Chemical constituents from *Piper caninum* and antibacterial activity

Wan Mohd Nuzul Hakimi Wan Salleh[1], Farediah Ahmad[1], Khong Heng Yen[2]

[1]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.
[2]School of Chemistry and Environmental Studies, Faculty of Applied Sciences, Universiti Teknologi MARA Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.

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ABSTRACT

Six flavonoids and two amides, characterized as 5,7-dimethoxyflavanone (**1**), 5,7-dimethoxyflavone (**2**), 4',5,7-trimethoxyflavone (**3**), 4'-hydroxy-5,7-dimethoxyflavone (**4**), 5-hydroxy-7-methoxyflavone (**5**), 2'-hydroxy-4',6'-dimethoxychalcone (**6**), *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicosatrienamide (**7**) and *N*-isobutyl-15-(3',4'-methylenedioxyphenyl)-2*E*,4*E*,12*E*-pentadecatrienamide (**8**) have been isolated and identified from the stem of *Piper caninum*. The identification of all compounds was achieved by physical properties and spectroscopically. These data were also confirmed by comparison with previously reported spectral data. Antibacterial activity of the extracts and isolated compounds was determined by disc diffusion method and minimum inhibitory concentration (MIC). All of the extracts displayed weak antibacterial activity against the tested bacteria. Compound (**7**) showed good activity towards *Bacillis subtilis* with MIC value of 125 μg/mL. Flavonoid compounds with high content in *P. caninum* can probably be used as a chemical marker for this *Piper* species.

INTRODUCTION

The genus *Piper* has the greatest diversity in geographical distribution with about 700 species in American tropic and nearly 340 species in Asian tropic including Indonesian and Malaysian tropical rainforests. There was an estimated total of 400 species of *Piper* were recorded from the Malaysian region alone (Parmar *et al.,* 1997). In past studies, the phytochemical investigations from *Piper* species have led to the isolation of a large number of physiologically active compounds such as alkaloids/amides, lignans/neolignans, propenylphenols, flavonoids, triterpenes, steroids, kawapyrones and piperolides (Sengupta *et al.,* 1987; Jensen *et al.,* 1993; Wu *et al.,* 1997), with various biological activities, such as antioxidant, antimicrobial, antifungal, antityrosinase, anticholinesterase, antituberculosis, antiplasmodial, anti-inflammatory, antileishmanial, and insecticidal activities (Yamaguchi *et al.,* 2006; Lie *et al.,* 2006; Hardik *et al.,* 2007; Siddiqui *et al.,* 2004; Salleh *et al.,* 2014b). *Piper caninum* Bl., or locally known as "*cabai hutan*"or"*lada hantu*", can be found throughout the tropic mostly in South East

Asia. The leaves are chewed by the locals to replace betel-leaves and the betel-quid is used for treating hoarseness and also for antiseptic (Burkill, 1966). Previous phytochemical studies have shown the presence of stilbene, flavonoids, phenolic acid amides, alkaloids and bornyl hydroxycinnamic esters (Ahmad *et al.,* 1997; Ma *et al.,* 2004a; Setzer *et al.,* 1999). *P. caninum* extracts were found to induce the relaxation of supercoiled pBR322 plasmid DNA in the presence of Cu^{2+}. Another study revealed the crude extract as a putative inducer of double-strand DNA damage (Ma *et al.,* 2004b). Besides, the crude chloroform bark extract of *P. caninum* exhibited antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus pneumonia* (Setzer *et al.,* 1999).

In recent times, anti-hypertensive effect of *P. caninum* extracts on normotensive rats (WKY) and spontaneously hypertensive rats (SHR), were also reported (Kim, 2000). In our previous work on this species, we have been report on the essential oil composition, together with their antioxidant and antimicrobial activities. A total of forty eight constituents were identified in the leaves (77.9%) and stems (87.0%) oil which were characterized by high proportions of phenylpropanoid, safrole (17.1 - 25.5 %). Stems oil showed the highest inhibitory activity towards lipid peroxidation (114.9 ± 0.9 %), compared to BHT (95.5 ± 0.5 %),

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* *Corresponding Author*
FAREDIAH AHMAD, Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia. Email: farediah [at] kimia.fs.utm.my

while leaves oil showed significant total phenolic content (27.4 ± 0.5 mg GA/g) equivalent to gallic acid. The leaves oil showed strong MIC value, 62.5 µg/mL towards *Escherichia coli* while the stems oil exhibited MIC value 125 µg/mL against *Staphylococcus aureus* and *Escherichia coli* (Salleh *et al.,* 2011). In this article we aim to report detailed phytochemical study and antibacterial activity of this species collected from Sarawak, Malaysia.

MATERIAL AND METHODS

Plant materials

Sample of *Piper caninum* were collected from Sarawak, Malaysia, in July 2010. This species was identified by Mohizar Bt Mohamad from the Forest Research Centre, Kuching, Sarawak and the voucher specimen (UiTMKS3003) was deposited at Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

General experimental procedures

Melting points were determined using a Leica Gallen III hot stage melting point apparatus, and were uncorrected. Ultraviolet (UV) spectra were recorded on Shimadzu UV-VIS 1601PC spectrophotometer in methanol. Infrared (IR) spectra were recorded on Perkin Elmer 1650 FTIR spectrophotometer as KBr disc and thin film. The ^{1}H NMR spectra were recorded on Bruker Avance 300 and 400 Spectrometer measured at 300 and 400 MHz and the ^{13}C NMR spectra were measured at 75 and 100 MHz, respectively. Deuterated solvents of chloroform ($CDCl_3$), methanol (CD_3OD) and acetone (CD_3COCD_3) were used as solvents. Mass spectra data were obtained from UCL Spectrometry Services, United Kingdom. Vacuum liquid chromatography (VLC) was performed using Merck silica gel 230-400 mesh, while column chromatography (CC) was performed using Merck silica gel 70-230 mesh. Preparative thin layer chromatography was prepared using silica gel 60 PF$_{254}$. Thin layer chromatography (TLC) aluminum sheets precoated with silica gel 60 F$_{254}$ (0.2 mm thickness) was used to detect and monitor components presence in the crude samples or fractions. The spots were visualized under UV light at 254 and 365 nm, incorporated with spraying reagent vanillin-sulphuric acid. Organic solvents were used for extraction and purification of the extracts. The organic solvents were *n*-hexane, EtOAc and MeOH.

Extraction and isolation of compounds

Soxhlet extraction of the powdered stems (500 g) of *P. caninum*, sequentially with *n*-hexane, EtOAc and MeOH yielded the crude *n*-hexane (7.12 g, 2.37%), EtOAc (9.24 g, 3.70%) and MeOH (8.65 g, 3.46%) extracts, respectively. The EtOAc extract (8.00 g) was fractionated by VLC on silica gel 60 (230–400 mesh) and eluted with *n*-hexane and EtOAc to afford 8 fractions (PCH1-PCH8). Fractions PCEA4-PCEA6 were combined and subjected to CC to yield (**1**). The MeOH extract (140.0 g) was subjected to VLC on silica and eluted with *n*-hexane, CHCl$_3$ and EtOAc. Fractions with similar TLC profiles were combined to form four

fractions (PCM1-PCM4). Purification of PCM 2 by washing with *n*-hexane yielded (**2**) (350.10 mg, 4.92%) as white crystalline needles. PCM 4 was washed with *n*-hexane to yield (**3**). Fraction PCM6-PCM8 was further subjected to CC on silica gel and eluted with the gradient solvent system of *n*-hexane and EtOAc to yield (**4**). Fraction PCM9-PCM11 was further subjected to CC on silica gel and eluted with the gradient solvent system of CHCl$_3$ and EtOAc to yield (**5**). Fraction PCM13-PCM15 was subjected to CC on silica gel and eluted with gradient solvent system of CHCl$_3$ and EtOAc to yield (**6**). The *n*-hexane extract (7.00 g) was fractionated by VLC on silica gel 60 (230–400 mesh) and eluted with *n*-hexane and CHCl$_3$ to afford 6 fractions (PCH1-PCH6). Fractions PCH2-PCH3 (1.52 g) were combined and purified by CC to yield compound (**7**). Fractions PCH4-PCH5 (1.52 g) were combined and separated by CC on silica gel 70–230 mesh and eluted with *n*-hexane and CHCl$_3$ to give (**8**).

Antibacterial activities
Disc diffusion assay

The test microorganisms used were *Staphylococcus aureus* (ATCC29737), *Bacillus subtilis* (ATCC6633), *Pseudomonas aeruginosa* (ATCC9027), *Pseudomonas putida* (ATCC49128) and *Escherichia coli* (ATCC10536). The strains were grown on Nutrient broth (Oxoid, Italy). Antimicrobial activity of the extracts/compounds of *P. caninum* was carried out by the disc diffusion method (Murray *et al.,* 1999; Salleh *et al.,* 2014a). The extracts/compounds were dissolved in DMSO (1 mg/mL). Inocolumn of 400 µL suspension containing 10^8 CFU/mL of bacteria was spread on the nutrient agar (NA) medium. The discs (6 mm diameter) impregnated with 10 µL of the extract/compound and DMSO (negative control) was placed on the inoculated agar and incubated for 24 h at 37°C. Streptomycin sulfate (10 µg/mL) was used as the positive control for bacteria. Clear inhibition zones around the discs indicated the positive antimicrobial activity. All tests and analysis were carried out in triplicates.

Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by broth micro dilution method using 96-well microplates (Gulluce *et al.*, 2004; Salleh *et al.*, 2014b). The inocula of the microbial strains were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Extract/compound (1 mg) was dissolved in DMSO (1 mL) to get 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to well from row B to H. The stock solutions of samples (100 µL) were added to wells at row A and B. Then, the mixture of samples and sterile broth (100 µL) at row B were transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.63 and 7.81 µg/mL). The inoculated bacteria (100 µL) were added to each well. The final volume in each well
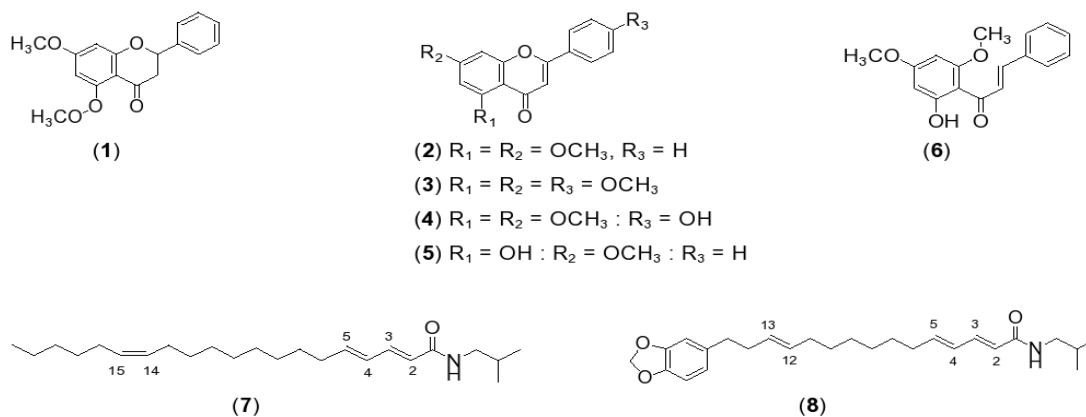
Fig. 1: Structure of compounds (1-8) isolated from *P. caninum*.

Table 1: Antimicrobial activity of the extracts and isolated compounds of *P. caninum*.

Microbes		*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Pseudomonas putida*	*Escherichia coli*
Extracts						
n-Hex	DD	ni	ni	ni	ni	ni
	MIC	>1000	>1000	>1000	>1000	>1000
EtOAc	DD	ni	ni	ni	ni	ni
	MIC	>1000	>1000	>1000	>1000	>1000
MeOH	DD	8.4 ± 0.5	8.5 ± 0.4	ni	ni	7.5 ± 0.4
	MIC	1000	1000	>1000	>1000	1000
Compounds						
(1)	DD	ni	ni	10.1 ± 0.4	ni	ni
	MIC	>1000	>1000	1000	>1000	>1000
(2)	DD	11.2 ± 0.3	ni	ni	ni	11.8 ± 0.5
	MIC	1000	>1000	>1000	>1000	500
(3)	DD	10.1 ± 0.5	ni	ni	ni	ni
	MIC	1000	>1000	>1000	>1000	>1000
(4)	DD	10.5 ± 0.5	ni	10.2 ± 0.3	9.5 ± 0.3	9.8 ± 0.4
	MIC	500	>1000	1000	1000	1000
(5)	DD	8.2 ± 0.4	ni	ni	ni	ni
	MIC	250	>1000	>1000	>1000	500
(6)	DD	9.1 ± 0.5	9.0 ± 0.4	8.2 ± 0.5	7.8 ± 0.3	ni
	MIC	250	250	250	250	>1000
(7)	DD	ni	ni	10.2 ± 0.6	ni	9.4 ± 0.4
	MIC	>1000	500	250	>1000	250
(8)	DD	10.4 ± 0.5	ni	ni	ni	ni
	MIC	125	500	>1000	1000	1000
SS	DD	16.4 ± 0.2	16.8 ± 0.2	16.2 ± 0.2	16.4 ± 0.2	16.5 ± 0.2
	MIC	7.8	7.8	7.8	7.8	7.8

Data represent mean ±SD of three independent experiments; DD – disc diffusion (zone of inhibition including the diameter of disc: 6 mm); MIC – Mininum inhibitory concentration (µg/mL); SS – streptomycin sulphate; ni – no inhibition

was 200 µL. Streptomycin sulfate were used as the positive control. Plates were incubated at 37°C for 24 h. Microbial growth was indicated by the turbidity and the presence of pellet at the bottom of the well.

Statistical analysis

Data obtained from antibacterial activities were stated in mean ±SD. Analysis of variance was performed by ANOVA procedures (SPSS 14.0 for Windows). Data were considered statistically different at $p < 0.01$.

RESULTS AND DISCUSSION

Eight compounds have been isolated from the stems of *P. caninum* (**Figure 1**). They are identified as 5,7-dimethoxy flavanone (**1**), 5,7-dimethoxyflavone (**2**), 4',5,7-trimethoxyflavone

(**3**), 4'-hydroxy-5,7-dimethoxyflavone (**4**), 5-hydroxy-7-methoxyflavone (**5**), 2'-hydroxy-4',6'-dimethoxy chalcone (**6**), N-isobutyl-(2E,4E,14Z)-eicosatrienamide (**7**) and *N*-isobutyl-15-(3',4'-methylenedioxyphenyl)-2E,4E,12Zpentadecatrienamide (**8**). The structural elucidation of these compounds was based on their spectroscopic data and by comparison of these data with the literatures (Kikuzaki *et al.,* 1993; Ahmad *et al.,* 1995; Sirat *et al.,* 1996; Ahmad *et al.,* 1997; Chavi *et al.,* 2007; Xuan *et al.,* 2008; James *et al.,* 2009; Rajudin *et al.,* 2010). Many of these flavonoids were isolated previouly from *Piper* genus. Compound (**1**), (**2**), (**3**), and (**6**) have been isolated from *P. porphyrophyllum*, *P. ungaromense*, *P. methysticum*, whilst compound (**5**) has been isolated from *P. sylvaticum*, compound (**7**) from *P. chaba*, *P. methysticum*, *P. retrofractum*, compound (**8**) from *P. ridleyi* and compound (**4**) isolated for the first time from *Piper* species (Haensel *et al.,* 1963; Banerji and Das, 1977; Ahmad *et al.,* 2002;

Wu *et al.,* 2002; Morikawa *et al.,* 2004; Matsuda *et al.,* 2008). All the flavonoid compounds were also reported from plants of various families such as Zingiberaceae, Winteraceae, Asteraceae and Lamiaceae. The antibacterial activities of the extracts/compounds are showed in **Table 1**. The extracts which showed 7.0-10.0 mm inhibition zone were considered weak towards all the tested bacteria. The MeOH extract was active against *B. subtilis*, *S. aureus* and *E. coli.* In general, the Gram-positive bacteria showed better antimicrobial activity rather than the Gram-negative bacteria. Gram-positive bacteria, *B. subtilis* and *S. aureus*, were the most susceptible to the extracts, with inhibition zone of 8-10 mm. Gram-negative bacteria were the most resistant to the extracts with inhibition zones between 7-8 mm diameters.

These results are similar to those of previous reports in the literature, indicating that Gram-negative bacteria are more resistant than Gram-positive bacteria due to the thickness of its outer membrane (Randrianarivelo *et al.,* 2009). Antibacterial screening of the pure compounds showed that they gave greater inhibition zone towards *B. subtilis* except (**7**) and (**1**), which showed no inhibition towards these bacteria. Compound (**4**) and (**6**) inhibited four out of five tested microorganisms. Compound (**4**) was active against the Gram-positive bacteria, *B. subtilis* and all the Gram-negative bacteria whereas (**6**) was found to be active towards *B. subtilis, S. aureus, P. aeruginosa* and *P. putida*. The MIC values of the isolated compounds were in the range of 125-1000 µg/mL. The significant antibacterial activity was shown by (**7**) towards *B. subtilis* with MIC value 125 µg/mL. Compound (**2**) is the major compounds in this species and has been isolated previously from the same species collected from Tanah Rata, Cameron Highland, Pahang, Malaysia (Ahmad *et al.,* 1997). It has been shown to be a potential chemopreventive agent in human cancer originating from the liver, mouth, esophagus and lung (Wen *et al.,* 2005).

Most of the flavonoids isolated from this species are methoxylated flavones which may have the ability to increase metabolic stability (Tsuji *et al.,* 2006). Compound (**7**) was found to have hepatoprotective effect on D-galactosamine (D-GaIN)-induced cytotoxicity in primary cultured mouse hepatocytes (Matsuda *et al.,* 2008). Compound (**5**) had shown chemokine and chemotactic agent (Liao *et al.,* 2012).

5,7-Dimethoxyflavanone (1)

White crystals, (10.32 mg, 2.58%); m.p 98-99°C; IR (KBr) υ_{max} cm^{-1}: 2937, 1673, 1602, 1570, 1486, 1241; ^{1}H NMR (400 MHz, CDCl$_3$): δ 2.84 (1H, dd, J = 13.0 and 3.3 Hz, H-3a), 3.07 (1H, dd, J = 13.0 and 10.3 Hz, H-3b), 3.83 (3H, s, 7-OCH$_3$), 3.91 (3H, s, 5-OCH$_3$), 5.44 (1H, dd, J = 10.3 and 3.3 Hz, H-2), 6.11 (1H, d, J = 2.4 Hz, H-6), 6.18 (1H, d, J = 2.4 Hz, H-8), 7.36 (5H, m, Ph); ^{13}C NMR (100 MHz, CDCl$_3$): δ 45.5 (C-3), 55.5 (7-OCH$_3$), 56.1 (5-OCH$_3$), 79.2 (C-2), 93.1 (C-8), 93.5 (C-6), 106.0 (C-10), 126.1 (C-2′, C-6′), 128.6 (C-4′), 128.7 (C-3′, C-5′), 138.7 (C-1′), 162.3 (C-9), 164.9 (C-5), 166.0 (C-7), 189.2 (C-4); EIMS *m/z* (rel.int.): 284 [M$^+$, C$_{17}$H$_{16}$O$_4$] (11), 207 (5), 195 (100), 180 (16), 149 (37).

5,7-Dimethoxyflavone (2)

White crystalline needles, (350.1 mg, 4.92%); m.p 158-159°C; IR (KBr) υ_{max} cm^{-1}: 2921, 1643, 1618, 1603, 1452, 1121; ^{1}H NMR (400 MHz, CDCl$_3$): δ 3.92 (3H, s, 7-OCH$_3$), 3.96 (3H, s, 5-OCH$_3$), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.58 (1H, d, J = 2.0 Hz, H-6), 6.68 (1H, s, H-3), 7.50 (3H, m, H-3′, H-4′, H-5′), 7.87 (2H, dd, J = 8.0 and 3.6 Hz, H-2′, H-6′); ^{13}C NMR (100 MHz, CDCl$_3$): δ 55.7 (5-OCH$_3$), 56.4 (7-OCH$_3$), 92.8 (C-6), 96.1 (C-8), 109.0 (C-3), 109.3 (C-4a), 125.9 (C-2′, C-6′), 128.9 (C-3′, C-5′), 131.1 (C-4′), 131.5 (C-1′), 159.9 (C-2), 160.6 (C-5), 160.9 (C-7), 164.0 (C-8a), 177.5 (C-4); EIMS (% rel int.): *m/z* 282 [M$^+$, C$_{17}$H$_{14}$O$_4$] (100), 253 (38), 224 (14), 209 (27), 150 (21).

4',5,7-Trimethoxyflavone (3)

Colourless crystalline needles, (220.58 mg, 3.08%); m.p 154-155°C; IR (KBr) υ_{max} cm^{-1}: 3019, 2923, 1642, 1599, 1567, 1258; ^{1}H NMR (400 MHz, CDCl$_3$): δ 3.89 (3H, s, 4'-OCH$_3$), 3.92 (3H, s, 7-OCH$_3$), 3.96 (3H, s, 5-OCH$_3$), 6.38 (1H, d, J = 2.4 Hz, H-8), 6.56 (1H, d, J = 2.4 Hz, H-6), 6.62 (1H, s, H-3), 7.02 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 7.84 (2H, d, J = 8.8 Hz, H-2′ and H-6′); ^{13}C NMR (100 MHz, CDCl$_3$): δ 55.4 (5-OCH$_3$), 55.7 (7-OCH$_3$), 56.3 (4′-OCH$_3$), 92.8 (C-6), 96.0 (C-8), 107.6 (C-3), 109.2 (C-4a), 114.3 (C-3′, C-5′), 123.8 (C-1′), 127.6 (C-2′, C-6′), 159.8 (C-4′), 160.7 (C-8a), 160.9 (C-5), 162.0 (C-2), 163.9 (C-7), 177.6 (C-4); EIMS (% rel int.): *m/z* 312 [M$^+$, C$_{18}$H$_{16}$O$_5$] (100), 283 (35), 266 (42), 239 (16), 180 (2).

4'-Hydroxy-5,7-dimethoxyflavone (4)

White crystalline needles, (15.24 mg, 4.35%); m.p 288-289°C; IR (KBr) υ_{max} cm^{-1}: 3084, 2947, 1679, 1584, 1508, 1266; UV (MeOH) λ_{max} nm (45 to 65 nm): 328 (band II), 370 (band I), 45-65 nm (addition AlCl$_3$/HCl); ^{1}H NMR (400 MHz, CD$_3$OD): δ 3.92 (3H, s, 5-OCH$_3$), 3.95 (3H, s, 7-OCH$_3$), 6.53 (1H, d, J = 2.4 Hz, H-8), 6.79 (1H, d, J = 2.4 Hz, H-6), 6.59 (1H, s, H-3), 6.92 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 7.85 (2H, d, J = 8.8 Hz, H-2′ and H-6′); ^{13}C NMR (100 MHz, CD$_3$OD): δ 55.1 (2 × OCH$_3$), 92.8 (C-6), 95.94(C-8), 105.3 (C-3), 105.3 (C-4a), 115.5 (C-3′, C-5′), 121.7 (C-1′), 127.7 (C-2′, C-6′), 159.8 (C-4′), 160.6 (C-8a), 162.5 (C-5), 161.0 (C-2), 165.0 (C-7), 178.7 (C-4); EIMS *m/z* (rel.int.): 298 [M$^+$, C$_{17}$H$_{14}$O$_5$] (8), 218 (5), 149 (12), 109 (11), 97 (13).

5-Hydroxy-7-methoxyflavone (5)

Colourless crystalline needles, (12.12 mg, 2.69%); m.p 162-163°C; IR (KBr) υ_{max} cm^{-1}: 3445, 2973, 1614, 1541, 1448, 1252; UV (MeOH) λ_{max} nm (46 to 68 nm): 270 (band II) and 340 (band I), 46-68 nm (addition AlCl$_3$/HCl); ^{1}H NMR (400 MHz, CDCl$_3$): δ 3.90 (3H, s, 7-OCH$_3$), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.53 (1H, d, J = 2.0 Hz, H-6), 6.69 (1H, s, H-3), 7.56 (3H, m, H-3′, H-4′, H-5′), 7.90 (2H, dd, J = 2.0 and 1.6 Hz, H-2′, H-6′), 12.74 (1H. s, 5-OH); ^{13}C NMR (100 MHz, CDCl$_3$): δ 55.8 (7-OCH$_3$), 92.7 (C-6), 98.1 (C-8), 105.7 (C-3), 105.9 (C-4a), 126.3 (C-2′, C-6′), 129.0 (C-3′, C-5′), 131.3 (C-4′), 131.8 (C-1′), 157.8 (C-2),

162.2 (C-5), 164.0 (C-7), 165.6 (C-8a), 182.5 (C-4); EIMS (% rel int.): m/z 268 [M$^+$, C$_{16}$H$_{12}$O$_4$] (100), 239 (16), 225 (13), 149 (27), 111 (10).

2'-Hydroxy-4',6'-dimethoxychalcone (6)

Yellow rhombics, (10.83 mg, 2.40%); m.p 87-89°C; IR (KBr) υ_{max} cm^{-1}: 3445, 2920, 2850, 1623, 1508, 1440, 1260; UV (MeOH) λ_{max} nm: 338 nm; ^{1}H NMR (400 MHz, CDCl$_3$): δ 3.85 (3H, s, 4'-OCH$_3$), 3.93 (3H, s, 6'-OCH$_3$), 5.98 (1H, d, J = 2.4 Hz, H-5'), 6.12 (1H, d, J = 2.4 Hz, H-3'), 7.41 (3H, m, H-3, H-4, H-5), 7.61 (2H, m, H-2, H-6), 7.78 (1H, d, J = 15.6 Hz, H-7), 7.90 (1H, d, J = 15.6 Hz, H-8), 14.30 (1H, s, 2'-OH); ^{13}C NMR (100 MHz, CDCl$_3$): δ 55.5 (4'-OCH$_3$), 55.8 (6'-OCH$_3$), 91.0 (C-5'), 94.2 (C-3'), 105.6 (C-7), 126.3 (C-1), 127.5 (C-3, C-5), 128.3 (C-2, C-6), 128.9 (C-1'), 130.0 (C-4), 142.4 (C-8), 161.1 (C-6'), 166.5 (C-4'), 169.0 (C-2'), δ 193.1 (C-2); EIMS m/z (rel. int.): 284 [M$^+$, C$_{17}$H$_{16}$O$_4$] (57), 283 (50), 207 (5), 208 (12), 207 (100).

N-isobutyl-(2E,4E,14Z)-eicosatrienamide (7)

Colourless crystalline needles (6.50 mg, 2.60%); m.p 60-61°C; IR (KBr) υ_{max} cm^{-1}: 3301, 2917, 2851, 1654, 1609; ^{1}H NMR (400 MHz, CDCl$_3$): δ 0.92 (3H, t, J = 6.8 Hz, H-20), 0.87 (6H, d, J = 6.8 Hz, H-3', H-4'), 1.26-1.33 (16H, m, H-7 – H-12, H-17 – H-19), 1.81 (1H, septet, J = 6.8 Hz, H-2'), 2.03 (2H, m, H-13, H-16), 3.17 (2H, t, J = 6.4 Hz, H-1'), 5.35 (2H, dt, J = 9.2 and 5.6 Hz, H-14, H-15), 5.47 (1H, br. s, N-H), 5.77 (1H, d, J = 15.2 Hz, H-2), 6.07 (1H, dt, J = 16.4 and 6.0 Hz, H-5), 6.13 (1H, dd, J = 16.4 and 9.6 Hz, H-4), 7.20 (1H, dd, J = 15.2 and 9.6 Hz, H-3); ^{13}C NMR (100 MHz, CDCl$_3$): δ 14.0 (C-20), 20.0 (C-3', C-4'), 22.6 (C-19), 26.8 (C-12), 26.8 (C-16), 27.1 (C-11), 28.6 (C-2'), 28.6 (C-10), 28.7 (C-9), 29.1 (C-8), 29.3 (C-7), 29.6 (C-17), 31.9 (C-18), 32.9 (C-6), 46.9 (C-1'), 121.6 (C-2), 128.1 (C-4), 129.7 (C-14), 129.8 (C-15), 141.3 (C-5), 143.1 (C-3), 166.3 (C-1); EIMS (% rel int.): m/z 361 [M$^+$, C$_{24}$H$_{43}$NO] (11), 333 (18), 203 (35), 202 (100), 152 (32).

N-isobutyl-15-(3',4'- methylenedioxyphenyl)- 2E,4E,12Z-pentadecatrienamide (8)

Colourless crystalline needles, (7.20 mg, 2.57%); m.p 95-96°C; IR (KBr) υ_{max} cm^{-1}: 3300, 1655, 1625, 1611, 1505, 1487, 1250, 1190; ^{1}H NMR (400 MHz, CDCl$_3$): δ 0.87 (6H, d, J = 6.8 Hz, H-3'', H-4''), 1.28 (4H, m, H-8, H-9), 1.42 (4H, m, H-7, H-10), 1.79 (1H, septet, J = 6.4 Hz, H-2''), 1.96 (2H, m, H-11), 2.15 (2H, m, H-6), 2.25 (2H, quartet, J = 6.8 Hz, H-14), 2.59 (2H, t, J = 6.8 Hz, H-15), 3.17 (2H, t, J = 6.4 Hz, H-1''), 5.42 (2H, dt, J = 7.2 Hz, H-12, H-13), 5.48 (1H, br.s, N-H), 5.73 (1H, d, J = 15.2 Hz, H-2), 5.92 (2H, s, OCH$_2$O), 6.09 (1H, dt, J = 13.6 and 7.2 Hz, H-5), 6.13 (1H, dd, J = 13.6 and 10.0 Hz, H-4), 6.61 (1H, d, J = 8.0 Hz, H-5'), 6.68 (1H, d, J = 1.2 Hz, H-2'), 6.71 (1H, dd, J = 8.0 and 1.2 Hz, H-6'), 7.19 (1H, dd, J = 15.2 and 10.0 Hz, H-3); ^{13}C NMR (100 MHz, CDCl$_3$): δ 20.1 (C-4''), 20.1 (C-3''), 28.6 (C-2''), 28.7 (C-9), 28.8 (C-10), 28.9 (C-7), 29.3 (C-8), 32.4 (C-6), 32.9 (C-11), 34.6 (C-14), 35.8 (C-15), 46.9 (C-1''), 100.6 (OCH$_2$O), 108.0 (C-

2'), 108.9 (C-5'), 121.1 (C-6'), 121.7 (C-2), 128.2 (C-4), 129.2 (C-12), 131.1 (C-13), 136.0 (C-1'), 141.3 (C-5), 143.1 (C-3), 145.4 (C-3'), 147.4 (C-4'), 166.3 (C-1); EIMS (% rel int.): m/z 411 [M$^+$, C$_{26}$H$_{37}$NO$_3$] (7), 276 (5), 152 (8), 136 (9), 135 (100).

CONCLUSIONS

Phytochemical investigation from the stems of *P. caninum* furnished six methoxylated flavonoids including flavanone, flavones and chalcone together with two long chain isobutyl amides. Thus, flavonoids may be considered potential therapeutic compounds for infections that may be caused by these pathogenic bacteria in the future. Additionally, the antimicrobial activity of some antibacterials in combination with flavonoids against the pathogenic bacteria may also need to be evaluated for the treatment of infections.

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