ISOLATION AND CHARACTERISATION OF BERGENIN FROM ETHYL ACETATE EXTRACT OF *FLUEGGEA VIROSA* LEAVES

C. T. Agber¹, T. A. Tor-Anyiin^{1*}, J. O. Igoli¹, E. M. Khan¹ and J. V. Anyam¹

¹Phytochemistry Research Group, Department of Chemistry, University of Agriculture Makurdi, Nigeria.

*Corresponding Author: toranyiint@yahoo.com

Received 27 July 2019; accepted 02 October 2019, published online 10 October 2020

ABSTRACT

Bergenin is an important constituent of *Flueggea virosa* (Euphorbiaceae), a tropical plant with several traditional uses. While there are numerous reports on the isolation and characterization of bergenin, a rapid, high-throughput, readily accessible method for the isolation and characterization of the compound locally has not been reported. Isocratic elution of ethyl acetate extract via vacuum liquid chromatography (VLC) with methanol produced a white amorphous solid (100 mg), which was successfully isolated from 250 g of the plant. On the basis of spectral data (¹H, ¹³C NMR, COSY, HSQC and HMBC) and comparison with literature reports, the structure of this solid was shown to be bergenin, a dihydroisocoumarin derivative of glucopyranosyl gallic acid.

Key words: *Flueggea virosa*, bergenin, Isocoumarins, NMR, Nigeria

INTRODUCTION

Flueggea virosa (Family: *Euphorbiaceae*) grows wild in tropical Africa and most parts of the world. It is a woody shrub with many branches that are erect or arching; lower branches often have thorny end. It has grey or brown bark that is smooth, fissured or rough [1]. *Flueggea virosa* is found throughout the tropical regions of Africa and Asia where it is often found along water courses and in fallow, disturbed lands up to 2300 m above sea level [2].

Different parts of the plant are used to treat many diseases like diabetes, HIV – AIDS and related infection, arrhythmia, malaria, fever, hepatitis and epilepsy [3]. It has been used in a number of biological assays such as antiarrhythmic, antioxidant, trypanocidal and antiplasmodial [3, 4]. In Benue State of North Central Nigeria, aerial parts of the plant are used for the treatment of malaria while leaves are used as animal feed. The plant leaves are also used to check bleeding and toothache among the Tiv of North-Central Nigeria. Inhalation of smoke from the burning leaves of *Flueggea virosa* is believed to ease rheumatoid pain (*Akpiti*) [5]. The compounds 11-O-acetyl bergenin, bergenin, virosecurinine, ent-phyllanthidine, kaempferol, quercetin, gallic acid, daucosterol, β -sitosterol along with securiniga alkaloids and flueggenoids have been isolated from the twigs and leaves of *Flueggea virosa* [6-10]. There are several ethno-

medicinal claims on the leaves of the plant here in Nigeria [11-12]. Therefore, chemical studies on this plant will be of importance in its chemotaxonomy as well as identification of compounds of potential value. Also, while there are many reports on the isolation and characterization of Bergenin from *Flueggea virosa* and some of these show a rapid and high-throughput method, a facile, rapid, accessible high-throughput report on its isolation has not carried out, to the best of our knowledge. Thus, we report, herein, a rapid high-throughput method for the isolation and structural elucidation of bergenin from leaves of *F. virosa*.

MATERIALS AND METHODS

Sample collection, Pretreatment and Microwave Assisted Extraction

Leaves of *F. virosa* were collected around Makurdi Metropolis, Benue State Nigeria in August 2016, air-dried for three weeks and ground to powder. Powdered sample (250 g) were placed in a Winchester bottle (2.5 L) and extracted sequentially with 1 L each of n-hexane, ethyl acetate and methanol via microwave assisted extraction. For each extraction, a bottle was placed in a microwave oven, set at the defrost function (70 Kw), and irradiated for 3 minutes after which it was allowed to cool for one minute; also, pent pressure was released by partially opening the

bottle cap. This was repeated 10 times for each solvent as described by Otto [13].

Isolation

Ethyl acetate extract (5 g), pre-adsorbed on Celite®, was allowed to dry and loaded on to vacuum liquid chromatography column (VLC) (packed with 30 g of silica gel (TLC grade), for purification as described by Tor-Anyiin *et al.*, [14]. After washing severally with hexane (20 washes), the column was eluted with a gradient of n-hexane in ethyl acetate as mobile phase, collecting 30 fractions. Thereafter, 100% methanol (mobile phase) was utilized to collect 10 more fractions, affording a total of 40 fractions (20 mL each). The column flow rate was at about 8 mL/minute. Fractions 31 – 40, eluted isocratically with methanol gave a white solid on drying which was washed severally with n-hexane to obtain a neat solid.

TLC of the white solid was performed on a pre-coated silica gel plate (silica gel GF 254, Merck®) using chloroform: methanol(9:1v/v) solvent system as mobile phase. The plate was sprayed with 10% sulphuric acid in methanol and then heated to about 120 °C for 1 minute to visualize spots from which R_f was determined. Melting point of the isolated compound was determined based on the procedure by Philip [15].

A portion of the solid (10 mg) was dissolved in methanol (5 mL) and from this, an aliquot (2 mL) was treated with 4 drops of 10 % ferric chloride solution (w/v). The formation of a bluish black colour indicated presence of a gallic tannin [16].

Characterization of the Isolate

Nuclear magnetic resonance experiments were carried out using a Bruker machine at 400 MHz (Proton) and 125 MHz (Carbon-13) using DMSO- d_6 as solvent. Both 1D and 2D (HSQC, HMBC and COSY) spectra were acquired.

MestReNova® and ChemsSketch® (ACDLABs) softwares were used in elucidating signal properties [2D NMR correlations were illustrated using the ACD labs ChemSketch (2015) software].

RESULT AND DISCUSSION

A white amorphous solid was obtained from the VLC of ethyl acetate crude extract of *F. virosa*. TLC of the solid gave a single dark spot (R_f = 2.3) when the plate was sprayed with 10% sulfuric acid in methanol and heated at 120 °C. This is suitable for detection of most polar compounds. The solid melted at a temperature of 237 – 240 °C.

A blue black colouration obtained from $FeCl_3$ test suggested that the white solid could be a galloyl tannin [9]. The proton NMR spectrum (summarized in Table 1) showed two phenolic-OH downfield signals at δ_H (9.75, 8.47) as singlets; three hydroxyl signals at δ_H (5.65, 5.45, 3.86), all singlets, which suggested the presence of an aromatic and an aliphatic group in the compound. Seven oxymethine signals (at δ_H 3.85, 3.65, 3.44, 3.21, 4.99, 3.57, 4.02) (*d*, *dd* and *ddd*) were also observed from the spectrum which corresponded to signals due to H-11, H-4, H-11, H-3, H-10b, H-2 and H-4a, respectively. These signals are similar to reports of Nguyen *et al.*, [17] for bergenin. The positions of these protons were confirmed by analysis of its 1H - 1H COSY and HSQC spectra. In addition, two singlet signals at δ_H 3.78 and 6.99 were assigned to a methoxy group (OCH_3) and an aromatic proton (H-7), respectively, in accordance to Lin *et al.* [18].

^{13}C -NMR spectrum (summarized in Table 1) showed methoxy and lactone carbon signals at δ_C 60.3 and 164.3 (C-6), respectively. The remaining twelve signals were characteristic of carbons from a glucosyl unit and an aromatic nucleus [18]. These data were comparable to those published for bergenin by Nguyen *et al.* [17] and Silva *et al.* [19].

HSQC spectrum showed the following correlations between C and H atoms:

δ_{CH} : 3.57, 82.3 (CH aliphatic, C-2); 3.21, 71.2 (CH aliphatic, C-3); 3.65, 74.2 (CH, aliphatic, C-4); 4.02, 80.3 (CH aliphatic, C-4a); 6.99, 110.2 (CH aromatic, C-7); 4.98, 72.6 (CH aliphatic, C-10b); 3.85 and 3.44, 61.7 (CH_2 aliphatic, C-11) and 3.78, 60.3 ($-OCH_3$).

The HSQC correlations confirmed that only one proton (δ_H 6.99, H-7) was attached to the aromatic carbon (δ_C 110.2, C-7). HSQC also revealed correlations typical of an aliphatic nucleus. A correlation typical of anomeric

proton (δ_H 4.98, H-10b) and carbon (δ_C 72.6, C-10b) was also observed. This further suggested the presence of an aromatic and sugar nucleus [20].

It was evident from the HMBC spectrum that the aromatic proton signal at δ_H 6.99 (H-7) correlated to a carbonyl carbon at δ_C 164.3 (C-6) and two oxygenic aromatic carbons at δ_C ; 141.3 (C-9) and 151.9 (C-8). The aromatic proton (H-7) also correlated to a tertiary aromatic carbon at δ_C 116.6 (C-10a). Therefore, a carbonyl group could be attached at C-6a that is ortho to the carbon bearing the only aromatic proton. Furthermore, the methoxy proton signal at δ_H 3.78 correlated to an aromatic carbon at δ_C 141.3 (C-9), thus implying that, the methoxy group could be attached on the aromatic ring at C-9

which was meta to C-7 but para to C-6a- bearing the benzoyl carbon (C-6, δ_C 118.6).

It was also noted from HMBC spectrum that H-10b, δ_H 4.99 (anomeric proton) correlated to one oxygenic aromatic carbon at δ_C 148.6 (C-10) and two other aromatic carbons at δ_C 116.6 (C-10a) and δ_C 118.6 (C-6a) [11]. The later (C-6a) was observed to correlate to H-4a (δ_H 4.02) which suggested that the sugar unit was C-C joined at C-10a of the aromatic ring via anomeric carbon (δ_C 72.6, C-10b) [18].

The downfield signal of H-4a (δ_H 4.02) appeared to be higher than those of other sugar protons except the anomeric proton (δ_H 4.99). This suggested that C-4a (δ_C 80.3) could be linked to C-6 (carbonyl carbon- electron withdrawing) through ethereal oxygen in a cyclic ester (lactone) formation.

Table 1: ^1H NMR and ^{13}C NMR Experimental and Literature Data of Bergenin (400 MHz, DMSO- d_6 , J in Hz)

Position	Exp. Data		Nguyen <i>et al.</i> , [17]		Silva <i>et al.</i> , [19]	
	^1H (δ)	^{13}C (δ)	^1H (δ)	^{13}C (δ)	^1H (δ)	^{13}C (δ)
2	3.57 (ddd; 9.7, 7.5, 2.1, 1H)	82.3	3.64	82.0	3.85	81.9
3	3.21 (dd; 9.8, 8.4, 1H)	71.2	3.37	70.8	3.43	70.7
4	3.65 (d, 9.0, 1H)	74.2	3.68	74.1	3.68	74.4
4a	4.02 (dd; 10.5, 9.5, 1H)	80.3	4.02	80.2	4.03	80.2
6	-	164.3	-	163.4	-	164.6
6a	-	118.6	-	118.1	-	118.2
7	6.99 (s 1H)	110.2	7.08	109.9	7.09	109.9
8	-	151.9	-	151.0	-	151.1
9	-	141.3	-	140.7	-	141.1
10	-	148.6	-	148.09	-	148.2
10a	-	116.6	-	115.51	-	116.1
10b	4.98 (d, 10.5, 1H)	72.6	4.86	72.89	4.94	73.1
11	3.85 (dd; 11.7, 2.1, 1H)	61.7	3.97	61.42	4.07	61.5
11	3.44 (dd; 11.9, 7.5, 1H)	61.7	3.60	61.42	3.81	61.5
9-OCH ₃	3.78 (s, 1H)	60.3	3.77	60.0	3.75	59.8
3-OH	5.45 (s, 1H)	-	5.59	-	5.39	-
4-OH	5.65 (s, 1H)	-	5.26	-	-	-
8-OH	8.47 (s, 1H)	-	8.35	-	-	-
10-OH	9.75 (s, 1H)	-	9.36	-	-	-
11-OH	3.86 (s, 1H)	-	3.88	-	-	-

^1H and ^{13}C chemical shifts data (Tables 1) of the white solid obtained from leaves of *Flueggea virosa* were in agreement with literature data reported by Silva *et al.*, [19] and Nguyen *et al.*, [17]. Similarly, melting point (237-240 °C) of the isolated compound was in agreement with that (238-240 °C) reported by Raj *et al.*, [21]. Based on these experimental analyses and comparison with literature reports, the white solid was identified as bergenin. Bergenin is a dihydroisocoumarin derivative of glucopyranosylgallic acid [22].

Bergenin has been isolated from several plant species including; *Ardisia crenata*, *Rodgersias ambucifolia*, *Bergenia aligulata*, *Ficus glomerata*, *Peltophorum africanum* and *Peltophorum pterocarpum* etc. It has been reported that bergenin displays several pharmacological properties such as antiplasmodial, antimicrobial, antiviral and antifungal; as well, exhibits antioxidant activity [4, 13, 21-26].

Fig. 1: **A**; IUPAC Numbering of Bergenin, Nguyen *et al.*, [17]; **B**: Important COSY and **C**: HMBC- Correlations of Bergenin

Conclusion

Motivated by lack of information on a facile method for isolation and purification of bergenin, a compound with high therapeutic

potential, a method for isolating it from leaves of *Flueggea virosa* was sought. A rapid protocol for its isolation was established using VLC and Microwave Assisted Extraction. The extensive treatment of NMR data and isolation protocol will also contribute baseline data to the phytochemistry of *Flueggea virosa*.

REFERENCES

1. Ezeonwumelu, J.O.C., Matuki, E.R., Ajayi, A.M., Okoruwa, A.g., Tanayen, J.K., Adiukwu, C.P., Goji, A.D.T., Dare, S. And Okonkwo, C.O. (2013). Phytochemical Screening, Acute Toxicity and Analgesic Properties of Aqueous Extract of *Flueggea virosa*'s Root in Rats. *Ibnosa Journal of Medicine and Biomedical Sciences*, **5**(1): 15-21
2. Monkodkaew, S., Loetchutinat, C., Nuntasaeen, N and Wilart, P. (2009). Identification and Antiproliferative Activity Evaluation of a Series of Triterpenoids Isolated from *Flueggea virosa* (Roxb. ex Willd.). *American Journal of Applied Sciences*, **6**(10): 1800-1806.
3. Nasir, A.S., Perwez, A., Adnan, J.A., Mai, M.A. and Mohammad, K.P (2015). Simultaneous Quantification of Biomarkers Bergenin and Menisdaurine in the Methanol Extract of Aerial Parts of *Flueggea virosa* by Validated HPTLC Densitometric Method. *Journal of Chromatographic Science*, **53**: 824-829

4. Chih-Hua, C., Ju-Chen, C., De-Yang, S and Tian-Shung, W (2014). Anti-Hepatitis C Virus Dinortepenes from the Roots of *Flueggea virosa*. *Journal of Natural Products*, **77**(1): 22-28
5. Shomkegh, S.A., Mbakwe, R. and Dagba, B.I. (2013). Ethnobotanical Survey of Edible Wild Plants in Tiv communities, Benue State of Nigeria. *Journal of Natural Science Research*, **3**(7): 2224-3186
6. Wang, G., Liang, J., Wang, Y., LI, Q. and Ye, W., (2008) Chemical Constituents from *Flueggea virosa*. *Chinese Journal of Natural Medicines*. **6**(4): 251-253
7. Wang, X., Liu, F., Zhu, Z., Fang, Q., Qu, S., Zhu, W., Yang, L., Zuo, J. and Tan, C. (2019). Flueggenoids A – E, new dinorditerpenoids from *Flueggea virosa*. *Fitoterapia*. **133**, 96-101
8. Zhang, H., Han, Y., Wainberg, M. A. and Yue, J. (2016). Flueggethers B–D, Securinega alkaloids with rare oligomerizing pattern from *Flueggea virosa*. *Tetrahedron Letters*, **57**(16): 1798-1800
9. Chao, C., Cheng, J., Shen, D., Huang, H., Wua, Y. and Wu, T. (2016). Terpenoids from *Flueggea virosa* and their anti-hepatitis C virus activity. *Phytochemistry*. **128**, 60-70
10. Hussain, A., Alam, P., Siddiqui, N. A., Alajmi, M. F., Rehman, T., Kalam, M. A. and Al-Rehaily, A. J. (2018). Development and validation of UPLC-PDA method for concurrent analysis of bergenin and menisdaurin in aerial parts of *Flueggea virosa* (Roxb. ex Willd.). *Saudi Pharmaceutical Journal*. **26**(7): 970-976
11. Magaji, M. G., Musa, A. M., Abdullahi, M. I., Ya'u, J. and Hussaini, I. M. (2015). Isolation of bergenin from the root bark of *Securinega virosa* and evaluation of its potential sleep promoting effect. *Avicenna journal of phytomedicine*, **5**(6): 587
12. Magaji, M. G., Anuka, J. A., Abdu-Aguye, I., Yaro, A. H., & Hussaini, I. M. (2008). Behavioural effects of the methanolic root bark extract of *Securinega virosa* in rodents. *African Journal of Traditional, Complementary and Alternative Medicines*, **5**(2): 147-153.
13. Otto, S (2007). Natural Products Isolation. *Natural Product Reports*, **25**: 517-554
14. Tor-Anyiin, T.A., Igoli, J.O., Anyam, J. and Anowu, J.N. (2015). Isolation and Antimicrobial Activities of Sarracenin from Root Bark of *Strychnos spinosa*. *Journal of Chemical Society of Nigeria*, **40**(1): 71-75
15. Philip, M. (1996). Advanced Chemistry. *Low Price Edition*, Cambridge, University Press, P 340
16. Sabri, F.Z., Belarbi, M., Sabri, S and Alsayadi, M.M (2012). Phytochemical Screening and Identification of some Compounds from Mallow. *Journal of Natural Products and Plant Resources*. **2**(4): 512-516
17. Nguyen, T.D., Pham, D.Y., Tailor, W.C and Hoang, V.D (2004). Bergenin Isolated from *Ficus glomerata* Bark. *Journal of Chemistry*, **42**(2): 250-25
18. Lin, C., Hwang, T., Lin, S. and Huang, Y. (2001). Bioactive Isocoumarins from *Cissus pteroclada*. *Journal of Chinese Medicine*, **23**(1): 41-49
19. Silva, S.L., Oliveira, V.G., Yano, T and Nunomura, R.S. (2009). Antimicrobial Activities of Bergenin from *Endopleura uchi* (Huber) Cuatrec. *Acta Amazonica*, **39**(1): 187-192

20. Agrawal, P.K. (1992). NMR Spectroscopy in the Structural Elucidation of Oligosaccharides and Glycosides. *Phytochemistry*, **31**(10): 3307-3330
21. Raj, M.K., Duraipaniyan, V., Agastian, P and Ignacimunthu, S (2012). Antimicrobial Activity of Bergenin Isolated from *Peltophorum pterocarpum* (DC) Flowers. *Asian Journal of Tropical Biomedicine*, **12**: 901-904
22. Zamani, K., Faghihi, L. and Ebrahimi, S. (2005). Synthesis of Some Novel Optically Active Isocoumarins and 3,4-dihydroisocoumarin Containing L-valine and L-leucine Moities. *Turkey journal of Chemistry*, **29**: 171-175
23. Jianchao, D., Xiaohua, X., Xing, T and Gongke, L (2010). Preparation of bergenin from *Ardisia crenatasims* and *Rodgersias ambucifolia* hemsl based on microwave-assisted extraction/high-speed counter-current chromatography. *Separation and Purification Technology*, **74**: 155-159
24. Ghias, U., Awar, S and Bina, S.S (2013). Comparative Antioxidant and Antiplasmodial Activities of 11-O-galloylbergenin and Bergenin Isolated from *Bergenia ligulata*. *World Applied Sciences Journal*, **27**(8): 977-981
25. Bizimenyera, E.S., Swan, G.E., Samdumu, F.B., Kamara, I.B and Eloff, J.N (2007). Isolation and Bioassey Characterisation of Bergenin from the Root Extract of *Peltophorum africanum* Sond. (Fabaceae). *South African Journal of Sciences*, **8**: 79-91
26. Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J and Folb, P.I, (2004). In vitro Antiplasmodial Activity of Medicinal Plants Native to or Naturalized in South Africa. *Journal of Ethnopharmacology*, **92**: 177–191.