Bioactive compounds from *Tipuana tipu* growing in Egypt

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Abstract: Six compounds were isolated from methanolic extracts of the leaf and the bark of *Tipuana tipu* growing in Egypt. The isolated compounds were identified as 1-Nonadecanol **(1)**, *β*-sitosterol **(2)**, Alpinumisoflavone **(3)**, *β*-sitosterol glucoside **(4)**, Protocatechualdehyde **(5)** and Protocatechuic acid **(6)**, based on different spectroscopic data (UV, IR, NMR and MS). This is the first report for the isolation of compounds **1** & **3-6** from *Tipuana tipu*. Compound **1** is isolated for the first time from family Fabaceae. Compounds **2** and **3** showed a promising anticancer activity, while compound **5** displayed a remarkable *in vivo* anti-inflammatory activity compared to indomethacin.
[Amen YM, Marzouk AM, Zaghloul MG, Afifi MS. **Bioactive compounds from *Tipuana tipu* growing in Egypt.** *J Am Sci* 2013;9(10):334-339]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 44

Keywords: *Tipuana tipu*, 1-Nonadecanol, Alpinumisoflavone, Protocatechualdehyde, Protocatechuic acid, anticancer, anti-inflammatory.

1. Introduction

Family Leguminosae (Fabaceae) is one of the three largest families of flowering plants, exceeded only by the Compositae and Orchidaceae (Harborne *et al.*, 1971). This family comprises 727 genera and ca. 19325 species. It was divided into three subfamilies; Mimosoideae, Caesalpinioideae and Papilionoideae (Lewis *et al.*, 2005). The Genus *Tipuana* (Benth.) Benth. (subfamily Papilionoideae) was originally assigned to *Machaerium*, but now is excluded from that genus (Ducke, 1930). It comprises only one species: *Tipuana tipu* (Hickey and king, 1997). *Tipuana tipu* (Benth.) Lillo [syn *T. tipu* (Benth.) Kuntze] is a South American tree which is indigenous to Argentina, Uruguay, Paraguay, Bolivia and Brazil. The tree is mainly an ornamental tree. It can be used as a supplementary food to small ruminants (*e.g.* goats), mainly the dried leaves proved to have high nutritive value (Norton and Waterfall, 2000). Plants in genus *Machaerium* are used in South America by indigenous population to treat diarrhea (Heinrich *et al.*, 1992), menstruation cramps (Ginzbarg, 1977), coughs (Joly *et al.*, 1987) and aphthous ulcers of the mouth. Certain *Machaerium* species were reported to have antimicrobial (Waage *et al.*, 1984), antigiardial (ElSohly *et al.*, 1999) and antitumor activities (Seo *et al.*, 2001). Little phytochemical studies were carried out on *T. tipu* including those of Braga De Oliveira *et al.* (1971) who reported the isolation of formononetin, β-sitosterol and stearic acid, while Kansoh *et al.* (2009) studied the volatile oil content and lipoidal matter of the flowers and pods. The present research was conducted to isolate the chemical constituents of *Tipuana tipu* growing in Egypt and evaluate its potential use in pharmacy and medicine.

2. Results and Discussion

2.1. Characterization of compounds

Six compounds (Fig. 1) were isolated from methanolic extracts of the leaf and the bark of *Tipuana tipu* (Benth.) Lillo growing in Egypt. Compounds **2** and **4** were identified by m.p., co-chromatography with authentic samples and comparing to literature data.

Compound **1** was isolated as colorless needle crystals. Its EI-MS showed a quasi-molecular ion peak at *m/z* 285 $[M+ H]^+$, compatible with the molecular formula $C_{19}H_{40}O$. APT spectrum showed nine signals, which were discriminated into a CH_3 resonance at δ 14.1 and a signal due to a primary alcoholic group at δ 62.5. The other signals were assigned to aliphatic CH_2 groups, which resonated at δ 22.7 – 32.5 (see experimental section 3.3.1). These data suggested compound **1** to be an aliphatic straight chain primary alcohol. ^{1}H-NMR spectrum revealed a downfield signal at δ 3.47 (t, *J* = 6.9 Hz, 2H) assigned for CH_2OH group that was confirmed by absorption band at 3423 cm^{-1} (alcoholic OH stretching) and 1062 cm^{-1} (C-O stretching of primary alcohol) in IR spectrum (Silverstein *et al.*, 2005), in addition to a signal at δ 0.78 (t, *J* = 7.3 Hz, 3H) assigned for the terminal CH_3 group. An upfield broad signal resonated at δ 1.16 (34H, m) was assigned to H3-H18 and confirmed by absorption band at 720 cm^{-1} (C-H bending of repeated methylene groups of straight chain paraffins) in IR spectrum (Silverstein *et al.*, 2005). From the above data, compound **1** was concluded to be 1-Nonadecanol which was previously isolated from *Convolvulus lanatus*, family Convolvulaceae (El-Nasr *et al.*, 1984), but isolated here for the first time from *T. tipu* as well as from family Fabaceae.

Compound **3** was isolated as yellow needles. EI-MS spectrum (positive mode) showed a molecular ion peak at *m/z* 336 $[M]^+$ corresponding to a molecular formula $C_{20}H_{16}O_5$. It displayed an intense UV absorption maximum at λ_{max} 283 nm, typical of an isoflavone nucleus (Mabry *et al*., 1970). This was supported by ^{1}H- and ^{13}C-NMR spectral data (Table 1). In ^{1}H-NMR spectrum, the most downfield of aromatic signals (δ 7.80, s, H-2) and signals at δ 152.6 for C-2, 123.1 for C-3 and 181.0 for C-4 in ^{13}C-NMR spectrum (Table 1) (Agrawal *et al*., 1989; Mabry *et al*., 1970). A pair of doublets in the ^{1}H-NMR spectrum centered at δ 5.59 and 6.69 (*J* = 10.1 Hz) together with a sharp singlet integrated for six protons at δ 1.45, suggested the presence of 2", 2"- dimethylpyrano- substituent and the set of signals at δ 28.4, 77.4, 128.3 and 115.6 in ^{13}C-NMR spectrum provided further support for the presence of *gem*-dimethylpyran moiety. The failure of NaOAC to produce a significant bathochromic shift (λ_{max} 282 nm) in the UV absorption maximum compared to MeOH spectrum, together with the ^{1}H- NMR singlet at δ 6.31 assigned to H-8 indicated a trisubstituted A ring. The signals at δ 106.0, 159.6 and 95.0 could be assigned to carbons C-6, C-7 and C-8 respectively. Thus, the *gem*-dimethylpyran moiety was suggested to be fused in a linear manner on ring A not in angular position, and this assignment was supported by inspection of the characteristic ^{13}C-NMR signals for C-5, C-6, C-7 and C-8 at the two possible positions (Agrawal *et al*., 1989). The 5-hydroxy substituent was deduced from the bathochromic shift (+12 nm) in the UV absorption maximum observed with $AlCl_3$ after 30 min. ^{1}H-NMR spectrum also displayed a pair of broad doublets (*J* = 8.2 Hz), each integrating for two protons, at δ 6.85 and 7.35, which were assigned to H-3′, H-5′ and H-2′, H-6′ of the *para*-disubstituted ring B. The relatively upfield resonance (δ 6.85) of H-3′ & H-5′ suggested the presence of an oxygenated substituent at C-4′, most probably as a hydroxyl group. The structure of **(3)** was confirmed by comparison with the reported data (Stewart *et al*., 2000) as alpinumisoflavone. It was first isolated by Jackson *et al*. (1971) from *Laburnum alpinum* J. Presl. (Fabaceae).It was further reported in different species of the genus *Erythrina* (Fabaceae): from *E. lysistemon*, *E. stricta*, *E. variegata*, *E. poeppigiana*, *E. senegalensis*, *E. indica* and *E. arborescens* (Nkengfack *et al*., 2001; Rahman *et al*., 2010); from other genera belonging to family Fabaceae as *Calopogonium mucunoides*, *Crotalaria ferruginea* and *Millettia thonningii* (Perrett *et al*., 1995). Alpinumisoflavone was also reported from family Moraceae from *Ficus chlamydocarpa*, *F. nymphaefolia* and from *Rinorea welwitschii*, family Violaceae (Stewart *et al*., 2000).

Table 1.^{13}C and ^{1}H-NMR spectral data of compound **3***

#	APT	^{13}C	^{1}H
2	CH	152.7	7.80, s
3	C	123.1	--------
4	C	181.0	--------
5	C	156.0	
6	C	106.0	--------
7	C	159.6	--------
8	CH	95.0	6.31, s
9	C	157.0	--------
10	C	105.7	--------
1'	C	123.6	--------
2'	CH	130.4	7.35 (d, *J*= 8.2)
3'	CH	115.7	6.85 (d, *J*= 8.2)
4'	C	157.4	--------
5'	CH	115.7	6.85 (d, *J*= 8.2)
6'	CH	130.4	7.35 (d, *J*= 8.2)
2"	C	77.4	--------
3"	CH	128.3	5.59 (d, *J*= 10.1)
4"	CH	115.6	6.69 (d, *J*= 10.1)
Me_2-2"	CH_3	28.4	1.45, s

*Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. ^{13}C and ^{1}H-NMR were measured in $CDCl_3$ at 100 and 400 MHz, respectively.

Compound **5** was isolated as colorless needles. Absorption bands at 1595 and 1451 cm^{-1} (C=C aromatic) in IR spectrum and UV absorption maxima at λ_{max} 313, 278 and 233 nm suggested the presence of aromatic ring (Silverstein *et al*., 2005). ^{1}H-NMR spectrum (Table 2) clearly indicated the presence of three aromatic signals in the range of δ 6.91 – 7.32. This was confirmed by ^{13}C-NMR spectrum which revealed the presence of signals due to a benzene ring resonating between δ 115.2 – 153.5, in addition to a signal at δ 192.9 assigned to a carbonyl carbon of aldehydic group.This was supported by absorption bands at 1670 cm^{-1} (aryl aldehyde), a pair of bands at 2734 and 2853 cm^{-1} (C-H stretching vibrations in aldehydes) in IR spectrum. A doublet observed at δ 6.91 (*J*= 8.0 Hz, 1H) was coupled to another doublet at δ 7.31 (*J*= 8.0 Hz, 2H) and could be attributed to two *ortho*-coupled protons at C-5 and C-6. The other singlet at δ 7.32 which was coincident with H-6 (d, *J*= 8 Hz) could be assigned to proton at C-2 and supported by two signals in ^{13}C-NMR spectrum for two oxygenated carbons at δ 147.0 and 153.5. By comparing these NMR spectral data (Table 2) with those reported by Pauletti *et al*. (2012), the structure of compound **5** was confirmed to be protocatechualdehyde which was previously isolated from the leaf of *Musanga cecropioides*, family Urticaceae (Ayinde *et al*., 2007).

Compound **6** was isolated as white crystals. EI-MS spectrum showed a molecular ion peak at *m/z* 154 corresponding to a molecular formula $C_7H_6O_4$. The UV absorption maxima in MeOH at λ_{max} 307, 298 and 260 nm and the absorption bands in IR at 1600 and 1468 cm^{-1} suggested also an aromatic nucleus. All the NMR spectral data of **6** (Table 2) almost resembled those of **5** described above. The differences appeared in ^{13}C-NMR spectrum which displayed a quaternary carbon signal at δ 170.7 assigned to a carbonyl function of carboxylate supported by

IR absorption bands at 3213 cm^{-1} (br, OH) and 1677 cm^{-1} C=O carboxylate) (Silverstein *et al*., 2005). Based on ^{1}H-NMR spectrum of both compounds, the substitution pattern of the aromatic ring remains the same except for the presence of a carboxyl group replacing the aldehyde group. The identity of **(6)** was confirmed as Protocatechuic acid through comparison with published literature data (Nova *et al*., 2012).

Table 2.^{13}C and ^{1}H-NMR spectral data of compounds **5** and **6****

#	5		6	
	^{13}C	^{1}H	^{13}C	^{1}H
1	130.6	--------	123.7	--------
2	116.0	7.32, s	117.7	7.46, s
3	147.0	--------	145.9	--------
4	153.5	--------	151.2	--------
5	115.2	6.91 (d, *J*= 8.0)	115.6	6.81 (d, *J*= 8.0)
6	126.2	7.31 (d, *J*= 8.0)	123.7	7.44 (d, *J*= 8.0)
7	192.9	--------	170.7	--------

** Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. ^{13}C and ^{1}H-NMR were measured in CD_3OD at 100 and 400 MHz, respectively.

2.2. Biological activity

2.2.1 Anticancer activity

β-sitosterol **(2)** showed a weak activity towards CNS SF-539, ovarian OVCAR-4 and renal UO-31 cancer cell lines with cell promotion percentages of 82.09, 83.24 and 85.91% respectively, while it showed a fairly good activity against Non-small cell lung cancer HOP-62 cell line with cell promotion percentage 73.78%. Alpinumisoflavone **(3)** was active against different leukemia cell lines. It showed a high activity against CCRF-CEM (acute lymphoblastic leukemia, nuclear lysate) with cell promotion percentage 48.83%, while it was less active against MOLT-4 (acute lymphoblastic leukemia, of peripheral blood origin) and HL-60(TB) (promyelocytic leukemia) cell lines with cell promotion percentages of 73.85% and 84.51%, respectively. It also, exhibited a good activity against renal SN12C cancer cell line with cell promotion percentage 67.33 % and a weak activity against breast MCF7 cancer cell line with cell promotion percentage of 82.82%.

2.2.2 Anti-inflammatory activity

The results of the anti-inflammatory screening (Table 3) revealed variable activities of the different compounds. Protocatechualdehyde **(5)** displayed a remarkable anti-inflammatory activity comparable to that of indomethacin while protocatechuic acid **(6)** was less active. 1-Nonadecanol, β-sitosterol, alpinumisoflavone and β-sitosterol glucoside displayed weak activity compared to the standard drug.

Table 3. Results of screening of the anti-inflammatory activity of the isolated compounds†

Treatment	% of increase in paw thickness	% of inhibition of edema
Control (Carrageenan only)	141.38 ± 4.33	-
Indomethacin (10 mg/kg)	79.44 ± 1.44	41.67 ± 0.21
1-Nonadecanol	106.5 ± 2.34	25.5 ± 0.22
β-sitosterol	97.35 ± 1.84	30.17 ± 0.17
Alpinumisoflavone	103.27 ± 3.83	29.17 ± 0.17
β-sitosterol glucoside	124.69 ± 4.05	7.83 ± 2.83
Protocatechualdehyde	69.54 ± 0.56	42.17 ± 0.17
Protocatechuic acid	90.42 ± 1.1	35.17 ± 0.17

† Values are expressed as mean ± SEM, n = 6 in each group, *P<0.01* compared with the control.

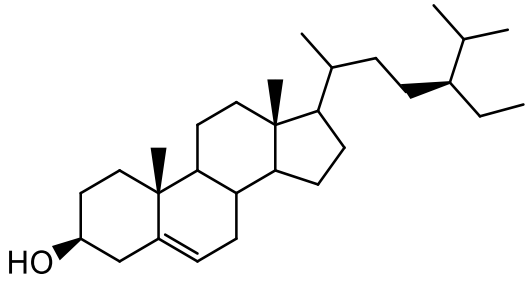
3. Experimental

3.1. General

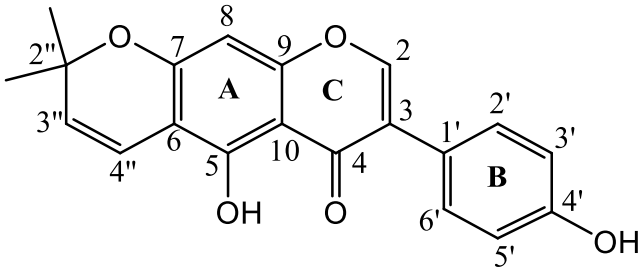
^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 spectrometer (400 and 100 MHz for ^{1}H and ^{13}C respectively). The melting points were measured on melting point apparatus Fisher-johns scientific Co., USA and were uncorrected. UV spectra were performed on a Shimadzu UV-1201 spectrophotometer (Japan). IR spectra were recorded in KBr disks using Infra-red spectrophotometer, Mattson 5000 FTIR (England). EI mass spectra (positive mode) were carried out on a JOEL JMS600 spectrometer (Japan). Male *Wistar albino* rats were provided by the Experimental Animal House, Pharmacology Department, Faculty of Pharmacy, Mansoura University. λ-carrageenan from Sigma-Aldrich (USA). All other chemicals used were of analytical grade.*3.2. Plant material* The leaves were collected from trees growing on Mansoura University campus, Egypt on May, 2011. The freshly collected leaves were air-dried in shade at room temperature. The plant identity was confirmed by staff members at Department of Horticulture, Faculty of Agriculture, Mansoura University, Egypt. A representative specimen deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. The bark was collected from the same trees on August, 2012.

Figure 1. Structures of isolated compounds

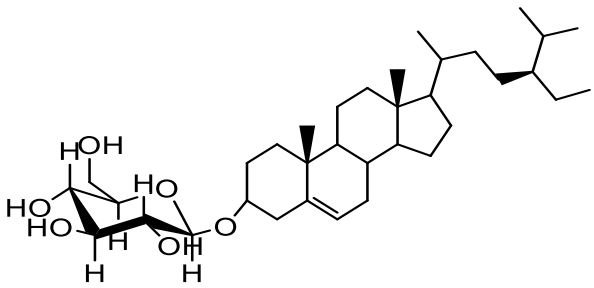
(1)



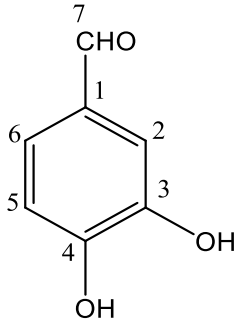
(2)



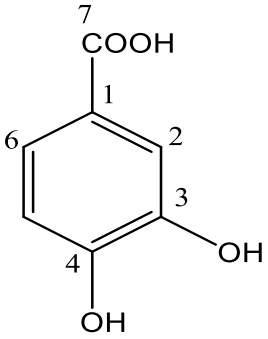
(3)



(4)



(5)



(6)

3.3. Extraction and isolation

The dried powdered leaves (6000 g) were percolated with MeOH (9 x 10 L). The combined extracts were concentrated to a syrupy consistency under reduced pressure at 40 ^{o}C. The residue (629.8 g) was suspended in distilled water and extracted successively with petroleum ether, methylene chloride, ethyl acetate and then finally with *n*-butanol. The different extracts were, separately, evaporated under reduced pressure to obtain petroleum ether fraction (fraction A, 70 g), methylene chloride fraction (fraction B, 7 g), ethyl acetate fraction (fraction C, 15 g) and *n*-butanol fraction (fraction D, 30 g).

The dried powdered bark (8000 g) was percolated with MeOH (8 x 18 L). The combined extracts were concentrated to a syrupy consistency under reduced pressure at 40 ^{o}C. The residue (170 g) was suspended in distilled water and extracted successively with petroleum ether, methylene chloride, ethyl acetate and then finally with *n*-butanol. The different extracts were, separately, evaporated under reduced pressure to obtain petroleum ether fraction (fraction E, 27 g), methylene chloride fraction (fraction F, 22 g), ethyl acetate fraction (fraction G, 9 g) and *n*-butanol fraction (fraction H, 28 g).

Fraction A was subjected to silica gel column chromatography and eluted with Petroleum ether – EtOAc gradient (0-80%). These subfractions were pooled based on similar R_f values and purified by rechromatography on silica gel columns and PTLC and by repeated crystallization to afford 1-Nonadecanol **1** (59 mg), β- sitosterol **2** (250 mg), Alpinumisoflavone **3** (26 mg) and *β*-sitosterol glucoside **4** (232 mg).

Fraction G was subjected to silica gel column chromatography and eluted with Petroleum ether –

EtOAc gradient (50-100%) and EtOAc – MeOH (0-60%). Collected fractions were pooled based on similar R_f values and subjected to purification through column chromatography and repeated crystallization to afford Protocatechualdehyde **5** (24 mg) and Protocatechuic acid **6** (28 mg).

3.3.1 1-Nonadecanol ***(1)*** colorless needle crystals (R_f 0.51) on precoated silica gel plates GF_{254} using 10% EtOAC /pet. ether and colored brown upon spraying with vanillin/ H_2SO_4 spray reagent and heating at 110 ˚C for 1 min., m.p. 70-71˚C (lit. 60-61˚C), ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm): 3.47 (t, *J* = 6.9 Hz, H-1), 1.44 (m, H-2), 1.16 (m, H-3 to H-18), 0.78 (t, *J* = 7.3 Hz, H-19).^{13}C-NMR ($CDCl_3$, 100 MHz, δ ppm): 62.5 (C-1), 32.5 (C-2), 31.9 (C-3), 29.4 – 29.7 (C-4 to C-16), 25.8 (C-17), 22.7 (C-18), 14.1 (C-19).

3.3.2 Alpinumisoflavone ***(3)*** yellow needles, (R_f 0.22) on precoated silica gel plates GF_{254} using 20% EtOAC /pet. ether, m.p. 210-212˚C (lit. 210-212˚C), UV λ_{max} MeOH 283 nm, λ_{max} NaOAC 282 nm, λ_{max} $AlCl_3$ 295 nm after 30 min.; ^{1}H and ^{13}C NMR (Table 1).

3.3.3 Protocatechualdehyde ***(5)*** colorless needles, (R_f 0.50) on precoated silica gel plates GF_{254} using 10% MeOH /CH_2Cl_2, m.p. 152-154 ^{o}C (lit. 152-154 ˚C), UV λ_{max} MeOH 313, 278, 233 nm; IR (KBr, ν_{max}): 3439, 2853, 2734, 1670, 1595, 1451 cm^{-1}; ^{1}H and ^{13}C NMR (Table 2).

3.3.4 Protocatechuic acid ***(6)*** white crystals, (R_f 0.30) on precoated silica gel plates GF_{254} using 10% MeOH / CH_2Cl_2, m.p. 196-198 ^{o}C (lit. 198-200˚C). UV λ_{max} MeOH 307, 298 and 260 nm; IR (KBr, ν_{max}): 3213, 1677, 1600, 1468 cm^{-1}; ^{1}H and ^{13}C NMR (Table 2).

3.4. Biological activity

3.4.1. Anticancer activity

Compounds **2** and **3** were submitted to National Cancer Institute (NCI), USA, for evaluating their antitumor activities under the Developmental Therapeutic Program (DTP). The operation of this screen utilizes 60 different human tumor cell lines. The compounds were tested at a concentration of 10^{-5} M in the full NCI 60 cell-line panel. The cultures were incubated for 48 h. End point determinations were made with a protein binding dye, *Sulforhodamine B.* Results for each compound are reported as mean of the percentage growth of the treated cells when compared to the untreated control cells (Monks *et al*., 1991; Grever *et al*., 1992; Boyd and Paull, 1995).3.4.2. Anti-inflammatory activity The antiinflammatory activity of the isolated compounds (**1**-**6**) was carried out using carrageenan-induced rat paw edema model (Winter *et al.,* 1962) by employing carrageenan solution as the phlogistic agent. Male *Wistar albino* rats weighing 150–180 g were kept in the animal house under standard conditions of light and temperature with free access to food and water. After overnight fasting, the animals were randomly divided into three groups each consisting of six rats. The first group received only carrageenan. Another group received the standard drug indomethacin (10 mg/kg, i.p., 1% w/v in normal saline). The last group of rats was administered the test compounds in equal doses (25 mg/kg, i.p.; w/v). Thirty minutes after administration of the standard drug and test compounds, 0.1 ml of 1% w/v of λ-carrageenan (Type IV) in sterile normal saline was injected into the subplanter region of the right hind paw of each rat. After 4 hr, the paw thickness was measured with electronic digital calipers, and the difference in the paw thickness between the injected and the control was recorded for each rat. The edema was expressed as the percentage of increase in the paw thickness compared to the positive control and the percentage of inhibition of edema for each group. Data were expressed as mean ± standard error (S.E.M.). The statistical significance of difference between groups were assessed by means of analysis of variance (ANOVA) followed by Dunnet's test.

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9/2/2013