Three - (-) Catechin-O-Rhamnosides from the Eastern Nigeria Mistletoe with Potent Immunostimulatory and Antioxidant Activities

Omeje Edwin Ogechukwu1*, Osadebe Patience Ogoamaka1, Akira Kawamura2, Amal Hassan3, Abdessamad Debbab3, Esimone Charles Okechukwu4, Nworu Chukwuemeka Sylvester2, Nwodo Ngozi1 and Proksch Peter2

1Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. 410001, Enugu, Nigeria
2Department of Chemistry, Hunter College of CUNY, The City University of New York, USA
3Department of Pharmaceutical Biology, Heinrich-Hein University, Bilk, 40225 Düsseldorf, Germany
4Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, PMB 6025, Awka

Keywords: Immunomodulatory; Catechin; Rhamnoside; Mistletoe; Antioxidant; Eastern Nigeria; C57BL/6 splenocytes

Introduction

The unmatched availability and chemical diversity characterizing natural products provide unlimited opportunities for development of new drug leads [1]. Beside small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases [2]. Despite the huge resources on research involving the use of conventional targets and therapeutic approaches to fight diseases of man and animal, mortality and morbidity rates resulting from some of these diseases are still unacceptable. There are today, newer frontiers and paradigm shifts in therapy being investigated as better alternatives to conventional therapy. Modulation of the immune system as well as optimizing oxidative processes of the body with the aid of natural products represents a field of drug development-based research witnessing unprecedented upsurge in recent times [3]. In addition, the human immune system is intricately interwoven with oxidative processes in the body. High oxidative stress usually breaks down immune system, precipitates radicals as well as severe diseases and this must be prevented [4]. Even though, the body has developed a variety of ways to deal with damaging free radicals, antioxidants from dietary sources also play important role in their control, thus limiting cellular damage [5]. More recent studies have also emphasized the therapeutic importance of plant derived immunomodulators and antioxidants [6,7]. A newer approach to therapeutics is now the search for safe and potent immune modulating substances preferably with synergistic antioxidant activity. Interestingly, there has been growing interest in isolating and characterizing natural compounds with immunomodulatory and antioxidant activities [8]. It has also been established that most pharmacological activities are related to the immunostimulatory and antioxidant properties of plant secondary metabolites [9]. Mistletoes grow in different continents of the world and in Eastern Nigeria, a species, Loranthus micranthus have been used traditionally for the management of various ailments, notably diabetes, high blood pressure and conditions affecting human immune system for many years [10,11]. We recently reported the immunomodulatory potentials of mistletoes of Eastern Nigeria origin, Loranthus micranthus, parasitic on five different host trees with mistletoe from Kola acuminata exhibiting the highest activity [12]. A preceding study had established a potent up-regulatory immune response activity of Loranthus micranthus and indicated that these species of mistletoe is highly safe and obviously contains no active proteins or lectins [13]. Further preliminary studies indicated that steroids, triterpenoids, alkaloids and flavonoids were the possible immunostimulants in Loranthus micranthus [14]. Unpublished data (using the radical scavenging (DPPH)-based model) also suggested that all the mistletoes possessed moderate antioxidant activity, with that from Citrus sp. exhibiting highest activity. The present study was aimed at isolating and characterizing the immunomodulatory and antioxidant activities of catechin rhamnosides from the eastern Nigeria mistletoe.

Materials and Methods

Collection and identification of plant material

Loranthus micranthus Linn. (Loranthaceae) leaves parasitic on the...
host tree (*Kola acuminata*) were collected in April, 2007 from different locations in Nsukka LGA, Enugu State. The leaves were identified and certified by Mr. AO Ozioko, a taxonomist of the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State. Voucher specimens were kept at the BDCP Centre with the number BDCP-532-07 for reference purposes.

**Instruments**

Gallenkamp melting point apparatus (England; used uncorrected), HREIMS and EIMS (mass spectrometers) linked to a MATT 8200 recorder, ¹H NMR, ¹³C NMR and correlation studies were recorded with Bruker Spectrometer (600 MHz spectrophotometer in CDOD or CDCl₃, with or without internal standards at the Institute of Anorganic Chemistry and Structure Chemistry, Heinrich-Heine-Universität, Düsseldorf, Germany or the Department of Chemistry, City University of New York (CUNY), USA. FT-IR spectrometer (Shimadzu, Japan) at the Department of Chemistry, Usmanu Dan Fodiyo University, Sokoto. UV/visible spectra were obtained in a UV2102PC spectrophotometer with integrated data station (UNICO, USA) at the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. Others were electronic analytical balance (Metler Toledo, 0.001 max, England), glass columns (4x150 cm; 2.7x70 cm), silica gel G₂₅₀ and precoated G₂₅₀ plates.

**Solvents and reagents**

Analar grade methanol, n-hexane, ethylacetate, acetone, chloroform (Sigma Aldrich; Germany). Distilled water, normal saline (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England), silica gel (70-230 and 60-120 mesh sizes), silica gel (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England), silica gel (70-230 and 60-120 mesh sizes), silica gel (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England), silica gel (70-230 and 60-120 mesh sizes), silica gel (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England).

**Preparation of standard antioxidant solution**

This was carried out using the DPPH radical-based assay procedure. Exactly 15 mg or 7.5 mg of DPPH was weighed on an analytical balance and dissolved in 10 ml of Analar methanol contained in a 250 ml volumetric flask. The volume was made up to mark with fresh methanol to produce a concentration of 0.4 or 0.2 mM solution respectively. This solution was kept in the dark to avoid degradation.

**Antioxidant studies on crude extract and solvent fractions**

This was carried out using the DPPH radical-based assay procedure. Exactly 15 mg or 7.5 mg of DPPH was weighed on an analytical balance and dissolved in 10 ml of Analar methanol contained in a 250 ml volumetric flask. The volume was made up to mark with fresh methanol to produce a concentration of 0.4 or 0.2 mM solution respectively. This solution was kept in the dark to avoid degradation.

**Antioxidant studies**

Stock solutions of isolated compounds under study were carefully made in Analar methanol. Serial dilutions (2 or 10 fold) were achieved for each stock and labeled appropriately. The initial absorbance, (A₀) of the DPPH solution was taken at 517 nm using the JENWAY 680 spectrophotometer. Similarly, the absorbance of the standard ascorbic acid or pure compounds were taken [15]. Then the final absorbance (Aₜ) of the fraction from the crude aqueous methanol extract of the Eastern Nigeria Mistletoe with Potent Immunostimulatory and Antioxidant Activities. J Biomol Res Ther 1:102. doi:10.4172/2167-7956.1000102

![Proposed structures of isolated catechin rhamnosides.](image-url)

**Figure 1:** Proposed structures of isolated catechin rhamnosides.
Statistical analyses

The results obtained (analysed by SPSS version 11), were recorded as the mean values with the standard error in mean (SEM) and statistical significance between treated and control groups were evaluated by the Student’s t-test and one way analysis of variance (ANOVA; Fischer LSD post hoc test). Differences between means of treated and control groups were considered significant at p<0.05, 0.01 or 0.001.

Results and Discussion

The proliferative effects of different concentrations (10, 25 and 100 µg/ml) of compounds I, II and III (Figure 1) on the C57BL/6 mice splenocytes are shown in Table 1. The compounds produced a dose-dependent and highly significant (p<0.001) stimulation of the target cells when compared to the response from the untreated group. At a 100 µg/ml concentration, compounds II and III produced stimulations of 91.49%, 95.17% and 94.23% respectively compared to 2.65% recorded for the untreated group. This implies an over 30 times potency of the compounds compared to the controls. Strikingly too, these compounds also performed better than the positive controls, LPS (10 µg/ml) and Con A (2 µg/ml) which exhibited stimulations of 16.09% and 20.57% respectively. The stimulation of C57BL/6 mice splenocytes suggests an up regulation of the immune function indicating an immunostimulatory potential of the compounds on these mice immune cells. It can be inferred from these data that the isolated catechin rhamnosides are potent immunostimulating on C57BL/6 mice splenocytes. It appears that the hydroxyl group at position 3 of the catechin nucleus is essential for activity as compounds II and III which had the position unsubstituted showed slightly higher activities than compound I with the position containing the glycone (rhamnoside). Methylation of the 4’-position did not produce any significant difference (p<0.05) in activity. These assumptions are however, subject to further detailed studies. Similarly, the stimulatory effects of the isolated compounds on the early activation marker, CD69 is shown in Table 2. The data revealed a moderate dose-dependent stimulation of this marker in manner higher than the unstimulated controls, however, lower than the positive control agents. CD69 (Cluster of Differentiation 69) is a human transmembrane C-Type lectin protein encoded by the CD69 gene. The activation of T lymphocytes, both in vivo and in vitro, induces expression of CD69. This molecule, which appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation, is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, including natural killer (NK) cells, and platelets [18]. CD69 is also a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor [19]. The enhancement of expression of this early activation marker by molecules is therefore a direct measurement of the immunomodulatory potentials of such molecules. Although the stimulation produced by these compounds in their present molecular structure is moderate, detailed structure activity studies would likely produce analogues with higher potencies. Furthermore, the antioxidant potentials of the isolated catechin rhamnosides are depicted in Table 3. The antioxidant potentials, which were estimated by the DPPH free radical method, showed that the test-
Table 3: Antioxidant potentials of crude extract, solvent fractions and catechin rhamnosides.

| Compound | **EC50 value (mg/ml)** | ***Potency factor** |
|----------|------------------------|---------------------|
| Compound I | 55.42 ± 0.99 | 3.14 |
| Compound II | 58.45 ± 1.07 | 3.32 |
| Compound III | 59.71 ± 1.63 | 3.39 |
| Compound III | 59.71 ± 1.63 | 3.39 |
| Ascorbic acid (standard) | 17.6 ± 1.78 | 1.00 |

**EC50** is the effective concentration of a substance estimated to cause a 50% reduction in the total free radical activity of DPPH. ***Potency factors between 1.00 and 3.50 were considered suitable candidates or sources for possible antioxidant development.

ed compounds were reasonably active compared to the standard drug, ascorbic acid. However, the standard, ascorbic acid was generally, three times more active than the respective compounds in their present structural forms. It is important to emphasize that the assessment model in this experiment is an in vitro-based type suggesting that a possible potentiation of activity when assayed by in vivo models. It is further envisaged that guided structural modifications of the compounds will afford more potent analogues. The very many documented biological activities of the eastern Nigeria mistletoe may be attributed largely to this established antioxidant property. In concrete terms, it is needful to assess this property with several other models depicting both in vivo and in vitro environment. Compound I was isolated and purified as yellow semi solid compound; yield (35 mg) and exhibited a positive reaction with FeCl₃, a yellow colour which deepens in alkaline solution. The UV λ max (in methanol) nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride AlCl₃ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH in A-ring of the flavonoid nucleus. The FT-IR v max KBr showed values at 3971-3400 (OH), 2941 (CH₃), 1701 and 1614 (C=C), 1022-613 (aromatic fingerprint). These were assigned to position 6 and 8 in ring A of the flavonoid nucleus. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of a carbonyl functional group in both C-13 NMR and FT-IR suggesting the absence of the usual ketonic group at position 4 of most flavonoids. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of a carbonyl functional group in both C-13 NMR and FT-IR suggesting the absence of the usual ketonic group at position 4 of most flavonoids. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of a carbonyl functional group in both C-13 NMR and FT-IR suggesting the absence of the usual ketonic group at position 4 of most flavonoids. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of a carbonyl functional group in both C-13 NMR and FT-IR suggesting the absence of the usual ketonic group at position 4 of most flavonoids.

The HMBC spectra suggested that the sugar linkage was at the position 7 of the A-ring of the flavonoid nucleus. DEPT-135 studies of compound I also supported the proposed structure. The proposed structure for compound I was elucidated as catechin-7-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. This was typical of 7-O-based catechin rhamnosides because they are known to have their rhamnose methyl groups resonating above δ value of 1.00 [21]. In addition there were no further signals suggesting the presence of extra sugar moiety. Compound II was isolated and purified as a yellow semi solid compound; yield (115 mg). It showed a positive reaction with FeCl₃, a yellow colour which deepens in alkaline solution. The UV λ max (in methanol) nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride AlCl₃ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372, and 382 (5-OH and 7-OH free). The FT-IR v max KBr showed values at 3971-3400 (OH), 2941 (CH₃), 1701 and 1614 (C=C), 1022-613 (aromatic fingerprint). The DEPT-135 studies of compound III also supported the proposed sugar portion and the aromatic region. Connectivities were confirmed by the 2-D correlation. In addition, the DEPT-135 studies of compound II also supported the proposed structure. The proposed structure for compound II was elucidated as catechin-7-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. This was typical of 7-O-based catechin rhamnosides because they are known to have their rhamnose methyl groups resonating above δ value of 1.00 [21]. In addition there were no further signals suggesting the presence of extra sugar moiety. Compound III was isolated as a yellow semi solid compound; yield (11 mg). It showed positive reaction with FeCl₃, a yellow colour which deepens in alkaline solution. The UV λ max (in methanol) nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride AlCl₃ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372, and 382 (5-OH and 7-OH free). The FT-IR v max KBr showed values at 3971-3400 (OH), 2941 (CH₃), 1701 and 1614 (C=C), 1022-613 (aromatic fingerprint). The DEPT-135 studies of compound III also supported the proposed structure. The proposed structure for compound III was elucidated as 4’-methoxy catechin-7-O-α-rhamnoside based on available spectral data [21]. In addition to the above arguments in favour of the catechin nucleus based rhamnosides, compound III showed a downfield shift of some B-ring protons indicating a methylation of the B-ring [26]. The signal at δ 3.82-3.90 was assignable to 4’-methoxy group [25,26]. For all these catechin-based flavonoids, the use of chemical shift reagents afforded further evidence that the 5-OH position of ring A was free for compound I, compound II and compound III. In compound I and compound III, the 7-OH of ring A was not free. The free 5-OH position was confirmed from the observation that addition of AlCl₃ (5 %) to the ethanol solution of the flavonoid glycosides caused a bathochromic shift in the UV absorption band [25]. In addition, a free 7-OH position is confirmed by the bathochromic shift caused by the introduction sodium acetate powder to an ethanolic solution of the flavonoid glycoside [25]. Although overwhelming evidence has been provided in this discuss supporting the proposed structures, further studies may be necessary to confirm an unequivocal proof of structure.

Spectral data of isolated compounds

Compound I was isolated as a yellow semi solid compound; yield (35mg). It showed positive reaction with FeCl₃, yellow colour deepens in alkaline solution an indication of a phenolic and or a flavonoidal compound.

CH₃OH

UV λ max nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride AlCl₃ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH in A-ring of the flavonoid nucleus. IR v max KBr 3971-3400 (OH), 2941 (C=H), 1701 and 1614 (C=C), 1022-613 (aromatic fingerprint).
The proposed structure for compound 1 was elucidated as catechin-7-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. DEPT-135 studies of compound 1 also supported the proposed structure. Compound 2 was isolated as a yellow semi solid compound; yield (115 mg). A positive reaction with FeCl₃, yellow colour which deepens in alkaline solution was observed and indicated phenolic and/or a flavonoidal compound.

CH₃OH

UV λ, nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride AlCl₃ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372 and 382 (5-OH and 7-OH free).

IR νmax,KBr (cm⁻¹): 3100-2700 (OH), 2927 (CH₃), 1701 and 1614 (C=C), 1022-613 (aromatic finger print).

'H-NMR (DMSO, 500 MHz): δ 2.45 (1H, dd, H4), δ 2.75 (1H, dd, H4), δ 1.25 (1H, d, H of a 7-O-rhamnoside), δ 6.60 (1H, dd, H-6), δ 6.80 (1H, dd, J=1.2-H8), δ 3.84 (H of 4-rhamnoside) δ 5.25 (1H, dd, H1 on anemic carbon of sugar) δ 3.92 (1H, dd-H2 of sugar), δ (3.70, 1H, dd, H3 of sugar), δ (6.70, 1H, d-H2'), δ 7.10 (1H, d, H5'), δ 7.25 (1H, H6'), δ 8.1 (1H, d, H3'-OH), δ 8.75 (1H, d,H4'-OH), δ 9.70 (1H, d, 5-OH). 13CNMR (DMSO, 500 MHz): δ 60.58 (C2), δ 60.32 (C3), δ 59.90 (C4', δ 115.38 (C6'), δ 110.60 (C6'), δ 39.73 (C7'), δ 39.27 (C6'). The proposed structure for compound 3 was elucidated as 4'-methoxy-catechin-7-O-α-rhamnoside based on available spectral data. DEPT-135 studies of compound 3 also supported the proposed structure.

Conclusion

The present study has demonstrated the in vitro antioxidant and immunostimulatory properties of three catechin rhamnosides obtained from the eastern Nigeria mistletoe. It has provided some logical evidence supporting the traditional uses of the eastern Nigeria mistletoe as an immunomodulants and antioxidant. Furthermore, this study suggests that, antioxidant; in addition to immunomodulation may be one of the mechanisms of action of mistletoe as an established antidiabetic agent.

Acknowledgements

The authors wish to thank Professor Dr. Peter Procksh, his sponsors and colleagues in Düsseldorf for their efforts and resources that led to the structural assignments of these compounds. Dr. CS Nworu is highly appreciated for performing the in vitro screening and FACS analysis on the compounds.

References

1. Cos P, Viletinck AJ, Vanden Berghe D, Maes L (2006) Anti-infective potential of natural products: How to develop a stronger in vivo ‘proof-of-concept’. J Ethnopharmacol 106: 290-302.
2. Clardy J, Walsh C (2004) Lessons from natural molecules. Nature 432: 829-837.
3. Nworu CS (2007) Evaluation of the immunomodulatory effects of their seed extracts of Garcia kola Heckel (Clusiaceae). A PhD thesis submitted to the University of Nigeria, Nsukka.
4. Halliwell B (2008) Reactive species and Antioxidants: Redox Biology is a fundamental theme of aerobic life. Plant Physiol 141: 312-322.
5. Sue C, Marc S, Gary Y, Karen N, Lynn M, et al. (2004) Pre-clinical study: Antioxidant and immunomodulatory effects of Wolfberry juice and other juice mixtures in mice. JAMA 37: 32-38.
6. Allam G (2009) Immunomodulatory effects of curcumin treatment on murine Schistosomiasis mansoni. Immunobiol 214: 712-727.
7. Guo L, Xie J, Ruan Y, Zhou L, Zhu H, et al. (2009) Characterization and immunostimulatory activity of a polysaccharide from the spores of Ganoderma lucidum. Int Immunopharmacol 9: 1175-1182.
8. Wang M, Guilbert Li, Li J, Wu Y, Pang P, et al. (2004) A proprietary extract from North American ginseng (Panax quinquefolium) enhances IL-2 and IFN-γ productions in murine spleen cells induced by Con-A. Int Immunopharmacol 4: 311-315.
9. Okonji CO, Schancy DJ, Iwu MM (1999) Challenges and issues involved in the standardization of Garcia kola formulations. Abstract of International Conference on Ethnomedicine and drug Discovery; Silver spring, Maryland, USA, Nov 3-5, Pp.29.
10. Osadebe PO, Okide GB, Akabogu IC (2004) Study on anti-diabetic activities of extracts of Garcinia kola (mistletoe) in hypertensive rats. Indian J Pharmacol 36: 287-293.
11. Ofem OE, Eno AE, Imoru J, Nikanu E, Unoh F, et al. (2007) Effect of crude aqueous leaf extract of Vaccum album (mistletoe) in hypertensive rats. Indian J Pharmacol 39: 15-19.
12. Osadebe PO, Omeje EO (2009) Comparatve acute toxicities and immunomodulatory potentials of five Eastern Nigeria mistletoes. J Ethnopharmacol 126: 287-293.
13. Omeje EO, Osadebe PO, Omeje CE (2008) Extracts of Eastern Nigeria mistletoe up regulates cellular and humoral immune response in mice. Recent progress in Medicinal Plants; Ethnomedicine; Source and Mechanism 1 27: 473-485.
14. Osadebe PO, Omeje EO (2009) Main immunomodulatory constituents of...
Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. Asian Pacific Journal of Tropical Medicine 2: 11-18.

15. Zhu QY, Hackman RM, Emsunsia JL, Holt RR, Keen CL (2002) Antioxidative activities of oolong tea. J Agric Food Chem 50: 6929-6934.

16. Lebeau J, Furman C, Bernier JL, Duriez P, Teissier E, et al. (2000) Antioxidant properties of di-tert-butyldihydroxylated flavonoids. Free Radic Biol Med 29: 900-912.

17. Ogechukwu OE, Ogoamaka OP, Sylvester NC, Kawamura A, Proksch P (2011) Immunomodulatory activity of a lupane triterpenoid ester isolated from the eastern Nigeria mistletoe, *Loranthus micranthus* (Linn). Asian Pac J Trop Med 4: 514-522.

18. Hamann J, Fiebig H, Strauss M (1993) Expression cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain. J Immunol 150: 4920–4927.

19. Borrego F, Robertson MJ, Ritz J, Peña J, Solana R (1999) CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor. Immunol 97:159-165.

20. Zou Y, Tan C, Zhu D (2009) A new acetylated flavonoid glycoside from *Myrsine africana* L. Bull Korean Chem Soc 30: 2111-2113.

21. Lin JH, Lin YT (1999) Flavonoids from the leaves of *Loranthus kaoi* (Chao) Kiu. Journal Food and Drug Analysis 7: 185-190.

22. Choi HJ, Kim JH, Lee CH, Ahn YJ, Song JH, et al. (2009) Antiviral activity of quercetin-7-rhamnoside against porcine epidemic diarrhea virus. Antiviral Res 81: 77-81.

23. Li Y, Zhang DM, Yu SS, Li JB, Luo YM (2006) A novel phenylpropanoid-substituted catechin glycoside and a new dihydrochalcones from *Sarcandra glabra*. Chinese Chemical Letters 17: 207-210.

24. Harborne JB (1994) The flavonoids advances in research since 1986. Chapman and Hall, New York.

25. Markham KR, Geiger N (1994) 1H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuteriodimethylsulfoxide. In: The Flavonoids advances in research since 1986, J.B. Harborne, ed. Chapman and Hall, New York, 441-473.

26. Harborne JB (1998) Phytochemical Methods: A guide to modern techniques of plant analysis, 3rd ed, Chapman and Hall, London.