African Journal of Biotechnology Vol. 7 (11), pp. 1727-1730, 3 June, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.194
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Steroids isolated from *Millettia versicolor* Baker (Fabaceae)

Ongoka, P. R.[1,2*], Banzouzi, J. T.[3,4], Poupat, C.[3], Ekouya, A.[2], Ouamba, J. M.[2] and Moudachirou, M.[5]

[1]Département des Sciences Exactes, Ecole Normale Supérieure, Université Marien Ngouabi, BP 69, Brazzaville – Congo.
[2]Unité de Chimie du Végétal et de la Vie, Faculté des Sciences, Université Marien Ngouabi, BP 69, Brazzaville – Congo.
[3]Institut de Chimie des Substances Naturelles (CNRS), 1, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex – France.
[4]Centre d'Etudes et de Recherches Médecins d'Afrique (CERMA), BP 45, Brazzaville – Congo.
[5]Université d'Abomey Calavi, 01 BP 526, Cotonou – Bénin.

Accepted 9 April, 2008

The objective of this investigation was to isolate and determine the chemical constituents of the leaves of *Millettia versicolor* Baker, a medicinal plant used in the traditional pharmacopoeias of Central Africa, essentially for its pain-relieving and anti-parasitic properties. A methanol extract of the leaves was made. The chemical compounds isolated were analyzed by HPLC/MS and GC/MS. The structures were elucidated on the basis of spectral studies (IR, RMN ^{1}H, ^{13}C) and confirmed by comparison with published data. Seven known compounds (two sterols, one stanol and four triterpene alcohols) were determined, the major compound being stigmasterol. Except lupeol, previously isolated from *M. versicolor* aerial parts, these compounds are isolated from this plant for the first time. Their presence supports the pain-relieving use of the plants, since 5 of the 7 compounds have reported anti-inflammatory activity, and 2 of these 5 had also an anti-nociceptive action.

Key words: Medicinal plant, *Millettia versicolor*, anti-inflammatory, anti-nociceptive, phytosterols.

INTRODUCTION

Millettia versicolor Baker (Fabaceae) is a medicinal plant used in African traditional medicine (Angola Congo, D.R. Congo and Gabon) to relieve pain and cure parasitosis. An aqueous decoction of stem bark is employed in Congo for intestinal parasitoses, kidney pains, cough, female sterility, senile impotence of men. An infusion is used in DR Congo to rub the syphilitic wounds. The aqueous decoction of leaves is taken against feverish rheumatisms, headache, kidney pains, intestinal parasitoses, and cough (Congo). It is also used in bath against syphilis (Gabon). The trunk bark has anthelminthic applications (Angola, Congo, and Gabon) (Bouquet, 1969; Adjanohoun et al., 1988).

Pharmacological studies confirmed the anthelminthic potential of the plant roots and leaves (Kasonia et al., 1989; Ongoka et al., 2004) but the active compounds responsible for this activity have not yet been determined. The stem bark has reported anti-inflammatory properties, attributed to a furoquinone (Fotsing et al., 2003). From the leaves, which are the major plant part used for relieving pain, only lupeol had yet been isolated (Ekouya et al., 1990). However, our preliminary chemical screening (Ongoka et al., 2004) indicated the presence of numerous secondary metabolites in the aqueous and alcoholic fractions: flavonoids, tanins, polyphenols, saponins, terpenes and steroids. The present study aims to separate and identify the chemical constituents of the leaves of *M. versicolor*, to try and support its traditional use.

MATERIAL AND METHODS

Plant material

M. versicolor leaves were collected from Mossaka area in the Cuvette region (North of Republic of Congo) in 2004. Botanical identification was confirmed by the Department of Plant Biology and

*Corresponding author. E-mail: ongokapascal@yahoo.fr.

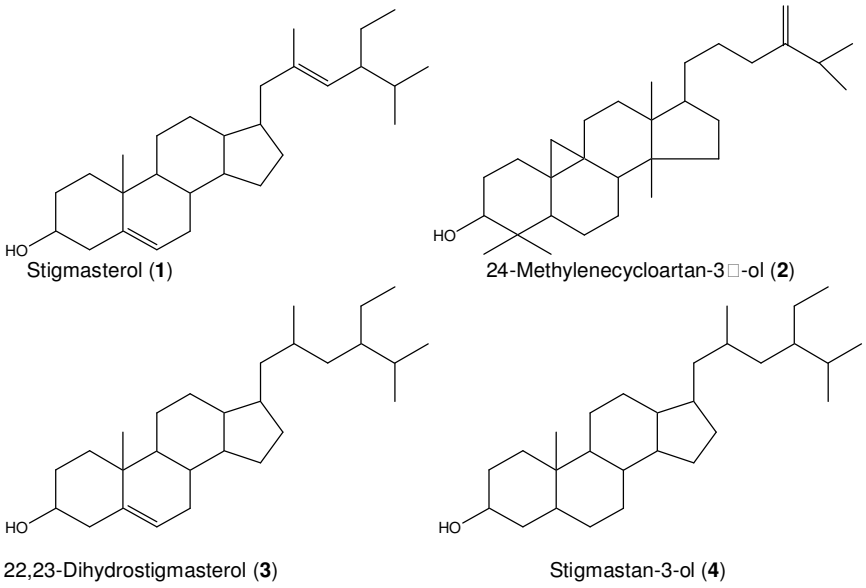

Figure 1. Structures of the compounds isolated from the leaves of *Millettia versicolor.*

Physiology of Université Marien Ngouabi (Brazzaville, Congo) where a voucher specimen has been deposited under number OP2004-1. The leaves were dried under shed then ground to powder prior to extraction.

Preparation of the extracts

70 g of dried leaves powder were extracted with methanol (soxhlet). The solvent was evaporated *in vacuo* and yielded 10 g of crude extract.

Isolation and determination of the steroids and triterpenes

5 g of the crude extract were separated on Sephadex LH20 column (200 g, methanol) and 5 fractions were obtained. Fraction 3 was chromatographed on silica gel column (200-300 mesh) with the following solvent systems: heptane-EtOAc (50:50), EtOAc (100) and EtOAc-MeOH (80:20), then by preparative silica TLC (Merck). Isolated compounds were analyzed by HPLC/MS (Symmetry column (C18), isocratic: H_2O-ACN (60:40), flow rate 1 ml/min) and GC/MS (GC TRACE Thermo 2000, solvent: CH_2Cl_2; Supelco Equity 5 silica capillary column 28089-U (30 m x 0.25 mm x 0.25 □m), carrier gas: helium, at a flow rate of 2 ml/min, column held initially at 160°C for 2 min and then increased to 280°C with a 5°C/min heating ramp, injection performed in split mode (50:1) at 280°C) to assess their purity. The chemical structures were elucidated on the basis of IR, RMN ^1H, ^{13}C spectral studies and confirmed by comparison with published data.

RESULTS AND DISCUSSION

The spectral analysis enabled us to identify 4 known compounds: 2 phytosterols: stigmasterol (1), 24-methylenecycloartan-3β-ol (2), 22,23-dihydrostigmasterol (3) and a phytostanol, stigmastan-3-ol (4) (Figure 1). Three triterpenes were also identified: lupeol, taraxasterol and β-amyrin. We have isolated these triterpenes from extracts of *M. versicolor* in previous studies (Ekouya et al., 1990; Alphonse et al., 2006).

Compound 1 is the major composite of the leaves methanol extract. The structure of these compounds was confirmed by comparison with published data (Toshihiro et al., 1988; Kojima et al., 1990) and by the use of authentic samples. With the exception of lupeol, all these compounds are isolated from *M. versicolor* for the first time and compounds 1, 2, 3, 4, had not yet been isolated in the *Milletia* genus, though all are already known from the *Fabaceae* family.

Pain-relieving activity is well supported by our study, since 5 of the 7 isolated compounds have a reported anti-inflammatory effect (stigmasterol) (Garcia et al., 1999; Gomez et al., 1999), (lupeol) (Akihisa et al., 1996; Della et al., 1994; Ramirez et al., 2004; Fernandez et al., 2001; Fernandez et al., 2001; Geetha and Varalakshmi, 2001), (24-methylene-cycloartan-3β-ol) (Akihisa et al., 1996; Yasukawa et al., 1998), (taraxasterol and β-amyrin) (Akihisa et al., 1996; Della et al., 1994) and 2 of these have also antinociceptive activity (3□-amyrin) (Lima et al., 2006; Oliveira et al., 2005; Otuki et al., 2005), (stigmasterol) (Santos et al., 1995). Lupeol and β-amyrin both have a hepatoprotective effect (Preetha et al., 2006; Sunitha et al., 2001; Oliveira et al., 2005) and lupeol also has a nephroprotective effect (Nagaraj, 2000). None of the 7 determined compounds had a reported anthelmintic activity.

Some biological activities that do not appear in traditional medicine are also reported, such as chemoprevention (taraxasterol) (Yasukawa et al., 1996; Takasaki et al., 1999; Ovesna et al., 2004), lupeol (Sultana et al., 2003; Saleem et al., 2004; Saleem et al., 2003; Hata et

al.,2000; Miles and Kokpol, 1976), stigmasterol (Awad and Fink, 2000; De Stefani et al., 2000), anti-hypercholesterolemia (stigmastan-3-ol) (Plat and Mensink, 2000; Plat and Mensink, 2001; Ramjiganesh et al., 2001; Jones et al., 1999) and 24-methylenecycloartan-3□-ol (Kiribuchi et al., 1983), and anti-malaria (lupeol) (Ziegler et al., 2004; Ziegler et al., 2002; Alves et al., 1997). It would be therefore interesting to test *M. versicolor* for hypocholesterolemic and chemopreventive activity. Antimalarial assay has already been done but revealed only a moderate activity against *Plasmodium falciparum in vitro* (Mbatchi et al., 2006).

Conclusion

This study permitted the isolation of seven known compounds from the methanolic extract of the leaves of *M. versicolor* Bak, six of them being new for the species. A majority of them has reported analgesic or anti-inflammatory activities, which support the traditional use of the plant for pain relief. Some of the compounds have other interesting biological effects, for which the leaves of *M. versicolor* could be investigated.

ACKNOWLEDGEMENTS

We thank ICSN and the French Embassy in Congo for their financial support of our study. Thanks also to Ms Aline Prost of CERMA for her help in the writing and in the translation of our paper.

REFERENCES

Bouquet A (1969). Féticheurs et médicine traditionnelle du Congo Brazzaville. Mémoires ORSTOM, Paris. 282 p.

Adjanohoun EJ, Ahyi AMR, Ake Assi L, Moutsambote JM, Mpati J, Doulou V, Baniakina J (1988). Médecine traditionnelle et pharmacopée: contribution aux études ethnobotaniques et floristiques en République Populaire du Congo. Rapport ACCT, Paris., 605.

Kasonia K, Kaba S, Kirikughundi N, Essai du Zengaver (1989). (décocté des racines de *Millettia versicolor* Welw.) sur les verminoses des animaux domestiques. Bull. Méd. Trad. Pharm. 3(2) : 199-202.

Ongoka PR, Ekouya A, Diatewa M, Bakoumasse-Ngamba G, et Atti R (2004). Etude chimique des plantes médicinales: cas des plantes anthelminthiques du Congo Brazzaville. Rev. Méd. Pharm. Afr. 18: 161-167.

Fotsing MT, Yankep E, Njamen D, Fomum ZT, Nyasse B, Bodo B, Recio MC, Giner RM, Rios JL (2003). Identification of an anti-inflammatory principle from the stem bark of *Millettia versicolor.*, Planta Med. 69(8): 767-770.

Ekouya A, Tchissambou L, Onanga M, Ouabonzi A, Ongoka P, Bayitoukou A (1990). *Millettia versicolor* : Etude chimique et pharmacologique. Discov. Innovat. 2(2): 45-47.

Toshihiro A, Parthasarathi G, Swapnadip T, Satoshi O, Toshitake T, Taro M (1988). 24-methylcholesta-5, 22E, 25-trien-3-ol and 24-ethyl-5-cholest-22E-en-3-ol from *Clerodendrum fragrans*. Phytochemistry 27: 241-244.

Kojima H, Sato N, Hatano A, Ogura H (1990). Sterol glucosides from *Prunella vulgaris*. Phytochemistry 29(7): 2351-2355.

Garcia MD, Saenz MT, Gomez MA, Fernandez MA (1999). Topical anti-inflammatory activity of phytosterols isolated from *Eryngium foetidum* on chronic and acute inflammation models. Phytother. Res. 13(1): 78-80.

Gomez MA, Saenz MT, Garcia MD, Fernandez MA (1999). Study of the topical anti-inflammatory activity of *Achillea ageratum* on chronic and acute inflammation models. Z Naturforsch [C]. 54(11): 937-941.

Akihisa T, Yasukawa K, Oinuma H, Kasahara Y, Yamanouchi S, Takido M, Kumaki K, Tamura T (1996). Triterpene alcohols from the flowers of Compositae and their anti-inflammatory effects. Phytochemistry, 43(6): 1255-1260.

Della Loggia R, Tubaro A, Sosa S, Becker H, Saar S, Isaac O (1994). The role of triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* flowers. Planta Med. 60(6): 516-520.

Ramirez Apan AA, Perez-Castorena AL, de Vivar AR (2004). Anti-inflammatory constituents of *Mortonia greggii* Gray. Z Naturforsch [C]. 59(3-4): 237-243.

Fernandez MA, de las Heras B, Garcia MD, Saenz MT, Villar A (2001). New insights into the mechanism of action of the anti-inflammatory triterpene lupeol. J. Pharm. Pharmacol. 53(11): 1533-1539.

Fernandez A, Alvarez A, Garcia MD, Saenz MT (2001). Anti-inflammatory effect of *Pimenta racemosa* var. ozua and isolation of the triterpene lupeol. Farmaco 56(4): 335-338.

Geetha T, Varalakshmi P (2001). Anti-inflammatory activity of lupeol and lupeol linoleate in rats. J. Ethnopharmacol. 76(1): 77-80.

Yasukawa K, Akihisa T, Kimura Y, Tamura T, Takido M (1998). Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumor promotion in two-stage carcinogenesis in mouse skin. Biol. Pharm. Bull. 21(10): 1072-1076.

Lima-Junior RC, Oliveira FA, Gurgel LA, Cavalcante IJ, Santos KA, Campos DA, Vale CA, Silva RM, Chaves MH, Rao VS, Santos FA (2006). Attenuation of visceral nociception by alpha- and beta-amyrin, a triterpenoid mixture isolated from the resin of *Protium heptaphyllum*, in mice. Planta Med. 72(1): 34-39.

Oliveira FA, Costa CL, Chaves MH, Almeida FR, Cavalcante IJ, Lima AF, Lima RC, Silva RM, Campos AR, Santos FA, Rao VS (2005). Attenuation of capsaicin-induced acute and visceral nociceptive pain by alpha-and beta-amyrin, a triterpene mixture isolated from *Protium heptaphyllum* resin in mice. Life Sci. 77(23): 2942-2952.

Otuki MF, Ferreira J, Lima FV, Meyre-Silva C, Malheiros A, Muller LA, Cani GS, Santos AR, Yunes RA, Calixto JB (2005). Antinociceptive properties of mixture of alpha-amyrin and beta-amyrin triterpenes: evidence for participation of protein kinase C and protein kinase A pathways. J. Pharmacol Exp Ther. 313(1): 310-318.

Santos AR, Niero R, Filho VC, Yunes RA, Pizzolatti MG, Delle Monache F, Calixto JB (1995). Antinociceptive properties of steroids isolated from *Phyllanthus corcovadensis* in mice. Planta Med. 61(4): 329-332.

Preetha SP, Kanniappan M, Selvakumar E, Nagaraj M, Varalakshmi P (2006). Lupeol ameliorates aflatoxin B(1)-induced peroxidative hepatic damage in rats. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. [Epub ahead of print]

Sunitha S, Nagaraj M, Varalakshmi P (2001) Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. Fitoterapia 72(5): 516-523.

Oliveira FA, Chaves MH, Almeida FR, Lima RC Silva RM, Maia JL, Brito GA, Santos FA, Rao VS (2005). Protective effect of alpha- and beta-amyrin, a triterpene mixture from *Protium heptaphyllum* (Aubl.) March. trunk wood resin, against acetaminophen-induced liver injury in mice. J. Ethnopharmacol. 98(1-2): 103-108.

Nagaraj M, Sunitha S, Varalakshmi P (2000). Effect of lupeol, a pentacyclic triterpene, on the lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure. J. Appl. Toxicol. 20(5): 413-417.

Yasukawa K, Akihisa T, Oinuma H, Kaminaga T, Kanno H, Kasahara Y, Tamura T, Kumaki K, Yamanouchi S, Takido M (1996). Inhibitory effect of taraxastane-type triterpenes on tumor promotion by 12-O-tetradecenoylphorbol-13-acetate in two-stage carcinogeneis in mouse skin. Oncology, 53(4): 341-343.

Takasaki M, Konoshima T, Tokuda H, Masuda K, Arai Y, Shiojina K, Ageta H (1999). Anti-carcinogenic activity of Taraxacum plant. II. Biol. Pharm. Bull. 22(6): 606-610.

Ovesna Z, Vachalkova A, Horvathova K (2004). Taraxasterol and beta-sitosterol: new naturally compounds with chemoprotective/ chemopreventive effects. Neoplasma 51(6): 407-414.

Sultana S, Saleem M, Sharma S, Khan N (2003). Lupeol, a triterpene, prevents free radical mediated macromolecular damage and allevia-

tes benzoyl peroxide induced biochemical alterations in murine skin. Ind. J. Exp. Biol. 41(8): 827-831.
Saleem M, Afaq F, Adhami VM, Mukhtar H (2004). Lupeol modulates NF-kappaB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. Oncogene 23(30): 5203-5214.
Hata K, Ishikawa K, Hori K, Konishi T (2000). Differentiation-inducing activity of lupeol, a lupane-type triterpene from Chinese dandelion root (Hokouei-kon), on a mouse melanoma cell line. Biol. Pharm. Bull. 23(8): 962-967.
Miles DH, Kokpol U (1976). Tumor inhibitors II: constituents and antitumor activity of *Sarracenia flava*. J. Pharm. Sci. 65(2): 284-285.
Awad AB, Fink CS (2000). Phytosterols as anticancer dietary components: evidence and mechanism of action. J. Nutr. 130(9): 2127-2130.
De Stefani E, Boffetta P, Ronco AL, Brennan P, Deneo-Pellegrini H, Carzoglio JC, Mendilaharsu M (2000). Plant sterols and risk of stomach cancer: a case-control study in Uruguay. Nutr. Cancer. 37(2): 140-144.
Plat J, Mensink RP (2002). Increased intestinal ABCA1 expression contributes to the decrease in cholesterol absorption after plant stanol consumption. FASEB J. 16(10): 1248-1253.
Plat J, Mensink RP (2001). Effects of plant sterols and stanols on lipid metabolism and cardiovascular risk. Nutr. Metab. Cardiovasc. Dis. 11(1): 31-40.
Ramjiganesh T, Roy S, McIntyre JC, Luz Fernandez M (2001). The hypocholesterolaemic effects of sitostanol in the guinea pig are in part related to changes in hepatic lipids and lipoprotein composition. Br. J. Nutr. 85(2): 165-172.
Jones PJ, Ntanios FY, Raeini-Sarjaz M, Vanstone CA (1999). Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. Am. J. Clin. Nutr. 69(6) : 1144-1150.
Kiribuchi M, Miura K, Tokuda S, Kaneda T (1983). Hypocholesterolemic effect of triterpene alcohols with soysterol on plasma cholesterol in rats. J. Nutr. Sci. Vitaminol 29(1): 35-43.
Ziegler HL, Franzyk H, Sairafianpour M, Tabatabai M, Tehrani MD, Bagherzadeh K, Hagerstrand H, Staerk D, Jaroszewski JW (2004). Erythrocyte membrane modifying agents and the inhibition of *Plasmodium falciparum* growth: structure-activity relationships for betulinic acid analogues. Bioorg. Med. Chem. 12(1): 119-127.
Ziegler HL, Staerk D, Christensen J, Hviid L, Hagerstrand H, Jaroszewski JW (2002). *In vitro Plasmodium falciparum* drug sensitivity assay: inhibition of parasite growth by incorporation of stomatocytogenic amphiphiles into the erythrocyte membrane. Antimicrob. Agents Chemother. 46(5): 1441-1446.
Alves TM, Nagem TJ, de Carvalho LH, Krettli AU, Zani CL (1997). Antiplasmodial triterpene from *Vernonia brasiliana*. Planta Med. 63(6): 554-555.
Mbatchi SF, Mbatchi B, Banzouzi JT, Bansimba T, Nsonde Ntandou GF, Ouamba JM, Berry A, Benoit-Vical F (2006). *In vitro* antiplasmodial activity of 18 plants used in Congo Brazzaville traditional medicine. J. Ethnopharmacol. 104(1-2): 168-174.
Alphonse Ekouya, Ongoka PR, Bedel GI, Antoine O, Jean M, Ouamba MD, Ange AA, (2006). Isolement de trois triterpènes de *Milletia versicolor* Baker. J. Soc. Afr. Chim. 21: 73-76.