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Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. *angustifolia* leaf extracts

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Abstract

Fractionation of dichloromethane and acetone fractions obtained by serial extraction from the leaf powder of *Dodonaea viscosa* Jacq. var. *angustifolia* (Sapindaceae) resulted in the isolation of four kaempferol methyl ethers. The compounds were identified by spectral data (^1H NMR, ^{13}C NMR and MS) as: 3, 5, 7-trihydroxy-4'-methoxyflavone (**1**); 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone (**2**); 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (santini) (**3**); and 5-hydroxy-3, 7, 4'-trimethoxyflavone (**4**) together with 3,4',5,7-tetrahydroxy flavone (kaempferol) (**5**). Antioxidant potential of the compounds was evaluated using a DPPH spectrophotometric assay, while antibacterial activity was determined using a serial dilution microplate technique. The isolates demonstrated varying degrees of antioxidant and antibacterial activities. Of all the compounds investigated, compounds **1** and **5** demonstrated some antioxidant activity ($\text{EC}_{50} = 75.49 \pm 1.76 \mu\text{M}$ and 35.06 ± 0.85 respectively) but lower than L-ascorbic acid ($\text{EC}_{50} = 13.55 \pm 0.28 \mu\text{M}$) used as a standard antioxidant agent. The minimum inhibitory concentration (MIC) of isolated compounds against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* varied from 16 $\mu\text{g/ml}$ to more than 250 $\mu\text{g/ml}$. Some structure activity relationships could be established for these compounds.

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Keywords: Antibacterial activity; Antioxidant activity; *D. viscosa*; DPPH; Flavonoids; Free radical; MIC

1. Introduction

Dodonaea viscosa Jacq. var. *angustifolia* (Sapindaceae) is a medicinal plant used in folk medicine to treat various ailments; the leaves have traditionally been administered to treat the following: sore throat, wounds, fever (Jansen, 1981); piles (Tadese, 1994); fever, malaria, angina, cold, arthritis, sinusitis flu (Lemordant, 1971); and boils, dressing for skin diseases of the head and face (Sandhya et al., 2006; Abate, 1989). Getie et al. (2003) reported that a methanol leaf extract of *D. viscosa* from Ethiopia demonstrated antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Corynebacterium diphtheriae* and exhibited antiviral activity against Cocksackie virus B3 and Influenza A virus. However, their quantitative data were obtained by using the agar diffusion

technique. This method is not useful to quantify antimicrobial activity of plant extracts for comparative purposes. We therefore decided to reinvestigate the antibacterial activity of *D. viscosa* leaf extracted with solvents of various polarities by using a microplate serial dilution assay (Eloff, 1998). that would provide MIC data.

Antioxidants are important in maintaining good health and there is a growing interest in the investigation of antioxidant activity of secondary metabolites from medicinal plants for compounds with higher potency and lower toxicities than the synthetic ones currently available (Thabrew et al., 1998). Plants offer a wide range of natural antioxidants due to the structural diversities of their secondary metabolites. Many medicinal plants have now been recognized as sources of natural antioxidant compounds which are mainly phenolic compounds (Wangensteen et al., 2004; Langley-Evans, 2000; Aruoma, 2003).

There has been much phytochemical work done on *D. viscosa*. We were interested in the interaction between edible insects feeding mainly on this plant on the possibility that the

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antibacterial compounds could be sequestered by the insect (Teffo, 2006; Teffo et al., 2007). There has been some reports on the antibacterial activity of *D. viscosa* extracts but we found no publication on the compounds responsible for the antibacterial and antioxidant activities of this widely used medicinal plant. To evaluate the medicinal potential of compounds present in *D. viscosa* and validate the traditional use of extracts to treat infections, we used bioassay guided fractionation method to isolate and characterize antibacterial compounds. Because plant extracts may also play an indirect role in protecting humans against infections by stimulating the immune system through antioxidant constituents (Craig, 1999); we also isolated antioxidant compounds present in the leaf extracts.

2. Methodology

2.1. Chemicals

All chemicals used were of analytical grade purchased from Sigma, Merck and Fluka.

2.2. Test micro-organisms

Gram-positive micro-organisms used were *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212); Gram-negative micro-organisms used were *Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853). These strains are responsible for nosocomial infections and are recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1990), Villanova, Pennsylvania, USA for comparing antibacterial activity of different compounds.

2.3. Plant collection and identification

Leaves of *D. viscosa* were collected from Modjadji in Limpopo Province in August 2004 and allowed to dry under the shade. The identification and voucher specimen number of the plant was sorted out and deposited at the National Botanical Institution, Pretoria with voucher specimen no. 1 Gen Spec no. 48310004. The plant was identified as *Dodonaea viscosa* Jacq. var. *angustifolia*.

2.4. Isolation of the antioxidant and antibacterial compounds

D. viscosa leaf powder (5 kg) was serially extracted with 3 × 5 L of hexane, dichloromethane (DCM), acetone and methanol respectively. This afforded four crude extracts on evaporation of the solvents using a rotary evaporator at 40 °C. Qualitative antioxidant assay on TLC plate with 0.2% DPPH in methanol (Glavind and Holmer, 1967) and bio-autography (Begue and Kline, 1972) showed that the DCM and acetone fractions (results not shown) mainly contained the antioxidant and antibacterial compounds.

2.4.1. Fractionation of the DCM extract

The DCM extract (30 g) was fractionated on a silica gel column eluted with hexane (100%); followed by a mixture of hexane/ethylacetate (EtOAc) with an increasing gradient of EtOAc up to 100% and an increasing gradient of methanol (MeOH) up to 100% into EtOAc. The eluent was collected in 50 ml test tubes and analysed by TLC sprayed with vanillin-sulphuric acid (Stahl, 1969). The fractions were pooled into 15 fractions based on the TLC profiles and concentrated. The active fractions were detected by MIC, antioxidant analysis and by bio-autography.

2.4.1.1. Purification of pooled fractions 4+5. A mixture of fractions 4 and 5 (2.1 g) was fractionated on a silica gel column using an increasing gradient of EtOAc in hexane up to 100%. The eluent was collected in 50 ml test tubes and a total of 80 test tubes were collected. Yellow crystals were formed in test tubes 48–60. The crystals were collected and allowed to dry. This afforded compound (3) (8.0 mg) with R_f 0.79 when developed in chloroform/ethylacetate/formic acid (CEF) (10:8:2) solvent system.

2.4.1.2. Purification of pooled fractions 7+8+9. Fractions 7, 8 and 9 had similar TLC profiles. They were combined (1.033 g) and fractionated using the same solvent mixtures as above in 50 ml test tubes into 115 fractions. Fractions 72–78 yielded yellow crystals of compound (2) (25.0 mg) with R_f 0.61 in the CEF solvent system.

2.4.1.3. Purification of fraction 6. Fraction 6 (1.8 g) was fractionated as above into 60, 50 ml fractions. Fractions 23–25 had one major spot on TLC plate. These were pooled and purified on Sephadex LH-20 using DCM:MeOH (2:1). The pure compound, a yellow powder (7.0 mg) was coded compound (1) with R_f 0.61 in CEF solvent system.

2.4.1.4. Purification of fraction 3. Fraction 3 (0.89 g) was purified as above into 50, 50 ml fractions. Yellow crystals formed in fraction 3 were collected and afforded compound 4 (9.0 mg) which had an R_f 0.84 in CEF solvent system.

2.4.2. Fractionation and purification of the acetone serial extract

The serial acetone crude extract (3.2 g) was fractionated on silica gel as in above into 80 and 50 ml fractions. Fractions 24–26 were combined and purified on Sephadex LH-20 using DCM:EtOAc (2:1). A pure (yellow powder) compound 5 (9 mg) was obtained; with an R_f 0.41 in CEF solvent.

2.5. DPPH spectrophotometric (quantitative) assay

Antioxidant activity was determined as described by Mensor et al. (2001) and modified by Aderogba et al. (2007). Sample stock solution of each of the isolated compounds (200 μM) from *D. viscosa* leaf extracts was diluted to a final concentration of 125, 100, 50, 25 and 12.5 μM in MeOH. Ten microlitres of 0.2 mM DPPH MeOH solution was added to 50 μl of sample

stock solution of different concentration and allowed to react at room temperature for 30 min in a dark chamber. The blank solutions were prepared with sample solution 50 μ l and 20 μ l of MeOH only while the negative control was a DPPH solution, 20 μ l plus 50 μ l MeOH. The absorbance values of the compounds changing from violet to yellow were measured at 515 nm on a microplate reader and converted to percentage antioxidant activity (AA%) using the formula:

$$AA\% = 100 - \left\{ \frac{[(Abs_{\text{sample}} - Abs_{\text{blank}}) \times 100]}{Abs_{\text{control}}} \right\}$$

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and Abs_{control} is the absorbance of the control). L-ascorbic acid (vitamin C) diluted to final concentrations of 50, 25, 12.5, 6.25 and 3.125 μ M in MeOH was used as a positive control (antioxidant agent).

EC₅₀ is the concentration of the test sample leading to a 50% inhibition of the DPPH free radicals. Its value was calculated from the separate linear regression of plots of the mean percentage of the antioxidant activity against concentration of the test compounds (μ M) obtained from three replicate assays.

2.5.1. Statistical analysis

The results were expressed as mean \pm SEM (standard error of mean). The software for regression plots (SigmaPlot® 2001, SPSS Science) was used.

2.6. Antibacterial activity assay

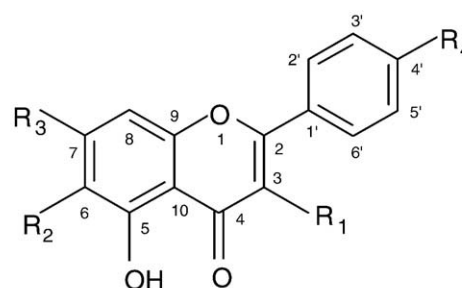
To determine the minimum inhibitory concentration (MIC) values, the microdilution assay as described by Eloff (1998) was followed. Gentamicin (SIGMA Aldrich, Germany) was used as a positive control; acetone and water were used as negative controls. Isolated compounds were tested at an initial concentration of 1000 μ g/ml (1 mg/ml) and the experiment was done in triplicate. The following test micro-organisms were used: Gram-positive—*S. aureus* and *E. faecalis*; Gram-negative—*P. aeruginosa* and *E. coli*. Briefly, 100 μ l of sterile distilled water was added to 96-well microtitre plates followed by the addition of 100 μ l of 1000 μ g/ml isolated compounds and serially diluted two fold after which 100 μ l of the above mentioned 4 actively growing test micro-organisms were added to each microtitre plates in all the wells to give the final volume of 200 μ l. The prepared microtitre plates were sealed so that they do not dry and incubated overnight at 37 °C in 100% relative humidity. After overnight incubation an indicator of bacterial growth, 40 μ l of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) (SIGMA Aldrich, Germany) dissolved in water was then added to all the microtitre plate wells and incubated for a further 30 min–2 h. Bacterial growth was indicated by the red colour and in the wells that were colourless or the colour intensity decreased the indicated inhibition of growth. The MIC values were then recorded as the lowest concentration at which a decrease in red colour is apparent compared to the next dilution after approximately 24 h of incubation.

3. Results and discussion

3.1. Structure elucidation

The compounds were identified and characterized by nuclear magnetic resonance analysis and comparison of the spectral data of each of the isolated compounds with the literature (Fig. 1).

- 3.1.1 Compound (1) was identified as 4'-O-methylkaempferol (3, 5, 7-trihydroxy-4'-methoxyflavone) by comparison of its spectral data with the literature (Harborne, 1982). Though there has been much phytochemical work done on *D. viscosa*, this compound 3, 5, 7-trihydroxy-4'-methoxyflavone is reported for the first time from this species (Latha and Daniel, 2001).
- 3.1.2 Compound (2) was identified as 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone when spectral data were compared with the literature (Van Heerden et al., 2000; Rashid et al., 1992).
- 3.1.3 Compound (3)-the spectral data were in agreement with that of 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (santin) reported in the literature (Rashid et al., 1992).
- 3.1.4 Compound (4)-the spectral data were in close agreement with those reported in the literature of 5-hydroxyl-3, 7, 4'-trimethoxyflavone (Rossi et al., 1997; Horie et al., 1998; Leitao et al., 2000).
- 3.1.5 Compound (5) was identified as 3, 4', 5, 7-tetrahydroxyflavone (kaempferol). The spectral data were in agreement with the literature (Markham, 1982). Kaempferol



	R ₁	R ₂	R ₃	R ₄
1	OH	H	OH	OMe
2	OMe	OMe	OH	OH
3	OMe	OMe	OH	OMe
4	OMe	H	OMe	OMe
5	OH	H	OH	OH

Fig. 1. Isolated compounds from *D. viscosa* leaf extracts.

was previously isolated from *D. viscosa* (Wollenweber et al., 1986; Getie et al., 2000).

3.2. DPPH qualitative assay

All the isolated compounds except compound **4** bleached the DPPH solution (0.2% in MeOH) purple colour in the TLC autography qualitative assay. Quantitative assay revealed varying degrees of antioxidant activity of the compounds. Kaempferol with free phenolic -OH groups (without any methoxyl substitution) was the most active. Compounds **1–4** are kaempferol derivatives with different methoxylation patterns. Of the four derivatives, compound **1** 4'-OMe derivative was the most active. Compound **1** has the same oxygenation pattern as the remaining derivatives but with only C-4'-methoxylation. The presence of these free-OH groups could be linked to higher antioxidant activity of the compound compared to other derivatives with more methoxylated groups. Methylation of free-OH groups in flavonoids substantially reduces the antioxidant activity of the compounds (Op de Beck et al., 2003). In line with this observation, compound **2** with 3-OMe and 6-OMe and also compound **3** with 3-OMe, 4'-OMe and 6-OMe did not inhibit 50% of DPPH free radical hence their EC₅₀ was higher than the highest concentration tested. The weak activity exhibited by these compounds could be linked to the methylation of some of their phenolic-OH groups (Martini et al., 2004; Op de Beck et al., 2003). At the highest concentration (200 µM) tested, compounds **2** and **3** had 19.06 and 8.23% antioxidant activities respectively. The LD 50 values were therefore substantially higher than 200 µM. It appears that methylation of 3-OH in both compounds reduced activity significantly when compared with compound **1** with free 3-OH. Compound **5** has the same oxygenation pattern as its four derivatives (**1–4**). All its -OH groups are free and this significantly enhances its antioxidant activity. This observation is in agreement with the earlier structural activity study (Op de Beck et al., 2003; Rice-Evans et al., 1996; Saskia et al., 1996). The effect of 6-OMe on activity is not clear. Compound **4** did not demonstrate activity in the qualitative assay apparently due to extensive methoxylation (3-OMe, 4'-OMe and 7-OMe). This observation suggests that 5-OH does not play a strong role in antioxidant activity of the investigated compounds. This is also in line with the earlier report on flavonoids (Rice-Evans et al., 1996). Our results indicate that only compounds **1** and **5** had strong activity (EC₅₀ = 75.49 ± 1.76 µM and 35.06 ± 0.85 respectively) but it was still less than that of the positive control L-

Table 1
Antioxidant activities (EC₅₀ in µM) of compounds **1** to **5** isolated from *D. viscosa* leaf extracts.

Compound	EC ₅₀	Correlation coefficient (r ²)
1	75.49 ± 1.76	0.947
2	> 200	NA
3	> 200	NA
4	> 200	NA
5	35.06 ± 0.85	0.952
L-ascorbic acid	13.55 ± 0.28	0.999

Table 2

MIC in µg/ml of the isolated compounds from *D. viscosa* leaf extracts against Gram-negative (*E. faecalis* and *S. aureus*) and Gram-positive (*E. coli* and *P. aeruginosa*) microorganisms.

Compound	<i>E. faecalis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
1	23*	250	125	250
2	32	125	26*	32
3	63	63	63	125
4	> 250	> 250	> 250	> 250
5	32	63	16	63
Gentamicin	6.3	0.2	0.8	0.8

Standard deviation 0 in all cases.

*Standard deviation 0.01.

ascorbic acid (EC₅₀ = 13.55 ± 0.28 µM) (Table 1). The EC₅₀ values obtained from the regression plots (SigmaPlot[®] 2001, SPSS Science) showed good correlation coefficient (r² ≥ 0.947).

3.3. Antibacterial assay

The MIC values for the isolated compounds on the four bacteria varied from 23 to more than 250 µg/ml (Table 2). Compound **5** was in general the most active against all the test organisms with MIC values between 16 and 63 µg/ml. Compound **2** was the second most active and was the only isolated compound with reasonable activity against *P. aeruginosa*. Compound **1** had the best activity (23 µg/ml) against *E. faecalis*, but had very low activity against the other organisms. Compound **3** had poor activity against all the test organisms (MIC between 63 and 125 µg/ml) and compound **4** had no activity at the highest concentration tested (250 µg/ml).

The antimicrobial activity of the isolated compounds against some of the tested organisms could be linked to varying degrees of methylation of the -OH groups of these compounds. This is consistent with earlier structure activity related study on the antimicrobial activity of flavonoids (Cushnie and Lamb, 2005).

The activity of the different pathogens was not associated with the Gram-positive-Gram-negative classification indicating that the effect was not cell-wall related. In all cases a hydroxyl was required at C-7 for antibacterial activity. A hydroxyl was required at C-4' for activity with *P. aeruginosa* but this was apparently not the case with *S. aureus*. It does not seem as if substitution at C-6 plays an important role.

No clear correlation between antioxidant and antibacterial activities of these compounds was apparent. Flavonoids are known for substantial antimicrobial activity (Alcaraz et al., 2000). Low antibacterial activity demonstrated by some of these compounds could be ascribed to methylation of the phenolic -OH groups in our compounds.

4. Conclusion

The compounds responsible for the antibacterial and antioxidant activity in *D. viscosa* leaf extracts are all flavonoids related to kaempferol. Some conclusions on the relationship between structural and biological activity could be drawn that supported earlier reports. The data also confirm the rationale for the traditional use of *D. viscosa* extracts to treat infections.

Compound **5** was in general the most active against all the test organisms with MIC values between 16 and 63 µg/ml. Compound **2** was the second most active and was the only isolated compound with reasonable activity against *P. aeruginosa*. Compound **1** had the best activity (23 µg/ml) against *E. faecalis*, but had very low activity against the other organisms. Compound **3** had poor activity against all the test organisms (MIC between 63 and 125 µg/ml) and compound **4** had no activity at the highest concentration tested (250 µg/ml).

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