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Antioxidant Activities of Flavonoid Aglycones from Kenyan *Gardenia ternifolia* Schum and Thonn

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Abstract: *Phytochemical investigation of surface exudates of the leaves of Gardenia ternifolia resulted to characterization of four flavonoids; 3,5,3'-trihydroxy-7,4'-dimethoxyflavone (**1**), 5,7-trihydroxy-4'-methoxyflavone (**2**), 5,7-dihydroxy-3,4'-dimethoxyflavone (**3**), 5,4'-dihydroxy-7-methoxyflavanone (**4**) and two tritepenoids; β-sitosterol (**6**) and stigmasterol (**7**). Compound **1** exhibited the highest antioxidant activity with IC_{50} = 40.3± 1.55 μM. The rest of the flavonoids showed minimal activities with IC_{50} values of 75.5±1.75, 89±0.22, 94±0.11 μM for **2-4**, respectively. The antioxidant activities of **1** was substantially lower than the standard, quercetin (IC_{50} = 20.1±1.34 μM). Methoxylation of quercetin at 7 and 4'-position in **1** substantially reduced antioxidant potential. Lack of oxygenation at 3' position, as observed for kaempferol derivatives was responsible for further reduction in the radical scavenging potential as observed for **2** and **3**. Furthermore, methylation of 3-OH position in kaempferol derivatives further reduced the antioxidant activities as exhibited by **3** with an oxygenation pattern similar to **2** except for the methylation at 3-position. The results of this study are consistent with previous findings that revealed that flavonols, exhibited better anti-oxidant activities as compared to 3-methoxyflavones. Acetylation of **3** at the 5 and 7 positions resulting to 3,4'dimethoxy-5,7-diacetylflavone (**5**), substantially reduced the activity of this compound. The triterpenoids exhibited were inactive as expected.*

Keywords: *Antioxidant activities, Gardenia ternifolia, surface exudates*

I. Introduction

Gardenia ternifolia (Schum and Thonn) (Rubiaceae) is a shrub or tree growing to upto about 10 meters in height. This species occurs mainly in the woodland or riverine, in Kenya, it occurs in Nyanza, Central, Eastern, Coast and the Western provinces [1]. In East African traditional medicine it is used for the management of a number of diseases including; malaria, ulcers, coughs, syphilis, stomachache, arthritis, asthma, epilepsy and other mental problems, fevers, pain, paludism and as purgative and astringent, anti-snake venom, laxative [2-9].

Different parts of this plant have exhibited various biological activities from separate studies including; anthelmintic, [10], bronchodilator and antiviral activities [4], antiplasmodial and mosquito larvicidal activities [11]. *Gardenia* spp elaborates several classes of compounds ranging from flavonoids [12-17], alkaloids, iridoids and even terpenoids [16, 17-22].

Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell [23-24]. Oxidative stress is caused by an imbalance between the generation of ROS by endogenous/exogenous pro-oxidants and the defense mechanism against ROS by antioxidants. ROS, are highly reactive molecules which are naturally occurring by-products of normal biological processes within the body or from exogenous factors. When antioxidants are not enough or during diseases, the amount of ROS increase and react with DNA, lipids and proteins to causing cell death due to necrosis [25].

There is substantial evidence implicating excess levels of ROS and the resulting oxidative stress in the etiology of a number of degenerative and chronic disorders in humans including; cardiovascular diseases, arthritis, diabetes, renal disorder, inflammatory, autoimmune, cancer, atherosclerosis, asthma, autism and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and several other human ailments [26-33].

Potent scavengers of free radicals (antioxidants) may serve as a possible preventative intervention for these diseases [34]. Antioxidants act as radical scavengers through the donation of one or more of their own electrons thereby neutralizing the free radical and thus terminating the chain reactions, which could otherwise damage cells and tissues. The commonly used synthetic antioxidants include; butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, which have been implicated in liver damage and carcinogenesis in laboratory animals [35].

Recently, there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants, in reducing free radical induced tissue injury. Antioxidant activities of plants are attributed to the presence of phenolic compounds which terminates the free radicals via various processes [36]. Flavonoids are a group of polyphenolic compounds with known properties which include; free radical scavenging activities,

inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [37]. They exhibit different biological activities influencing on numerous metabolic pathways. Due to their radical scavenging, antioxidant, anti-inflammatory, anti-allergic, anticancer, antiatherosclerotic, anti-aggregational and detoxification activities they might be useful for prevention and treatment of many human diseases [38]. In this study, four related flavonoid aglycones, one derivative and two steroids isolated from *G. ternifolia* were evaluated for their *in vitro* antioxidant potential. The effect of methoxyl, acetyl groups in place of hydroxyl in the flavone skeleton was also ascertained.

II. Materials and methods

2.1 General Experimental Procedures

The proton (1H) NMR and carbon (^{13}C) NMR spectra were acquired on a Varian 400 MHz spectrometer and the chemical shifts values were obtained in ppm (δ) with TMS as the internal standard. The compounds were revealed under UV light at 254 and 366 nm and on exposure to ammonia fumes and iodine vapour. Quantitative isolation of the compounds was achieved by normal phase liquid column chromatography on activated silica gel 60-120 mesh with gradient elution, while Kieselgel 60 H was used to make the preparative thin layer chromatography (TLC) plates. The solvents which were used for the extraction of the surface exudates and column chromatography were purified by fractional distillation and a thermometer was used in monitoring the distillation process. Preparative thin layer chromatography (PTLC) plates were prepared on glass plates (20 cm by 20 cm). This was done by measuring 80 g of preparative silica gel and mixing with 200 ml of distilled water for six plates. 40 ml of the resultant slurry was measured and poured onto each plate then was spread uniformly using a flat spatula and left overnight to dry resulting to plates of 2 mm thickness. The plates were eventually activated in an oven set at 110 ºC for 30 minutes and allowed to cool before they were used for the separation process.

2.2 Collection of Plant Material

The aerial parts of *Gardenia ternifolia* were collected from Machakos district in Kenya in December 2014. The plant materials were identified by Mr. Patrick Mutiso from the University of Nairobi herbarium, School of Biological Sciences where a voucher specimen number EOA 2014/UoN was deposited.

2.3 Extraction and Isolation Procedures

Crude leaf surface extract was obtained by dipping about 200 g of fresh leaves into about 5 litres of acetone for a short period of 15 seconds hence avoiding the extraction of internal tissue components [39]. This process was repeated until the colour of acetone changed to yellowish when fresh acetone was used. The surface extract was then filtered and evaporated *in vacuo* using a rotary evaporator under reduced pressure giving a yellowish black gummy extract of 85 g. 70 g portion of the resultant extract was adsorbed onto 70 g of silica gel, dried *in vacuo*, ground into fine powder and loaded onto a column packed with 700 g of silica gel under *n*-hexane. This was subjected to column chromatography by gradient elution initially with *n*-hexane with increasing amounts of CH_2Cl_2 up to 100 % followed by 1 and 2 % MeOH in CH_2Cl_2. This yielded one hundred and ten (110) fractions of 250 ml each which were combined based on similarities of their thin layer chromatography (TLC) profiles resulting into only eleven fractions (15A-15K).

Fraction 15A eluted with 10% CH_2Cl_2 in *n*-hexane yielded β-sitosterol (**6**) (65 mg, R_f value of 0.69 in 50% CH_2Cl_2 in *n*-hexane) which recrystallized out of CH_2Cl_2/*n*-hexane. The second fraction of the major column, 15B eluted with 20% CH_2Cl_2 in *n*-hexane showed the presence of one spot on the TLC profile on exposure to iodine. This fraction was thereafter recrystallized from this solvent system to obtain white crystalline solids of stigmasterol (**7**) (50 mg, R_f value of 0.5 in 50% CH_2Cl_2 in *n*-hexane). Fractions 15C, 15D and 15E eluted with 50% CH_2Cl_2 in *n*-hexane were further combined and purified through a minor silica gel column eluting with varying polarities of CH_2Cl_2 in *n*-hexane beginning with 50% upto 100%. This minor column resulted into only four fractions labeled 16 A-D. The fraction eluted with 60% CH_2Cl_2 in *n*-hexane resulted into white solids of 5,4′-dihydroxy-7-methoxyflavanone (**4**, 80 mg, R_f value of 0.3 in neat CH_2Cl_2).

Fractions 15F, 15G and 15H (60 mg) of the main column eluted with up to 60-70% CH_2Cl_2 in *n*-hexane revealed the presence of two spots on analytical TLC plate. Purification of this combined fraction was achieved by preparative-TLC (PTLC) with multiple development using 60% CH_2Cl_2 in *n*-hexane resulting to two flavonoids namely; 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (**1**, 15 mg) and 5,7-dihydroxy-3,4′-dimethoxyflavone (**3**, 25 mg). The last three fractions, 15I, 15G and 15K (150 mg) eluted with 80% CH_2Cl_2 in *n*-hexane up to 1% MeOH/CH_2Cl_2, which revealed one major spot was similarly subjected to separation using PTLC with multiple development in 80% CH_2Cl_2 This afforded compound 3,5,7-trihydroxy-4′-methoxyflavone (**2**, 120 mg, R_f value of 0.1 in neat CH_2Cl_2).

2.4 Structural Modification

3,4′dimethoxy-5,7-diacetylflavone (**5**) was obtained by acetylating 10 mg of 5,7-dihydroxy-3,4′-dimethoxyflavone (**3**) using 1 ml of pyridine and 5 ml of acetic anhydride. The reaction mixture was left at room temperature for 24 hours. The reaction mixture was then poured in ice cold water in 250 ml beaker then stirred for two minutes and left to settle for 20 minutes from which a white precipitate was formed. This was filtered over a sunction pump and dried affording 11.54 mg of **5**.

2.5 Radical Scavenging Test Using DPPH

A preliminary radical scavenging activities, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) was done by spotting the isolated compounds on a TLC plate followed by spraying with 0.2 mg/ml DPPH solution to view the active compounds which displayed white or yellowish sports on a purple background. The active compounds were quantitatively analyzed by UV-VIS spectrometry method adopted from Hou *et al*., 2002 [40] with modifications made on the concentrations of the samples. For each compound, the concentration of the sample was varied by serial dilutions to give concentrations of 160, 80, 40, 20, 10, 5.0, 2.5, 1.25 µM while the concentration of DPPH was kept constant at 100 µM. The reaction mixture consisted of adding 0.5 ml of sample, 3 ml of absolute ethanol and 0.3 ml of 100 µM/ml DPPH radical solution in ethanol. These solutions were then measured for UV-VIS absorbance at DPPH absorbing wavelength (517 nm) half an hour after adding the DPPH. The absorbance measured at each of these intervals was converted into percentages of scavenged DPPH radicals using the following equation. In all the cases, the mean values were used from triplicate experiment.

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

Where Ablank is the absorbance of DPPH solution without sample, Asample is the absorbance of the sample. The percentages of scavenged DPPH were then plotted against concentration of the compound to give graphs from which concentrations (µM) at half inhibition (IC_{50}) were determined. The tests were done in triplicates.

III. Results And Discussion

3.1 Isolated Compounds

In a search for more effective antioxidant principles from Kenyan ethnomedicinal flora, chromatographic separation of the surface exudates of the leaves of *G. ternifolia* obtained using acetone yielded four known flavonoid aglycones namely; 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (1) [41-42], 3,5,7-trihydroxy-4′-methoxyflavone (**2**) [11], 5,7-dihydroxy-3,4′-dimethoxyflavone (**3**) [43-45], 5,4′-dihydroxy-7-methoxyflavanone (**4**) [45], two common tritepenoids; β-sitosterol (**6**) [42], stigmasterol (**7**) [42, 46-47]. One of the flavonoids, **3** was acetylated at 5 and 7 positions in the flavone skeleton to obtain 3,4′dimethoxy-5,7-diacetylflavone (**5**) (Fig. 1). The structures of these compounds were determined using 1D and 2D NMR spectroscopy and MS, and supported by literature values. The compounds were investigated for *in vitro* antioxidant potencies, especially using DPPH.

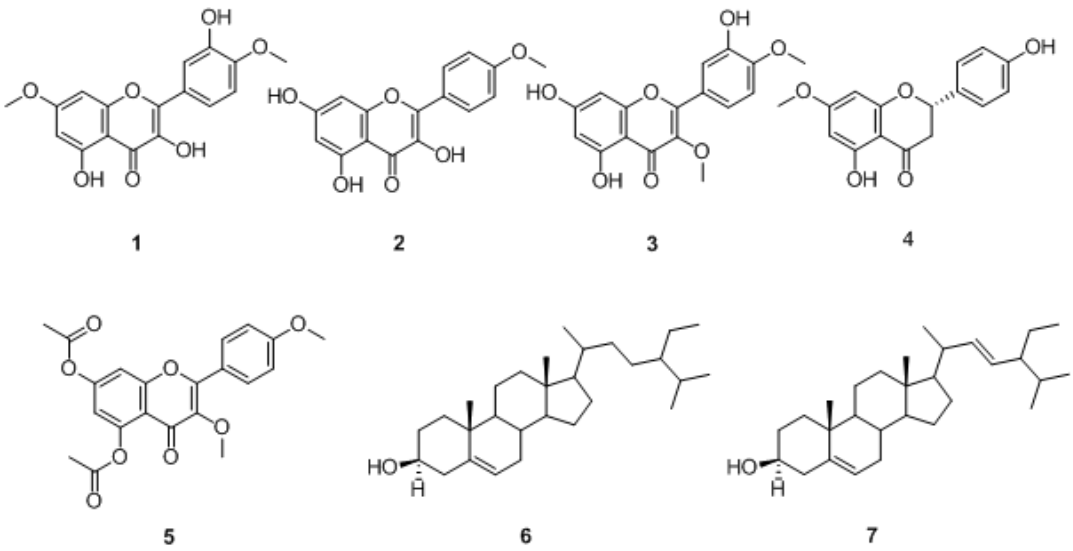

Figure 1: Compounds isolated from the surface exudates of *Gardenia ternifolia*

3.2 Antioxidant Assay Results

All the compounds isolated were tested for anti-oxidant activities. The compounds were first subjected to preliminary qualitative testing on TLC plate where the steroids, **6** and **7** did not turn white after spraying with DPPH reagent. However, the flavonoids, **1**-**5** showed varied degree of activities after spraying their spots on TLC with 2,2-diphenylpicrylhydrazyl (DPPH) reagent. The radical scavenging activities of the flavonoids and their acetylated derivative were quantitatively analyzed using UV-VIS absorption measurements (517nm). The flavonoid aglycones exhibited varying antioxidant activities mainly dependent on the substitution pattern of the hydroxyl and methoxyl groups on the flavone skeleton. The antioxidant activities of the most active flavonoid, 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (**1**) with an IC_{50} value of 40.3±1.55 μM was substantially lower than the standard, quercetin which exhibited an IC_{50} value of 20.1±1.34 μM. Previous studies by Akimanya *et al* (2014) [48] revealed that the presence of hydroxyl groups at 4′ position and oxygenation at 3′-position in quercetin derivatives are necessary for good antioxidant activities. In the current study, methoxylation of quercetin at 7 and 4′-position substantially reduced its antioxidant potential. Lack of oxygenation at 3′ position, as observed for kaempferol derivatives was responsible for further reduction in the radical scavenging potential as observed for compounds **2** (IC_{50} = 75.5±1.75) and **3** (IC_{50} = 89± 0.22).

Furthermore, methylation of 3-OH position in kaempferol derivatives further reduced the antioxidant activities as exhibited by **3** with an oxygenation pattern similar to **2** except for the methylation of the hydroxyl group at 3-position. The weak activity exhibited by the flavonoid aglycones could be linked to the methylation of some of their phenolic-OH groups [49]. The more the reduction in the hydroxyl moieties of the compound, the lesser the anti-oxidant activities.

Table 1: Anti-oxidant activity of flavonoids with DPPH experiment

Compound	**TLC Assay results**	**IC_{50} (μM)**
3,5,3′-trihydroxy-7,4′-dimethoxyflavone (**1**)	+	40.3± 1.55
3,5,7-trihydroxy-4′-methoxyflavone (**2**)	+	75.5± 1.75
5,7-dihydroxy-3,4′-dimethoxyflavone (**3**)	+	89± 0.22
5,4′-dihydroxy-7-methoxyflavanone (**4**)	+	94± 0.11
3,4′dimethoxy-5,7-diacetylflavone (**5**)	+	> 100
3,5,7,3′4′-Penta-hydroxyflavone (Quercetin, **standard**)	+	20.1± 1.34

IV. Conclusion

The phytochemical investigation of Kenyan *Gardenia ternifolia* surface exudates led to characterization of six compounds including three flavones (**1**-**3**), one flavanone (**4**) and two phytosterols (**6**-**7**). Acetylation of compound **3** resulted to **5** with completely reduced antioxidant activity.

The antioxidant activities of the most active flavonoid, 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (**1**) with an IC_{50} value of 40.3±1.55 μM was substantially lower than the standard, quercetin which exhibited an IC_{50} value of 20.1±1.34 μM. Previous studies by Akimanya *et al* (2014) [48] revealed that the presence of hydroxyl groups at 4′ position and oxygenation at 3′-position in quercetin derivatives are necessary for good antioxidant activities. In the current study, methoxylation of quercetin at 7 and 4′-position in **1** substantially reduced its antioxidant potential. Lack of oxygenation at 3′ position, as observed for kaempferol derivatives was responsible for further reduction in the radical scavenging potential as observed for compounds 2 (IC_{50} = 75.5±1.75) and **3** (IC_{50} = 89±0.22). Furthermore, methylation of 3-OH position in kaempferol derivatives further reduced the antioxidant activities as exhibited by **3** with an oxygenation pattern similar to **2** except for the methylation of the hydroxyl group at 3-position. The results of this study are consistent with Omosa *et al*., 2016 [50] that revealed that flavonols exhibited better anti-oxidant activities as compared to 3-methoxyflavones. Comprehensive phytochemical studies of the surface exudates of the aerial parts of *G. ternifolia* using HPLC should be carried out in order to isolate all the compounds that might have greater structural diversity for antioxidant structural activity relationship studies.

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Conflict of Interests

The authors declare no conflict of interest.

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