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Suppression of cytokine production by newly isolated flavonoids from pepper

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ABSTRACT

New flavonoid glycoside, kaempferol 3-*O*-*α*-[(6-P-coumaroyl galactopyranosyl-*O*-*β*-(→4)-*O*-*α*-rhamnopyranosyl-(1→4)]-O-α-rhamnopyranoside **1**, in addition to five known flavonoid glycosides (**2–6**) kaempferol 3-*O*-[*α*-rhamnopyranosyl-(1→4)-*O*-*α*-rhamnopyranosyl-(1→6)-*O*]-*β*-galactopyranoside (kaempferol 3-*O*-*β*-isorhamninoside) **2**, quercetin 3-*O*-[(2,3,4-triacetyl-α-rhamnopyranosyl)-(1 → 6)-*β*-galactopyranoside **3**, quercetin 3-*O*-[(2,4-diacetyl-α-rhamnopyranosyl)-(1 → 6)]-3,4-diacetyl-*β*-galactopyranoside **4**, quercetin 3-*O*-[(2,4-diacetyl-α-rhamnopyranosyl)-(1→6)]-2,4-diacetyl-*β*-galactopyranoside **5**, quercetin 3-*O*-[(2,3,4-triacetyl-α-rhamnopyranosyl)-(1 → 6)-3-acetyl-*β*-galactopyranoside **6** were isolated from bell pepper (*Capsicum annum* L.) fruits and tested for both anti-inflammatory activity through cytokine production (TNF-α and IL-1β) and antioxidant activity through scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Compounds **1–3** significantly suppressed production of TNF-α / IL-1β in cultured THP-1 cells previously co-stimulated by LPS in a dose-dependent manner (10.2/49.1, 28.1/55.7, and 35.2/57.5 μM respectively) whereas compounds **4–6** have relatively weaker inhibitory activity. (45.3/73.5, 48.2/65.6, and 42.2/67.4 μM respectively). All compounds **1–6** showed no cytotoxic activity against the growth of THP-1where the percentage of cell viability was (127.4, 108.5, 105.4, 103.9, 103.4, and 104.2 μM respectively). All isolated compounds exhibited higher radical scavenging activity than ascorbic acid in (DPPH) assay. These results indicated that bell pepper fruits could be an effective candidate for ameliorating inflammatory-associated complications.

1. Introduction

Because the discovery of new drugs requires a long time and expense, searching for new compounds from natural sources known for their high safety and applicability will be a good avenue to deal with inflammatory-associated complications. From which, bell pepper (*Capsicum annum* L.), a member of the family Solanaceae is the most commonly consumed fruity vegetable well known for its nutritional and antioxidant significance [1]. Bell pepper exhibits medicinal as well as food value all over the world. Both warm and dry climates are suitable for its cultivation [2]. It is a rich source of neutral phenolic compounds, especially luteolin, quercetin, capsaicinoids, nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin, norcapsaicin, and nornorcapsaicin [3,4]. Carotenoids exhibit antioxidant properties and prevent tissue damage by acting as singlet molecular oxygen; reactive oxygen species (ROS); peroxyl radicals and reactive nitrogen species (RNS) scavengers [5]. The utilization of bioactive compounds plays key health-promoting functions such as protecting against oxidative cell damage, cancer insurgence, diabetes prevalence, cardiovascular disorders, Alzheimer's, and Parkinson's disease [6,7] that evoked us to carry out this study.

2. Materials and methods

2.1. General

The optical activity was determined with Horiba SEPA-3000 high-sensitivity polarimeter (Horiba). UV analysis was determined on Shimadzu UV-1600 UV–visible spectrometer, IR analysis was operated on Shimadzu FTIR-8400. NMR analysis was carried out on JEOL GSX-600 spectrometer in CD_3OD. Fast atom bombardment (FABMS) and high-resolution fast atom bombardment (HRFABMS) were carried out on JEOL JMS SX-102 mass spectrometry. Reversed-phase high-performance liquid chromatography (HPLC) was undertaken on an ODS column (particle size: 5 μm, TOSO, 18 × 250 mm) RP-23 (5 μm; Waters). Diaion HP-20 (Mitsubishi) (Tokyo Japan), silica gel (63–210 μm; Kanto Kagaku), and ODS (63–212 μm; Wako Pure Chemical) (Tokyo Japan) were used for open column chromatography. Thin-layer

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chromatography (TLC) was carried out on silica gel (SiO2, 60–100 mesh; Wako Pure Chemical) 60 F254 and RP-18 F254S (Merck).

2.1.1. Extraction and isolation

Air-dried bell pepper fruits (2 kg) were extracted thrice with MeOH (5 l each) to yield methanol extract (310 g) which was partitioned between distilled water, chloroform, ethyl acetate, and n-butanol (1 l each) to yield chloroform fraction (90 g), ethyl acetate fraction (60 g), the n-butanol fraction (50 g) and the rest aqueous fraction (100 g). All fractions were screened for the antioxidant and cytokine production in cultured THP-1 cells activities where the ethyl acetate was the most active fraction and hence, and hence it was fractionated by ODS column using six mobile phase systems of CH_3CN-H_2O (10, 25, 40, 50, 70 and 90% *v*/v; elution volume: 500 ml of each) to give six corresponding subfractions. Subfraction eluted with 40% CH_3CN (3.8 g) was further isolated by silica gel column chromatography with gradient elution by $CHCl_3$:MeOH (ratios of 9:1, 6:1, 4:1, 3:1 and 1:1, *v*/v, elution volume: 200 ml each) to give five corresponding subfractions. The subfraction eluted by 6:1 CHCl3: MeOH was further chromatographed by preparative HPLC, ODS column equipped with a UV detector (210 nm) with mobile phase 20% CH_3CN in H_2O which afforded compounds **3–6**, (15, 18, 22, and 9 mg respectively). These preparative HPLC conditions were also used after gradually increasing the mobile phase to 50% CH_3CN in H_2O to isolate the same fraction to afford compounds **1** and **2**, (13 and 24 mg respectively).

2.1.2. Acid hydrolysis

Acid hydrolysis of the flavonoid glycosides was carried out by refluxing 5 mg of the compound in 5 ml of 6% HCl in MeOH for 3 h. The reaction mixture was partitioned against EtOAc (3 × 10 ml). The aglycones were obtained from the EtOAc layer and identified as kaempferol and quercetin by co-chromatography on silica gel with reference samples (Sigma) (Tokyo, Japan). Identification of galactose and rhamnose present in the sugar fraction was carried out by comparison with authentic samples, galactose (Rf 0.41), glucose (Rf 0.46), and rhamnose (Rf 0.66) (Sigma) (Tokyo, Japan) in TLC on silica gel ($CHCl_3$-MeOH-H_2O 8:5:1) using 5% H_2SO_4 in MeOH as spraying reagent followed by heating the plates at 120°C for 15–20 min.

2.1.3. Measurements of the optical rotation of D-galactopyranose and L-rhamnopyranose tetrabenzoate derivatives

Benzoyl chloride (0.5 ml) was added to each ice-cooled solution of either D–galactopyranose (6.0 mg) or L-rhamnopyranose (4.0 mg) in dry pyridine (1.0 ml) and each mixture was stirred at room temperature for 15 h. MeOH (1.0 ml) was added dropwise to the reaction mixture, stirred for 30 min, and then diluted with EtOAc and aqueous Na_2CO_3, and the layers were separated. Each organic layer was washed with brine and the combined aqueous layers for each was extracted with EtOAc. Each combined organic extract was dried over MgSO4 and concentrated. The corresponding residual dark brown oil fractions were individually purified by silica gel cc (eluted by hexane/EtOAc 5:1) to give either D–galactopyranose tetrabenzoate $[\alpha]_D^{31} + 53.5$ (c= 1.2, $CHCl_3$) or L-rhamnopyranose tetrabenzoate $[\alpha]_D^{29.6} + 75.0$ (c =1.6, $CHCl_3$) as a colorless oil, respectively [8,9].

2.1.4. Evaluation of cytokine production in cultured THP-1 cells and cytotoxic assay

To determine the effect of both fractions and the isolated compounds (1–6) on the production of inflammatory cytokines in monocytes THP-1 cells (Dainippon Pharmaceutical Company), a method modified by (Bornstein et al; 2004 and Nehmé et al.; 2008) was used [10,11].

2.1.5. MTT in vitro assay

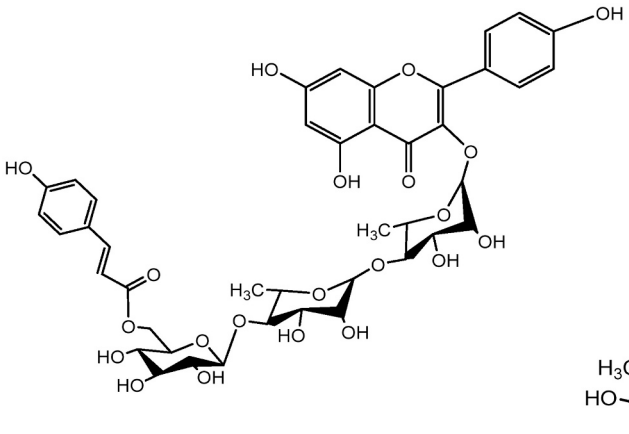
To determine the cytotoxic activity of the tested samples, THP-1 cells (180 µl) were seeded in 96-well plates at 1.0 × 105 cells per well with tested samples (purity >93%) (20 µl in DMSO/ PBS) at various concentrations. After 48-h cultivation, supernatants were removed, non adherent cells (THP-1) incubated with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 10 µl, 5 mg/mL in PBS) for 4 h, and then solubilized with 10% (*w/v*) sodium dodecyl sulfate (SDS; in 60% [*v*/v] dimethyl formamide) solution (100 µl) for 18 h. The absorbance was measured at 570 nm using a microplate reader, and the cytotoxicity was calculated by comparing absorbance with that of the non-treated control culture. The cell growth curve was graphed using statistical analysis software (Kaleida Graph version 4.00; Synergy Software), and IC50 values calculated using simple linear regression. The cytotoxic activity of all of isolates was determined by MTT colorimetric assay (Segun, et al., 2019; Alley et al., 1988) [12,13].

Table 1
^{13}C and ^{1}H NMR assignments for compound 1 recorded in CD_3OD.

Position	^{13}C NMR (δ, mult.)	^{1}H NMR [δ, mult, *J* (Hz)]	Position	^{13}C NMR (δ, mult.)	^{1}H NMR [δ, mult, *J* (Hz)]
2	159.2,s	–	**Galactopyranosyl**		
3	134.1,s	–	1‴	100.5,d	5.62,d,7.5
4	179.4,s	–	2‴	72.4,d[b]	3.82,dd, 9.9,7.5
5	163.1,s		3‴	73.8,d	3.87,dd, 9.9,3.4
6	99.8,d	6.09,d, 2.06	4‴	72.0,d[b]	5.20,d,3.4
7	165.7,s	–	5‴	77.7,d	3.84,m
8	94.7,d	6.29,d, 2.06	6‴	67.1,t	3.41,m, 3.13,m
9	158.3,s	–	***P*-coumaroyl**		
10	105.9,s	–	1⁗	127.6,s	–
1′	123.1,s	–	2⁗	116.0,d	6.66,2H, d,8.0.9
2′	132.2, d	7.97, 2H,d, 8.9	3⁗	134.0,d	7.65,2H,d,8.9
3′	115.9,d	6.82, 2H,d, 8.9	4⁗	160.0,s	–
4′	161.3,s	–	5⁗	134.0,d	7.65,2H,d,8.9
5′	115.9,d	6.82, 2H,d, 8.9	6⁗	116.0,d	6.66,2H, d,8.0.9
6′	132.2,d	7.97, 2H,d, 8.9	7	146.1,d	6.86,d,13.0
Rhamnopyranosyl			8	115.9,d	5.77,d,13.0
1″	102.7,d	4.36, brs.	C=O	167.6,s	
2″	72.1,d[a]	3.88,dd, 3.4, 1.7			
3″	72.3,d[a]	3.39,dd, 9.6, 3.4			
4″	74.0,d	3.25,dd, 9.9, 9.6			
5″	69.9,d	4.0,dd,9.9,6.1			
6″	17.4,q	0.85,d, 6.1			
Rhamnopyranosyl					
1‴	102.6,d	5.10,brs.			
2‴	71.9,d	3.71,m			
3‴	73.7,d	3.74,m			
4‴	74.0,d	3.12,m			
5‴	69.9,d	3.29,m			
6‴	17.88,q	0.96,d, 6.1			

2.1.6. DPPH radical scavenging activity

DPPH assay was performed by a method previously reported by (Kumar et al.; 2011) [14]. 100 µl of the tested samples at different concentrations in MeOH and 1.0× 10–4 M DPPH in MeOH (300 µl) were added to the 96-well microtiter plate. The plate was shaken for 1 min on a plate shaker and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was recorded at 517 nm. The tested samples at different concentrations without DPPH solution were used as a blank control to eliminate the influence of sample color. Ascorbic acid was used as a positive control [15] and DPPH solution in MeOH served as a negative control.



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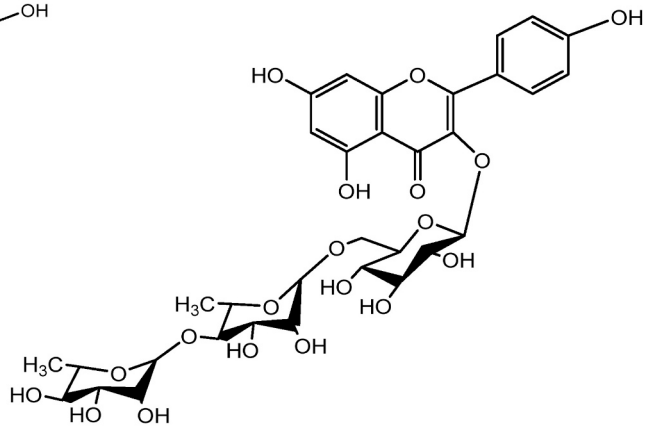

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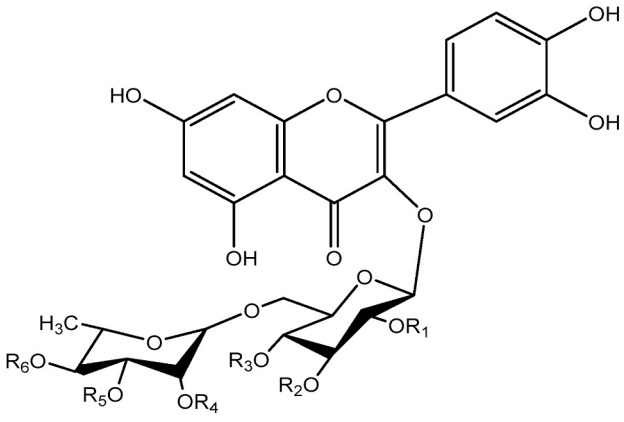

3-6

Cpd. NO.	R1	R2	R3	R4	R5	R6
3	H	H	H	CH_3COO	CH_3COO	CH_3COO
4	H	CH_3COO	CH_3COO	CH_3COO	H	CH_3COO
5	CH_3COO	H	CH_3COO	CH_3COO	H	CH_3COO
6	H	CH_3COO	H	CH_3COO	CH_3COO	CH_3COO

Fig. 1. Structure of compounds **1–6.**

3. Results and discussion

3.1. Structure elucidation of compound 1

Compound **1** (Table 1, Fig. 1) (13 mg) was obtained as yellow amorphous powder, soluble in methanol, with $[\alpha]_D^{30.1}$ -54.5° (c = 0.333, MeOH). The structure of compound 1 was elucidated by UV, IR, one- and two dimensional NMR spectroscopy including ^1H, ^{13}C NMR, DEPT-135H-H COSY, HMQC, and HMBC experiments, as well as HRFAB mass spectrometry. UV spectrum (in MeOH) exhibited absorption maxima at 256 nm (band-II) and 352 nm (band-I) indicating a flavonol type. IR spectrum of **1** (in $CHCl_3$) indicated the presence of hydroxyl (3445 cm-1), carbonyl (1782 cm-1) and phenyl (2980, 1640, 1533 cm-

Table 2
Effects of methanol extract and fractions isolated from a particular extract on TNF-α production in cultured THP-1.

Extract/ fraction	Inhibitory activity (IC50) (μg/ml)		DPPH radical scavenging activity	
	TNF-α production	Dexamethasone; positive control 5 μM	Ascorbic acid positive control 12 μM	n
Total methanol extract	52 ± 8		32 ± 8	3
Chloroform fraction	130 ± 6		45 ± 5	3
Ethyl acetate fraction	19 ± 9		21 ± 8	3
n-butanol fraction	75 ± 3		65 ± 4	3

Table 3
Activities of compounds 1–6 on the production of inflammatory cytokines and their cytotoxic activity in vitro.

Compound	Inhibitory activity (IC_{50}) (μM) Cytokines (TNF-α/IL-1β)	% of Cell viability Cell lines THP-1	n
1	10.2/49.1	127.4	**2**
2	28.1/55.7	108.5	**2**
3	35.2/57.5	105.4	**2**
4	45.3/73.5	103.9	**2**
5	48.2/65.6	103.4	**2**
6	42.2/67.4	104.2	**2**
Dexamethasone	42.1/40.5	ND	**2**

Values are mean values from two experiments (n = 8 in total) n: number of experiments (one experiment: n = 4).
Dexamethasone (positive control for the activity of cytokine inhibition) ND: not determined.

1). ^{1}H and ^{13}C NMR spectra of **1** indicated the presence of kaempferol moiety, three sugar moieties (one hexose unite and two pentose unites) in addition to the presence of *P*-coumaroyl moiety attached to a terminal hexose. ^{1}H NMR spectrum showed pair of doublets at δ_H 6.09 (H-6) and δ_H 6.29 (H-8) and two-spin system with the typical coupling pattern of 1,4-disubstituted benzene ring [a pair of doublets each is equivalent to two protons at δ_H 7.97 (H-2′, H-6) and δ_H 6.82 (H-3′, H-5)] which are two features characteristic of a flavonol with hydroxyl functionality at positions 5, 7 and 4. ^{1}HNMR spectrum showed also signals characteristic to *p*-coumaroyl moiety, where there is a typical coupling pattern of 1,4-disubstituted benzene ring [a pair of doublets each is equivalent to two protons at δ_H 7.65 (H-2‴,H-6‴) and at δ_H 6.66 (H-3‴,H-5‴)] in addition to a trance olefinic protons at δ_H 6.86 and 5.77 (H-7,H-8) respectively. ^{13}C NMR agreed with 3-substituted kaempferol moiety. Substitution of kaempferol in C-3 was evident from the chemical shift of neighboring C-2 (δ_C 159.2 ppm) whereas in flavonols with unsubstituted hydroxyl functionality at C-2 was detected around δ_C 147 ppm [16]. A long-range correlation was observed in HMBC experiment between C-3 of kaempferol (δ_C 134.1 ppm) and the anomeric proton of rhamnose (δ_H 4.36 ppm) confirmed that this was the site of glycosylation and rhamnose was the first sugar. Other long-range correlations observed in HMBC experiment, between C-4 of the first rhamnose (δ_C 74 ppm) and the anomeric proton of the middle one (δ_H 5.1 ppm) and between C-4 of the middle rhamnose (δ_C 74 ppm) and the anomeric proton of the terminal galactose (δ_H 5.62 ppm) confirmed that the attachment between the middle rhamnose and first one is (1 → 4) and that between the terminal galactose and the middle rhamnose is (1 → 4). Another HMBC correlation was observed between H-6 protons of the terminal galactose (δ_H 3.41 and 3.13 ppm) and the carbonyl group of *P*-coumaroyl moiety (δ_C 167.6 ppm), confirming that the *P*-coumaroyl moiety is attached to the terminal galactose. Also, ^{13}C- downfield shift of both C-6 of

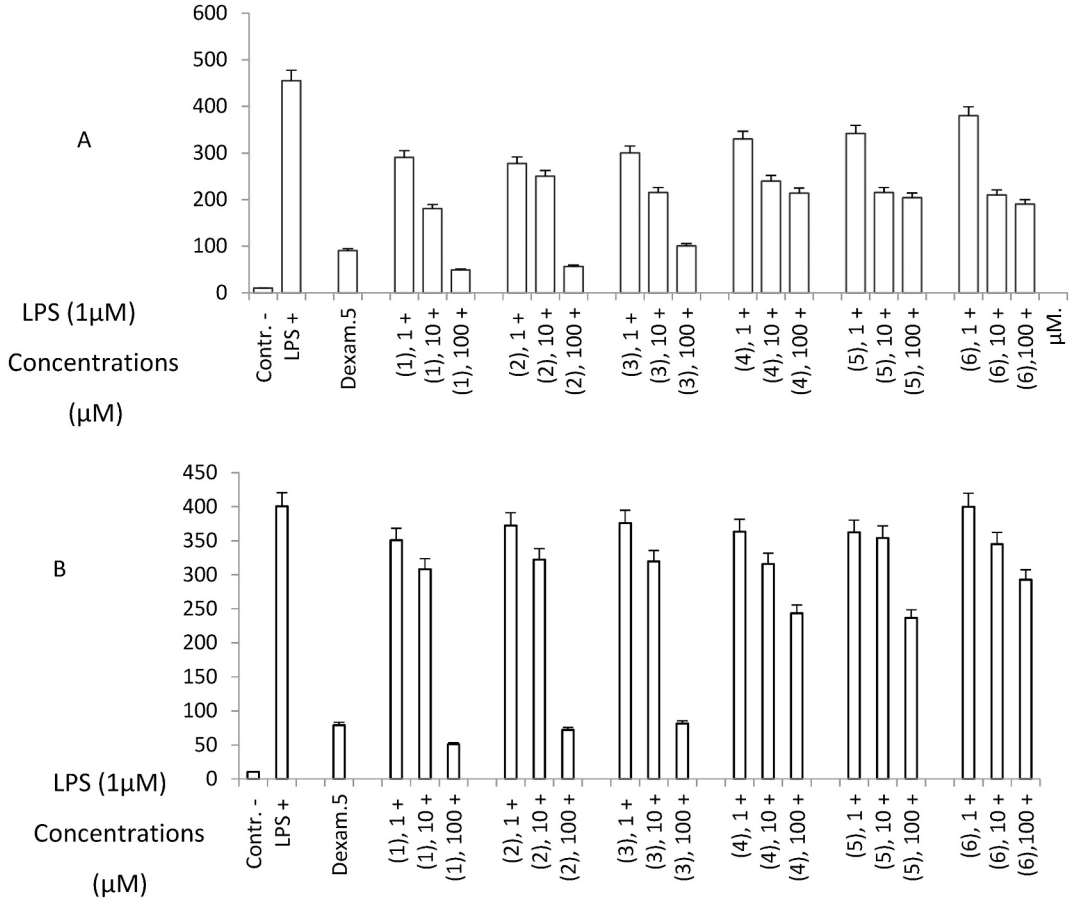

Fig. 2. Effects of flavonoid glycosides on the production of inflammatory cytokines in cultured THP-1 cells.
Cells were directly treated with various concentrations (1, 10 and100μM) of compounds **1–6** co-stimulated with 1 μM LPS. Culture supernatants were collected 24 h after LPS stimulation and cytokine levels such as TNF-α (**A**) and IL-1β (**B**) were measured using ELISA. Data are means ± SD of quadruplicate cultures. Correlation coefficients for TNF-α production co-stimulated with LPS: r^2 = 0.89629 (**1**), r^2 = 0.96711 (**2**), r^2 = 0.92035 (**3**), r^2 = 0.95413 (**4**), r^2 = 0.99115 (**5**), r^2 = 0.87086 (**6**). Correlation coefficients for IL-1β production co-stimulated with LPS: r^2 = 0.99760 (**1**), r^2 = 0.99454 (**2**), r^2 = 0.99024 (**3**), r^2 = 0.90043 (**4**), r^2 = 0.99944 (**5**), r^2 = 0.95169 (**6**).
Data are means ± SD of three independent experiments (n = 12 in total); n: numbers of experiment (one experiment: n = 4). [a] Original methanol extract. [b] Original extract was loaded onto HP-20, and successive elution with water, methanol and acetone were carried out yielding three fractions.

Table 4
DPPH radical scavenging activity of compounds 1–6.

Compound	IC_{50} (μM)[a]
1	1.37
2	1.42
3	3.62
4	3.29
5	1.87
6	7.21
Ascorbic acid	12

[a] IC_{50} values were determined by regression analysis and expressed as the mean of four replicates.

immunosuppressive glucocorticoid agent dexamethasone was used as a positive control for inhibition of cytokine production at 5 μM, dexamethasone strongly decreased levels of TNF-α, and IL-1β in culture supernatants of THP-1 cells co-stimulated with LPS [21]. It was noticed that compounds **1–3** strongly suppressed the production of TNF-α / IL-1β in THP-1 cells in a dose-dependent manner (10.2/49.1, 28.1/55.7, and 35.2/57.5 μM respectively) whereas compounds **4–6** have relatively weaker inhibitory activity.(45.3/73.5, 48.2/65.6, and 42.2/67.4 μM respectively), These represents results more or less more potent than the positive control Dexamethasone.

A possible mechanism for flavonoids used to alleviate some inflammation-related symptoms is to act by inhibiting cyclooxygenase

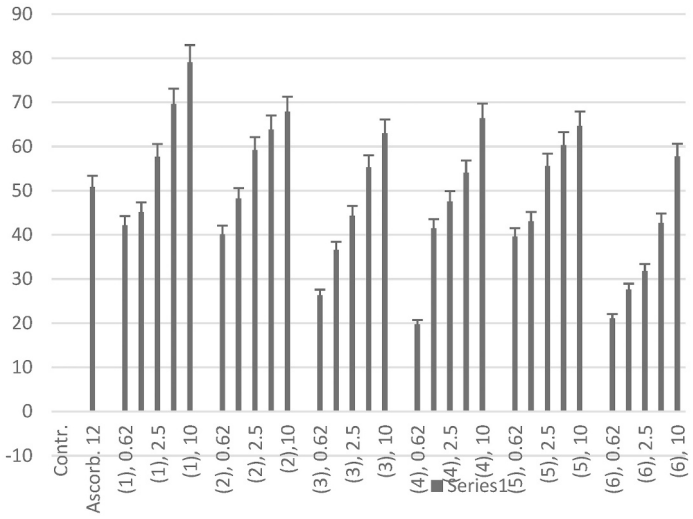

Fig. 3. DPPH radical scavenging activity of compounds 1–6.

galactose, C-4 of the middle rhamnose, and C-4 of the first rhamnose confirmed the mentioned site of attachment [17]. The HRMS spectrum showed a quasi-molecular ion peak at *m/z* 887.2629 [M + H] + calculated as 886.2532 per the molecular formula $C_{42}H_{46}O_{21}$. Hence **1** could unequivocally be identified as Kaempferol 3-*O*-*α*-[(6-*P*-coumaroyl galactopyranosyl-*O*-*β*-(1 → 4)-*O*-*α*-rhamnopyranosyl-(1 → 4)]-*O*-*α*-rhamnopyranoside which is a new compound.

In addition, five known falvonoid glycosides (**2–6**) Fig. 1, Kaempferol 3-*O*-[*α*–rhamnopyranosyl-(1 → 4)-*O*-*α*-rhamnopyranosyl-(1 → 6)-*O]*-*β*-galactopyranoside (kaempferol 3-*O*-*β*-isorhamninoside) **2**, quercetin 3-*O*-[(2,3,4-triacetyl-*α*-rhamnopyranosyl)-(1 → 6)-*β*-galactopyranoside **3**, quercetin 3-*O*-[(2,4-diacetyl-*α*-rhamnopyranosyl)-(1 → 6)]-3,4-diacetyl-*β*-galactopyranoside **4**, quercetin 3-*O*-[(2,4-diacetyl-*α*-rhamnopyranosyl)-(1 → 6)]-2,4-diacetyl-galactopyranoside **5**, quercetin 3-*O*-[(2,3,4-triacetyl-α-rhamnopyranosyl)-(1 → 6)-3-acetyl-*β*-galactopyranoside **6** were isolated and their structure were identical with the reported data [18–20].

3.2. Inhibition of production of inflammatory cytokines in THP-1 cells

Both fractions and isolated compounds (1–6) were subjected to 24-h stimulation by lipopolysaccharide (LPS) at 1μM (Tables 2, 3; Fig. 2) where ethyl acetate fraction showed the strongest inhibitory activity. This fraction inhibited TNF-α production and DPPH radical scavenging activity with IC50 values of 19.9 and 21 μg/ml respectively. Its inhibitory activity was 2.5–1.5 times stronger than those of the original methanol extract.

Ethyl acetate fraction was subjected to isolation by HPLC yielding the previously mentioned six flavonoids 1–6 which in turn were subjected to the same assay as the parent fraction. Putative activity or by blocking cytokine receptors [22,23].

As SAR study, it was noticed that the effect of kaempferol and quercetin on cytokine-induced pro-inflammatory status of cultured human endothelial cells was always more strongly inhibited in kaempferol-treated than in quercetin-treated cells [24], Kaempferol can modulate the Th1/Th2 balance and could be useful for the treatment of cell-mediated immune diseases [25] which may explain the strong suppression activity of compounds (**1–2**) (kaempferol derivative) over compounds (**4–6**) (quercetin derivatives), for compound **3**, it has a relatively weaker activity.

3.3. DPPH radical scavenging activity

The isolated flavonoid compounds **1–6** exhibited free radical scavenging activity with IC50 values of 1.37 and 1.42 μM for 1and 2 (kaempferol derivatives) and 3.62, 3.29, 1.8, and 7.21 μM for 3–6 (quercetin derivatives), (Table 4; Fig. 3). This may be attributed to the differences in the sites of the acetyl groups in compounds **3–6**.

4. Conclusion

Based on the previous results, ball pepper fruit extracts may be safely considered potential anti-inflammatory and antioxidant candidates for inflammatory diseases.

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Conflicts of interests

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.104903.

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