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Variability of Surface Exudates of *Dodonaea angustifolia* L.f, Antioxidant and Antiplasmodial activities of the compounds

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Abstract

Dodonaea angustifolia L.f is a medicinal plant used in the Kenyan traditional system of medicine to cure human diseases. The secondary metabolites present in the surface exudates, showed phytochemical diversity of this plant from different geographical conditions in Kenya. There was great geographical variability in the composition of the surface exudates in *D. angustifolia* populations as reflected in the antiplasmodial and radical scavenging activities (RSA) of this substance from the Ngong forest, Nairobi and Taita hills, Voi populations. Most of the compounds from the exudates of *D. angustifolia* and *Senecio roseiflorus* showed moderate anti-plasmodial activities against chloroquine sensitive (D6) and resistant (W2) strains of *P. falciparum* with IC_{50} values ranging between 3.2 and 23.6 μg/ml. Of the pure compounds tested flavonols *ca* kaempferol (**1**) and rhamnocitrin (**2**) exhibited radical scavenging activities (RSA) comparable to the standard, quercetin (**3**) at 50 μM. These studies showed that flavonoids exhibited both antiplasmodial and RSA although there was no direct relationship between these activities. This is elaborated in 5,4´-dihydroxy-7-methoxyflavanone (**4**), exhibiting good antiplasmodial activities but minimal % RSA. The structure-activity relationship of the active flavonoids showed that flavonols had appreciable RSA activities as compared to 3-methoxy flavones from the surface exudates.

Keywords: *Dodonaea angustifolia,* geographical variations, antioxidant activities, antiplasmodial activities.

1. Introduction

Dodonaea angustifolia L.f belongs to the family Sapindaceae distributed in the tropical and subtropical regions of the world (Beentje, 1994). Traditionally this plant and others from the same genus are used as analgesic, laxative, antipyretic, in rheumatism, eczema, and skin ulcers (Dominguez *et al*. 1980 and Watt and Breyer-Brandwijk, 1981). The most common secondary metabolites of *Dodonaea* species are terpenoids and flavonoids, among other minor compounds usually deposited on the surface of the leaves (Ghisalberti, 1998) and are known to have antioxidant, antibacterial, antiviral and recently antimalarial activities (Yenesew *et al*. 2012). A number of 3-methoxyflavones from *Dodonaea* species derived from quercetin and kaempferol have been shown to exhibit pronounced antiviral activity and to be active in tissue cultures against picornavirus (Vlietinak et al., 1995). From a large screening programme, some 3-methoxyflavones, including penduletin (**9**), from *Dodonaea angustifolia* and *D. viscosa* emerged as active *in vitro* compounds against polio and rhinovirus.

There is great geographical variability in the composition for this substance in *Dodonaea* species and population as is reflected in the antiplasmodial and radical scavenging activities (RSA) of the extracts and therefore the interest to study this plant. The thin layer chromatography (TLC) analysis shows variation between the *D. angustifolia* collected from Ngong forest and Taita hills near Voi town. In Kenya, there are two major populations, the ones from the upland region represented by the Ngong population and those from the more coastal locale represented by the plant growing in Taita hills near Voi town in this study (Beentje, 1994).

Senecio roseiflorus R.E Fries on the other hand is a weak shrub, with very sticky leaves of up to 30% dry leaf weight of surface exudates. It is endemic to Kenya and is found in the drier alpine zone, at an altitude of 3100-4200 m (Agnew, 1994). It belongs to the genus *Senecio*, composed of about 1500 species, of which about 33 are found in Kenya (Nordenstam, 1977, Agnew, 1994). The characteristic phytochemical features of plants from this genus include mainly pyrrolizidine alkaloids which are poisonous and hence inedible to many herbivores. The other compounds that have been characterized from these plants include; sesquiterpenoids mainly with an eremophilane skeleton, diterpenoids and flavonoids, with hepatotoxic, carcinogenic, insecticidal, antimicrobial, antitumor, antiviral, antiulcer, and immunosupressing properties (Robins *et al*. 1977, Zhao *et al*. 2004, Tonia *et al*.

2004; Lee *et al*. 2005, Chen *et al*. 2009).

Flavonoids are known to have good antiplasmodial activity against chloroquine-sensitive Sierra Leone I (D6) and chloroquine-resistant Indochina I (W2) Q strains of *Plasmodium falciparum* (Yenesew, 2003, Andayi *et al*. 2006). There is the generation of reactive oxygen species (ROS) during malarial infection and antioxidants, both enzymatic and non-enzymatic, would contribute towards limiting this oxidative damage (Muller, 2004). Flavonoids which are the main phytochemical feature of *Dodonaea* species and are present in the exudates of *S. roseiflorus*, display a wide range of biological and pharmacological activities such as antiplasmodial activities most of which could be attributed to their radical scavenging activities (RSA) (Fotie, 2008). There is speculation that the antiplasmodial activities of extracts and pure compounds could be attributed to the ability of the phenolic compounds in the extracts to wipe out reactive oxygen species (ROS) generated in different stages of malaria infection (Yenesew *et al*. 2012). In this study the chemical viariability of *D. angustifolia* from different geographical locations in Kenya was investigated. The RSA and antiplasmodial activities of the crude extracts and compounds from *D. angustifolia* and flavonoids from *Senecio roseiflorus* were also compared.

2. Materials and Methods

2.1 Plant Material

The fresh leaves of *Dodonaea angustifolia* L.f were collected from three different geographical locations in Kenya including Taita hills in Voi (400 km from Nairobi) on 30th March 2013, Ngong forest (6 km from Nairobi) on 23rd July 2013 and Kilifi (450 km from Nairobi) on 15th August 2013. The plant parts were also collected from Antananarivo, Madagascar (in the environs of the Antananarivo University) on 3rd November 2013 and Gaborone, Botswana (within the University of Gaborone grounds) on 18th July 2012. The plants were identified by Mr. Patrick Mutiso from the University of Nairobi (UoN) Herbarium, School of Biological Sciences (SBS), where voucher specimen labeled LKO-DA Taita hills 30/March/2013, LKO-DA Ngong forest 23rd/July/2013, LKO-DA Kilifi 15th/August/2013, LKO-DA Antananarivo 03rd/November/2012, LKO-DA Gaborone 18th/July/2012 are deposited. The aerial parts of *Senecio roseiflorus* were collected from Mt. Kenya, Meru, at about 1300ft-1500ft and identified by Mr. S. G. Mathenge of the SBS, UoN, where a voucher specimen labeled LKO-SR Meru 18/05/2012 is deposited**.**

2.2 Extraction and Isolation procedure

The leaves of this plant were shinny and gummy indicating that they were coated with surface compounds. Extraction of the surface exudates on the aerial parts (mainly leaves) was done after plucking out the flowers. The surface exudates of the leaves, were washed out into solvent by successive dipping into fresh portions of acetone for short periods (*ca* 15 seconds) to yield the crude extract, thus avoiding the extraction of the internal tissue components. A portion of the crude extracts, was kept aside for bioassays and the rest was subjected to separation by column chromatography using silica gel as the stationary phase and stepwise gradient elution with mixtures of dichloromethane (CH_2Cl_2) in *n*-hexane and then with methanol (MeOH) in CH_2Cl_2 in increasing polarities, Sephadex-LH eluting with 50% MeOH in CH_2Cl_2, Preparative TLC and crystallization.

2.3 Biological Activity Studies

2.3.1 In vitro Anti-plasmodial Test

The crude extract and the pure compounds were assayed using an automated micro-dilution technique to determine 50 % growth inhibition of cultured parasites (Desjardins *et al*. 1979, Chulay *et al*. 1983). Two strains of *Plasmodium falciparum* parasites, from the Walter Reed Army Institute of Research that are commonly used in drug sensitivity assays, were cultured. The chloroquine sensitive Sierra Leone I (D6) and chloroquine-resistant Indo-china I (W2) strains were grown in continuous culture supplemented with mixed gas (90 % nitrogen, 5 % oxygen, 5 % carbon dioxide), 10 % human serum, and 6 % hematocrit of A+ red blood cells. Once cultures reached a parasitemia of 3 % with at least a 70 % ring development stage present, parasites were transferred to a 96 well microtiter plate with wells pre-coated with compound. The samples were serially diluted across the plate to provide a range of concentration used to accurately determine IC_{50} values. Plates were incubated in a mixed gas incubator for 24 hours. Following the specified incubation time, 3H-hypoxanthine was added and parasites allowed to grow for an additional 18 hours. Cells were processed with a plate harvester (Tom Tec) onto a filter paper and washed to eliminate unincorporated isotope. Filters were measured for radioactivity in a microtiter plate scintillation counter (Wallac). Data from the counter was imported into a Microsoft Excel spreadsheet, which was then imported into an Oracle database/program to determine IC_{50} values.

2.3.2 Radical Scavenging Test Using DPPH

Preliminary radical scavenging activities, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical as a spray reagent on thin layer chromatography (TLC) plates, of the crude acetone extract of fresh leaves of *D. angustifolia*-Ngong forest, Nairobi and *S. roseiflorus* indicated that the extract contained compounds with radical scavenging activities. Radical scavenging activities were observed in the crude extract and some compounds tested. The results are summarized in Table 2. Using spectrophotometric method, the radical scavenging activity of the surface exudates of *D. angustifolia* and *S. roseiflorus* were tested at 11.4 µg/ml while the pure compounds were tested at 50 µM. The compounds that showed antioxidant potential comparable to quercetin at that concentration were tested further at lower concentrations. The scavenging activities of the samples were measured as the percentage decrease in absorbance (at 517 nm) of DPPH radical after mixing the sample with DPPH.

To a MeOH solution (3 ml) of DPPH (100 µM), 0.5 ml of test compounds at 50 µM (10 µg/ml for crude extract) were added and the mixture was shaken and left to stand for 30 min. The RSA were estimated as the percentage decrease of absorbance of DPPH (100 µM) at 517 nm (Ohinishi *et al*. 1994). For 3,5,4´-trihydroxy-7-methoxyflavone (rhamnocitrin) (**5**), 5,7,4´,-trihydroxy-3-methoxy flavone (isokaempferide) (**6**), and quercetin (**4**), tests were done at six different concentrations (50, 25, 12.5, 3.13 and 1.56 µM). In all cases the mean values were used from triplicate experiments. These solutions were then measured for UV-VIS absorbance at DPPH absorbing wavelength (517 nm) half an hour after adding the DPPH solution. The absorbance measured at each of these concentrations were converted into percentage radical scavenging activity (RSA) calculated as follows;

RSA (%) = (Ablank - Asample) / Ablank)* 100

Where Ablank refers to the optical density (OD) of the control (DPPH solution) while Asample refers to the OD of the test sample (Erasto *et al*. 2011). The percentages of scavenged DPPH were then plotted against concentration of the compound to give graphs from which concentrations at half inhibition (IC_{50}) were determined. The tests were done in triplicates.

3. Results and Discussion

3.1 TLC analyses

The TLC analyses of crude extracts (acetone) obtained from the three populations of *D. angustifolia* from Taita hills, Voi, Kilifi, and Ngong forest, Nairobi from Kenya and from other parts of Africa ca Antannarivo, Madagascar and Garborone, Botswana were compared and all showed variation in the exudates components and flavonoids composition (Figure 1). The flavonoid and diterpenoid profiles which from chemotaxonomic assumption constitute the major compounds of the surface exudates do not completely match each other (Figure 1). From the above overview, different *D. angustifolia* populations from different geographical regions of Kenya probably elaborate different sets of flavonoids and terpenoids. All collected populations differed from each other based on phytochemical markers. The population from Taita hills, Voi showed more phytochemical diversity as compared to those of Ngong, Nairobi which was evaluated by TLC fingerprinting, RSA and anti-plasmodium activities.

3.2 Anti-plasmodial activities

The criteria used for evaluation of *in vitro* antimalarial activities was based on their IC_{50} values categorized as follows: IC50 < 0.1 µg/ml (very good); 0.1–1 µg/ml (good); 1.1–10 µg/ml (good to moderate); 11–25 µg/ml (weak), 26–50 (very weak), >100 µg/ml (inactive) (Willcox ., 2004).

The acetone extract of the fresh leaves (surface exudates) of *D. angustifolia*-Ngong forest, Nairobi and *D. angustifolia*-Taita hills, Voi showed anti-plasmodial activities with IC_{50} values of 41.5±3.9 and 56.3±4.2 µg/ml against the chloroquine-sensitive (D6) respectively and 38.9±4.9 and 54.4±2.3 µg/ml against chloroquine-resistant (W2) strain therefore inactive against the two strains of the *P. falciparum*. The compounds tested had good to moderate activities (IC_{50} values between 3.2 and 23.6 µg/ml) consistent with earlier studies on flavone methyl ethers (Kraft *et al*. 2003, Casanoa *et al*. 2010, Kerubo *et al*. 2013). This implies that the activities of the compounds were considerably more than those of the crude extracts and hence the need to re-isolate the compounds obtained in low yields in order to determine their antiplasmodial potential (Table 1.0).

3.3 Antioxidant Activities

The surface exudates and some compounds isolated from aerial parts of *Dodonaea angustifolia* (from two

locations Ngong forest, Nairobi and Taita hills, Voi) and *Senecio roseiflorus* showed radical scavenging activities using 3,5,7,3,4-pentahydroxyflavone (quercetin, **3**) as the standard. Using DPPH free radical as a spray reagent on TLC plates, crude extract of the leaves of *D. angustifolia*-Ngong forest, Nairobi and *S. roseiflorus* indicated that the extract contained compounds with radical scavenging activities (Table 1.0). The surface exudates of *D. angustifolia* from Ngong forest, Nairobi and Taita hills, Voi showed modest RSA of 54.6 and 34.7%, respectively at 11.4 µg/ml while that of *S. roseiflorus* exhibited minimal activity of 9.25 % at the same concentration. All the flavonoids tested showed varied activities at 50 µM (Table 1.0). The flavonol, 3,5,7,4´-tetrahydroxyflavone (kaempferol, **1**) was found to be the most active with RSA of 96.8% at 50 µM followed by 3,5,4´-trihydroxy-7-methoxyflavone (**2**) and 3´,5,7-trihydroxy-3,4´ -dimethoxyflavone (quercetin-3,4´ -dimethyl ether)(**6**) with RSA of 96.2 and 77.1 % respectively at the same concentration. The % RSA of 3,5-dihydroxy-7,4´-dimethoxyflavone (**7**) was 25.4 % while the flavanones, 5,4´ -dihydroxy-7-methoxyflavanone (**4**) and 5,7-dihydroxyflavanone (**8**) were the least active of all the flavonoids tested with RSA of only 1.22 and 2.31 % respectively. The RSA of 5,4´-dihydroxy-3,6,7-trimethoxyflavone (**9**), 5,7-dihydroxy-3,6,4´-trimethoxyflavone (**10**), 5,7,4´-dihydroxy-3-methoxyflavone (**5**) and 5,4´-dihydroxy-3,7-dimethoxyflavone (**11**) were 2.55%, 6.67%, 11.2% and 18.4% respectively at 50 µM. The activity of kaempferol (**1**) was comparable to that of quercetin (**3**) (96.7%) at 50 µM. The activities of compounds **1**, **2** and **6**, which were comparable to the standard were ascertained at lower concentrations to determine their IC_{50} value. At these concentrations the activity of quercetin (**3**) was higher than that of kaempferol (**1**). The activity of 3,5,4´-trihydroxy-7-methoxyflavone (**2**) and kaempferol (**1**) were comparable at all concentrations. Flavonols exhibited appreciably good anti-oxidant activities compared to the 3-methoxyflavones, indicating the importance of the hydroxyl group at C-3 consistent with studies by Harborne, (1983) and Rice-Evans *et al*. 1996 and Saskia *et al*. 1996, Op de Beck *et al*. 2003, Teffo *et al*. 2010). The diterpenoids tested; 2β-hydroxyhardwickiic acid (**12**), and dodonic acid (**13**) showed no radical scavenging activity (RSA) at 50 µM as expected. Some flavonoids exhibited both antiplasmodial and radical scavenging activities although there was no clear correlation between the activities.

4. Conclusion

The surface exudates of the *D. angustifolia* Ngong forest, Nairobi and Taita hills, Voi exhibited different antiplasmodial and radical scavenging activities using DPPH reagent, which could be attributed to the flavonoids composition, the major class of phytochemicals found in these plants. The major differences in % RSA of the two populations could be attributed to the qualitative and quantitative composition of the flavonoids in the exudates. This observation supports the great variability in the composition and yield of surface exudate compounds in the two geographical locations in Kenya. The surface exudates of *D. angustifolia* from Ngong, Nairobi, that exhibited higher activity, could be richer in flavonoids that have higher radical scavenging activity, in this case the flavonols, as compared to the population from Taita hills, Voi. Furthermore the percentage yield of the flavonols content in the total extract would be higher in the Ngong forest population hence the higher potencies. The pure compounds from the two plant species were also tested for anti-oxidant activities and some of the flavonoids showed RSA activity. The potential use of the surface exudates and flavonoids from the two populations of *D. angustifolia* as radical scavenger was established. The structure-activity relationship of the active flavonoids showed that flavonols had appreciable antioxidant activities as compared to 3-methoxy flavones isolated from the surface exudates. It was evident from this studies that the antiplasmodial and radical scavenging activities of the pure compounds were considerably more than those of the crude extracts and hence the need to test the minor compounds from these plants to establish their activities. Some flavonoids exhibited both antiplasmodial and radical scavenging activities although there was no clear correlation between the activities.

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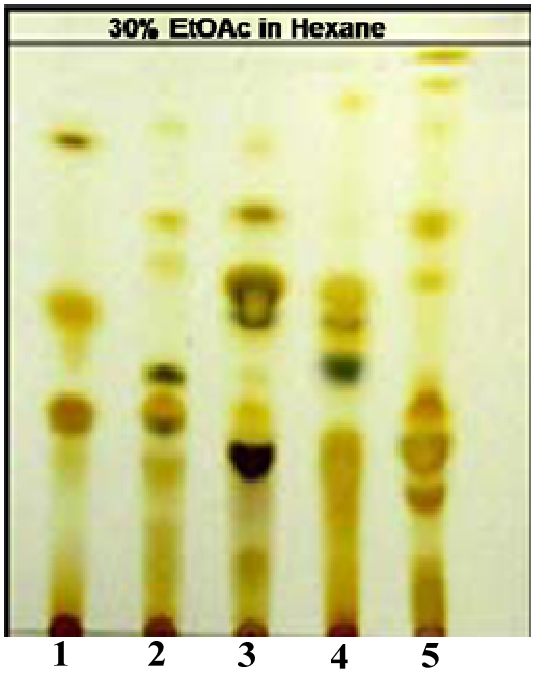
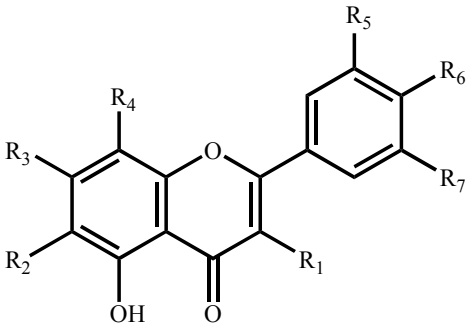


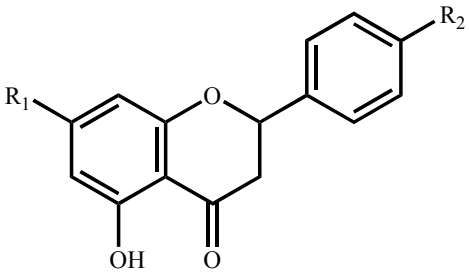
Figure 1. *Dodonaea angustifolia* collections from the following locations in Africa; 1, Taita hills near Voi, Kenya; 2, Kilifi, Kenya; 3, Ngong forest, Kenya; 4, Antananarivo, Madagascar; 5, Gaborone, Botswana.

Table 1. *In vitro* antiplasmodial and % radical scavenging activities (RSA) of compounds from *D. angustifolia* and *S. roseiflorus*

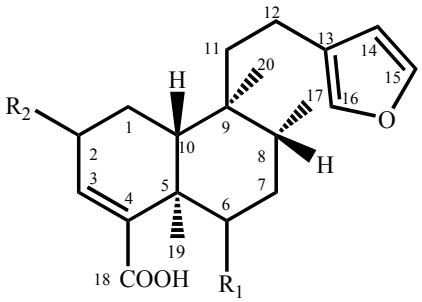
Sample	Source	Antiplasmodial activity IC_{50} (µg/ml)		% RSA (50 µM) DPPH[b]
		D6[a]	W2[a]	
Crude extracts				
D. angustifolia-Ngong forest		41.5± 3.9	38.9 ± 4.9	54.6 %
D. angustifolia-Voi		56.3± 4.2	54.4± 2.3	34.7%
Flavonols				
Kaempferol (**1**)	*D. angustifolia*	NT	NT	96.8
Rhamnocitrin (**2**)	*D. angustifolia*	17.4 ± 3.9	14.4 ± 3.3	96.2
3,5-Dihydroxy-7,4´-dimethoxyflavone (**7**)	*D. angustifolia*	13.0 ± 2.4	8.7 ± 2.4	25.4
6-Methoxykaempferol (**14**)	*D. angustifolia*	18.4 ± 4.8	15.3 ± 1.2	NT
Rhamnazin (**15**)	*S. roseiflorus*	18.6 ± 7	12.1 ± 3.3	NT
Quercetin (**3**)				96.7
3-methoxyflavones				
5-Hydroxy-3,7,4´-trimethoxyflavone (**16**)	*D. angustifolia*	13.8 ± 4.2	10.6 ± 2.3	NT
Kumatakenin (**17**)	*D. angustifolia*	7.6 ± 2.3	9.9 ± 1.6	18.4
Santin (**18**)	*D. angustifolia*	8.6 ± 1.7	12.5 ± 2.4	6.67
Isokaempferide (**5**)	*D. angustifolia*	11.1 ± 4.0	14.3 ± 2.1	11.2
Quercetin-3,4´-Dimethyl Ether (**6**)	*S. roseiflorus*	18.2 ± 3.5	28.9 ± 2.3	77.1
5,4´-Dihydroxy-3,6,7-trimethoxyflavone (**9**)	*S. roseiflorus*	8.5± 3.5	18.3± 1.5	NT
5,7-Dihydroxy-3,4´-dimethoxyflavone (**19**)	*S. roseiflorus*	8.9 ± 1.7	8.5 ± 1.4	NT
Retusin (**20**).	*S. roseiflorus*	10.7 ± 5.7	9.4 ± 3.7	NT
5,4´-Dihydroxy-3,7,3´-trimethoxyflavone (**21**)	*S. roseiflorus*	10.9 ± 2.1	NT	NT
Flavanone				
5,4´-Dihydroxy-7-dimethoxyflavanone (**4**)	*S. roseiflorus*	3.2 ± 0.8	4.4 ± 0.01	1.22
Pinocembrin (**8**)	*D. angustifolia*	10.7 ± 1.3	17.6 ± 3.4	2.31
Terpenoids				
2β–Hydroxyhardwickiic acid (**12**)	*D. angustifolia*	10.8 ± 2.2	16.5 ± 1.7	NA
Dodonic acid (**13**)	*D. angustifolia*	9.7 ± 2.8	8.3 ± 3.1	NA
Chloroquine		0.43 ± 0.002	0.51 ± 0.004	NT
Quinine		0.069 ± 0.001	0.073± 0.002	NT
Mefloquine		0.004 ± 0.001	0.002 ± 001	NT



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	OH	H	OH	H	H	OH	H
2	OH	H	OMe	H	H	OH	H
3	OH	H	OH	H	OH	OH	H
5	OMe	H	OH	H	H	OH	H
6	OMe	H	OH	H	OH	OMe	H
7	OH	H	OMe	H	H	OMe	H
9	OMe	OMe	OMe	H	H	OH	H
10	OMe	OMe	OH	H	H	OMe	H
11	OMe	H	OMe	H	H	OH	H
14	OH	OMe	OH	H	H	OH	H
15	OH	H	OMe	H	OH	OMe	H
16	OMe	H	OMe	H	H	OMe	H
17	OMe	H	OMe	H	H	OH	H
18	OMe	OMe	OH	H	H	OMe	H
19	OMe	H	OH	H	H	OMe	H
20	OMe	H	OMe	H	OMe	OMe	H
21	OMe	H	OMe	H	OMe	OH	H



	R_1	R_2
4	OH	H
8	OH	H



	R_1	R_2
12	H	OH
13	OH	H

Figure 1. Compounds from *Dodonaea angustifolia* and *Seneio roseiflorus*