

CHAPTER 7

7. BIOLOGICAL ACTIVITY OF ISOLATED COMPOUNDS FROM *D. VISCOSA*

7.1 Biological assays

For the bioassays procedures (Bioautography and MIC assays) of the isolated compounds, the procedure as described under Methodology in Chapter 2 was followed. The following two Gram-positive bacteria (*S. aureus* and *E. faecalis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were used.

7.2 Results and Discussion

7.2.1 Biological assays

7.2.1.1 Bioautography assay

Compound 1

Compound 1 exhibited no antibacterial activity against all bacteria tested (data not shown).

Compound 2

At concentrations of 10 µg/ml, Compound 2 showed various level of bacterial inhibition. A clear zone of growth inhibition was highly evident with *P. aeruginosa*, less growth inhibition was observed on *S. aureus* whereas slight activity was observed with *E. coli* and *E. faecalis* (Fig 7.1).

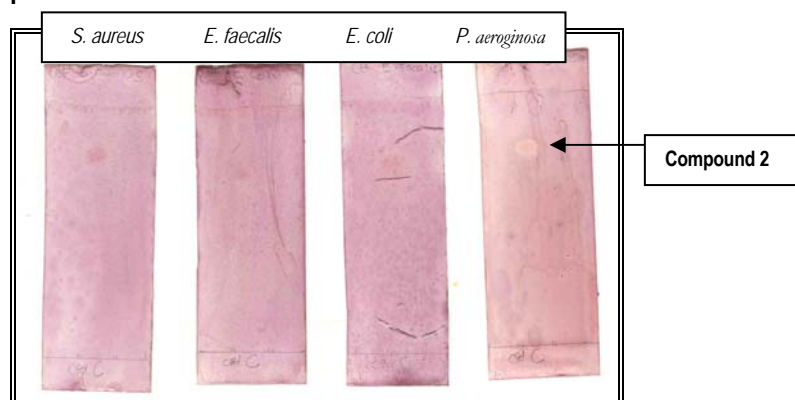


Figure 7.1: TLC chromatograms of Compound 2 against four test microorganisms, (an arrow points the area of growth inhibition).

Compound 3

Compound 3 (Fig. 7.2) showed a good activity against *E. faecalis* at concentrations of 5 and 10 μg . Good antibacterial activity was also found with *E. coli*, whereas *S. aureus* and *P. aeruginosa* were not sensitive when 10 μg was separated.

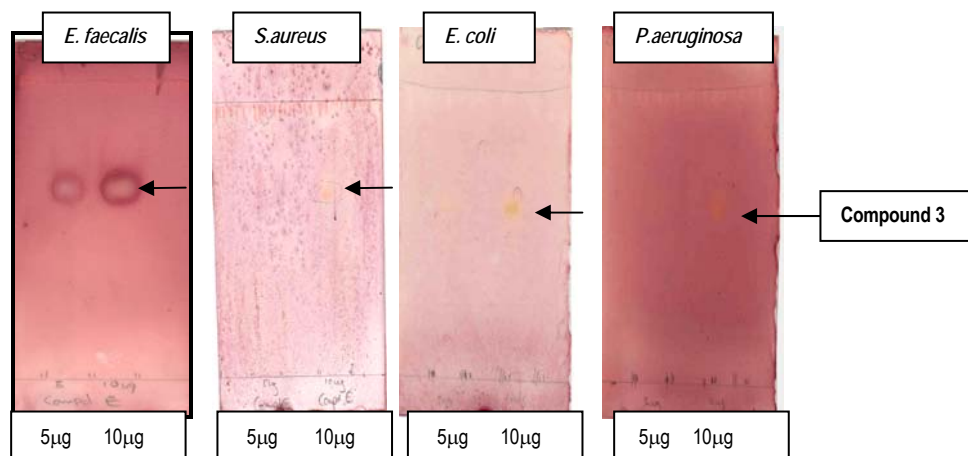


Figure 7.2: Bioautography of Compound 3 against four test microorganisms.

Compound 4

The bioautography results gave clear spots as zone of inhibition against *S. aureus* and *E. coli* (Fig 7.3). *P. aeruginosa* and *E. faecalis* did not grow well on the TLC plates (data not shown).

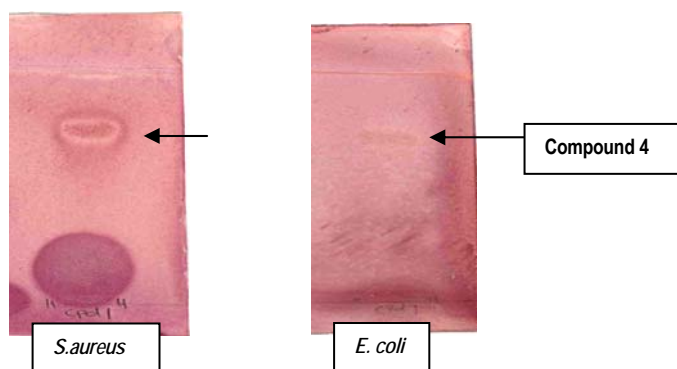


Figure 7.3: Bioautography activity of Compound 4 against *S. aureus* and *E. coli*.

Compound 5

There was a clear zone of inhibition against *S. aureus*. Clear yellow zones were less evident against the pink background for *E. coli* and *P. aeruginosa*. This implied that there was growth inhibition by Compound 5 against *S. aureus*, *E. coli* and *P. aeruginosa* (Fig. 7.4)

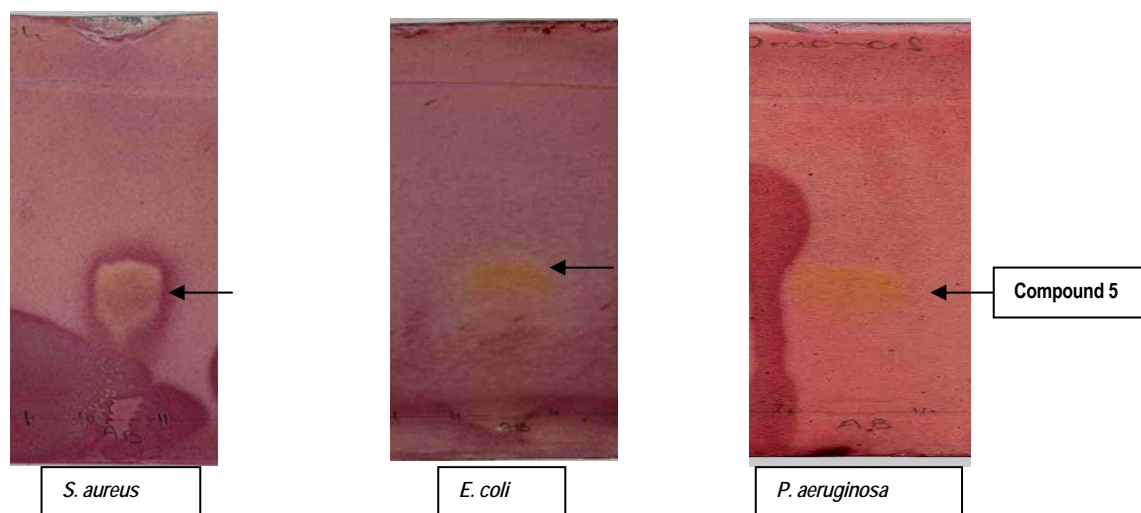


Figure 7.4: TLC chromatograms of Compound 5 activity against three test microorganisms.

7.2.1.2 Antioxidant assay

Qualitative assay

In the TLC autographic DPPH assay, Compound 1 did not show any antioxidant activity (results not shown). The TLC chromatograms in Fig 7.5 indicates that the compounds possess antioxidant activities. Compound 2 showed a clear yellow band 15 minutes after spraying with 0.2% DPPH in methanol depicting a good level of antioxidant activity. Compound 3 also tested positive for antioxidants showing a clear yellow band 5 minutes after spraying with DPPH. Compound 4 revealed a yellow band after 15 min whereas Compound 5 showed a yellow band immediately after spraying with DPPH. All the compounds except Compound 1 had antioxidant activities. The level of antioxidants in these compounds was quantified using a DPPH Spectrophotometer assay.

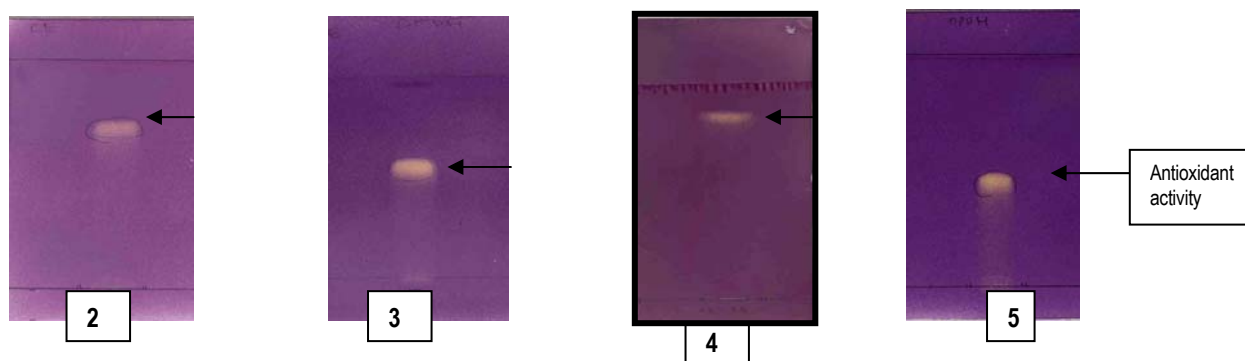


Figure 7.5: TLC chromatogram separated by CE indicating the level of antioxidant activity of Compound 2, 3, 4 and 5.

DPPH Spectrophotometric assay

Quantitative assay has revealed a varying degree of antioxidant potential of the compounds. Kaempferol without any methoxyl substitution was the most active. Compounds 1-4 are kaempferol derivatives with different methoxylation patterns. Of the four derivatives, compound 4 which is a 4'-OMe derivative was the most active. Compound 3 with 3-OMe and 6-OMe and also compound 2 with 3-OMe, 4'-OMe and 6-OMe did not inhibit 50% of DPPH free radical. At the highest concentration (200 μM) tested compound 2 and 3 had 19.06 and 8.23 percentage antioxidant activity (Table 7.1). It appeared that 3-OMe reduced activity significantly when compared with compound 4 with free 3-OH. This observation is in agreement with the earlier structural activity study (Op de Beck, 2003). The effect of 6-OMe on activity is not clear. Compound 1 was not quantified since it 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 play strong role in antioxidant activity of the investigated compounds since the level of antioxidant activity demonstrated by these compounds correlated in the number of free-OH groups in the rings. This is also in line with the earlier report on flavonoids (Rive-Evans *et al.*, 1996). Our results have shown that only Compound 5 (kaempferol) and Compound 4 have demonstrated strong activity ($\text{EC}_{50} = 35.06 \pm 0.85$ and 75.49 ± 1.76 μM respectively) but lower than L-ascorbic acid ($\text{EC}_{50} = 13.55 \pm 0.28$ μM) used as a standard antioxidant agent (Table 7.2).

Table 7.1: Percentage antioxidant activity of Compounds 2-5

Conc.(μM)	Mean absorbance (%) (3x experiments) (SD)			
	Compound 2	Compound 3	Compound 4	Compound 5
200.0	19.06 \pm 0.001	8.28 \pm 0.002	95.22 \pm 0.16	-
100.0	0.00	0.00	65.36 \pm 7.63	92.37 \pm 0.66
50.0	0.00	0.00	48.92 \pm 1.97	62.83 \pm 8.97
25.0	0.00	0.00	27.93 \pm 3.42	49.25 \pm 5.11
12.5	0.00	0.00	16.50 \pm 3.45	27.87 \pm 2.43

Table 7.2: EC₅₀ of compound 4 and 5 from *D. viscosa* leaf extracts

Compound	EC ₅₀ \pm SEM μM	Correlation coefficient (r ²)
4	75.49 \pm 1.76	0.947
5	35.06 \pm 0.85	0.952
L-ascorbic acid	13.55 \pm 0.28	0.999

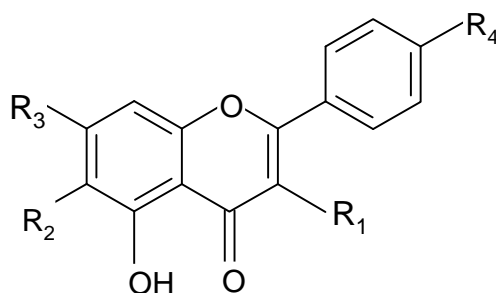
7.2.1.3 Microdilution assay- structure activity relationship

In the bioassay used (Eloff, 1988b) the initial concentration is reduced four fold. The MIC values of the isolated compounds were determined in triplicate using the four nosocomial bacterial pathogens (Table 7.3). In Table 7.3 the number of methoxy, hydroxyl and free hydrogen atoms on R1-R4 is also provided (also refer to Fig 7.6). The structure activity relationship differed for the different pathogens. This was not associated with Gram positive-Gram negative classification indicating that the effect was not cell wall related. In all cases a hydroxyl was required at R3 for antibacterial activity. An hydroxyl was required at R4 for activity with *P. aeruginosa* but this was apparently not the case with *S. aureus*. It does not seem as if substitution at R2 plays an important role. The data available did not make it easy to evaluate the contribution of substitution on R1 to the activity. Flavonoids are known for potent

multiple biological actions (Alcaraz *et al.*, 2000). However, low antibacterial activity demonstrated by these compounds could be ascribed to methylation of the phenolic -OH groups in our compounds.

Table 7.3: MIC values in $\mu\text{g/ml}$ of the isolated pure compounds determined in triplicate and a positive control. Structural information is also provided.

Pure Compounds	Methoxy groups	OH groups	Free H atoms	MIC values ($\mu\text{g/ml}$)			
				<i>E. faecalis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
1	3	0	1	$>250 \pm 0.00$	$>250 \pm 0.00$	$>250 \pm 0.00$	$>250 \pm 0.00$
2	3	1	0	62.5 ± 0.00	62.5 ± 0.00	62.5 ± 0.00	125 ± 0.00
3	2	2	0	31.25 ± 0.00	125 ± 0.00	26 ± 9.01	31.25 ± 0.00
4	1	2	1	23.44 ± 9.01	250 ± 0.00	125 ± 0.00	250 ± 0.00
5	0	3	1	31.25 ± 0.00	62.5 ± 0.00	15.63 ± 0.00	62.5 ± 0.00
Gentamicin				$6.3 \mu\text{g/ml}$	$0.2 \mu\text{g/ml}$	$0.8 \mu\text{g/ml}$	$0.8 \mu\text{g/ml}$



Compound Name	Number	R1	R2	R3	R4
5-hydroxy -3, 7, 4'-trimethoxyflavone	1	OMe	H	OMe	OMe
5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone	2	OMe	OMe	OH	OMe
5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone	3	OMe	OMe	OH	OH
4'-O-methylkaempferol	4	OH	H	OH	OMe
Kaempferol	5	OH	H	OH	OH

Figure 7.6: Structure of the five isolated compounds (Compound 1-5) from *D. viscosa* leaf extracts.

7.3 Conclusion

According to the MIC results, Compound 1 possessed no activity against the tested microorganisms. Compound 2 exhibited a poor bacterial activity against all the tested microorganisms. Compound 3 showed a high level of activity against *E. coli*, *P. aeruginosa* and *E. faecalis*, however *S. aureus* was resistant. Only *E. faecalis* was sensitive towards Compound 4. However, Compound 5 exhibited a good level of activity against *E. coli* and *E. faecalis*. The most sensitive bacteria against isolated compounds were *E. coli* and *E. faecalis*. The structure activity relationship differed for the different pathogens. This was not associated with Gram positive-Gram negative classification indicating that the effect was not cell wall related. In all cases a hydroxyl was required at R3 for antibacterial activity. An hydroxyl was required at R4 for activity with *P. aeruginosa* but this was apparently not the case with *S. aureus*. It does not seem as if substitution at R2 plays an important role. The data available did not make it easy to evaluate the contribution of substitution on R1 to the activity.

From the qualitative antioxidant assay, all the compounds except for compound 1 had good antioxidant activity. After spraying with 0.2% DPPH in methanol, good level of antioxidant activity was revealed immediately with Compound 3 and 5, whereas with Compound 2 and 4; the antioxidant activity was observed after 15 min. Based on the literature, antioxidant potential of the compounds correlates to the number of free-OH groups in the rings. In this case the relationship did not hold well because Compound 4 had no free hydroxyl groups (Table 7.1) A quantitative DPPH spectrophotometric assay has demonstrated that only compound 4 and 5 have strong antioxidant activities. The presence of these antibacterial and antioxidant compounds although they have demonstrated moderate activities, they could provide rationale for the ethnomedicinal use of *D. viscosa* leaf extract in traditional medicine.

CHAPTER 8

8. GENERAL DISCUSSION AND CONCLUSION

In western countries, entomophagy is not highly appreciated. Consumption of insects is more common in Africa and Asia, where insects are widely consumed by the rural population. The problem with eating of insects is that many of educated people are ashamed of their culture and they eat insects secretly whereas some regard insects as food for the poor people. It is necessary to educate people and promote eating of insects as they are cheap source of protein and some families harvest and sell insects for income.

The main aim of this study was to investigate possible interaction between thongolifha (*E. delegorguei*) and its host plant *D. viscosa*. Because thongolifha feeds mainly on *D. viscosa* with known antibacterial activity, it may be possible that some compounds that are present in *D. viscosa* are also present in the insect. Thongolifha is an important cultural food of the vhaVhenda tribe and many families rely on selling of the insects for income. It was therefore necessary in this study to also determine the nutritional composition of thongolifha since it was never determined before.

8.1 *E. delegorguei* extracts

It is well documented that some insects ingest secondary metabolism compounds from plants and store them as part of its defence mechanism (Harborne, 1993). In our study, this was tested by comparing hexane, DCM, acetone and methanol extracts obtained from *D. viscosa* and *E. delegorguei* loaded onto TLC plates and developed in three eluent systems, EMW, CEF and BEA. Few similarities of compounds separated from both the plant and insect extracts were observed; implying that the sequestered compounds from host plant may be present in lower quantities in the insects extract, or the insect might not have sequestered components or have metabolised the sequestered compounds. The feeding mechanism of the bug from the host plant is by sucking the sap which is mainly the liquid from the leaves of the host plant. In that case they might not be much of the secondary plant compounds that are being ingested by the insect. Bioautography had revealed that two of the insect extracts compounds (Rf 0.79 and 0.66) had antibacterial against *S. aureus* and only one compound (Rf 0.66) showed activity against *E. faecalis*. The quantitative assay using the microplate dilution assay had MIC. 2.5 mg/ml indicating that the insect extracts had low antibacterial activity.

To determine the nutritional composition of *E. delegorguei*, ground material of the insect was extracted using standardised techniques. Thongolifha can be utilised as a protein supplement diet as it contains good quantity of protein (35. 2%) with high energy content of 2599 k.cal/100 g. The total mineral content of the thongolifha was at 1.2

g/100g and is a level comparable to other insects. Furthermore relatively high concentrations of B, Ba, Mn, Sr and Zn were detected in *D. viscosa* (Nagaraju and Karimulla, 2002) indicating that thongolifha obtained most of its minerals from its host plant.

The level of carbohydrate was at an acceptable level of 7.63 g/100 g. The insect contained high levels of fat (50.5%) and its amino acids content showed good levels of essential amino acids which varied from 0.82 mg/100 g (threonine) to 1.32 mg/100 g (valine). However to satisfy the daily nutritional need for the essential amino acids (phenylalanine and threonine), 680 and 3400 of thongolifha has to be consumed which can be quite a large number, and may be difficult to attain.

8.2 *D. viscosa* extracts

Methanol leaf extracts of *D. viscosa* possess antibacterial activity (Getie *et al*, 2003). However, their agar diffusion method did not provide any quantitative data regarding the level of activity of the extract against *S. aureus*. *E. coli* was found to be resistant. In this study, the antibacterial activity of *D. viscosa* crude leaf extracts was quantified by using serial dilution microplate assay and the total activity (TA) was also calculated. The microplate assay has confirmed that *D. viscosa* leaf extract had strong activity against *S. aureus* and even other test bacteria such as *E. coli*, *E. faecalis* and *P. aeruginosa* were susceptible to the extract MIC value (0.04-0.28 mg/ml). The data for total activity has indicated that acetone extract exhibited the highest total activity for all test microorganisms tested ranging from 48.4-750 ml/g. The antibacterial compounds extracted from 1 g of leaves could therefore be diluted up to 750 ml and still inhibit bacterial growth. *D. viscosa* extracts were also found to have reasonable antioxidant properties. Both the qualitative and quantitative DPPH methods have reported ethyl acetate and acetone extracts to have good level of antioxidant activities.

Based on the above findings with regard to *D. viscosa* extracts, a bioassay-guided fractionation silica gel chromatography was performed and four flavonoids were isolated from dichloromethane fraction of crude *D. viscosa* leaf extract: (5-hydroxyl- 3, 7, 4'-trimethoxyflavone (**Compound 1**); 5,7-dihydroxy-3, 6, 4'-trimethoxyflavone (**Compound 2**); 3,6-dimethoxy-5, 7, 4'-trihydroxyflavone (**Compound 3**); 4'-O-methylkaempferol (**Compound 4**). Only one compound, kaempferol (**Compound 5**) was isolated from the acetone crude leaf extract fraction. We report for the first time isolation of 4'-O-methylkaempferol from *D. viscosa*, this compound is not novel since it was isolated before from other plant species such as *Lens culinaris* (Latha and Daniel, 2001). Only three of the isolated compounds (i.e. **Compound 3, 4 and 5**) exhibited good antibacterial activities against *E. coli* (15.63-26.5 µg/ml), *E. faecalis* (23.44-31.25 µg/ml) and *P. aeruginosa* (31.25 µg/ml). Furthermore, the TLC qualitative assay using 0.2% DPPH in methanol has shown that the four isolated compounds except for **Compound 1** have good level of antioxidant activity. The level of activity was observed from immediately to up to 15 min after spraying with 0.2% DPPH in methanol. Based on the literature, antioxidant potential of the compounds correlates to the number of free-

OH groups in the rings. The DPPH spectrophotometer assay indicated that, 4-methoxykaempferol and kaempferol have strong antioxidant activity ($EC_{50} = 35.06 \pm 0.85$ and $75.49 \pm 1.76 \mu\text{M}$ respectively) but lower than L-ascorbic acid ($EC_{50} = 13.55 \pm 0.28 \mu\text{M}$) used as a standard antioxidant agent. Although the isolated compounds **3**, **4** and **5** had moderate to good level of both antibacterial and antioxidant activities, it was difficult to establish clear structure function correlation between antioxidant and antibacterial activities of these compounds. Flavonoids are however known for potent multiple biological actions (Alcaraz *et al.*, 2000).

8.3 Insect-plant interaction

For the insect extracts there were compounds with antibacterial activity against *S. aureus* (Rf 0.79 and 0.66) and *E. faecalis* (Rf 0.66) based on bioautography when separated with CEF solvent system. Some of the isolated antibacterial compounds from *D. viscosa* leaf extracts had an Rf 0.79 (5,7-dihydroxy-3,6,4'-trimethoxyflavone) and Rf 0.61 (5,7,4'-trihydroxy-3,6-dimethoxyflavone) when separated with CEF eluent system. This indicates that thongolifha may have sequestered the two compounds from *D. viscosa* leaves, but this possibility has to be confirmed. This conclusion may be speculative, but there were only two antibacterial compounds in the insect extract. The close correlation of Rf values suggests that they may be identical to compounds isolated from the plant extract. Unfortunately bioautography is a difficult process that does not work with all TLC solvent systems otherwise it would have been easy to compare the Rf values of the antibacterial compounds of the insect extract co-chromatographed with the isolated compounds using other TLC solvent systems.

In conclusion, thongolifha has good protein content and it is therefore not just a traditional delicacy for the vhaVenda tribe, it contains a good source of protein, minerals and vitamins. It is also a good diet supplement and it provides a valuable source of income for the harvesters and therefore, sustainable harvesting is necessary. Although our study did not reveal any antibacterial activities of the insects, it contains a chemical defensive substance that can be toxic to human eyes. The insects may have medicinal value in folk medicine that is not published anywhere in the literature. The crude leaf extracts of the host plant *D. viscosa* on the other hand, possess good level of antibacterial and antioxidant activities. There are two compounds obtained from insect extracts that may be the same as the isolated compounds 5,7-dihydroxy-3,6,4'-trimethoxyflavone and 5,7,4'-trihydroxy-3,6-dimethoxyflavone from *D. viscosa*, implying the possibility of the insect to have sequestered the two compounds from *D. viscosa* leaves.

Investigation into the structure relationship activity of isolated compounds did not yield a clear structure function correlation. However, the presence of these antioxidant and antibacterial compounds although demonstrating moderate level of activities could provide rationale for the ethnomedicinal use of *D. viscosa* leaf extract in traditional medicine