

Antioxidant Activity of the Crude Extract of the Fruits of *Pycnanthus angolensis* and α -Glucosidase Inhibitory Activity of its Constituents

Alembert T. Tchinda^{a*}, Marguerite H. Tchuendem^b, Shamsun N. Khan^c, Iman Omar^c, François Ngandeu^d, Pepin, E.A. Nkeng^d, Iqbal M. Choudhary^c

^aCentre for Studies of Medicinal Plants and Traditional Medicine (CRPMT), Institute of Medical Research and Medicinal Plants Studies (IMPM), P.O. Box 6163, Yaounde, Cameroon

^bDepartment of Organic Chemistry, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon

^cH.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi 75270, Pakistan

^dDepartment of Chemistry, University of Dschang, P.O. Box 67, Dschang, Cameroon

Summary

Glyceryl-1,3-ditetradecanoate (**1**), sargaquinoic acid (**2**) and sargachromenol (**3**) have been isolated from the CH₂Cl₂-MeOH (1:1) extract of the fruits of *Pycnanthus angolensis*. The crude extract showed remarkable potency with 99.0 % Radical Scavenging Activity (RSA) in the nitric oxide scavenging assay compared to n-propyl gallate (90.3 % RSA) used as standard. Compounds **1-3** displayed significant α -glucosidase inhibitory activity with IC₅₀ 522.0, 3.0 and 4.6 μ M, respectively compared to the standards (deoxynojirimycin and acarbose). Sargaquinoic acid and sargachromenol were previously reported from the fruits of *P. angolensis*. However, this is the first report of glyceryl-1,3-ditetradecanoate from this plant species. The results obtained in the course of this work showed that extract of the fruits of *P. angolensis* may be used in the management of type-2 diabetes and related diseases. The structures of the compounds were determined by analysing their spectroscopic data.

Key words: *Pycnanthus angolensis*, antioxidant, α -glucosidase inhibition, diabetes.

*Corresponding author: E-mail: alembert2002@yahoo.fr; Tel: (237)77641965

Introduction

Oxidation in living organisms is essential for the acquirement of energy in catabolism. However, oxygen-centred free radicals and other reactive oxygen species, which are continuously, produced *in vivo* result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to ageing, and diseases such as atherosclerosis, diabetes, cancer and cirrhosis (1). Fruits contain a variety of scavenging molecules with antioxidant activity. Epidemiological studies have shown that regular consumption of these fruits may be useful in the prevention of these diseases (2). α -Glucosidase is an enteric enzyme attached to the brush border of the intestinal cells that breaks down complex starches, oligosaccharides and disaccharides to simple monosaccharides. This process increases postprandial hyperglycemia in patients with diabetes mellitus (3). Thus, inhibitors of that enzyme are of great interest in the management of diabetes mellitus. In a program aimed at discovering bioactive compounds in edible and medicinal fruits, CH₂Cl₂-MeOH (1:1) extract of the fruits of *Pycnanthus angolensis* (Well.) Warb (Myristicaceae) was studied. *P. angolensis* also known as "African nutmeg" is a tree of 35-40 m height growing in humid dense forests in tropical Africa. The oblong-shaped fruits, about 3.75 cm long, contain oil-rich seeds encased in a hard shell (4, 5). The seeds are aromatic and are used as soup condiment in many parts of Africa (6). The fat from the seeds are used as a mouthwash to cure thrush and as a topical treatment for fungal skin infections. The aril is used against hernia (6, 7). Previous compounds isolated from the seeds fat include the plastoquinones kombic acid, sargahydroquinoic acid, sargaquinoic acid and sargachromenol, high contents of tetradecanoic acid (60%) and (*Z*)-9-tetradecenoic acid (20%) (2,8,9). We herein report the isolation of glycerol-1,3-ditetradecanoate (**1**), sargaquinoic acid (**2**) and sargachromenol (**3**) from the fruits and the α -glucosidase inhibitory activity of compounds **1**, **2** and **3** as well as the antioxidant activity of the crude extract.

Methods

Plant materials

The fruits of *P. angolensis* were collected in March 2003 at Manjo (Littoral Province, Cameroon) and were identified by Mr Nana of the National Herbarium of Yaounde, Cameroon where a voucher specimen (No 2359/SRFK) was deposited.

Isolation of compounds

The air-dried and powdered fruits of *P. angolensis* (1.8 Kg) were ground and extracted with CH₂Cl₂-MeOH (1:1) (6 L x 2) to give 100 g of an orange fatty extract. Forty grams of this extract were applied to a silica gel chromatography column (CC). The column was eluted with increasing amounts of EtOAc in petroleum (pet.) ether. Fractions of 250 mL each were collected and similar fractions were combined based on their TLC profiles. From the fractions collected with pet. ether-EtOAc (19:1), glyceryl-1,3-ditetradecanoate (**1**, white amorphous solid, 20 g) crystallized and was filtered. Fractions collected with pet. ether-EtOAc (4:1) were subjected to repeated CC using a gradient of pet. ether-EtOAc to afford an oily mixture of two compounds which was further separated through a silica gel CC using the same solvent system. Hence, sargaquinoic acid (**2**, colorless oil, 18 mg) was obtained from fractions 5-14 while fractions 18-30 were purified through silica gel prep. TLC plates using pet. ether-EtOAc (4:1) as eluent to yield sargachromenol (**3**, colorless oil, 16 mg).

IR spectra were recorded on a Jasco 302-A spectrophotometer. UV spectra were obtained using a Hitachi UV 3200 spectrophotometer. EI and FAB MS (70 eV) were measured using a Varian MAT 312 A spectrometer. 1D and 2D spectra were recorded on Bruker AMX 300 and 400 MHz NMR spectrometers. The chemical shifts are given in ppm (δ), relative to TMS as internal standard, and coupling constants are in Hz. Column chromatography was carried out on silica gel (70-230 mesh, Merck). TLC and prep. TLC were performed on Merck precoated silica gel 60 F₂₅₄ aluminium foil and Merck silica gel 60 plates (0.5 mm layer) resp. and spots were detected using ceric sulphate as spray reagent. A Molecular Device spectrophotometer was used for measurement of enzyme inhibition.

α -Glucosidase inhibitory assay

The assay was performed according to the slightly modified method of Matsu *et al.* (10). α -Glucosidase (E.C.3.2.1.20) from *Saccharomyces* sp. was obtained from Wako Pure Chemical Industries Ltd. (Wako 076-0284). The enzyme inhibition studies were carried out spectrophotometrically in 96 well plates. The optimum pH 6.9 was maintained and the assay was carried out at 37° C. *p*-nitrophenyl α -D glucopyranoside (0.7 mM) as a synthetic substrate and 250 units/mL of enzyme, in 50 mM sodium phosphate buffer containing 100 mM NaCl were used. 1-Deoxyjirimycin (0.425 mM) and acarbose (0.78 mM) were used as positive controls. The increment in the absorption at 400 nm due to the hydrolysis of PNP-G by α -glucosidase was monitored continuously with a spectrophotometer (Molecular Devices, USA). The results are expressed with the Standard Error of the Mean (SEM) equals to standard deviation $\pm\sqrt{n}$ where n represents the number of replicates for IC₅₀ value. Three replicates were performed for each compound.

Antioxidant assay: Nitric oxide scavenging assay

Modified Griess Illosvoy reaction (11) involving naphthylethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine was used. The reaction mixture containing 10 μ L sample, 20 μ L potassium phosphate buffer (0.1 mM, pH 7.4), and 70 μ L sodium nitroprusside (10 mM) were incubated at 25° C for 90-100 min. Fifty microliters sulfanilic acid (0.33% in 20% glacial acetic acid) was added to the above mixture followed by 50 μ L naphthylethylenediamine dihydrochloride (0.1%) with shaking. Absorbance was recorded at 540 nm against the responding blank solution in microtiter plate by ELISA reader. A pink chromophore was then formed in diffused light. The change in absorbance or O.D. was studied in terms of %RSA (Radical scavenging Activity) using the formula $\%RSA = \{ O.D. (test) / O.D. (blank) \times 100 \} - 100$ (O.D. = Optical Density or absorbance). %RSA depends upon the concentration of the inhibitor.

Results

From the CH₂Cl₂-MeOH (1:1) extract of the fruits of *P. angolensis*, three compounds were isolated and identified by analysing of their 1D and 2D NMR and mass spectra. The NMR data of compounds **1** and **2** were identical to those previously reported (12, 13).

Structure **1** was assigned to glycerol-1,3-ditetradecanoate obtained as amorphous white solid; $[\alpha]_D^{26}$ 0 (*c* 0.08, CHCl₃); UV (CHCl₃) blank; IR (KBr) 3444, 2916, 2850, 1735, 1473, 1415, 1392, 1272, 1253, 1180, 1114, 1060; FAB MS *m/z* 513 [M+H]⁺; EI MS *m/z* (rel. int) 496 (3), 495 [M-OH]⁺ (9), 494 [M-H₂O]⁺ (3), 468 (3), 467 [M-(H₂O+C₂H₅)]⁺ (9), 466 (3), 339 [M-(H₂O+CH₃(CH₂)₉CH₂)]⁺ (5), 326 (3), 311 (3), 285 [M-CH₃(CH₂)₁₂COO]⁺ (11), 257 [M-(CH₃(CH₂)₁₂+C₂H₆)]⁺ (5), 211 [CH₃(CH₂)₁₂CO]⁺ (38), 183 [CH₃(CH₂)₁₁CH₂]⁺ (24), 171 (4), 158 (2), 129 (4), 113 (3), 112 (9), 99 (5), 98 (27), 97 (14), 85 (25), 84 (21), 83 (19), 71 (43), 70 (8), 69 (27), 57 [CH₃CH₂CH₂CH₂]⁺ (100); ¹H and ¹³C NMR data: see Table 1.

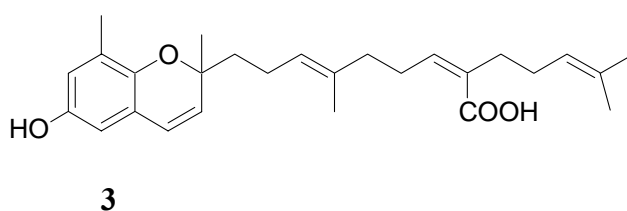
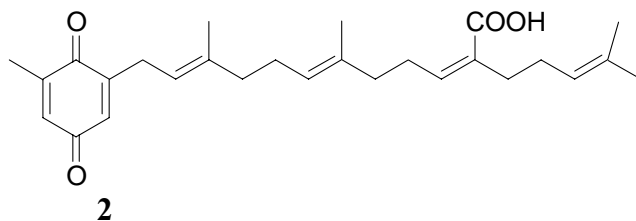
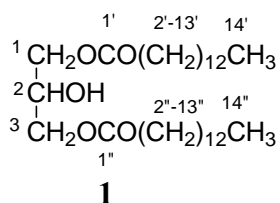


Table 1: NMR data of compound 1 in CDCl₃

Carbon no.	δ_C	DEPT 135	δ_H (mult.), J (Hz)	HMBC ^a correlations
1/3	62.1	t	4.26 dd (11.8, 4.2) 4.10 dd (11.8, 6.0)	1'/1'', 2
2	68.8	d	5.23 m	1/3
1'/1''	173.2	s		
2'/2''	34.0	t	2.27 t (7.4)	1'/1'', 3'/3'', 4'/4''
3'/3''	24.8	t	1.58 m	1'/1'', 2'/2'', 4'-12'/4''-12''
4'-12'/4''-12''	29.6-29.0	11 t	1.24-1.20 ov	3'/3'', 13'/13'', 14'/14''
13'/13''	22.6	t	1.21 ov	4'-12'/4''-12'', 14'/14''
14'/14''	14.0	q	0.84 t (6.4)	4'-12'/4''-12'', 13'/13''

^aThe protons 4'-12'/4''-12'' form an overlapping spot which shows cross-peaks with C-2'/C-2'', C-3'/C-3'', C-13'/C-13'' and C-14'/C-14''.

Compounds **1**, **2** and **3** showed significant α -glucosidase inhibitory activity with IC_{50} 522 ± 12 , 3 ± 0.123 and 4.6 ± 0.123 μ M respectively compared to the standards deoxynojirimycin (IC_{50} 425 ± 8.14 μ M), a potent α -glucosidase inhibitor and acarbose (IC_{50} 780 ± 28 μ M), a popular clinically used drug for type-2 diabetes.

At a concentration of 1 mg/ml, the crude extract of *P. angolensis* showed 99.0% Radical Scavenging Activity (%RSA) in the nitric oxide scavenging assay compared to n-propyl gallate (90.3% RSA) used as standard.

Discussion

Compound **1** was obtained as a white amorphous solid. The FAB MS spectrum displayed the pseudo-molecular ion peak at m/z 513 $[M+H]^+$ in agreement with the proposed molecular formula C₃₁H₆₀O₅. The IR spectrum showed absorption bands for hydroxyl (-OH) and carbonyl (C=O) groups at 3444 and 1735 cm^{-1} , respectively. The NMR data were typical of those of a glycerol ester (14). The symmetrical glycerol moiety was characterized by the chemical shifts at δ_H 5.23 (1H, m, H-2), 4.26 (2H, dd, J = 11.8, 4.2 Hz, H-

1A/H-3A) and 4.10 (2H, dd, $J = 11.8, 6.0$ Hz, H-1B/H-2B). The presence of a glycerol moiety in the molecule was further confirmed by the ^1H - ^1H COSY cross-peaks between H-2 and 2H-1/3. The DEPT NMR spectra of **1** showed signals for a fatty acid moiety [δ 14.0 (q), 22.6 (t), 29.0-29.6 (11 t), 24.8 (t), 34.0 (t), 173.2 (s)]. The HMBC spectrum (Table 1) showed unambiguously correlations between 2H-1/3, 2H-2'/2'' (δ 2.24, t, $J = 7.4$ Hz), 2H-3'/3'' (δ_{H} 1.58, m) and the ester carbonyl (δ_{C} 173.2). These correlations indicated that the fatty acid moieties are attached at C-1 and C-3. Tetradecanoic acid was suggested to be the fatty acid moiety from the analysis of the mass spectrum which showed prominent fragments at m/z 183 [$\text{CH}_3(\text{CH}_2)_{11}\text{CH}_2$] $^+$, 211 [$\text{CH}_3(\text{CH}_2)_{11}\text{CH}_2\text{CO}$] $^+$ and 285 [$\text{M}-\text{CH}_3(\text{CH}_2)_{11}\text{CH}_2\text{COO}$] $^+$. The previous report of high contents of tetradecanoic acid in the seed fat of *P. angolensis* (8) further supports the assignment of tetradecanoyl as the acidic moiety. Thus, the structure of compound **1** was established as glyceryl-1,3-ditetradecanoate (15).

Diacylglycerols (DAGs) such as compound **1** are abundant in various edible oils. When consumed in large amounts, they have metabolic characteristics that may be beneficial in preventing and managing obesity. Experimental studies in animals and humans showed that DAGs, mainly 1,3-diacylglycerols, decrease postprandial triglyceridemia. In addition consumption of DAGs results in an increase of serum concentrations of high density lipoprotein (HDL) cholesterol in patients with type-2 diabetes (16, 17). Moreover, the α -glucosidase inhibitors are used in the composition of anti-obesity drugs. Due to the significant α -glucosidase inhibitory activity of compound **1**, it may possess beneficial health effects in obese patients and those with type-2 diabetes. Compounds **1-3** are more potent than the standards deoxynojirimycin and acarbose. The activities of sargaquinoic acid (3 ± 0.123 μM) and sargachromenol (4.60 ± 0.123 μM) are close. The two compounds may bind on the active site of the enzyme by their lateral chains which are almost identical. Compound **1** is more than 100 times less active than compounds **2** and **3**. The high difference can be explained by the difference in their chemical structures and accordingly different action mechanisms.

The crude CH_2Cl_2 -MeOH (1:1) extract of the fruits of *P. angolensis* has shown 99.0% nitric oxide Radical Scavenging Assay activity. A number of studies have shown that free radicals, so called "reactive oxygen species (ROS)" are implicated in certain chronic and ageing diseases, including malaria, rheumatoid arthritis, cataracts acquired

immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer and neurodegenerative diseases (Parkinson's and Alzheimer's diseases) (18). Among ROS, superoxide plays an important role in the development and complications of diabetes. A sample that inhibits the production of those ROS can be helpful in the management of diabetes (5).

The study on plastoquinones as a source of new therapies is increasing. Sargaquinoic acid (**2**) has been found to be an effective and selective inhibitor of butyrylcholinesterase (BuChE), a new target for the treatment of Alzheimer's disease (AD) BuChE making it an interesting potential drug candidate for Alzheimer's disease (19). The anti-inflammatory and antioxidant activities as measured by the DPPH radical scavenging assay of sargachromenol (**3**) obtained from a different source exceeded that of α -tocopherol. Simon *et al.* (5) showed that the seed fat of *P. angolensis* (kombo butter), sargaquinoic acid and sargachromenol possess significant antioxidant activity as evidenced by their radical scavenging activities in both the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assays and also anti-inflammatory activity attested by their inhibitory effect on nitric oxide production in lipopolysaccharide-induced RAW 264.7 murine macrophage cells.

Results obtained during this work and previous studies on DAGs, plastoquinones and kombo butter showed that the extract of the fruits of *P. angolensis* may be effective in the management of diabetes, cardiovascular and related diseases. However, *in vivo* pharmacological studies need to be carried out on the fruits collected in Cameroon for their possible use as an alternative therapy against the above mentioned diseases.

Acknowledgements

We are grateful to TWAS (Academy of Sciences for the Developing World), Trieste, Italy and UNESCO, for the award of the 'TWAS-UNESCO Associateship Scheme at Centres of Excellence in the South' fellowship to T.T.A. at the H.E.J. Research Institute of Chemistry, ICCBS, University of Karachi, Pakistan. Part of this work was supported by the TWAS research grant No 07-145 RG/CHE/AF/AC.

References

1. Halliwell B., Gutteridge JMC. 1999. The definition and measurement of antioxidant in biological systems. *Free Radic Biol Med* 1999; 18: 125-126.
2. Masoko P, Eloff, JN. Screening of twenty-four South African Combretum and six Terminalia species (Combretaceae) for antioxidant activities. *Afr J Trad CAM* 2007; 4: 231-239.
3. Ortiz-Andrade RR, García-Jiménez S, Castillo-España P, Ramírez-Ávila G, Villalobos-Molina R, Estrada-Soto S. α -Glucosidase inhibitory activity of the methanolic extract from *Tournefortia hartwegiana*: An anti-hyperglycemic agent. *J Ethnopharmacol* 2007; 109, 48–53.
4. Adjanohoum JE, Aboubakar N, Dramane K et al. Traditional Medicine and Pharmacopeia. Contribution to Ethnobotanical and Floristic Studies in Cameroon. OAU/STRC, OAU. 1996: 309.
5. Simon JE, Wang M, Gbewonyo K, Rafi MM, Acquaye DF, Asianowa Y. United States Patent Application 20050003030. 2005: 23.
6. Iwu M.M. Handbook of African Medicinal Plants. CRC Press, Florida, US. 1993: 225.
7. Abondo A, Mbenkum F, Thomas D. Ethnobotany and the medicinal plants of the Korup rainforest project area. *Ethnobotany and Medicinal Plants. Proceedings of International Conference on Traditional Medicinal Plants. Arusha, Tanzania. February 18-23, 1990: 112-124.*
8. Lok CM, Groenewegen A, Stroink JBA, Ward JP. Kombic acid, a hydroquinone polyisoprenoic acid from *Pycnanthus kombo* seed fat. *Phytochemistry* 1983; 22: 1973-1976.
9. Kusumi, T., Shibata, Y., Kinoshita, T., Kakisawa, H. Structures of new plastoquinones from the alga *Sargassum terratifolium*. *Chem Lett* 1979: 277-278.
10. Matsu T, Yoshimoto C, Osajima K, Oki T, Osajima Y. In vitro survey of α -glucosidase inhibitory food components. *Biostat Biotech Biochem* 1996; 60: 2019-2022.
11. Badami S, Gupta MK, Suresh, B. Antioxidant activity of the ethanolic extract of *Striga ovabanchioides*. *J Ethnopharmacol* 2003; 85: 227-230.
12. Numata A, Kanbara S, Takahashi C, Fujiki R, Yoneda M, Usami Y, Fujita E. A cytotoxic principle of the brown alga *Sargassum tortile* and structures of chromenes. *Phytochemistry* 1992; 31: 1209-1213.

13. Segawa M, Shirahama H. New plastoquinones from the brown alga *Sargassum sagamianum* var. *yezoense*. *Chem Lett* 1987; 1365-1366.
14. Qi S-H, Zhang S, Huang J-S, Xiao Z-H, Wu J, Long L-J. Glycerol derivatives and sterols from *Sargassum parvivesiculosum*. *Chem Pharm Bull* 2004; 52: 986-988.
15. Dictionary of Natural Products (DNP) on CD-ROM. Version 16.1. Chapman & Hall, London, UK. 2007.
16. Yamamoto K, Takeshita M, Tokimitsu I, Watanabe H, Mizuno T, Asakawa H, Tokunaga K, Tatsumi T, Okazaki M, Yagi N. Diacylglycerol ingestion in type-2 diabetic patients with hypertriglyceridemia. *Nutrition* 2006; 22: 23-29.
17. Maki KC, Davidson MH, Tsushima R, Matsuo N, Tokimitsu I, Umponowicz DM, Dicklin MR, Foster GS, Ingram KA, Anderson BD, Frost SD, Bell M. Consumption of diacylglycerol oil as part of a reduced-energy diet enhances loss of body weight and fat in comparison with consumption of a triacylglycerol control oil. *Amer J Clin Nutr* 2002; 76: 1230-1236.
18. Olukemi OA, Olukemi IO, Oluwatoyin SM, Austin AO, Mansurat LB, Olufunmilola TI. Antioxidant activity of Nigerian dietary spices. *Electron J Environ Agric Food Chem* 2005; 4: 1086-1093.
19. Choi BW, Ryu G, Park SH, Kim ES, Shin J, Roh SS, Shin HC, Lee BH. Anticholinesterase activity of plastoquinones from *Sargassum sagamianum*: lead compounds for Alzheimer's disease therapy. *Phytother Res* 2007; 21: 423-426.