Chapter 7

Bioactive guided Isolation and identification of two flavonoids from *Hermannia incana* Cav. leaves

CHAPTER 7

Bioactive guided Isolation and identification of two flavonoids from *Hermannia incana* Cav. leaves

Introduction

The genus *Hermannia* has been used traditionally by a diversity of people such as the European, Tswana, Kwena, Southern Sotho, Xhosa and Zulu for the treatment of fever, cough, respiratory diseases such as asthma, wounds, burns, eczema, stomach-ache, as a purgative, diaphoretic, for heartburn, flatulence in pregnant women, colic and haemorrhoids (Essop et al., 2008). *Hermannia incana* is used for the treatment of diarrhoea, stomach-ache (Appidi et al., 2008), it is also used as emetic and purgative. The methanol extracts from the leaf and root of this plant showed appreciable antimicrobial activity against diarrhoea causative organisms (Appidi et al., 2009). The flavonoid, kaempferol and quercetin present in most of the Sterculiaceae species (Watson and Dallwitz, 1992), which were found to be responsible for the antidiarrhoeal activity (Palombo, 2006). Some antimicrobial compounds; Eicosane, Myristic acid, Palmitic acid and Stearyl alcohol were isolated from *Hermannia depressa* (Reid et al., 2005). No studies relating to the chemical composition or antimicrobial activities of isolated compounds in this species have previously been reported. This study therefore was undertaken to isolate and identify bio-active compounds from this plant.

Material and methods

Plant material

The leaves of *H. incana* were collected in November 2008 from a natural population growing near the University of Fort Hare in the Eastern Cape Province of South Africa. The plant was identified by Prof. D.S. Grierson at the Department of Botany, University of Fort Hare, and a voucher specimen (Jaipal Med 001) was deposited in the Griffen Herbarium.

Extraction and isolation of compounds

The leaves were air-dried at room temperature and dried leaves were ground into powder. The dried plant powder (1 kg) was extracted with methanol (2.5 L X 3 times, 24 hours, RT) by shaking on an orbital shaker (Stuart Scientific Orbital Shaker, UK) and the combined methanol extract were filtered using a Buchner funnel and Whatman no. 1 filter paper. The filtrate was then concentrated under reduced pressure at 40ºC using a rotary evaporator (Laborota 4000-efficient, Heildolph, Germany) and finally freeze dried to get methanol extract (152g). The methanol extract (145 g) was suspended in distilled water (200 ml) and successively partitioned with n-hexane, ethyl acetate and n-butanol respectively. This partitioned procedure resulted into four fractions, n-hexane, ethyl acetate, butanol and water fractions. Each fraction was tested for antibacterial activity, using bioautographic assay of Slusarenko et al. (1989) and the ethyl acetate soluble fraction was found to exhibit the stronger antibacterial activity.

The active ethyl acetate soluble fraction (HIE, 24 g) was fractionated by vaccum liquid chromatography (VLC) over silica gel and eluted with gradient mixture of ethyl acetate-methanol of increasing polarity to give 15 pooled fractions (HIE1-HIE15). All 15 subfraction were further tested for antibacterial activity using bioautographic assay and the subfraction HIE 3 and HIE 6 were showed strong antibacterial activity. The active HIE 3 (1.25 g) was further chromatographed over silica gel column and eluted with chloroform-methanol of increasing polarity to yield six subfractions (HIE 3-1 to HIE 3-6). The subfraction HIE 3-3 (40 mg) was showed single compound with small impurities and was washed with chloroform to give pure compound **1** (15 mg). Another active subfraction HIE 6 (1.55 g) was further fractionated over silica gel column using chloroform-methanol as an eluent to give

eight fraction (HIE 6-1 to HIE 6-8). The subfraction HIE 6-3 (250 mg) was further chromatographed over silica gel column and eluted with chloroform:methanol (9.2:0.8) as eluent to afford pure compound **2** (21 mg).

Bioautography on TLC plates

A modified autobiographic assay method of Slusarenko et al. (1989) was followed. A thin layer chromatography (TLC) plate (Merck silica gel F_{254}) was spotted with the test samples and sprayed with an actively growing suspension of *Baccillus subtilis* in nutrient broth. The plate was incubated at 37^{o}C for 24 h in a closed container to which 20 ml of distilled water had been added to provide a humid environment. After incubation, the plate was sprayed with a 2 $mg.ml^{-1}$ solution of *p*-iodonitrotetrazolium chloride (INT) and incubated for a further 30 minutes. The inhibition of bacterial growth was visible as a white spot against a deep violet/red background.

Antibacterial activity of the compounds

Micorplate dilution mothod was adapted to determine the minimum inhibitory concentration (MIC) of the pure compounds against two Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) and two Gram-negative (*Echerichia coli* and *Shigella flexneri*) bacteria. Each organism was maintained on nutrient agar plates and was recovered for testing by growth in nutrient broth for 24 h. Before use, each bacterial culture was diluted 1:100 with fresh sterile nutrient broth (Afolayan & Meyer, 1997). The microtiter plates were prepared using serial dilution (Eloff, 1998) and incubated for 24 h at 37 °C. As an indicator of bacterial growth 40µl of 0.2 mg/ml INT solution was added to each well and incubated at 37 °C for 30 min. The colorless tetrazolium salt was reduced to a red-colored product by biological activity of the

organisms, thereby making the inhibition of bacterial growth visible as clear wells. MIC values were recorded as the lowest concentration resulting in complete inhibition of bacterial growth. Each treatment was replicated three-times. Streptomycin, chloramphenicol, solvents and sample-free solutions were used as standard and controls.

Results and discussion

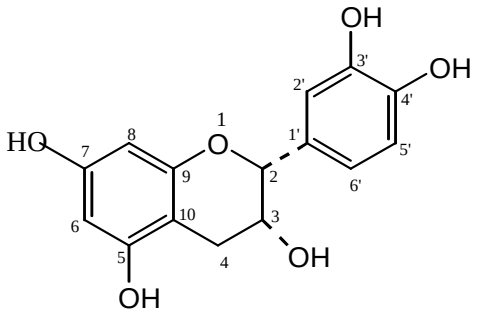
Two compounds were isolated from the ethyl acetate fraction of the leaves of *H. incana.* The structures of the isolated compounds were established by the spectroscopic analysis mainly NMR spectra in conjunction with 2D experiments, COSY, HMQC, HMBC and direct comparison with published spectroscopic data.

Compound 1 (Epicatechin): ^{1}H- NMR (400 MHz, CD_3OD) δ 4.26 (s, H-2), 3.88 (s, H-3), 2.56 (dd, *J*=4.54, 16,76 Hz, H-4), 2.41 (dd, *J*= 2.78, 16.76 Hz, H-4), 5.62 (d, *J*=2.08 Hz, H-6), 5.65 (d, *J*=2.08 Hz, H-8), 6.66 (s, H-2'), 6.46 (d, *J*= 8.12 Hz, H-5') and 6.49 (d, *J*= 9.77 Hz, H-6') ^{13}C- NMR (400 MHz, CD_3OD) δ 79.83 (C-2), 67.43 (C-3), 29.17 (C-4), 157.53 (C-5), 96.51 (C-6), 157.87 (C-7), 95.96 (C-8), 157.28 (C-9), 100.17 (C-10), 132.23 (C-1'), 115.53 (C-2'), 145.86 (C-3'), 145.72 (C-4'), 116.05 (C-5') and 119.46 (C-6').

Compound 2 (3, 5, 7, 2' tetra-hydroxy flavone-3- O-β-D-glucopyranoside): ^{1}H-NMR (400 MHz, CD_3OD) δ 6.13 (s, H-6), 6.36 (s, H-8), 6.84 (d, *J*= 8.4 Hz, H-3'), 7.97 (d, J= 8.3 Hz, H-4'), 7.34 (m, H-5'), 6.78 (d, *J*=8.1 Hz, H-6'), 5.43 (d, *J*= 7.06 Hz, H-1 of glucose), ^{13}C- NMR (400 MHz, CD_3OD) δ 133.04 (C-2), 124.91 (C-3), 177.30 (C-4), 159.78 (C-5), 98.88 (C-6), 166.15 (C-7), 93.93 (C-8), 159.96 (C-9), 103.69 (C-10), 120.78 (C-1'), 156.35 (C-2'), 115.76, (C-3'), 130.11 (C-4'), 115.07

(C-5'),), 130.11 (C-6'), 101.02 (C-1 of glucose), 73.90 , 69.97 , 76.23, 74.12, and 62.95 (CH_2OH)

Compound **1** was isolated from the ethyl acetate extract by column chromatography as brownish amorphous powder and was identified as epicatechein. The ^1H NMR spectrum of the compound showed a number of characteristic signals of epicatechein. Two signals at δ_H 5.62 (d, *J*=2.08 Hz) and 5.62 (d, *J*=2.08 Hz) were due to two phenyl protons situated at 1,3 position to each other of ring A. Two signals at δ_H 6.46 (d, *J*= 8.12 Hz) and 6.49 (d, *J*= 9.77 Hz) were due to two phenyl protons situated at ortho position and a signal at 6.66 (s) was due to one phenyl proton at para position of ring C a. A singlet at δ_H 3.86 was due to methine proton (H-3) having an adjacent –OH group and situated between methylene and a methine carbon. A singlet at δ_H 4.26 (s) for a methine proton attached with an oxygen atom and CHOH group. Two doublet of doublets resonating at δ_H 2.56 (dd, *J*=4.54, 16,76 Hz) and 2.41 (dd, *J*= 2.78, 16.76 Hz) were due to two methylene protons adjacent with a methine carbon.

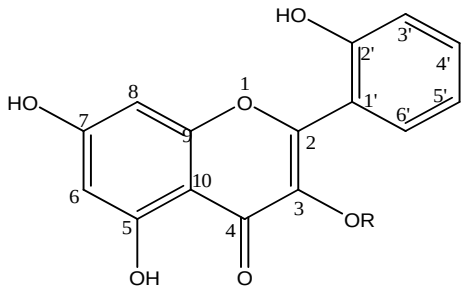


Epicatechin

The ^{13}C NMR data of compound **1** showed 15 carbons. The DEPT spectra revealed the presence of one methylene, seven methine and seven quaternary carbons. All connectivities between proton and carbon were determined from the COSY and HMQC experiment. The HMBC experiment of compound **1** showed strong cross peak

between H-2 (δ_H 4.26) with C-3 (δ_C 67.43), C-2′ (δ_C 132.23), C-6′ (δ_C 119.46) and C-9 (δ_C 157.87). By the study of the above ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC and comparison of the reported published spectral data (Agrawal, 1989), it was concluded that compound **1** was epicatechin.

Compound **2** was isolated from the ethyl acetate soluble fraction by column chromatography as a yellow amorphus powder. The ^{1}H NMR of the compound showed the presence of six protons signals in the aromatic region indicated that it might be a flavonoid. Two singlet signals at δ_H 6.13 and 6.36 were assigned to H-6 and H-8 respectively at ring B due to the two oxygen atom present at the geminal carbons. Four mutually coupled aromatic signals resonated at δ_H 6.84 (d, J= 8.4 Hz), 7.97 (d, J= 8.3 Hz), 7.34 (m'), 6.78 (d, *J*=8.1 Hz) were assigned to aromatic H-3', H-4', H-5' and H-6' respectively at ring C. The most downfield signal at δ_H 12.54 was due to the OH proton at C-5 involved in characteristic hydrogen bonding with carbonyl oxygen at C-4. The anomeric proton at δ_H 5.43 (1H, d, *J*=7.06 Hz) indicated the presence of β-D-glucose in compound **2**.



R= β-D-glucopyranoside

The ^{13}C NMR spectrum of compound **2** showed 21 carbon signals in the molecule. The DEPT spectra of the compound revealed the presence of six methine and nine

quaternary carbons in the aromatic region. A downfield quaternary signal at δ_C 177 was assigned to C-4 ketonic carbon indicated the presence of oxygen at C-3. The ^{13}C NMR also showed 12 aromatic carbons of which 6 were methine and 6 were quaternary carbons. The downfield signal at δ_C 159.78, 166.15 and 156.35 were oxygen bearing carbon to C-5, C-6 and C-2' respectively. The signals at δ_C 101.02, 73.90, 69.97, 76.23, 74.12, and 62.95 were assigned to C-1, C-2, C-3, C-4, C-5 and C-6 of glucose moiety. Connectivities of all protons and carbons of compound **2** were determined by ^{1}H-^{1}H COSY, HMQC and HMBC experiments. The study of ^{1}H-, ^{13}C NMR, COSY, HMQC and HMBC spectra and compared it with reported compounds (Shen et al., 1993) and the structure of compound **2** was elucidated as 3,5,7,2′, tetrahydroxy flavone-3- O-β-D-glucopyranoside.

Epicatechin and 3,5,7,2′, tetrahydroxy flavone-3- O-β-D-glucopyranoside showed appreciable antimicrobial activity against all four microorganisms (*Echerichia coli, Shigella flexneri, Bacillus cereus* and *Staphylococcus aureus*), exhibiting minimum inhibitory concentrations ranging from 12.5 to 100 µg/ml (Table 1). The antibacterial potency of these two compounds against *Echerichia coli, Shigella flexneri, Bacillus cereus* and *Staphylococcus aureus* is noteworthy, because all these bacteria have been implicated as causal agents of diarrhoea (Anne & Geboes, 2002; Krause et al., 2001; McGaw et al., 2000). Epicatechin has been reported by Esquenazi et al. (2002) to posses appreciable activity against acyclovirresistant herpes simplex virus type 1 (HSV-1-ACVr) and *Staphylococcus aureus*. Flavonoids are reported to have antidiarrhoeal activity by inhibit intestinal motility and hydroelectrolytic secretion (Venkatesan et al., 2005). It has been found that epicatechin can inhibit the development of fluids that result in diarrhoea by targeting the intestinal cystic fibrosis transmembrane conductance regulator Cl^- transport and

inhibiting cAMP-stimulated Cl^- secretion in the intestine (Schuier et al., 2005). No studies relating to the biological activity has been reported on 3,5,7,2′, tetrahydroxy flavone-3- O-β-D-glucopyranoside. However, in this study, the compound was found to be more active against all the organisms used compared to that of epicatechin. In recent years, flavonoids have attracted the interest of researchers because they show powerful antioxidant activities which protect the human body from free radicals (Bors et al., 1996; Halliwell and Gutteridge, 1999; Penga et al., 2003). The antibacterial activity of compound 1 and 2 isolated from this plant has validated the use of *H. incana* for the treatment of diarrhoea in the Eastern Cape Province.

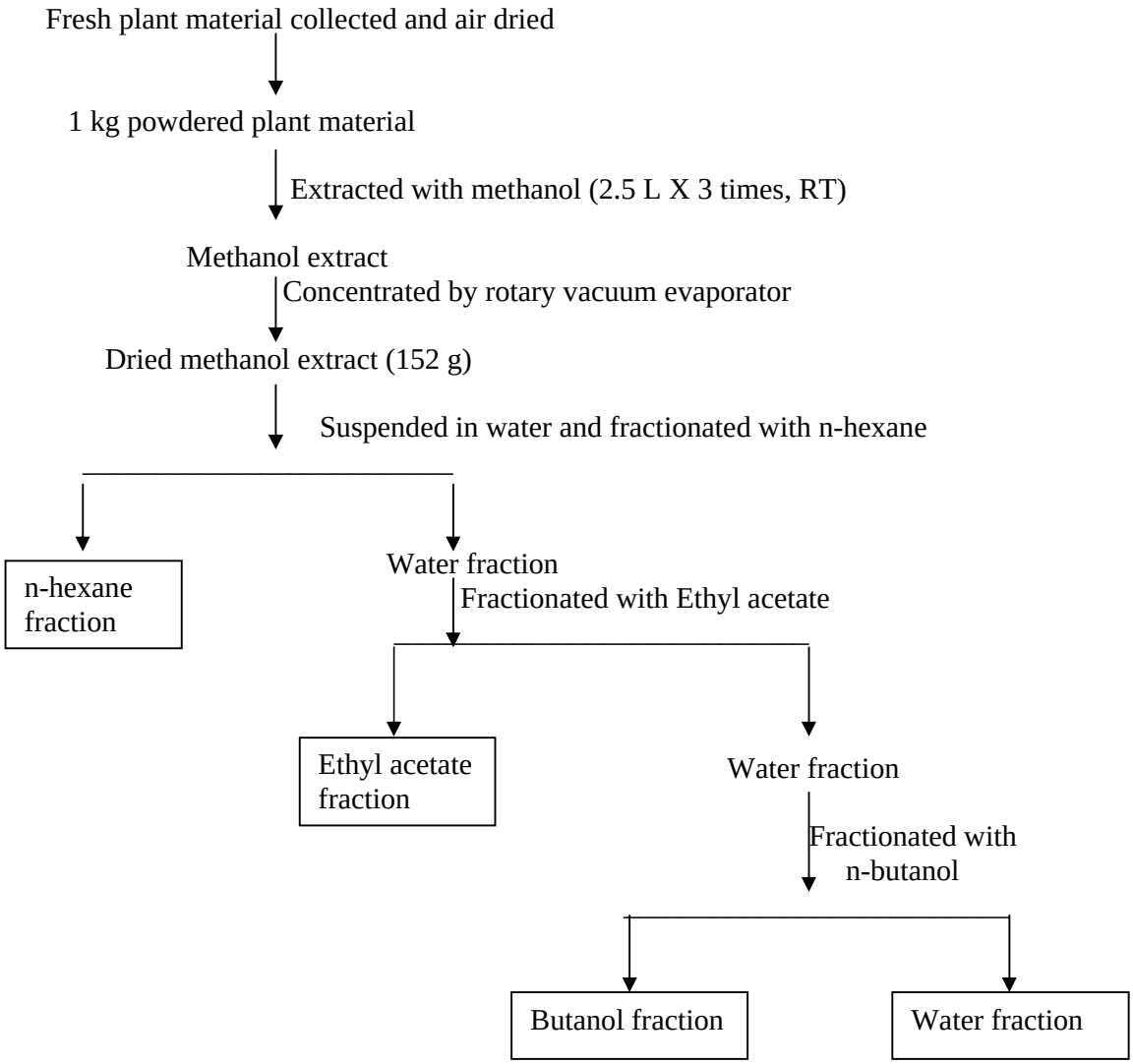


Figure 1: Extraction and partitioning method employed for the isolation of compounds from *H. incana*.

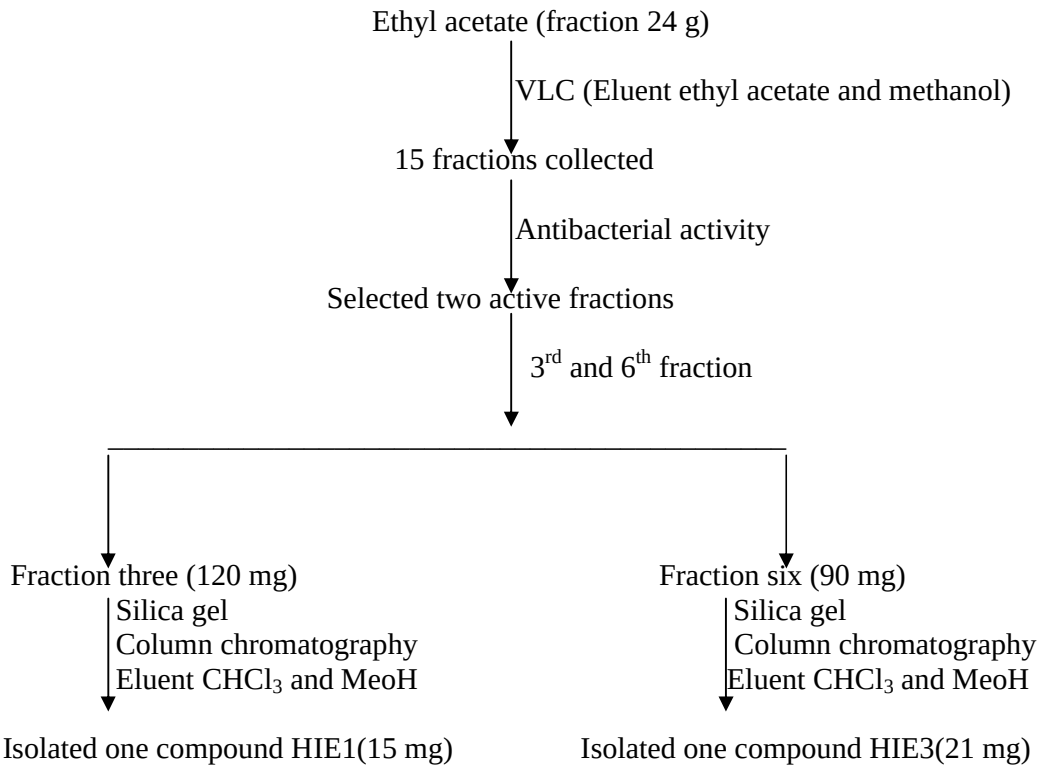


Figure 2: Isolation and purification of compounds from Ethyl acetate fraction.

Table 1. Antibacterial activity of epicatechin and 3,5,7,2′, tetrahydroxy flavone-3- O-β-D-glucopyranoside isolated from *H. incana*.

	Minimum inhibitory concentration (μg/ml) of compounds			
Bacteria	Epicatechin	R= β-D-glucopyranoside	Streptomycin	Chloramphenicol
B. subtilis	100	25	2	2
S. aureus	25	12.5	2	2
E. coli	25	12.5	2	2
S. flexneri	50	25	2	2

References

Afolayan, AJ., Meyer, J.J.M., 1997. The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoot of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57, 177-181.

Agrawal, P.K., 1989. P.K. Agrawal, Carbon-13 NMR of Flavonoids, Elsevier, New York (1989).

Ann, J.M., Geboes, K., 2002. Infectious colitis. Acta Endoscopica 32, 2-2.

Appidi, J.R., Grierson, D.S., Afolayan, A.J., 2009. Antimicrobial activity of *Hermannia incana.* Pharmaceutical biology 47, 615-619

Appidi, J.R., Grierson, D.S., Afolayan, A.J., 2008. Ethnobotanical study of plants used for the treatment of diarrhoea in the Eastern Cape, South Africa. Pakistan j biol sci. 11, 1961-1963.

Bors, W., Heller, W., Michel, C., Stettmaier, K., 1996. Flavonoids and polyphenols: chemistry and biology. In: Cadenas, E., Paker, L. (Eds.), Handbook of Antioxidants. Marcel Dekker Inc, New York. 409–466.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 65, 711–713.

Esquenazi, D., Wigg, M.D., Miranda, M.M.F.S., Rodrigues, H.M., Tostes, J.B.F., Rozental, S., da Silva, A.J.R., Alviano, C.S., 2002. Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. Res Microbiol 153, 647–652.

Essopa, A.B., Van Zyl, R.L., Van Vuurena, S.F., Mulhollandb, D., Viljoend, A.M., 2008. The *in vitro* pharmacological activities of 12 South African *Hermannia* species. Journal of Ethnopharmacology 119, 615-619.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press Inc., New York; 225-226.

Krause, R., Schwab, E., Bachhiesl, D., Daxbock, F., Krejs, G.J., Reisinger, E.C., Wenisch, C., 2001. Role of *Candida* in antibiotic-associated diarrhea. Journal of Infectious Diseases 184, 1065-1069.

Palombo, E.A., 2006. Phytochemicals from Traditional Medicinal Plants used in the Treatment of Diarrhoea: Modes of Action and Effects on Intestinal Function. Phytotherapy Research 20, 717–724.

McGaw, L.J., Jager, A.K., van Staden, J., 2000. Antibacterial, anthelmintic and antiamoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72, 247-263.

Reid, K.A., Jager, A.K., Light, M.E., Mulholland, D.A., Van Staden, J., 2005. Phytochemical and pharmacological screening of Sterculiaceae species and isolation of antibacterial compounds. Journal of Ethnopharmacology. 97, 285-291.

Shen, C.C., Chang, Y.S., Ho, L.K., 1993. Nuclear magnetic resonance studies of 5,7-Dihydroxyflavonoids. Phytochemistry 34, 843–845.

Schuier, M., Sies, H., Illek, B., Fischer, H., 2005. Cocoa-related flavonoids inhibit CFTR-mediated chloride transport across T84 human colon epithelia. Journal of Nutrition. 135, 2320–2325.

Slusarenko, A.J., Longland, A.C., Whitehead, I.M., 1989. A convenient, sensitive and rapid assay for antibacterial activity of phytoalexins. Botanica Helvetica. 99, 203-207.

Venkatesan, N., Vadivu, T., Sathiya, N., Arokya, A., Sundararajan, R., Sengodan, G., Vijaya, K., Thandavarayan, R., James, B.P., 2005. Anti-diarrhoeal potential of *Asparagus racemosus* wild root extracts in laboratory animals. Journal of Pharmaceutical Sciences 8, 39-45.

Watson, L., Dallwitz, M.J., 1992 onwards. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 14th February 2008.

Penga, Z.F., Strackb, D., Baumertb, A., Subramaniama, R., Goha, N.K., Chiaa, T.F., Tana, S.N., Chiaa, L.S., 2003. Antioxidant flavonoids from leaves of Polygonum hydropiper L. Phytochemistry 62, 219–228.