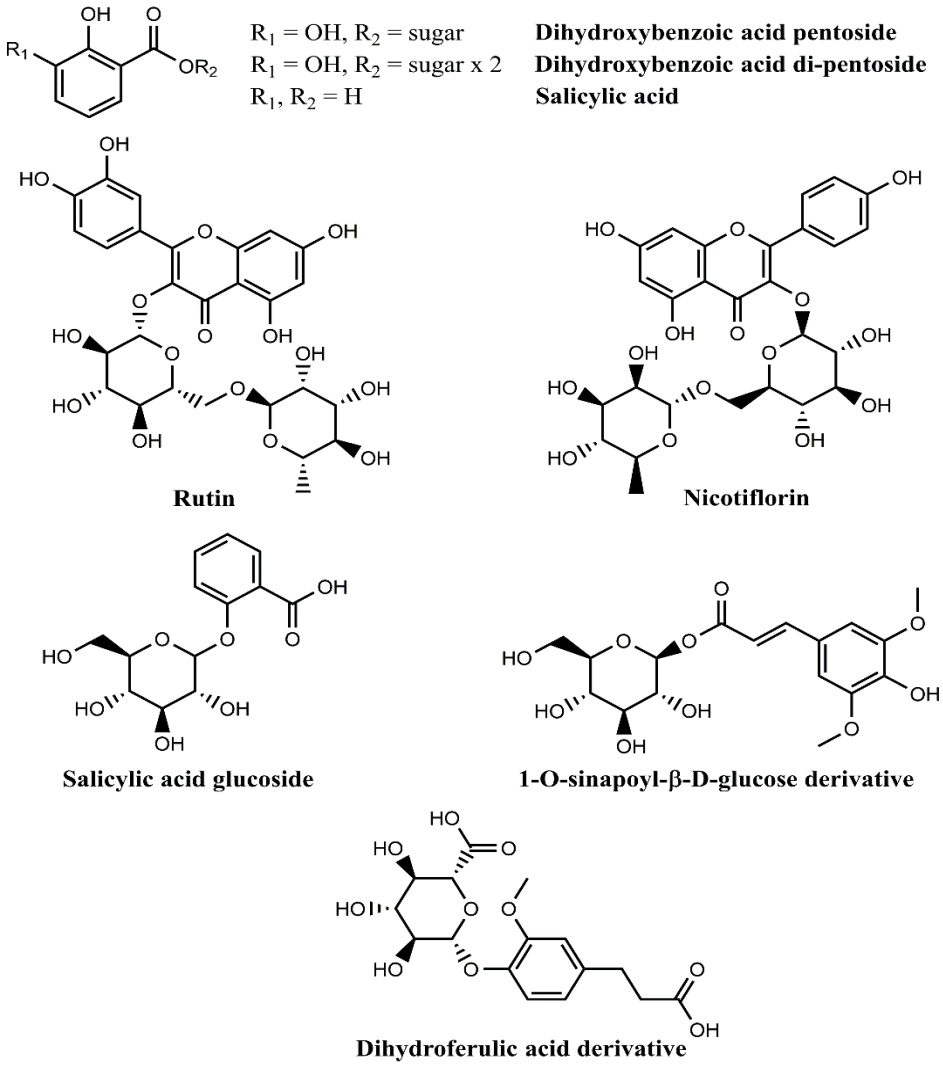
R1 = OH, R2 = sugar
Dihydroxybenzoic acid pentoside
R1 = OH, R2 = sugar x 2
Dihydroxybenzoic acid di-pentoside
R1, R2 = H
Salicylic acid
Rutin
Nicotiflorin
Salicylic acid glucoside
1-O-sinapoyl-β-D-glucose derivative
Dihydroferulic acid derivative

Fig. 2.