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Isolation and toxicity evaluation of feruloyl ester and other triterpenoids from *Synadenium glaucescens* Pax

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ABSTRACT

The use of plants as sources of drug agents is attributed by factors among which are the easy accessibility to plants, less toxicity and little or no drug resistance. An improvement in both traditional medicine and drug discovery field necessitates investigation of pure compounds in any plant with medicinal value. *Synadenium glaucescens* Pax of the family Euphorbiaceae is among the medicinal plant in Tanzania which are proven to contain bioactive compounds against microbial infections. Analysis of ethanolic and methanolic extracts of root and stem barks respectively aided to isolated six pure compounds (**SG1- 6**). These compounds were analyzed by both 1D, 2D NMR and GC-MS while their spectral processing was achieved in the Bruker TopSpin 3.6.2. Among these compounds, one was a phenolic (hemicosanyl ferulate-**SG1),** three triterpenoids, (lupeol- **SG2**, epifriedelanol- **SG4** and euphol- **SG5**), one steroid (β-sitosterol- **SG6**) and a long chain alkene (1-nonacosene- **SG2**). Cytotoxicity evaluation by Brine shrimp lethality test (BLST) indicated the compounds under report were practically non-toxic.

Keywords: Triterpene, Lupeol, Friedelanol, NMR, Euphorbiaceae, Brine shrimp.

INTRODUCTION

Natural products isolated from various sources especially plants, have long been used in treatment of human ailments. Despite estimates varying depending on the definition of what is considered a natural product-derived drug, between 25 and 50% of currently marketed drugs owe their origins to natural products [1]. Among these, the triterpenoids form diverse structures of secondary metabolites and are widely distributed in both edible and ethnomedicinal plants [2, 3]. Plant members of Euphorbiaceae are reported to contain these triterpenes which are known for pharmacological activities including anti-inflammatory, anticancer, anticarcinogenic [4, 5], antidiabetic [6,7] and antimicrobial activities [8]. *Synadenium glaucescens* Pax is a medicinal plant of Euphorbiaceae which is used against both animal and human ailments. Phytochemical screening by Mabiki et al., 2013 [9] for dichloromethane extracts indicated presence of more than one hundred compounds including triterpenoids (euphol, lanosterol and lupeol backborn), steroids, long chain hydrocarbons and fatty alcohols while ethanolic extracts contained polyphenolic compounds as major constituents. Despite the large number of compounds in *S. glaucescens* Pax (SG) and the reported pharmacological potential of this plant, the isolations and structure assignment of pure forms have sorely reported only six of them namely euphol, β-sitosterol [10], erythrinacinate C and octacosanol [11], β and α-friedelanol [12] from leaves. Additionally, there is evidence from bioactivity studies of the crude extracts that the most bioactive compounds were in the SG root and stem barks [13]. However, the number of pure compounds isolated from the rootbark was limited to only two. This study aimed at isolating more pure compounds which would be associated with the previously observed bioactivities. Phytochemical investigations of the ethylacetate (EtOAc) and dichloromethane (DCM) extracts of the root and stem barks respectively resulted into isolation of one phenolic compound (hemicosanyl ferulate), three triterpenoids (Lupeol, epifriedelanol, Euphol), one long chain alkene (1-nonacosene) and a sterol (β-sitosterol). Their toxicity evaluation indicated they are none toxic at the tested concentrations.

MATERIALS AND METHODS

Plant collection and Processing

Plant authentication in Njombe region was done by a botanist and the voucher specimen (voucher no. 3672) was stored in the herbarium of the Department of Botany- University of Dar es Salaam (UDSM). Sample were collected from mtulingala village of Njombe district (08°34′ to 08°49′ S and 034°55′ to 035°10′ E), December 2018. The roots (NSG2) and stems (NSG5) parts were peeled to separate barks and wood parts. The root and stem barks of *S. glaucescens* Pax were air dried in a cold dark room at 15 ^{o}C to retain the light and temperature sensitive compounds.

Chemicals and apparatus

All chemicals used in this study were of analytical grade. They were obtained from either Loba Chemie, Mumbai-India i.e. ethylacetate (EtOAc), Dichloromethane (DCM) and Petroleum ether (PE) or Finar Chemical, Gujarat-India i.e. Methanol (MeOH) and Ethanol (EtOH). Moreover, silica gel 60 (70- 230 mesh, 60 angstrom pore size) and Thin Layer Chromatography Aluminium sheets (TLC, silica gel 60 F254) were obtained from Merck KGaA group, Darmstadt, Germany. An ultraviolet lamp (wavelength λ; 254 and 365 nm) was used for illumination of the TLC sheets.

Extraction and isolation of pure compounds

Powdered 2500 g of root barks (SG2) and 1200 g of stem barks (SG5) were extracted by maceration using ethanol and methanol solvents respectively according to [14, 15]. Each sample was packed in opaque bottles in either 500 g or 600 g followed by 2 L solvent. Mixtures were shaken for 5 minutes before they were placed in a dark room. Filtration of the mixture was done thrice after every 72 hours' time until a clear solution was formed as a maximum extraction. All extracts were air dried to evaporate the solvents affording 300 g NSG2 brown residues and 185 g NSG5 dark green residues. Fractionation of 90 g for each sample by vacuum liquid chromatography (VLC) packed with 90 g of silica gel and eluted using 5 L of n-hexane, 10 L ethyl acetate and 5 L ethanol (from least to the most polar solvent) yielded 262 mg for hexane (He), 60 g for ethyl acetate (EtOAc) and finally 20.2 g for EtOH in root bark extract (SG2FE) while in the stem bark extract, only two solvents (12.5L of DCM and 7 L of MeOH) were used to afford 35.5g and 50 g respectively. The SG2FE column (58 g of EtOAc fraction) was eluted at a solvent gradient systems of petroleum ether (PE) to 50 % MeOH: DCM. Based on the TLC profiles of eluted vials, a total of 15 (*fr.* 1-15) sub fractions were collected from 155 vials. Purification of precipitates in **fr**.1, 5, 8 and 10 cleanup with MeOH until a single sport for each compound was observed on an eluted TLC plate afforded compound **SG1**, **SG3, SG4, SG5** and **SG6.** Isolation of pure compounds from the DCM fraction of the stem bark extract, (SG5FD), 32.5 g was achieved by column chromatography. The column was eluted at 30 % EtOAc/ PE along to 30 % MeOH/ EtOAc to afford 10 sub-fractions (*frb.* 1- 10). Repetitive cleanup of *frb.* 3 by vacuum suction on the filter paper, Whatman 1 afforded compound **SG2** and again **SG4**.

Spectroscopic analyses

Nuclear magnetic Resonance (NMR) spectroscopy experiments for compounds were obtained using a 600 MHz Bruker Avance III HD equipped with a cryogenically cooled 5 mm dual probe optimized for ^{13}C and ^{1}H, and TMS (as internal standard). The chemical shifts (□) were recorded in ppm. Both compounds were dissolved in deuterated solvents either dichloromethane, chlorofoam or acetone prior to analysis. Mass spectrometric data were acquired on a Gas Chromatograph (GC) machine (6890N, Agilent Technologies, Germany) coupled with an MS detector (5973, Agilent Technologies, Germany) using electron impact ionization. The obtained NMR spectral data were processed in Bruker TopSpin version 3.6.2.

Cytotoxicity evaluation of Pure compounds

The egges of brine shrimps (*Artemia salina*) were hatched and used. The brine shrimp lethality test was carried out using the standard procedure as described by [16], with slight modifications. The stock solution of the study compounds were prepared by dissolving 40 mg of each compound in 1ml of 20 % v/v DMSO. A serial dilution was made to afford final concentrations of 2400, 1200, 800, 400 and 240 μgml^{-1}. Each concentration was tested in duplicate making 10 vials per test compound and one set of vials was prepared using 20 % v/v DMSO as a negative control. Ten live naupuli were transferred into each vial containing test compound with sea salt solution using Pasteur pipettes, followed by immediate adjusting the volume of the sea salt solution to 5 mL mark. The vials were maintained at room temperature on a laboratory bench for 24 hours after which the survivors were counted. The concentration of the killing 50 % of the naupuli larvae (LC_{50}) was determined from the graph.

RESULTS AND DISCUSSION

Compound **SG1** ($C_{31}H_{52}O_4$, Fig. 1), 65 mg was isolated as white powder from the root bark extract. The ^{13}C NMR (150 MHz, CD30D, δppm) indicated benzylic signals at δ_C 127.6 (C-1), 111.4 (C-2), 150.2 (C-3), 148.9 (C-4), 116.1 (C-5), 124 (C-6), the olefinic carbons at 145.6 (C-**7),** 116.6 (C-8), carbonyl carbon (167.6, C-9), 56.4 (OMe), 64.8 (C-1'). Other aliphatic chain signals were 31.7 (C-19'), 30.4- 30.5 for the C-4' to C-18'), 23.4 (C-20'), 30.4 (C-2'**),** 26.8 (C-3'), and 14.4 (terminal CH_3, C-21'**).** The ^{1}H NMR (600 MHz, CD_3OD) afforded two trans-olefinic protons at δ_H 7.58 (1H, *d*, 16.0, H-7), 6.38 (1H, *d*, *J*=16.0, H-8) and three aromatic protons 7.14 (1H, *dd*, *J*=8.2, 2.2Hz, H-2), 6.90 (1H, *d*, *J*= 8.2, H-5) and 7.34 (1H, *d*, *J*= 1.9, H-6). It further exhibited a methoxy signal at 3.93 (3H, *s*, -OCH_3), and a methylene at δ 4.15 (2H, *t*, *J*= 6.6, C-1'), 1.65 (2H, *qui*, H-2'), 1.41 (2H, *m*, C-3') and 0.88 (3H, *t*, CH_3). These structural clues suggested a feruloyl ester moiety [17,18]. The presence of an aliphatic alcohol moiety was indicated by the triplet signal at δ 0.88 (terminal methyl), the broad singlet at δ 1.29 for CH_2 and the downfield triplet at δ 4.15 that corresponded to a methylene adjacent to an oxy-carbonyl function. The broad singlet in ^{1}H NMR (at 1.29 ppm, 34H) corresponded to a total of seventeen (17) CH_2 groups thus making 21 carbon atoms in the aliphatic chain. The larger coupling constant between H-7 and H-8 (*J*= 15.9) suggested a trans-geometry [17,18]. A key HMBC correlation was observed between the ferulic acid and the long chain alcohol at δ_C 167.8 (C-9) to δ_H 4.15 (H-1') Based on the 1D and 2D spectral data and in comparison to the available literature [18–21], the **SG1** was assigned as hemicosanyl ferulate. This compound was previously isolated from the stem bark of *Pavetta owariensis* of the family Rubiaceae and *Aristolochia kankauensis*.

Compound **SG2, (**$C_{30}H_{50}O$, 65 mg) was isolated as white powder from the stem bark extract**.** The ^{13}C-NMR (Table 1) indicated thirty (30) carbon signals while the ^{1}H NMR spectrum had many signals concentrated in the high field region which was a characteristic of triterpene skeleton. Two olefinic carbon signals in 13C-NMR spectrum were observed at □c□148.8 (C-20) and 109.8 (C-29) indicating the exocyclic double bond. The □c 78.7 was due to a carbon bearing OH group (C-3). The ^{1}H NMR spectrum indicated seven singlet methyls at □H 0.72, 0.74, 0.82, 0.97, 1.00, 1.18, and 1.74. These experimental data ^{1}H and ^{13}C NMR were compared and in agreement with the literature [22–26] and was finally concluded **SG2** to be Lupeol.

Compound **SG3**, ($C_{29}H_{58}$, 11 mg), white waxy solid was isolated from the root bark extract. Its TLC profile indicated a retention factor (R_f) value of 0.96 in PE and reacted bright purple with vanillin reagent. This was an indicator for non-conjugated moiety. The ^{13}C NMR spectrum indicated fourteen (14) carbon signals one of them being the longest and broad at □C 29.9□which indicated the CH_2 chain stretch. Two peculiar olefinic carbons were observed at 114.3 (C-1) and 139.5 (C-2) due to a terminal π-bond while □C 14.3 corresponded to the terminal CH_3. The ^{1}H NMR indicated key signals at □H 5.79 (*m*, 1H) corresponding to a proton attached to an olefinic β-carbon, 4.94 (*dd*, 2H, *J*= 17.1 Hz and 10.2 Hz) for the two terminal hydrogen atoms at the chiral carbon rendering them different chemical environment. The other signals at □H 2.02 (*q*, 2H, *J* = 7.2) and 1.35 (*qui*, 2H) were due to γ and δ- CH_2 attachments while 0.86 (*t*, 3H, *J*= 6.5 Hz) was terminal CH_3. The final structure was confirmed by 2D NMR data (HSQC and HMBC) together with the GC_MS data indicated m/z 405.4 which was finally assigned as 1-nonacosene (Fig. 1). To the best of our knowledge, this long chain alkene is reported for the first time from *S. glaucescens* Pax but had been isolated from *Cissampelos mucronata* [27].

Compound **SG4**, ($C_{30}H_{52}O$), was isolated from bark extracts of both root (155 mg) and stem (12 mg) as white powder. The ^{13}C NMR spectrum indicated thirty (30) carbon signals. Comparative analysis of ^{13}C and DEPT135 NMR identified signals were eleven CH_2, six quaternary, five (C-H) and eight CH_3 signals were observed. The peculiar carbon signal at 73.1 represented a C-O stretch at C-3 of a triterpene skeleton. ^1H NMR spectrum indicated many proton signals in the high field region. Peculiar signals of ^1H NMR included a broad singlet at δ_H 3.70 representing a proton at C-3 bearing the-OH group in a trans-orientation [27]. Seven more proton signals for seven methyls resonated in the high field region as singlets at δ 0.95 (3H, s, C-24), 0.86 (3H, s, C-25), 1.00 (3H, s, C-27), 1.02 (3H, s, C-26), 1.00 (3H, s, C-28), 0.94 (3H, s, C-29) and 1.18 (3H, s, C-30). One methyl doublets resonated at δ 0.92 (3H, d, J = 7.3 Hz) and was assigned to C-23). Based on the experimental data (Table 1) and the available literature data [12,29,30], the **SG4** (Fig. 1) was concluded to be an epi-friedelanol (also known as β-friedelanol). Despite epifriedelanol being reported for the first time from the root and stem regions of *S. glaucescens* Pax, it was previously isolated from its leaves [12], stem barks of *S. grantii* [30], *Euphorbia neriifolia* [31] and leaves of *Pouteria ramiflora.*

Compound **SG5**, ($C_{30}H_{50}O$), R_f 0.6 in DCM, and [M^+], m/z 426.4 was isolated from bark extracts of both root (1834 mg) and stem (21.5 mg) as white powder. Their spectral data (^{13}C NMR, ^1H NMR and GC-MS) were compared and concluded to be the same compound. The ^{13}C NMR spectrum indicated thirty (30) carbon signals, four (4) of which resonated in olefinic regions at δ_C 134.7 (C-8), 134.1 (C-9), 125.8 (C-24) and 133.1 (C-25). The ^1H NMR indicated seven singlets at δ_H 0.77, 0.78, 0.88, 0.95, 0.98 and 1.26 (*s*, 6H) representing seven methyls. Comparison of these experimental data with the literature [10,33] and the GC-MS data, the compound **SG5** was confirmed to be euphol (Fig.1)

Compound **SG6**, ($C_{29}H_{50}O$, 62 mg) and the R_f 0.36 in DCM was isolated from the root bark extract as white shiny crystals. The ^{13}C NMR spectrum indicated a total of twenty nine (29) carbon signals (Table 1), which is a sterol characteristic. Two important olefinic carbon signals resonated at high field δ_C 141.0 (C-5) and 122.0 (C-6) while a signal at δ_C 72.1 was due to a proton attached to a carbon bearing the OH group at C-3. Its ^1H NMR spectrum indicated two main singlets at δ_H 0.66 (*s*, 3H) and 0.99 (*s*, 3H) assigned to two methyl groups at C-18 and C-19. Four more signals assigned to four secondary methyls appeared as doublets at δ_H 0.79, 0.82, 0.83 and 0.90 (*J*= 6.7, 6.8, 7.4 and 6.5) respectively. An olefinic proton at δ_H 5.33 (*br d*, 1H, *J*= 5.3) was assigned to H-6 while a proton at C-3 resonated as a multiplet at δ_H 3.50. These experimental data were in agreement with [10,34–36] together with the GC-MS data, m/z 414.3, and a melting point of 134.6-136.1 ^oC helped to confirm **SG6** as β-sitosterol. This compound was earlier isolated from the SG leaves but it is for the first time isolated from the root barks of this plant.

Brine shrimp toxicity test

The degree of toxicity for each tested compound in the BLST was evaluated according to [33] in which the LC_{50} <1.0 µg/ml – highly toxic; LC_{50} 1.0-10.0 µg/ml – toxic; LC_{50} 10.0-30.0 µg/ml – moderately toxic; LC_{50} >30 <100 µg/ml – mildly toxic, while LC_{50} > 100 µg/ml as non-toxic.

The test results (Table 2) indicated that 100 % of the compounds had the LC_{50} values greater than 100 µg/ml suggesting them to be non-toxic. Despite their toxicity category, compound **SG1** (feruloyl ester) showed the least LC_{50} values (most toxic) of all whereas **SG2** (lupeol) was the least. Since the compounds were obtained from crude extract of the high polar solvents, these results (at maximum test concentration of 24000 µg/ml) support the safety in traditional use of extracts from root and stem regions of *S. glaucescens* Pax.

Table 1: ^{13}C NMR Experimental data for the isolated triterpenoids (**SG2, SG4,** and **SG5**) and a steroid (**SG6**) from the root and stem barks of Synadenium glaucescens Pax

Position	SG2 (150 MHz, CD_2Cl_2, δ ppm)	SG4 (150 MHz CD_2Cl_2, δ ppm	SG5 (150 MHz, CD_2Cl_2, δ ppm)	SG6 (150 MHz, $CDCl_3$, δ ppm)
1	39.3	16.4	35.8	36.7
2	27.9	35.8	28.4	37.5
3	79.3	73.1	79.3	72.1
4	42.3	49.8	39.4	42.5
5	55.6	37.7	51.5	141
6	19.0	42.3	19.2	122
7	33.9	18.1	28.2	32.1
8	42.6	53.7	134.7	31.9
9	55.4	38.9	134.1	50.4
10	37.7	61.9	37.8	36.4
11	22.2	35.9	22.1	21.3
12	24.5	30.5	31.5	40
13	39.2	38.4	44.7	42.6
14	45.3	40.2	50.6	57
15	25.3	32.9	30.3	26.3
16	34.3	36.7	28.7	28.5
17	47.1	30.3	50.3	56.3
18	50.9	43.4	15.9	34.2
19	50.1	36.1	20.5	19.3
20	149.4	28.6	36.5	32.2
21	28.4	33.4	19.5	24.5
22	42.4	39.8	36.0	46.1

23	28.1	12.0	25.3	23.3
24	16.2	16.8	125.8	12.2
25	17.0	18.6	131.3	29.4
26	16.5	19.0	17.9	20
27	15.7	20.5	26.0	19.6
28	17.0	31.2	28.6	19
29	110.4	35.3	15.9	12.1
30	21.6	32.1	24.7	

Reference for: **SG2-** *Lupeol* [22–26], **SG4-** *epifriedelanol* [12,18,19] , **SG5-** *Euphol* [10, 33], and **SG6-** *β-sitosterol* [10, 23-25]

Table 2: LC_{50} on Brine shrimps for the pure compounds isolated from the root and stem barks of *Synadenium glaucescens* Pax

Compound	**Concentration (µgmL1^{-1})**	**% Mortality**	**LC_{50} (µg/mL)**
SG1 (feruloylester)	2400	80	762.807
	1200	65	
	800	50	
	400	50	
	240	0	
SG2 (Lupeol)	2400	40	25813.42
	1200	20	
	800	10	
	400	0	
	240	0	
SG3 (1-nonacosene)	0	0	NT
	0	0	
	0	0	
	0	0	
	0	0	
SG4 (epifriedelanol)	2400	0	1088.48
	1200	0	
	800	0	
	400	0	
	240	0	
SG5 (Euphol)	2400	80	825.52
	1200	70	
	800	40	
	400	25	
	240	0	
SG6 (β-sitosterol)	2400	40	5688.20
	1200	25	
	800	0	
	400	0	
	240	0	

***** NT= not tested

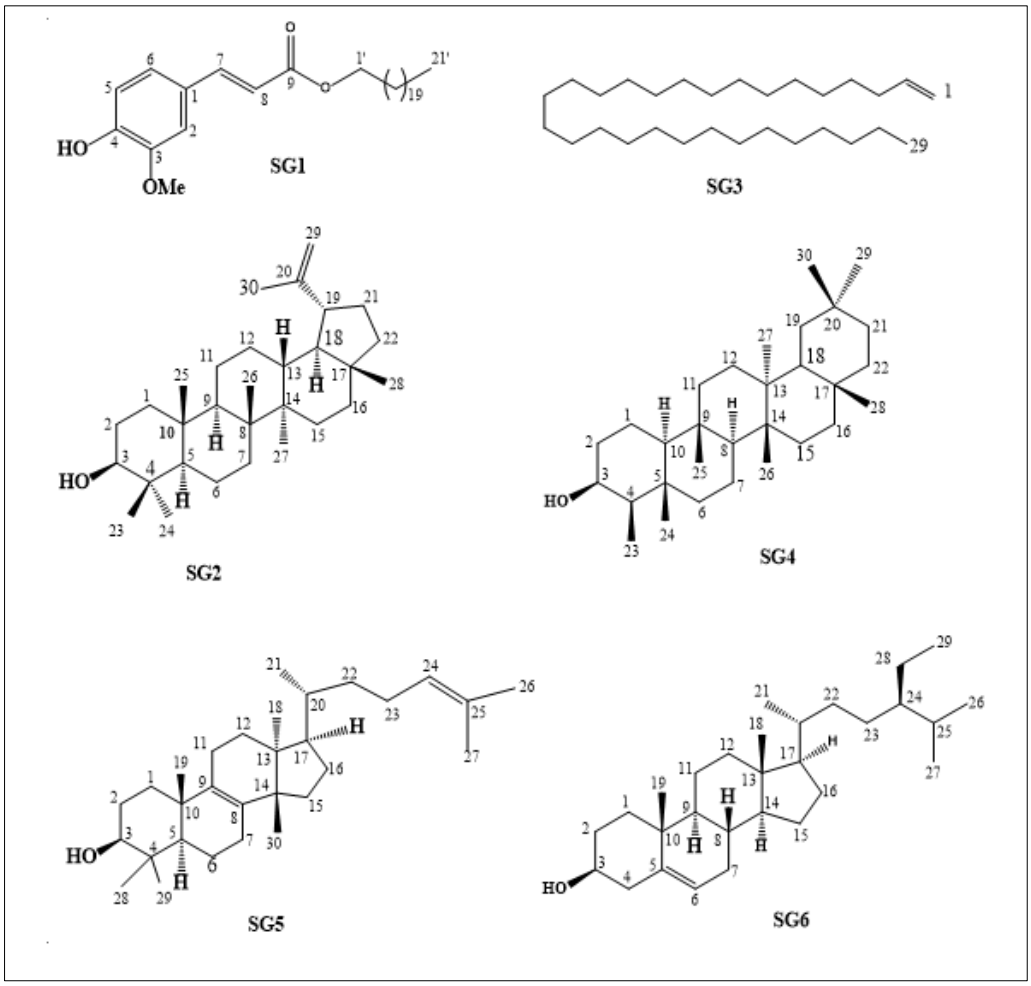

Figure 1: Structures of compounds isolated from the root barks (**SG1, SG3-6**) and stem barks (**SG2**) of *Synadenium glaucescens* Pax

Figure legend

Chemical structures of the compounds **SG1-6** were drawn using ChemDraw Professional 16.0 software.

CONCLUSION

This study (to the best of researchers' knowledge) reports for the first-time isolation of hemicosanyl ferulate (also known as eicosyl ferulate) from *S. glaucescens* Pax. Additionally, this is the first-time report on isolation of the triterpenoids, Lupeol, euphol (from the stem bark extracts) and epifriedelanol from both root and stem barks including the steroid β-sitosterol from the root regions of *S. glaucescens* Pax. These compounds demonstrated to be nontoxic to brine shrimps at the tested concentrations.

Limitation of the study

Cytotoxicity of 1-nonacosene was not done due to its insufficient weight.

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Conflict of Interest

None declared.

Supplementary information

Supplementary figures (Figure 1-23) can be downloaded by following the given link.

Link:
http://www.phytopharmajournal.com/Vol11_Issue5_06_SupFig.pdf

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