ISSN 1996-3351

Asian Journal of Biological Sciences

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Asian Journal of Biological Sciences 2 (4): 112-117, 2009
ISSN 1996-3351

Isolation of Stigmasterol and γ-Sitosterol from Petroleum Ether Extract of Woody Stem of *Abelmoschus manihot*

P.S. Jain, S.B. Bari and S.J. Surana
Department of Pharmaceutical Chemistry,
R.C. Patel Institute of Pharmaceutical Education and Research,
Karwand Naka, Shirpur Dist., Dhule 425405, Maharashtra, India

Abstract: The aim of this study is to identify and characterize the bioactive principles from the woody stem of *Abelmoschus manihot.* It has wide folk medicinal use. For isolation of the compounds, the dried woody stem's powder of *Abelmoschus manihot* was subjected to hot extraction with petroleum ether, this extract was saponified with alcoholic KOH and subjected to chromatography. Two compounds (PEA-2 and PEA-3) were isolated and purified by chloroform. Mass spectrum of PEA-2 and PEA-3 showed a parent molecular ion [M^+] peak at mlz 412 which corresponds to the molecular formula $C_{29}H_{48}O$ and 414 corresponds to $C_{29}H_{50}O$. In ^{1}H-NMR spectrum of PEA-2, H-3 proton appeared as a triplet of a double doublet (tdd) at S 3.62 and H-6 olefinic proton showed a multiplet at S 5.14. Two olefenic protons appeared downfield at S 4.16 (m) and S 4.14 (m) and in the ^{1}H-NMR data of PEA-3, H-3 proton appeared at S 3.51 as a triplet of a double doublet with a J value of 4.5 and 1.1 MHZ and H-6 olefinic proton showed a multiplet at S 5.10. From the physical, chemical and spectral characteristics, PEA-2 and PEA-3 were concluded as stigmasterol and γ-sitosterol.

Key words: Stigmasterol, γ-sitosterol, *Abelmoschus manihot*

INTRODUCTION

Research studies leading to extraction, isolation and biological study of plant constituents have now formed the major field of study. *Abelmoschus manihot,* Malvaceae is a large annual erect hairy plant, 1.2-1.8 m high. It is Native to China, introduced into India, near Calcutta and in coastal areas of Maharashtra. The plants mucilage contains polysaccharides and proteins (Kirtikar and Basu, 1994). The flower contain quercetin-3-robinoside, quercetin-3'-glucoside, hyperin, myrecetin and anthocyanins. The saturated acids and liquid acids such as linoleic and oleic acids were isolated from the seed fat and unsaponifiable matters. The different chromatographic methods have been developed on the flavones present in the plant (Liang *et al*., 2007; Lai *et al*., 2006; Yi *et al*., 2008). The ethanol extract of flower was screened for antiviral activity and it was observed that the hyperoside shown significant anti HBV activity (Lin-Lin *et al*., 2007). The flavones present in the plant showed preventive effect in the injury (Liu *et al*., 2009; Wen and Chang, 2007). The leaves were tested on bone loss in ovarectomised rats and it was observed that it was able to prevent the ovariectomy-induced femoral osteopenia (Puel *et al*., 2005). The modulatory

Corresponding Author: P.S. Jain, Department of Pharmaceutical Chemistry,
R.C. Patel Institute of Pharmaceutical Education and Research,
Karwand Naka, Shirpur Dist., Dhule 425405, Maharashtra, India

effect of Total Flavone of *Abelmoschus manihot* (TFA) on NMDA-activated current (I_{NMDA}) was investigated in cultured rat hippocampal neurons using the whole-cell patch-clamp technique. TFA rapidly and reversibly inhibited the I_{NMDA} in a concentration-dependent manner (Xin-Ping *et al*., 2006).

But these studies are not enough for identifying and characterizing the bioactive compounds in this plant. The purpose of this study is to identify and characterize the bioactive principles from the root bark of *Abelmoschus manihot*.

MATERIALS AND METHODS

Preparation of Plant Material

The woody stem of *Abelmoschus manihot* were collected during the month of May-June, 2007 from the Toranmal Hills of Maharashtra. The plant was taxonomically identified by Professor Dr. D.A. Patil, HOD Botany Dept, SSVPS College, Dhule, North Maharashtra University, Jalgaon. The dried woody stem powder (4 kg) was subjected to hot extraction with petroleum ether by Soxhlet extractor and after evaporation of the solvent 26 g extract was found. The extract then saponified with alcoholic KOH, to remove fatty material yields 12 g of unsaponified matter.

Isolation and Purification of Compounds

A small quantity of unsaponifiable matter was dissolved in chloroform and this solution was spotted on TLC plates. Then the TLC plates were run by specific solvent system and were viewed individually under UV light and also with the vanillin-H_2SO_4, reagent. Through several pilot experiments, it was found that the compounds of unsaponifiable fraction were separated by the solvent system of petroleum ether and ethyl acetate in the proportion of 7:3. The unsaponifiable fraction, 8 g, was subjected to column chromatography on a silica gel (60-120 mesh) with gradient elution using petroleum ether: ethyl acetate (Stahl, 1969).

Two fractions were found homogeneous on TLC plate by using petroleum ether: ethyl acetate (7:3), petroleum ether: chloroform (10:1), n hexane: ethyl acetate (9:1) and petroleum ether: methanol (9:1) solvent systems. These fractions were crystallized (Bahl and Bahl, 1992) and named as PEA-2 (Pet Ether Abelmoschus-2) and PEA-3 (Pet Ether Abelmoschus-3), respectively.

Test for Alcohol

Four gram of ceric ammonium nitrate was dissolved in 10 mL of 2 N HNO_3, on mild heating. A few crystals of PEA-2 and PEA-3 were dissolved in 0.5 mL of dioxane. The solution was added to 0.5 mL of ceric ammonium nitrate reagent and diluted to 1 mL with dioxane and shaken well. Both PEA-2 and PEA-3 developed yellow to red color indicating the presence of an alcoholic hydroxyl group (Harborne, 1998).

Test for Steroid

Salkowski Reaction: A few crystals of PEA-2 and PEA-3 were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to the solution. For both PEA-2 and PEA-3, a reddish color was seen in the upper chloroform layer (Harborne, 1998).

Liebermann-Burchard Reaction

A few crystals of PEA-2 and PEA-3 were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by the addition of 2-3 drops of acetic

anhydride. Solution for both PEA-2 and PEA-3 turned violet blue and finally green (Harborne, 1998).

Spectroscopic Characterization

Different spectroscopic methods were used to elucidate the structure of PEA-2 and PEA-3. Among the spectroscopic techniques IR, 'H-NMR and GMS were carried out. The infra red spectrum was recorded on FTIR 8400 s (Shimadzu) at NMU, Jalgaon, MS, India. ^{1}HNMR spectra were recorded on a Varian-500 MHZ NMR spectrometer (Shimadzu) at Cadila Pharmaceuticals, Ahmedabad, India. The ^{1}HNMR spectra were recorded using $CDC1_3$, as solvent with tetramethylsilane (TMS) as an internal standard. Mass spectrum was recorded at high resolution on a mass spectrometer (Perkin Elmer Autosystem XL with Turbomass) at Sophisticated Instrumentation Centre for Applied Research and Technology, Anand, Gujarat, India and the data are given in mlz values.

RESULTS

From the positive tests for steroids and alcohols given by the PEA-2 and PEA-3, they were assumed to be a sterol. The melting point of PEA-2 and PEA-3 were 176 and 137°C. The UV ëmax value of PEA-2 and PEA-3 is 257 and 251 nm, respectively. Mass spectrum of PEA-2 and PEA-3 showed a parent molecular ion (M^+) peak at mlz 412 and 414, respectively which corresponds to the molecular formula $C_{29}H_{48}O$ (Fig. 1) and $C_{29}H_{50}O$ (Fig. 2). In the IR spectrum

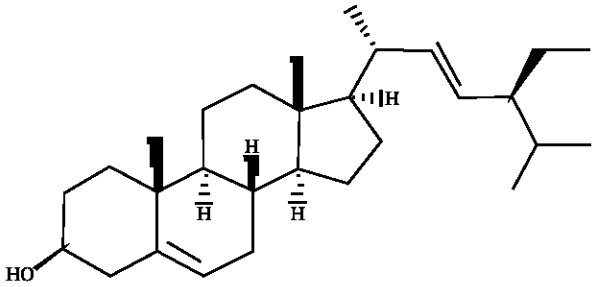

Fig. 1: The chemical structure of PEA-2 (Stigmasterol)

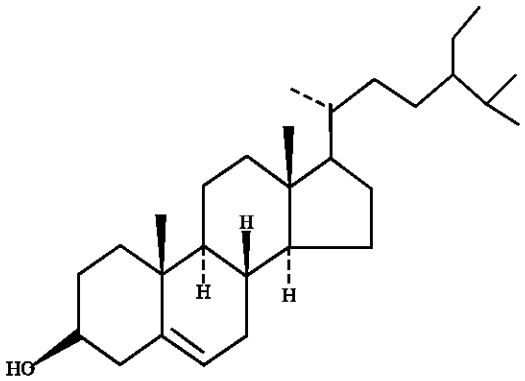

Fig. 2: The chemical structure of PEA-3 (γ-sitosterol)

Table 1: Spectroscopic data of PEA-2 (Stigmasterol)

Spectroscopic technique	Data
UV λ max:	257 nm
IR: ($CHCl_3$):	3320, 2946, 2854, 1648, 1460, 1450, 1220, 1189, 1050, 890, 740, 680 cm^{-1}
GCMS: m/z with	412 (M^+) 55(100), 394 (8), 255 (16), 213 (9), 199 (8),
(% abundance)	159 (25), 145 (29), 133 (26), 121 (19), 105 (32), 91 (34) 83 (64), 81 (59), 69 (52), 41(39)
1HNMR ($CDCl_3$)	δ 5.14 (m, 1H, H-6), δ 4.16 (s, 1H), δ 4.14 (s, 1H), δ 3.62 (tdd, OH, H-3), δ 1.27 (s, 3H), δ 1.19 (s, 3H), δ 1.07 (s, 3H), δ 1.00 (s, 3H), δ 0.99 (s, 3H), δ 0.91 (s, 3H)

Table 2: Spectroscopic data of PEA-3 (γ-sitosterol)

Spectroscopic technique	Data
UV λ max	251 nm
IR: ($CHCl_3$)	3319, 2946, 2854, 1640, 1470, 1460, 1189, 1060, 870, 720, 670 cm^{-1}
GCMS: m/z with	414 (M^+),43 (100), 396 (8), 381 (6), 329 (2), 303 (2), 275 (10),
(% abundance)	255 (14), 213 (13), 199 (8), 159 (25), 147 (34), 145 (48), 131 (25), 121 (32), 107 (34), 105 (43), 81 (61), 69 (36), 57 (55), 55 (64)
1HNMR ($CDCl_3$)	δ 5.10 (m, 1H, H-6),δ 3.51 (tdd, 1H, H-3),δ 1.26 (s, 3H),δ 1.17 (s, 3H),δ 1.03 (s, 3H),δ 1.00 (s, 3H),δ 0.91 (s, 3H),δ 0.90 (s, 3H)

of PEA-2 and PEA-3, an intensely broad band at 3320 and 3319 cm^{-1} showed presence of OH stretching and in the ^{1}H-NMR spectrum of PEA-2, H-3 proton appeared as a triplet of a double doublet (tdd) at S 3.62 and H-6 olefinic proton showed a multiplet at S 5.14. Two olefenic protons appeared downfield at S 4.16 (m) and S 4.14 (m) and of PEA-3, H-3 proton appeared at S 3.51 as a triplet of a double doublet H-6 olefinic proton showed a multiplet at S 5.10 and six methyl proton appeared at S 1.26, S 1.17, S 1.03, S 1.00, S 0.91 and S 0.90 for methyl group (Table 1, 2). From the above observations, PEA-2 and PEA-3 was found be stigmasterol and γ-sitosterol, respectively.

DISCUSSION

In IR spectrum of PEA-2, a very intensely broad band at 3320 cm^{-1} and moderately intense band at 1220 and 680 cm^{-1} were observed for the O-H bond vibrations of hydroxyl group. The out of plane C-H vibrations of the unsaturated part was observed at 890 cm^{-1}. The corresponding C-C vibrations was shown around 1648 cm^{-1} as weakly intense band. The stretching and bending vibrations of methyl part were noticed by the intense band 2946 cm^{-1} and medium intensity band at 1450 cm^{-1}. The vibration of the methylenic part was shown by the band at 2854 cm^{-1} and the medium band at 1460 cm^{-1}. The moderately intense band at 740 cm^{-1} was attributed to the rocking movement of methylenic part. The corresponding C-C vibration was shown as weak intense band at 1050 cm^{-1}. In ^{1}H-NMR spectrum of PEA-2, H-3 proton appeared as a triplet of a double doublet (tdd) at S 3.62 (J = 4.5 and 1.1 MHZ) and H-6 olefinic proton showed a multiplet at *S* 5.14. Two olefenic protons appeared downfield at S 4.16 (m) and S 4.14 (m) which were identical with the chemical shift of H-22 and H-23, respectively of stigmasterol (Li *et al.,* 2006). Six methyl protons also appeared at S 1.27, S 1.19, S 1.07, S 1.00, S 0.99 and *S* 0.91 (3H each, s, CH_3). These assignments are in good agreement for the structure of stigmasterol (Habib *et al*., 2007).

Similarly from the IR spectrum of PEA-3, a very intensely broad band at 3319 cm^{-1} and moderately intense band at 1189 and 670 cm^{-1} were observed for the O-H bond vibrations of hydroxyl group. The out of plane C-H vibrations of the unsaturated part was observed at 870 cm^{-1}. The corresponding C = C vibrations was shown around 1640 cm^{-1} as weakly intense band. The stretching and bending vibrations of methyl part were noticed by the intense band 2946 cm^{-1} and medium intensity band at 1460 cm^{-1}. The vibration of the

methylenic part was shown by the band at 2854 cm^{-1} and the medium band at 1470 cm^{-1}. The moderately intense band at 720 cm^{-1} was attributed to the rocking movement of methylenic part. The corresponding C-C vibration was shown as weak intense band at 1060 cm^{-1}. The ^{1}H-NMR data of PEA-3 it was seen that H-3 proton appeared at *S* 3.51 as a triplet of a double doublet with a J value of 4.5 and 1.1 MHZ and H-6 olefinic proton showed a multiplet at S 5.10. Moreover, Six methyl proton appeared at S 1.26, S 1.17, S 1.03, S 1.00, S 0.91 and S 0.90 (3H each, s, CH_3). These assignments are in good agreement for the structure of γ-sitosterol (Habib *et al*., 2007).

In the previous study on *Abelmoschus manihot* literature revealed that plant contains flavonoids are present in the flowers only, by present study it was confirmed that this plant also contains steroids.

CONCLUSION

From these physical, chemical and spectral evidences PEA-2 and PEA-3 were confirmed as Stigmasterol (Fig. 1) and γ-sitosterol (Fig. 2).

ACKNOWLEDGMENTS

Authors are thankful to the Principal, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, MS, India for the support and encouragement in the study.

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