

Tocopherols, Polyprenols and Steroids from *Passiflora edulis* and Bioactivities of its Extractives

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ABSTRACT: β -tocopherol (1), δ -tocopherol (2), polyprenol-12 (3), polyprenol-15 (4), stigmasterol and β -sitosterol were isolated from the yellow *flavicarpa* variety of leaves of *Passiflora edulis*. The structures of the isolated compounds were elucidated using ¹H-NR and ¹³C-NMR spectral analysis. The organic and aqueous soluble fractions of crude methanolic extract were evaluated for the antioxidant, cytotoxic, thrombolytic and antimicrobial activities. In DPPH free radical scavenging assay, the aqueous soluble fraction displayed maximum activity having IC₅₀ value of 139.56 μ g/ml. On the other hand, dichloromethane soluble fraction revealed maximum cytotoxic (LC₅₀ 24.17 μ g/ml) and thrombolytic (14.49% clot lysis) activities, when compared to the respective blanks.

Key words: *Passiflora edulis*, chromatography, tocopherol, polyprenol, DPPH, antioxidant, thrombolysis

INTRODUCTION

Passiflora edulis Sims (Family: Passifloraceae, Bengali name: Tang Phal; English name: Passion fruit) is a vine species of passion flower that is native to Brazil, Paraguay and Northern Argentina¹. Different parts of the plant have been used as medicines for many years. The leaves are reported to contain polyphenols, which act as natural antioxidant.² The aqueous extract of leaves has potent anti-inflammatory action.³ Several species of *Passiflora* have been employed widely as folk medicines because of sedative and tranquillizing activities.⁴ Traditionally, it has also been used as antihypertensive agent.⁵ It has also cholesterol and lipid lowering effects.⁶ Decoction of fruits of *P. edulis* inhibits metallo-proteases which are involved in the tumor invasions, metastasis and angiogenesis.⁷ As part of our continuing phytochemical research we studied bright yellow *flavicarpa* variety of *P. edulis* and herein, we report six compounds (1-6) from the methanolic extract of its leaves. The antioxidant,

thrombolytic, antimicrobial and other bioactivities of different fractions are also reported here.

MATERIALS AND METHODS

General experimental procedures. NMR spectra were recorded using Bruker (400 MHz) instrument in deuterated chloroform (CDCl₃) and the δ values are reported relative to the residual non-deuterated solvent signal. Buchi Rotavapor (Germany) was used for solvent evaporation. Vacuum liquid chromatography (VLC) and gel permeation chromatography (GPC) were performed on Kieselgel 60H (mesh 70-230) and Sephadex (LH-20). Analysis of the compounds were carried out over precoated thin layer chromatography plates (Silica gel 60 F₂₅₄, Merck). For the visualization of the spots on TLC plates, UV light and vanillin/H₂SO₄ reagents were used. All the other chemicals used in the research were of analytical grade.

Collection of plant sample. The leaves of yellow *flavicarpa* variety of *Passiflora edulis* Sims were collected from Barisal area, in the month of April, 2015 and it was identified in Bangladesh

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National Herbarium, where a voucher specimen (DACB number-42018) for this collection has been preserved for future reference.

Extraction and isolation. The air dried and powdered leaves (700 g) of *P. edulis* were soaked in 2.5 liters of methanol in an amber glass container. It was sealed by cotton plug and then kept for period of two weeks with occasional shaking and stirring. After two weeks, whole mixture was filtered off through a filter paper and the filtrate thus obtained was concentrated at 40°C using Buchi Rotavapor (Germany). Finally 34.17g (4.88%) of dried methanolic extract was obtained.

A portion of the crude extract (25 g) was then subjected to vacuum liquid chromatography (VLC) over silica gel.⁸ First the column was eluted using 100% petroleum then the polarity of the solvent was increased by adding dichloromethane and then ethyl acetate until it reached to 100% dichloromethane and ethyl acetate. Then the polarity of the solvent was further increased by adding methanol until it was reached to 100%. A total of 29 VLC fractions were collected. After careful TLC screening VLC fractions obtained with 30% dichloromethane in petroleum ether, 50% dichloromethane in petroleum ether, 100% dichloromethane, 5% ethyl acetate in dichloromethane, 8% ethyl acetate in dichloromethane, 10% ethyl acetate in dichloromethane and 15% ethyl acetate in dichloromethane were further subjected to gel permeation chromatography (GPC) using Sephadex (LH-20) column. The Sephadex column was eluted using 20% petroleum ether in chloroform, 10% petroleum ether in chloroform and 100% chloroform. The polarity was further increased by mixing methanol with chloroform.

A total of 169 sub-fractions of the VLC fraction eluted with 15% ethyl acetate in dichloromethane were collected from the Sephadex column. The sub-fractions were screened by TLC and sub-fractions 27-30 were mixed together from which crystal lineness were collected by washing with n-hexane. The crystal were a mixture of stigmasterol and β -sitosterol. Sub-fractions 38-40 were mixed together and subjected to

PTLC using 2% ethyl acetate in toluene to yield compound **2**. Again, sub-fractions 42-44 were mixed together and subjected to PTLC using 2% ethyl acetate in toluene to yield compound **1**.

VLC fraction eluted with 8% ethyl acetate in dichloromethane was subjected to the Sephadex column and a total of 33 sub-fractions were obtained. PTLC of sub-fractions 16-20 using 1% ethyl acetate in toluene provided compound **3** and **4**.

Preparation of different partitionates for biological tests. Crude methanolic extract (5 g) was fractionated by the modified Kupchan partitioning portocol.⁹ The resultant partitionates were evaporated to dryness to yield petroleum ether soluble fraction (PESF, 1.7 g), dichloromethane soluble fraction (DCMSF, 0.4 g), chloroform soluble fraction (CSF, 1.2 g), aqueous soluble fractions (AQSF, 1.1 g) and methanolic extract (0.6 g).

DPPH free radical scavenging assay. The antioxidant activity of different test samples was determined by evaluating the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by using standard antioxidant ascorbic acid as reference standard.¹⁰

Brine shrimp lethality bioassay. The general toxic properties of the dimethylsulfoxide (DMSO) solution of the plant extractives against *Artemia salina* were estimated in a 24 hrs assay by using vincristine sulphate as positive control.¹¹

Thrombolytic activity. The thrombolytic activity was assessed following the method developed by Prasad and Harbertson,¹² by using streptokinase as positive control.

Antimicrobial screening. Disc diffusion method¹³ was used to determine the antimicrobial activity of different partitionates of the crude extract.

Statistical analysis. For all bioassays, three replicates of each sample were used for statistical analysis and the values have been reported as mean \pm SD.

RESULT AND DISCUSSION

Six compounds were isolated from the crude methanol extract of leaves of *P. edulis* by using repeated chromatographic separations over the silica gel. The structure of isolated compounds were elucidated as β -tocopherol (**1**)^{14,15}, δ -tocopherol (**2**)^{15,16}, polyprenol-12 (**3**)¹⁷, polyprenol-15 (**4**)¹⁷, stigmasterol^{18,19} and β -sitosterol^{18,19} by analyzing the NMR spectral data and comparing those data with published values. In some cases, co-TLC was done with the reference sample which also ensure the identity of the compounds.

β -tocopherol (*Syn.* (2R)-2,5,8-Trimethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-3,4-dihydrochromen-6-ol) (**1**): yellow viscous liquid and soluble in ethyl acetate and chloroform; ¹H NMR (400 MHz, CDCl₃): δ 0.85 (3H, m, Me-4'), 0.85 (3H, m, Me-8'), 0.86 (6H, d, $J=6.8$ Hz, Me-12'), 1.22 (3H, s, Me-2), 1.74 (1H, m, H-3), 1.79 (1H, m, H-3), 2.08 (3H, s, Me-5), 2.10 (3H, s, Me-8), 2.59 (2H, t, $J=6.8$ Hz, H-4), 6.47 (1H, s, H-7); ¹³C NMR (100 MHz, CDCl₃): δ 11.0 (Me-5), 15.9 (Me-8), 19.8 (Me-4'8'), 20.8 (C-4), 21.0 (C-2'), 22.7 (Me-12'), 23.9 (Me-2), 24.5 (C-10'), 24.8 (C-6'), 28.0 (C-12'), 31.5 (C-3), 32.7 (C-8'), 32.8 (C-4'), 37.3 (C-9'), 37.4 (C-7'), 37.5 (C-3',5'), 39.4 (C-11'), 40.1 (C-1'), 74.5 (C-2), 115.3 (C-7), 119.2 (C-5), 120.4 (C-10), 124.1 (C-8), 145.7 (C-9), 146.0 (C-6).

δ -tocopherol (*Syn.* (2R)-2,8-dimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-6-chromanol) (**2**): brownish yellow liquid and soluble in ethyl acetate and chloroform; ¹H NMR (400 MHz, CDCl₃): δ 0.85 (3H, m, Me-4'), 0.85 (3H, m, Me-8'), 0.86 (6H, d, $J=6.8$, Me-12'), 1.23 (3H, s, Me-2), 1.72 (1H, m, H-3), 2.11 (3H, s, Me-8), 2.67 (2H, t, H-4), 6.37 (1H, br. s, H-5), 6.47 (1H, br. s, H-7).

Polyprenol-12 (*Syn.* tri-*trans* poly-*cis* prenol-12) (**3**): transparent oily liquid, soluble in ethyl acetate and chloroform; ¹H NMR (400 MHz, CDCl₃): δ 1.59 (12H, s, 4 *trans* methyls), 1.67 (24H, s, 8 *cis* methyls), 1.74 (3H, s, Me-3), 2.03 (-CH₂- 4, 5, 8, 9, 12, 13, 16, 17, 20, 21, 24, 25, 28, 29, 32, 33, 36, 37, 40, 41, 44, 45), 4.08 (2H, d, $J=7.2$ Hz, H₂-1), 5.12 (br. s, H-6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46), 5.43 (1H, b, t,

H-2). ¹³C NMR (100 MHz, CDCl₃): δ 59.05 (C-1), 139.94 (C-3), 131.29 (C-47), 39.79 (C of *cis-trans* linkage).

Polyprenol-15 (*Syn.* tri-*trans* poly-*cis* prenol-15) (**4**): transparent oily liquid, soluble in ethyl acetate and chloroform; ¹H NMR (400 MHz, CDCl₃): δ 1.59 (12H, s, 4 *trans* methyls), 1.67 (33H, s, 11 *cis* methyls), 1.73 (3H, s, Me-3), 2.03 (-CH₂- 4, 5, 8, 9, 12, 13, 16, 17, 20, 21, 24, 25, 28, 29, 32, 33, 36, 37, 40, 41, 44, 45, 48, 49, 52, 53, 56, 57), 4.08 (2H, d, $J=7.2$ Hz, H-1), 5.11 (br. s, H-6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58), 5.43 (1H, b, t, $J=7.2$, H-2).

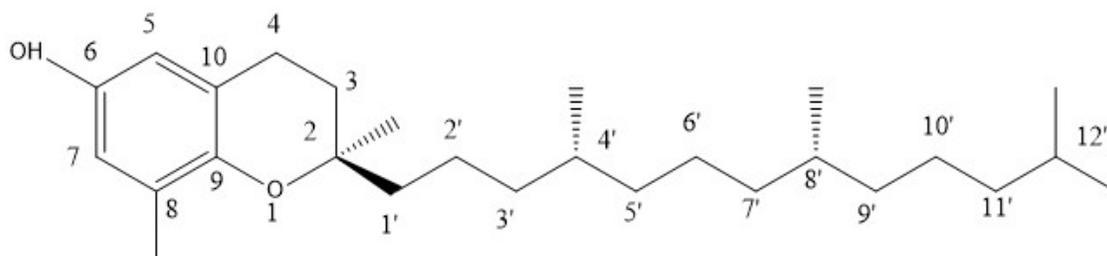
Stigmasterol: ¹H NMR (400 MHz, CDCl₃): δ 0.69 (3H, s, Me-18), 0.79 (3H, d, $J=6.5$ Hz, H-26), 0.81 (3H, t, $J=7.5$ Hz, H-29), 0.85 (3H, d, $J=6.5$ Hz, H-27), 0.99 (3H, s, Me-19), 1.016 (3H, d, $J=7.5$ Hz, H-21), 3.51 (1H, m, H-3), 5.01 (1H, m, H-22), 5.13 (1H, m, H-23), 5.35 (1H, br. s, H-6).

β -sitosterol: ¹H NMR (400 MHz, CDCl₃): δ 0.69 (3H, s, Me-18), 0.81 (3H, d, $J=6.4$ Hz, H-26), 0.83 (3H, d, $J=6.4$ Hz, H-27), 0.85 (3H, t, $J=7.4$ Hz, H-29), 0.916 (3H, d, $J=6.4$ Hz, H-21), 1.01 (3H, s, Me-19), 3.51 (1H, m, H-3), 5.35 (1H, br. s, H-6).

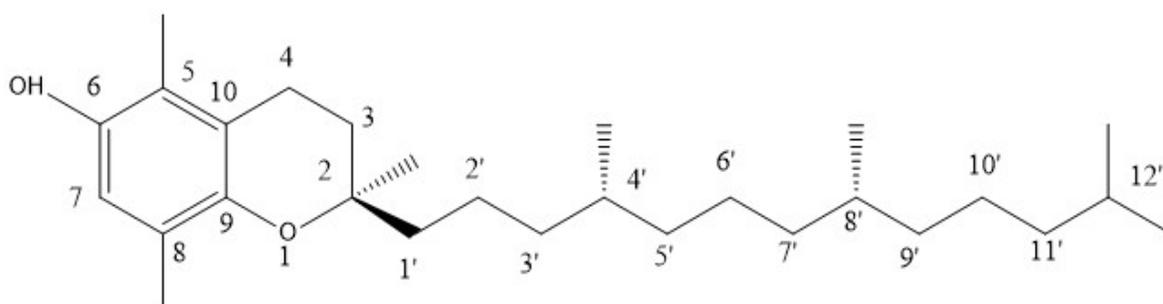
Compound **1** was obtained as yellow viscous liquid. The ¹H NMR spectrum (400 MHz, CDCl₃) of compound **1** showed the presence of three methyl signals at δ 1.22 (3H, s, Me-2), 2.08 (3H, s, Me-5) and 2.10 (3H, s, Me-8) and two characteristic signals at δ 6.47 (1H, s, H-7), δ 2.59 (2H, t, H-4) all of which indicate the presence of chromanering with three methyl substituents. The presence of methyl signals at position δ 0.85 (3H, m, Me-4'), 0.85 (3H, m, Me-8') and 0.86 (3H, m, Me-12') indicate that those methyl groups are in the aliphatic side chain. The ¹³C NMR spectrum (100 MHz, CDCl₃) of the compound **1** showed signals at position δ 11.0 (Me-5), 15.9 (Me-8), 19.8 (Me-4'8'), 20.8 (C-4), 21.0 (C-2'), 22.7 (Me-12'), 23.9 (Me-2), 31.5 (C-3), 37.5 (C-3'), 40.1 (C-1'), 74.5 (C-2), 115.3 (C-7), 119.2 (C-5), 120.4 (C-10), 124.1 (C-8), 145.7 (C-9), 146.0 (C-6) which are in close agreement with the observed NMR data of β -tocopherol.^{14,15}

The ^1H NMR spectrum of the compound **2** showed signals of two methyl groups at δ 1.23 (3H, s, Me-2) and 2.11 (3H, s, Me-8). All the NMR signals of compound **2** matched with the compound **1** except the absence of methyl group at C-5, instead of which it showed a signal at δ 6.37 (1H, br. s, H-5) which indicates the presence of chromane ring with two

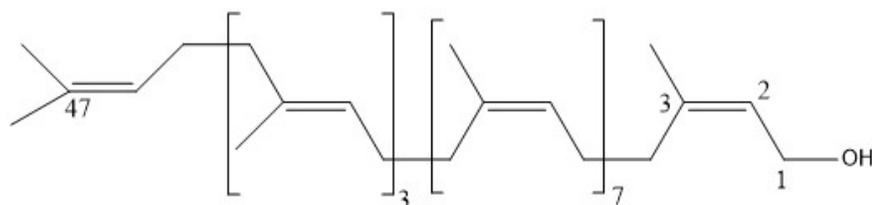
methyl substituents. The methyl signals at position 0.85 (3H, m, Me-4') and 0.86 (3H, m, Me-12') indicate that those methyl groups are in the aliphatic side chain. The NMR data of compound **2** was also compared with the reference values which confirmed that the compound **2** was δ -tocopherol.^{15,16}



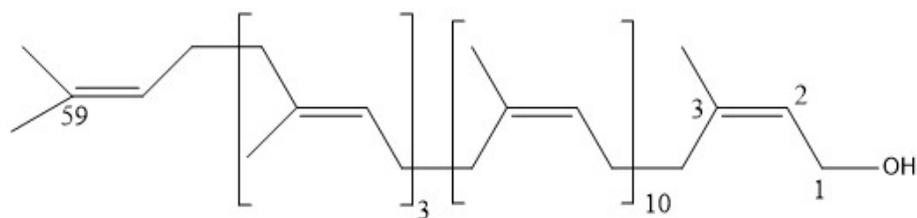
β -tocopherol (**1**)



δ -tocopherol (**2**)



tri-transpoly-cis prenil-12 (**3**)



tri-transpoly-cis prenil-15 (**4**)

Table 1. Free radical scavenging, cytotoxicity and thrombolytic activities of *P. edulis*.

	Sample	DPPH free radical scavenging activity (IC ₅₀ µg/ml)	Cytotoxicity (LC ₅₀ µg/ml)	% Clot lysis
Extract	ME	337.26 ± 2.32	188.56 ± 1.03	17.25 ± 0.39
	PESF	252.45 ± 1.21	92.92 ± 0.83	15.42 ± 0.63
	DCMSF	208.96 ± 2.78	24.17 ± 0.22	14.49 ± 0.53
	CSF	157.14 ± 1.53	39.60 ± 0.37	15.38 ± 0.45
	AQSF	139.56 ± 1.67	74.84 ± 0.87	16.95 ± 0.56
Standard	Ascorbic acid	37.22 ± 0.76	-	-
	VS	-	0.404 ± 0.03	-
	SK	-	-	33.60 ± 1.09

ME= Methanolic extract, PESF= Petroleum ether soluble fraction, DCMSF= Dichloromethane soluble fraction, CSF= Chloroform soluble fraction, AQSF= Aqueous soluble fraction, VS= Vincristine sulphate, SK= Streptokinase (Positive control); Water (Negative control for thrombolytic activity)

The ¹H NMR spectrum of the compound **3** and **4** showed signals at δ 5.43 (1H, br. t, *J*=7.2, H-2) and 4.08 (2H, d, *J*=7.2, H-1). The signal at δ 1.67 was the characteristic peak of *cis*-methyls and δ 1.59 was assigned to all *trans*-methyls. On the other hand, methyl at C-3 gave signal at δ 1.74. The signal at δ 2.03 indicates the methylene hydrogen. ¹³C NMR spectrum (100 MHz, CDCl₃) showed the signal at δ 40.0 which is the *cis-trans*-linkage carbon. All the signals are close agreement with the ¹H NMR and ¹³C NMR signals of polyprenols which were also confirmed with the reference value.¹⁷ Both compounds had same signals but they were different only in integration value which ensured that the compound **3** and **4** was identified as *tri-transpoly-cis* prenol-12 and *di-transpoly-cis* prenol-15 respectively.

Stigmasterol and β-sitosterol were identified after comparing their ¹H NMR spectral data with the reference values.^{18,19}

Bioactivities of the crude extract and its different fractions. In case of DPPH free radical scavenging assay, the aqueous soluble fraction of leaves of *P. edulis* showed moderate free radical scavenging activity having IC₅₀ value of 139.56 ± 1.67 µg/ml, while the standard ascorbic acid showed IC₅₀ value of 37.22 ± 0.76 µg/ml (Table 1).

In the brine shrimp lethality test, the dichloromethane soluble fraction displayed the maximum cytotoxic effect with LC₅₀ value of 24.17 ± 0.22 as compared to 0.404 ± 0.03 for vincristine

sulphate. On the other hand, the dichloromethane soluble fraction of the crude methanol extract showed the highest thrombolytic activity having the percentage clot lysis of 14.49 ± 0.53 where standard streptokinase showed 33.60 ± 1.09% of clot lysis. All the partitionates and methanolic extract were assayed for the antimicrobial activities against different gram-positive and gram-negative bacteria taking antibiotic vancomycin as a standard. None of the samples showed any antimicrobial properties where the standard vancomycin displayed significant antimicrobial activity.

CONCLUSION

The methanolic extract of the leaves of *Passiflora edulis* was investigated for isolation of the secondary metabolites. Successive chromatographic separation and purification yielded a total of six compounds namely β-tocopherol, δ-tocopherol, polyprenol-12, polyprenol-15, stigmasterol and β-sitosterol. In free radical scavenging activity assay, aqueous soluble fraction of *P. edulis* showed the maximum free radical scavenging activity with IC₅₀ value of 139.56 µg/ml. However, the dichloromethane soluble fraction (DCMSF) showed maximum thrombolytic and cytotoxic activities. Our findings justify the traditional uses of the plant. Therefore, considering the isolated compounds and the potential bioactivities of different fractions of the crude extract, this plant can further be studied to find

out the unrevealed scientific findings which might be helpful in medicinal chemistry.

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