

Isolation and characterization of spinasterol from *Crossopteryx febrifuga* stem bark

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ARTICLE INFO

Article history:

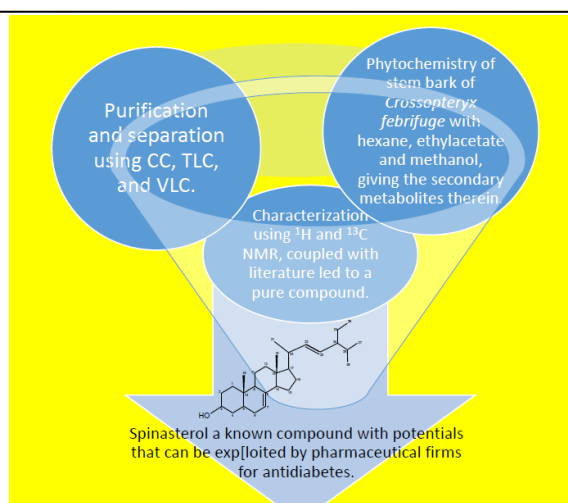
Received: 30 March 2019

Accepted: 29 June 2019

Available online: 31 July 2019

Manuscript ID: PCBR-1903-1028

GRAPHICAL ABSTRACT



KEYWORDS

Crossopteryx febrifuga
Phytochemistry bioactive component
Chromatography
Purification and isolation

ABSTRACT

In this study, stem bark of *Crossopteryx febrifuga* (Rubiaceae), a traditional medicinal, plant used for treatment of various diseases. The diseases was phytochemically screened by cold maceration using hexane, ethyl acetate, and methanol extracts. Results revealed the presence of bioactive compounds such as alkaloids, tannins, saponins, flavonoids, cardiac glycosides, and phytosterols. Separation and purification of the ethyl acetate extracts by column chromatography (CC), thin layer chromatography (TLC), vacuum liquid chromatography (VLC) and characterization with nuclear magnetic resonance (NMR) spectroscopic analyses, led to isolation of spinasterol, established on the basis of both ^{13}C and ^1H NMR spectral data and by comparison with literature. It was also confirmed that, spinasterol cures diabetes by reducing serum triglycerides. This is the first report on isolation of spinasterol from the stem bark of *Crossopteryx febrifuga*.

1. Introduction

Medicinal plants are a rich source of bioactive compounds which possess therapeutic properties that can be used in drug development and synthesis [1- 3]. Since creation, man has relied so much on medicinal plants for

health, food, and promotion of physical and spiritual well-being [4-8]. Due to the therapeutic nature of natural products, man has succeeded in alleviating and treating several diseases; however, some are yet to be properly and efficiently managed and as such they posed a great threat to humanity. Medicinal plants are thought to be effective and safer with less side effects and also available

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†Electronic Supplementary Information (ESI) available

to the common population in the underdeveloped and developing countries in the world. Due to the therapeutic nature of natural products, medicinal plants are being used as antidiabetic [9], antimicrobial [10, 11], anti-trypanosomal [12], antibacterial [13], antimalarial [14], antioxidant [15], anticonvulsant [16], and antihypertensive activities [17].

The plant *Crossopteryx febrifuga* (Rubiaceae) is a monotypic genus of flowering plants with a wide distribution which is found in East, West, Tropical and Central [18, 19]. In West Africa, it is widely distributed throughout the Savannah region including Nigeria [19]. It is commonly called "Ayeye" (Ebiras) and "Ii-kwar" (Tiv) in North-Central Nigeria, and its English name is "Ordeal tree". This plant has been traditionally used in Nigeria for several medicinal purposes such as antidiabetic, antihypertensive and antimicrobial activities. This research work was carried out to isolate the chemical compound(s) in the stem bark of the plant to identify the secondary metabolites responsible for the biological activity.

2. Materials and Methods

2.1. Collection and Identification of Plant material

The plant material was collected in April from the bush along Imoga road in Akoko-Edo L.G.A of Edo State, from Ere in Kogi State Nigeria. The plant was identified and authenticated by a botanist, Mr. Mark Uleh of Department of Social Forestry and Environmental Protection, University of Agriculture Makurdi, where a specimen with voucher number UAM/FH/0254 was deposited

2.2. Extraction of Plant Material

The plant sample (Stem bark) was dried at room temperature for 3 weeks and pulverized into a fine powder with a wooden mortar and pestle. The powdered sample (500 g) was macerated with 2.5 L of *n*-hexane for 72 h with manual agitation. The mixture was filtered, concentrated, and allowed to dry by evaporation through exposure to air at room temperature. This procedure was repeated to obtain ethyl acetate and methanol extracts.

2.3. Preliminary Phytochemical Screening

Phytochemical screening of the hexane, ethyl acetate, and methanol extracts were carried out to determine the presence of the secondary metabolites including alkaloids, saponins, tannins, steroidal compounds, flavonoids, cardiac glycosides, and terpenoids by following the standard procedures [20-23].

2.4. Vacuum Liquid Chromatography and Isolation of the Compound

The ethyl acetate extract was subjected to suction aided column chromatography. The stationary phase was a mixture of celite [pre-adsorbed with ethyl acetate extract (2.4 g)] and silica gel 60 G. Gradient elution was used throughout utilizing two solvent systems, hexane: ethyl acetate and ethyl acetate: methanol as mobile phases after washing the column repeatedly with *n*-hexane (20 washes). Thirty different combinations of hexane: ethyl acetate beginning with [100 percent hexane: 0 percent ethyl acetate; 99 percent hexane: 1 % ethyl acetate; 98% hexane: 2% ethyl acetate....] then with one hundred percent ethyl acetate and 30 different concentrations of ethyl acetate: methanol beginning from [100% ethyl acetate: 0% methanol] with one percent increases in polar methanol to [0% ethyl acetate: 100% methanol]; each fraction being a product of a unique solvent binary phase. Portions of fractions R-40 to R-42; containing fine, colourless, needle shaped, crystals were combined on the basis of their TLC similarity [R_f 0.42 in acetone: hexane (1:3)] and re-chromatographed using *n*-hexane alone for the first 10 fractions then hexane: ethyl acetate (19: 1). Fractions 22 to 30 were found to be identical on the basis of TLC and were combined. These on drying gave colourless needle shaped solids which were further recrystallized in methanol. The crystals gave a melting point ranging from 136 °C to 141 °C.

2.5. Proton Nuclear Magnetic Resonance Spectroscopy (1H -NMR)

The 1H -NMR (400 MHz) spectrum of fraction R-29 was run on a Bruker DPX 400 MHz NMR spectrometer using $CDCl_3$ as solvent and TMS as internal standard at the University of Strathclyde, Glasgow, Scotland, United Kingdom

3. Results and Discussion

3.1. Phytochemical Screening

Table 1 shows the phytochemical screening results.

Table 1. Phytochemical Constituents of *Crossopteryx febrifuga* Stem Bark

Phytochemicals	HE	EA	ME
Alkaloid	+	+	+
Saponins	-	-	+
Tannins	-	+	+
Phytosterols: (Sterols and triterpenes)	-	+	+
Flavonoids	-	+	+
Cardiac Glycosides	+	+	+

Key: HE = Hexane, EA = Ethyl acetate, ME = Methanol, - = absent, + = present

3.2. Spectral Data of Phytochemicals Isolated from *Crossopteryx febrifuga* Stem Bark

The NMR spectral data obtained from the isolated fraction is given below

¹H NMR (400 MHz, chloroform-D): δ (ppm) 5.15 (dd, J=15.1, 8.6 Hz, 1H), 5.02 (dd, J=15.2, 8.6 Hz, 1H), 3.60 (tt, J=10.8, 4.4 Hz, 1H), 1.25 (s, 2H), 1.02 (d, J=6.6 Hz, 1H), 0.92 (d, J=6.4 Hz, 1H), 0.85 (d, J=6.2 Hz, 1H), 0.80 (d, J=2.9 Hz, 2H), 0.55 (s, 1H).

¹³C NMR (101 MHz, chloroform-D): δ (ppm) 139.71, 138.32, 129.57, 117.60, 77.16 (solvent), 71.22, 56.03, 55.27, 51.39, 49.58, 43.43, 40.99, 40.39, 39.60, 38.11, 37.28, 34.36, 32.02, 31.60, 29.78, 28.66, 25.55, 23.16, 21.69, 21.52, 21.25, 19.14, 13.19, 12.40, 12.20.

Table 2. ¹³C & ¹H NMR Data of SCF 14 in CDCl₃ in Comparison with Literature

Position	Experimental		Literature [24]	
	δC	δH	δC	δH
1	37.28	1.02, 1.76	37.155	1.01, 1.74
2	31.70	1.37, 1.72	31.479	1.36, 1.72
3	71.28	3.59 (1H, tt)	71.079	3.52 (1H, m)
4	38.16	1.29, 1.52	37.997	1.18, 1.63
5	40.39	1.40	40.275	1.33
6	29.87	1.25, 1.53	29.69	1.15, 1.68
7	117.78	5.15	117.46	5.09 (1H, m)
8	139.71		139.56	
9	49.75	1.62	49.466	0.66
10	34.36		34.23	
11	21.53	1.40	21.555	1.50
12	39.60	1.25, 1.81,	39.479	1.18, 1.92
13	43.43		43.29	
14	55.45	1.77	55.135	1.74
15	23.59	1.38, 1.40	23.086	1.31, 1.44
16	28.66	1.25 (2H, s)	28.498	1.20, 1.66
17	55.27	0.80	55.918	1.18
18	12.20	0.55 (1H, s)	12.049	0.50
19	13.14	0.80 (2H, d)	13.037	0.72
20	40.99	0.92 (1H, d)	40.817	1.97
21	21.48	1.03 (1H, d)	21.372	1.50
22	138.43	5.19 (1H, dd)	138.158	5.1 (1H, dd)
23	129.65	5.05 (1H, dd)	129.457	4.983 (1H, dd)
24	51.44	1.53	51.253	1.45
25	31.59	1.41	31.874	1.48
26	21.21	0.86 (1H, d)	21.079	0.75
27	19.18	0.80, 0.84	18.99	0.72, 0.85
28	25.55	1.01, 1.40	25.393	1.09, 1.33
29	12.42	0.82	12.24	0.75

δ = Chemical shift (ppm)

A comparison of NMR spectra data with those reported in the literature [24] showed that the isolated compound is spinasterol as presented in Table 2.

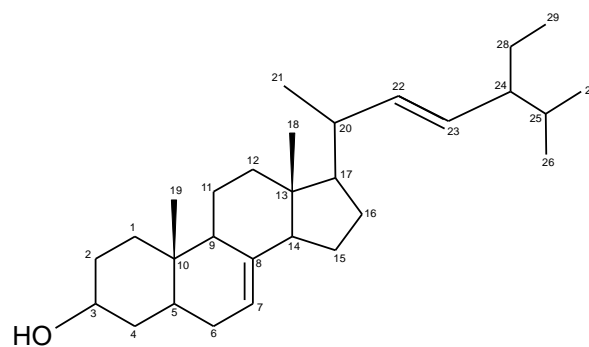


Fig 1. Spinasterol (stigmasta-7, 22-dien-3-ol)

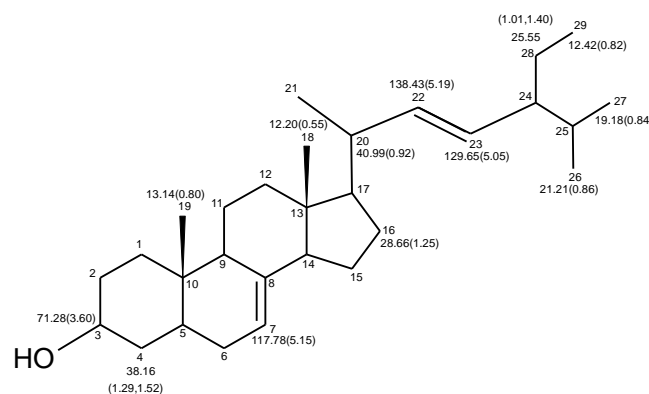


Fig. 2. Spinasterol showing carbon NMR chemical shifts values

3.3. Characterization and Structure Elucidation of the Isolated Compound (SCF 14)

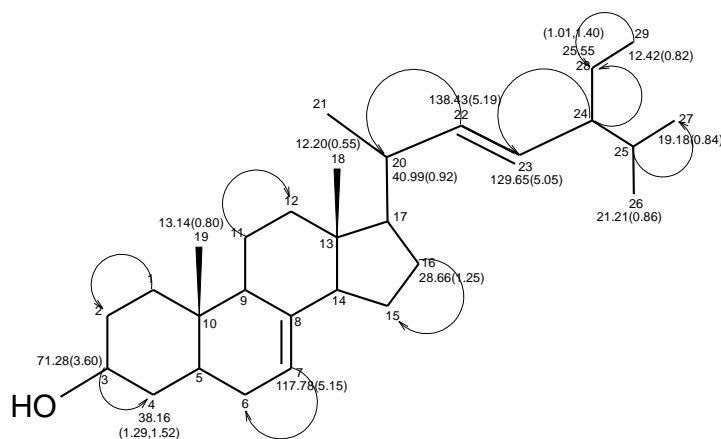


Fig. 3. COSY analysis for the identification of spinasterol

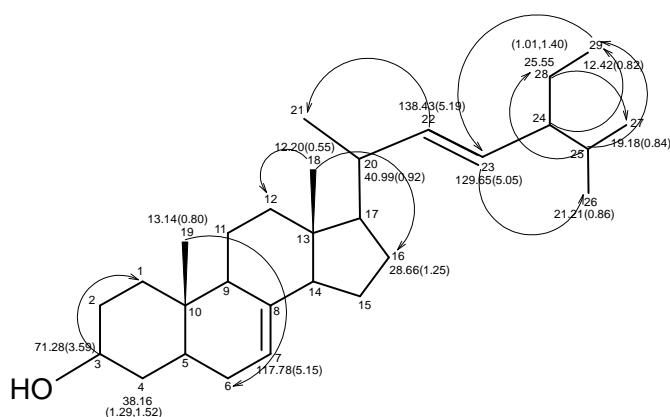


Fig. 4. HMBC analysis for the identification of spinasterol

The pure isolate obtained was labeled SCF 14 and had an R_f value of 0.42 in Hexane: Ethyl acetate (3:1) which was similar to that reported for spinasterol by [25]. It was determined that the fraction was a phytosterol

The ^1H NMR spectrum of the isolated compound displayed 48 proton signals, and the ^{13}C NMR spectrum 29 carbon resonances indicating the molecular composition $\text{C}_{29}\text{H}_{48}\text{O}$ for spinasterol.

For the compound spinasterol, the 400 MHz ^1H NMR spectrum showed the presence of three olefinic protons at δ 5.15 (1H, s H-7), 5.15 (1H, dd, $J = 15.1, 8.6$ Hz, H-22), 5.02 (1H, dd, $J = 15.2, 8.6$ Hz, H-23) respectively. The trans (E) configuration of the double bond in the compound was clearly shown by the chemical shifts of the carbons next to the double bond at δC 21.48 (C-21) and 51.44 (C-24) [26]

The 101 MHz ^{13}C NMR spectrum showed 4 signals at chemical displacements of 117.78, 139.71, 138.43, and 129.65 ppm indicating the presence of carbon-carbon double bonds, the 71.28 ppm signal represents a carbon atom connected with hydroxyl group which is assigned to C3. The two double bonds present in the compound was made evident from the appearance of three olefinic carbon signals attributed to C7 (δ 117.78), C22 (δ 138.43) and C23 (δ 129.65) [24]. The olefinic carbon resonance at δ 117.78 is a remarkable feature of sterols with Δ^7 bond that is very significant in the confirmation of the compound as spinasterol [27, 28].

The HMBC experiment showed the correlation between various carbons, 13.14 ppm and 1.25 ppm signals corresponding to C19 and C6, joined to 3 bonds of distance, 12.20 and 1.81, 1.25 showed the correlation between C18 and C12 at two bonds distance and C16 at three

bonds of distance signals at 21.48 ppm and 5.19 ppm represent the correlation between C21 and C22 at 2 bonds distance, 129.65 ppm and 0.82 ppm revealed the correlation between C23 and C29 at three bonds of distance, 31.59 and 1.01, 0.82 ppm showed the correlation between C25 with C28 at two bonds of distance and C29 at three bonds of distance, 129.65 and 0.82 ppm signals represent the correlation between C23 and C29 at three bonds of distance, 12.42 and 1.53 ppm correspond to C29 joined to C24 at two bonds of distance and signals 25.55 and 0.80 ppm showed the correlation between C28 and C27 at three bonds distance [24, 29].

The presence of quaternary carbon in the compound was shown by the signal 139.71 ppm as it was not joined to any proton signal. COSY analysis also revealed a correlation between the proton signals at 3.60 and 1.29 ppm which corresponds to the C3 and C4 bonds. The correlation of proton signals at 5.19 ppm and 5.05 ppm corresponding to C22 and C23 was also revealed by the COSY experiments, thereby confirming the presence of double bonds between the two carbon atoms with displacement signals 138.43 and 129.65, respectively. The 0.80 ppm and 0.92 ppm signals showed the direct correlation of C17 and C20, carbons linked together by the sterane nucleus and the aliphatic lateral chain [24].

1.1. Biological Activity of Spinasterol

It has been reported that alpha-Spinasterol has a significant therapeutic potential to modulate the development of diabetic nephropathy by showing a significant reduction in serum triglycerides increase, renal weight and urinary protein excretion in streptozotocin-induced diabetic mice [29].

Other biological activities exhibited by spinasterol include anti-inflammatory [30, 31], antiulcerogenic [32], antigenotoxic [33, 34] antidepressant [35, 36] and anti-tumor properties in breast, ovarian and cervical cancer cells [37].

4. Conclusion

The results of the phytochemical screening of the stem bark extract of the plant *Crossopteryx febrifuga* revealed the presence of tannins, phytosterols and flavonoids in the ethyl acetate and methanol, alkaloids and cardiac glycosides in all the extracts (n-hexane, ethyl acetate and methanol) while saponins were found exclusively in methanol extract. The presence of alkaloids, tannins, and saponins indicated that the plant is capable of acting as an anti-microbial reagents. Sterols and flavonoid have been known to have hypoglycemic activity as found that plants

containing natural antioxidants including, flavonoids and steroids can preserve β -cells function and prevent diabetes induced reactive oxygen species (ROS) formation.

Acknowledgements

Our sincere gratitude goes to Prof J.O Igoli and University of Strathclyde, Glasgow, UK. for their help in the analyses

Conflict of interest

The authors declare that they have no competing interests.

References

- [1] Mamta S., Jyoti S., Rajeev N., Dharmendra S. Abhishek G. *J. Pharmacogn. Phytochem.* 2013, **1**: 168
- [2] Himani R., Garima S., Nupur J., Neelam S., Navneeta B. *Turk. J. Bot.*, 2014, **38**: 1027
- [3] Singh R. *J. Plant Sci.*, 2015, **3**: 50
- [4] Nwachukwu C.U., Umeh C.N., Kalu I.G., Okere S., Nwoko M.C., *Report and Opinion*, 2010, **2**: 1
- [5] Chukwuemeka I.M., Udeozo I. P., Odo M. C., Oraekwute E. E., Onyeze R. C. Ugwu O.P.C. *Int. J. Res. Rev. Pharm. Appl. Sci.*, 2013, **3**: 470
- [6] Olasehinde G., Olusola O., Adegboyega O.A., Obasola E.F., Neena V., Isaac O. A., Adesola A.A., Louis, O.E., *Malar. J.*, 2014, **13**: 63
- [7] Emiola O. K.S., Abbah E.A., Odeyemi J.B., Taiwo T.B., *Int. res. j. eng. tech.*, 2015, **2**: 550
- [8] Mrinmay D., Ashok K.D., Mastanaiah K., Arup D., *Int. j. pharm. sci. res.*, 2015, **6**: 1027
- [9] Adikwu U.M., Uzuegbu D.B., Okoye T.C., Uzor P.F., Adibe, M.O., Amadi, B.V, *J. Basic. Clin. Pharm.*, 2010, **1**: 197
- [10] Khan M.E., Yakubu U.D., Kubmarawa D. *Br. J. Appl. Sci. Technol.*, 2015, **5**: 396
- [11] Tor-Anyiin T.A., Igoli J.O., Anyam J.N., *J. Chem. Soc. Nigeria*, 2015, **40**: 71
- [12] Rasooli I. (Ed.). *Phytochemicals: Bioactivities and Impact on Health*. BoD–Books on Demand, 2011
- [13] Shobha G.S.C., Shashidhara K.S., Vinutha M., *Int. Res. J.*, 2014, **5**: 273
- [14] Gbolahan W.A., Titus A.O., Isiaka A.A., Gbolahan-Ayoade E.E., *Int. J. Sci. Res.*, 2014, **4**: 1
- [15] Atawodi S., Samson Y., Emmanuel O., Precious I., Mercy O., Haruna Y., Sakina D. M., *Annu. Res. Rev. Biol.*, 2015, **5**: 501
- [16] Bassoueka D.J., Taiwe S.G., Nsonde N.G.F., Ngo B.E., *Int. J. Sci. Res.*, 2016, **5**:112
- [17] Tsabang N., Yedjou C.G., Tsambang L.W.D., Tchinda A.T., Donfagsiteli N., Agbor G.A., Tchounwou P.B.B., Nkongmeneck B.A., *Med. Aromat. Plants*, 2015, **S2**: 003
- [18] Orwa C., Mutua A., Kindt R., Jamnadass R., Anthony S., *Crossopteryx febrifuga Agroforest Tree Database: A Tree Reference and Selection Guide Version 4.0*. 1-5. 2009. Retrieved from [http://worldagroforestry.org/af/tree/\(Databases](http://worldagroforestry.org/af/tree/(Databases) 2009; 4/2/17)
- [19] Ojewale A.O., Olaniyan O.T., Yemitan O.K., Odukanmi O.A., Dare B.J., Nnaemeka W.S., Omoaghe O., Akingbade A.M., Ogunmodede O.S., Adebari A.O., *Mintage J. Pharm. Med. Sci.*, 2013, **2**: 48
- [20] Prashant T., Bimlesh K., Mandeep K., Gurpreet K., Harleen K., *Int. Pharm. Sci.*, 2011, **1**: 98
- [21] Anyam J.N., Tor-Anyiin T.A., Igoli J.O., *Res. Chem. Pharm. Sci*, 2015, **2**: 32
- [22] Khan M.E., Anyam J.V., Okwudili E.E., *J. Sci. Res.*, 2015, **3**: 252
- [23] Tor-Anyiin T.A., Shimbe R.Y., *J. Chem. Soc. Nigeria*, 2012, **37**: 69
- [24] Meneses-Sagrero S.E., Moisés N., Eduardo R., Carmen L.D., Manuel J., Ramón E.R., *Saudi Pharm. J.*, 2017, **25**:1137
- [25] El-Domiaty M.M., Mahmoud M.A.A., Zeinab I.A.E., Shima E., Nora T.W., *Biosci. biotech. res. asia*, 2013, **10**: 533
- [26] Turibio K.T., Ache R.N., Talontsi F., Tala M.F., Yeboah O.S., Ngadjui T.B., *Int. J. Chem. Pharm. Sci.*, 2015, **3**: 1764
- [27] Wilzer K.A., Spectral Studies of Constituents of Members of the Family Asteraceae. Louisiana State University (LSU) Historical Dissertations and Theses. 4214. 1986. <http://digitalcommons.lsu.edu/gradschool-disstheses/4214>. Pp 59-63
- [28] Yadessa M., Bioassay Directed Chemical Study of Antimalarial Substances from *Clerodendrum myricoides* and *Dodonaea angustifolia* and Comparative Chemical Studies of *Moringa stenopetala* and *Moringa oleifera*. Ph.D thesis. Department of Chemistry, College of Natural Sciences, Addis Ababa University. 2015, p 31

- [29] Ayu M., Adlis S., Djaswir D., *Int. J. Chem. Pharm. Anal.*, 2016, **3**: 1
- [29] Jeong S.I., Kim K.J., Choi M.K, Keum K.S., Lee S., Ahn S.H., Back S.H., Song J.H., Ju Y.S., Choi B.K., Jung K.Y., *Planta Med.*, 2004, **70**: 736
- [30] Jeong G.S., Li B., Lee D.S., Kim K.H., Lee I.K., Lee K.R., Kim Y.C., *Int. Immunopharmacol.*, 2010, **10**: 1587
- [31] Borges F., Silva M., Cordova M., Schambach T., Pizzolatti M., Santos A., *J. Ethnopharmacol.*, 2014, **151**: 144
- [32] Klein L.C., Gandolfi R.B., Santin J.R., Lemos M., Filho V.C., De Andrade S.F., *Naunyn Schmiedebergs. Arch. Pharmacol.*, 2010, **381**: 121
- [33] Villaseñor I.M., Lemon P., Palileo A., Bremner J.B., *Mutat. Res.*, 1996, **360**: 89
- [34] Ntie-kang F., Yong J.N., *RSC Adv.*, 2014, **4**: 61975
- [35] Socała K., Nieoczym D., Pieróg M., Wlaz P., *J. Neural. Transm.*, 2015, **122**: 1239
- [36] Socała K., Wlaz P., *Behav. Brain Res.*, 2016, **303**: 19
- [37] Gook-Che J., Myoung-Soon P., Do-Young Y., Chul-Ho S., Hong-Sig S., Soo-Jong U., *Exp. Mol. Med.*, 2005, **37**: 111