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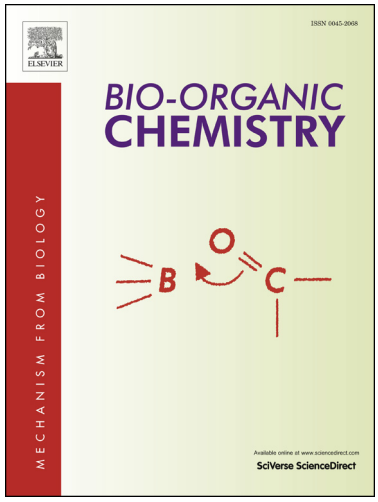

Synthesis, characterization, anti-inflammatory and anti-proliferative activity against MCF-7 cells of O-alkyl and O-acyl flavonoid derivatives

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Title :

Synthesis, characterization, anti-inflammatory and anti-proliferative activity against MCF-7 cells of O-alkyl and O-acyl flavonoid derivatives

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Abstract

A series of O-alkyl and O-acyl flavonoid derivatives was synthesized in high efficiency. Alkylation and acylation of 5-hydroxyflavonoids showed that the low reactivity hydroxyl group, 5-OH, well reacted with strong reagents whereas with weaker reagents, the different products were obtained dependently on structural characteristic of ring C of respective flavonoid. In order to evaluate anti-inflammatory activity, all compounds were tested for *in vitro* inhibition of bovine serum albumin denaturation and *in vivo* inhibition of carrageenan-induced mouse paw edema. Among them, the compounds **3**, **3b**, **4b** and **4c** demonstrated more effective anti-inflammatory activity than standard drugs (diclofenac sodium and ketoprofen) in both tests. Meanwhile, the flavonoids **2**, **2c**, **3a** and **4b** displayed anti-proliferative activity against MCF-7 cell lines. Triacetyl derivative of hesperetin **4b** inducing degradation of DNA in MCF-7 cells was observed.

Keywords :

Flavonoid, Synthesis, Anti-inflammatory, Anti-proliferative, MCF-7.

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Introduction

Flavonoids consisting of a large group of polyphenolic compounds present in almost plants where they are synthesized through the phenylpropanoid pathway. [1] The flavonoids possess a wide range of biological activities such as antioxidant, [2-4] antifungal,[5] antiviral,[6-8] anticancer[9-11] and anti-inflammatory agent.[12-14] In recent years, carrageenan-induced edema process is commonly used in study on *in vivo* of anti-inflammatory drugs. This edema formation is a biphasic event. The first phase of edema is attributed to the release of histamine and serotonin while the second phase is due to the release of prostaglandins. The drugs possessed anti-inflammatory activity may inhibit one of the two phases.[15] In addition, a common study method on *in vitro* is the interaction of drugs with normal proteins, which strongly inhibit heat coagulation of serum, and may cause the biological activity of anti-inflammatory drugs.[16, 17] These drugs have exerted an inhibitory activity on immune haemolysis and also have suppressive effect on vascular reactivity. Denaturation as one of the causes of inflammation is well documented. Anti-inflammatory drugs interact in some way with proteins. To cause the interaction between the drug and the proteins, the stability of proteins against heat coagulation can be measured. [18] Thus, the coagulated inhibitors originating flavonoids have recently been recognized as efficient anti-inflammatory drugs. [19-21]

Recently, flavonoid compounds which occur naturally and are widely distributed in plants have been reported as cancer chemopreventive agents.[22, 23] Many molecular mechanisms of inhibition of cancer cells have been proposed for flavonoids. For example, quercetin may inhibit the expression of mutant p53 to lead to arrest human breast cancer cells.[24] It has been experimentally proved that quercetin may also interact with nuclear type II estrogen binding sites in breast cancer cells.[25] Since MCF-7 cell line has become a prominent model system for the study of breast cancer, many flavonoid derivatives against MCF-7 cells and inducing apoptosis in the cells have been found as a potent source of new therapeutic anticancer drugs.[26, 27]

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Flavonoid structures are based upon a fifteen-carbon skeleton consisting of two benzene rings linked via a heterocyclic pyrane ring. They can be divided into a variety of classes such as flavones (e.g., flavone, chrysin, diosmetin and luteolin), flavonols (e. g., quercetin, kaempferol and galangin), flavanones (e.g. flavanone, hesperetin and naringenin) and others. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of pyrane ring, while individual compounds within a class differ in the pattern of substitution of the benzene rings.[28] The biological activities depend remarkably on their structural characteristics such as conjugations, degree of hydroxylation and substitutions. A small alternation in the chemical structure of flavonoids may lead to significant changes in biological activities. As a result, numerous flavonoid derivatives have recently been designed and synthesized with convenient procedures.[29-31] Due to specifically biochemical properties, methyl-O and acetyl-O flavonoid derivatives particularly have attracted significant attention of synthesis chemists.[32-35] In 5-hydroxyflavonoids including quercetin (**1**), luteolin (**2**), diosmetin (**3**) and hesperetin (**4**) (**Fig. 1**), the low reactivity of hydroxyl group at 5-position is specifically considered because of forming hydrogen bond with carbonyl.[36] The acylation or alkylation of such a proton often requires more drastic conditions. Some O-methyl and O- acetyl flavonoid derivatives have been previously reported in the literature. [37-39] However, it is not revealed influence of flavonoid structures and reagent on acylation and alkylation of these 5-hydroxyflavonoids. Our previous researches [40, 41] showed that acylation and alkylation of the natural products including flavonoid derivatives were carried out in high efficiency and they possessed significantly biological activities. In this paper, we describe the synthesis, NMR data, anti-inflammatory activity and anti-proliferative activity against breast cancer cells MCF-7 of O-alkyl and O-acyl flavonoid derivatives.

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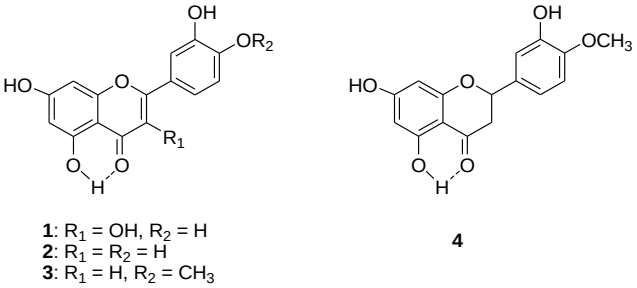

1: R_1 = OH, R_2 = H
2: R_1 = R_2 = H
3: R_1 = H, R_2 = CH_3

4

Fig. 1. Structures of four important flavonoids: quercetin (**1**), luteolin (**2**), diosmetin (**3**), and hesperetin (**4**)

Results and Discussion

Chemistry

The methods used for synthesis of flavonoid derivatives are simple and highly effective because the reagents are readily available and protocols are straightforward. The general synthetic routes are illustrated in **Scheme 1**. We first investigated the direct alkylation of the flavonoids with excess of alkylating reagent in order to get an insight into the reactivity of the 5-OH group. Dimethyl sulfate and allyl bromide were chosen as alkylating reagents with reactivity difference. The reaction was carried out in acetone with K_2CO_3 as a base. These derivatives were obtained in good yields. The methylation of quercetin (**1**) and luteolin (**2**) revealed that all of the hydroxyl groups including 5-OH were replaced in yield of 65.7 % and 90.7 %, respectively.

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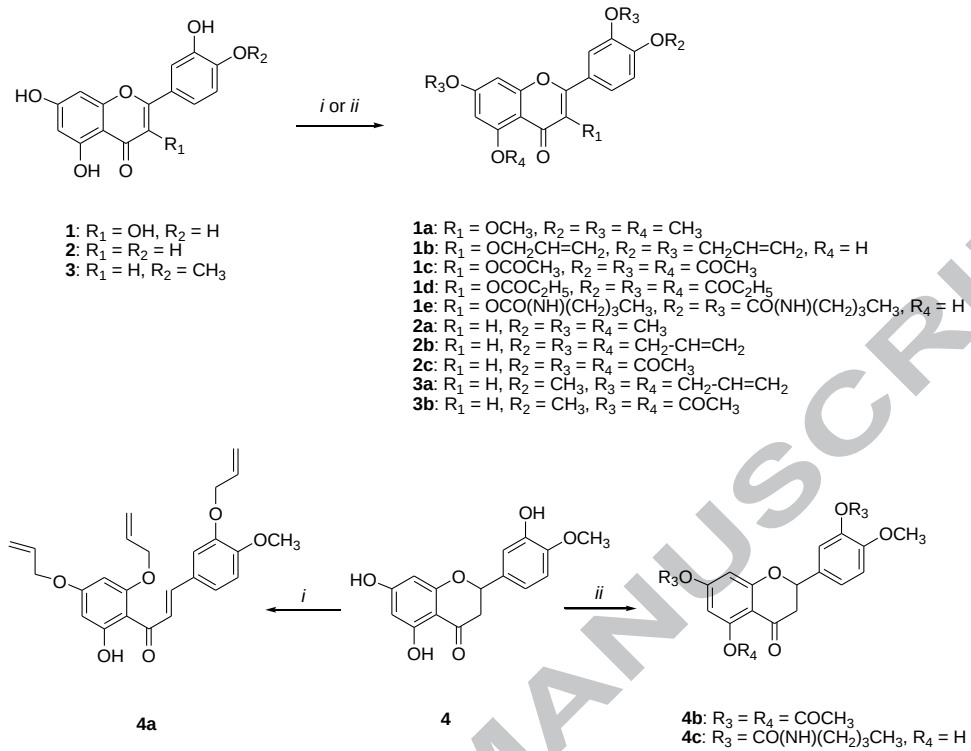

Scheme 1. Reagents. (i) Dimethyl sulfate or allyl bromide, K_2CO_3, acetone, reflux; (ii) CH_3COCl or C_2H_5COCl or $CH_3(CH_2)_3NCO$, Py

In a similar fashion, one interesting result had come from reaction of all four flavonoids with allyl bromide as a weak alkylating reagent. First, two flavones, luteolin (**2**) and diometin (**3**), were allylated at all positions of hydroxyl group to afford the perallylated derivatives **2b** and **3b**, respectively. In next consideration, allylation of quercetin (**1**) showed that the 5-OH group was not replaced to give 3,3',4',8-*tetra*-O-allylquercetin (**1b**) in 82.5 % yield. It may be because the electron pair of oxygen at 3-position stabilized the non-covalent six-membered ring (**fig 1**) which are formed from a hydrogen bond between 5-OH group and adjacent carbonyl. Finally, to understand influence of double bond of ring C to the flavonoid alkylation, hesperetin (**4**) was employed in comparison with allylation of **3**. Outstandingly, in the same conditions, allyl bromide opened the pyrane ring of hesperetin to afford a chalcone derivative (**4a**) bearing one hydroxyl group. Although the detailed mechanism has been unknown, some flavanones generally can be isomerized by ring opening into chalcones in alkaline media provided a hydroxyl

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substituent at position 6' (or 2') of the chalcone.[42, 43] These results indicated alkylation reaction of the 5-OH group depending not only on the alkylating reagent but also on structure of ring C of flavonoids.

The structure of **4a** was unambiguously determined by NMR spectra as described in **table 1**. In the HSQC spectrum, cross peaks between two ^{1}H peaks at 7.73 and 7.64 ppm and two ^{13}C peaks at 125.08 and 142.85 ppm, respectively, were observed. Moreover, these ^{1}H peaks were long-range coupled to the ^{13}C peak at 192.11 (C-1) in HMBC spectrum. Therefore, these shifts were assigned to H-2/C-2 and H-3/C-3, respectively. The coupling constant between H-2 and H-3 (15.5 Hz) indicated that the conformation of the bond between C-2 and C-3 was *trans*. The spectra demonstrated that compound **4a** is a derivative of tri-O-allylchalcone.

Employing a similar strategy for synthesis of O-acyl flavonoids, we used short fatty chloride acids as strongly acylating reagent and n-butyl isocyanate as weak acylating reagent in pyridine at room temperature. The reaction of all flavonoids with short fatty chloride acids, acetyl chloride and propyl chloride, generated peracyl derivatives **1c**, **1d**, **2c**, **3b** and **4b** whereas the 5-hydroxyl group of quercetin (**1**) and hesperetin (**4**) was not acylated by n-butyl isocyanate in pyridine as deduced by ^{1}H NMR (**1e** and **4c**, table **3**). All the synthesized compounds including three new derivatives **2b**, **4a** and **4c** were identified by ESI-MS, IR and NMR as shown in **table 2 – 4**.

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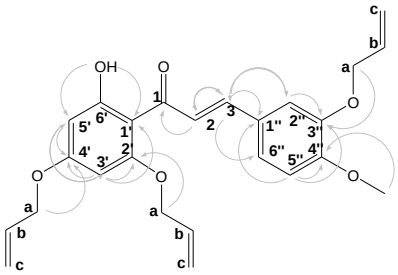
Table 1 ^{1}H, ^{13}C NMR, DEPT spectral data and HSQC and HMBC correlations in compound **4a**[*]



Position	^{1}H NMR (ppm)	^{13}C NMR (ppm)	DEPT	Correlations	
				HSQC	HMBC
1	-	192.11	C	-	-
2	7.73 (d, 15.5 Hz)	125.08	CH	C-2	C-1, C-1'', C-2'', C-3,C-6''
3	7.64 (d, 15.5 Hz)	142.85	CH	C-3	C-1, C-1'', C-3
1'	-	106.55	C	-	-
2'	-	160.63	C	-	-
3'	6.19 (d, 2.5 Hz)	92.39	CH	C-3'	C-1', C-2', C-4', C-5'
4'	-	165.39	C	-	-
5'	6.13 (d, 2.5 Hz)	94.79	CH	C-5'	C-1', C-3', C-4', C-6'
6'	-	164.16	C	-	
1''	-	127.45	C	-	
2''	7.27 (s)	112.01	CH	C-2''	C-1'',C-3,C-3'',C-4'',C-6''
3''	-	147.77	C	-	-
4''	-	151.45	C	-	-
5''	7.04 (d, 8.5 Hz)	112.01	CH	C-5''	C-1'', C-3'', C-4''
6''	7.25 (d, 8.0 Hz)	123.48	CH	C-6''	C-1'', C-3, C-4'', C-5''
OH	13.51 (s)	-	-	-	C-1', C5'
a	4.68 (d, 5.5 Hz), 4.64 (d, 4.0 Hz), 4.60 (d, 5.5 Hz)	69.46, 68.98, 68.59	CH_2	C-a	C-b, C-c, C-2', C-3'', C-4'
b	6.17 (m), 6.14-6.00 (m)	133.56, 132.96, 132.91	CH	C-b	-
c	5.47 (m), 5.44 (m), 5.41 (m), 5.30 (m), 5.29 (m), 5.27 (m)	118.30, 117.95, 117.71	CH_2	C-c	C-a, C-b
4''-OCH_3	3.82 (s, 3H)	55.65	CH_3	C-O	C-4''

[*]^{1}H-NMR and ^{13}C- NMR experimental conditions: 500 MHz and 125 MHz, respectively, in DMSO-d_6

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Table 2. physical data, IR and HR-MS spectra of O-alkyl and O-acyl flavonoid derivatives.

Sub.	M.p. (^{o}C)	Isolated Yield (%)	IR	HR-MS
1a	147-151	65.7	2921 (CH_3), 1650 (C=O), 1160 (C-O)	373.1 $[M+H]^+$
1b	112-115	82.5	2986 (CH_2), 1664 (C=O), 1160 (C-O)	429.4, 463.4 $[M+H]^+$
1c	194-197	84.0	2932 (CH_3), 1773 (C=O, ester), 1641 (C=O,), 1189 (C-O)	428.9, 470.9, 512.9 $[M+H]^+$
1d	118-122	41.2	2984 (CH_3), 1770 (C=O, ester), 1644 (C=O), 1121 (C-O)	471.1, 527.1, 583.2 $[M+H]^+$
1e	215-219	52.5	3335 (-CONH), 1725 (C=O, ester), 1657 (C=O), 1539 (N-H), 1251 (C-O)	134.1, 175.1 (100), 481.1, 675.3, 699.3 $[M+H]^+$
2a	192–194	90.7	3085 (CH_3), 1647 (C=O), 1163 (C-O)	343.1 $[M+H]^+$
2b	103–107	67.6	2982 (CH_2), 1634 (C=O), 1179 (C-O)	447.2 $[M+H]^+$
2c	223–225	53.2	1772 (C=O, ester), 1639 (C=O), 1167 (C-O)	413.06, 455.09 $[M+H]^+$
3a	100–104	80.4	2983 (CH_3), 1639 (C=O), 1178 (C-O)	421.2 $[M+H]^+$
3b	189–191	83.0	1770 (C=O, ester), 1645 (C=O), 1204 (C-O)	427.1, 449.1 $[M+H]^+$
4a	98-100	76.6	2916 (CH_2), 1623 (C=O), 1136 (C-O)	233.1, 423.2 $[M+H]^+$
4b	135-139	84.0	2957 (CH_3) 1771 (C=O, ester), 1619 (C=O), 1192 (C-O)	74.3, 88.2, 345.1, 387.1 (100), 429.1 $[M+H]^+$
4c	155-159	79.2	3332 (NH), 2916 (CH_3), 1716 (C=O, ester), 1665 (C=O), 1136 (C-O)	523.2 $[M+Na]^+$

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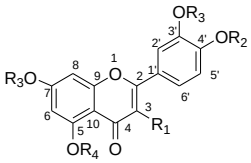
Table 3. Chemical shifts (*ppm*) of the protons in the ^{1}H NMR spectra of O-alkyl and O-acyl flavonoid derivatives.*



Sub.	Flavonoid moiety						O-alkyl or O-acyl groups			
	H-2	H-6	H-8	H-2'	H-5'	H-6'	R_1 (or H-3)	R_2	R_3	R_4
1a	-	6.48 (d, 2.5 Hz)	6.81 (d, 2.5 Hz)	7.63 (d, 2.0 Hz)	7.12 (d, 8,5 Hz)	7.66 (dd, 2.0, 8.5)	OMe: 3.89 (s, 3H), 3.85 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.75 (s 3H)			
1b	-	6.38 (d,2.0 Hz)	6.75 (d, 2.0 Hz)	7.21 (d, 2.0 Hz)	7.15 (d, 8.5 Hz)	7.70 (dd, 2.0, 8.5 Hz)	$OCH_2CH=CH_2$: 6.12 -6.03 (m, 3H), 6.02-5.94 (m, 1H), 5.46 -5.41 (m, 3H), 5.35-5.28 (m, 4H); 5.21-5.19 (dd, 11.0, 1.0Hz, 1H), 4.68- 4.56 (m, 8H)			12.58 (s, 1H)
1c	-	6.87 (d, 2.0 Hz)	7.33 (d, 2.0 Hz)	7.73 (d, 2.0 Hz)	7.37 (d, 8.0 Hz)	7.69 (dd, 2.0, 8.0 Hz)	$OCOCH_3$: 2.34 (s, 3H), 2.33 (s, 6H), 2.43 (s, 3H)			
1d	-	7.17 (d, 2.0 Hz)	7.65 (d, 2.0 Hz)	7.86 (d, 2.0 Hz)	7.55 (d, 8.0 Hz)	7.83 (dd, 2.0, 8.0 Hz)	$OCOCH_2CH_3$: 2.70-2.62 (m, 10H); 1.09-1.08 (m, 15H)			
1e	-	6.64 (d, 2.0 Hz)	7.10 (d, 2.0 Hz)	7.75 (d, 2.0 Hz)	7.40 (d, 8.5 Hz)	7.83 (dd, 2.0, 8.5 Hz)	$OCONH(CH_2)_3CH_3$: 8.07 – 8.06 (m, 1H), 8.02-8.00 (m, 1H), 7.88-7.85 (m, 2H); 3.15-3.05 (m, 8H); 1.54-1.44 (m, 8H); 1.37-1.29 (m, 8H); 0.92-0.89 (m, 12H)			12.18 (s, 1H)
2a	-	6.49 (d, 2.5Hz)	6.86 (d, 2.5 Hz)	7.52 (d, 2.0 Hz)	7.09 (d, 8.5 Hz)	7.63 (dd, 2.0, 8.5 Hz)	6.76 (s, 1H)	OCH_3: 3.90 (s, 3H), 3.87 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H)		
2b	-	6.53 (d, 2.5 Hz)	6.87 (d, 2.5 Hz)	7.57 (d, 2.0 Hz)	7.13 (d, 8.5 Hz)	7.61 (dd, 2.0, 8.5 Hz)	6.71 (s, 1H)	$OCH_2CH=CH_2$ 6.12-6.02 (m, 4H), 5.69-5.65 (m, 1H), 5.59-5.48 (m, 1H), 5.46-5.44 (m, 1H), 5.43-5.42 (m, 1H), 5.34-5.27 (m,4H), 4.72-4.71 (m, 4H), 4.68-4.64 (m, 4H)		
2c	-	7.09 (d, 2.5 Hz)	7.64 (d, 2.5Hz)	8.04 (d, 2.0 Hz)	7.50 (d, 9.0 Hz)	8.05 (dd, 2.0, 9.0 Hz)	6.95 (s, 1H)	$COCH_3$: 2.34 (s, 3H), 2.34 (s, 6H), 2.32 (s, 3H)		
3a	-	6.53 (d, 2.0 Hz)	6.87 (d, 2.0 Hz)	7.55 (d, 2.0 Hz)	7.13 (d, 8.5 Hz)	7.65 (dd, 2.0, 8.5 Hz)	6.71 (s, 1H)	3.85 (s, 3H)	$OCH_2CH=CH_2$: 6.12-6.02 (m, 3H), 5.69-5.27 (m, 1H), 5.48-5.43 (m, 2H), 5.33-5.27 (m, 3H), 4.72-4.68 (m, 4 H), 4.64-4.63 (m, 2H)	
3b	-	7.07 (d, 5.0 Hz)	7.64 (d, 5.0 Hz)	7.89 (d, 2.5Hz)	7.33 (d, 9.0 Hz)	8.02 (dd, 2.5, 9.0 Hz)	6.86 (s, 1H)	3.88 (s, 3H)	$COCH_3$: 2.34 (s, 3H), 2.33 (s, 3H), 2.31 (s, 3H)	
4b	5.60 (dd, 2.5, 13 Hz)	6.69 (d, 2.0 Hz)	6.87 (d, 2.0 Hz)	7.3 (d, 2.0 Hz)	7.17 (d, 8.5 Hz)	7.40 (dd, 2.0, 8.5 Hz)	3.24 (dd, 13.0, 17.0 Hz, 1H); 2.70 (dd. 2.5, 17.0 Hz, 1H)	3.79 (s, 3H)	2.30 (s, 3H), 2.27 (s, 3H), 2.26 (s, 3H)	
4c	5.63 (dd, 3.0, 13.0 Hz)	6.29 (d, 2.5 Hz)	6.31 (d, 2.0 Hz)	7.26 (d, 2.0 Hz)	7.14 (d, 9.0 Hz)	7.35 (dd, 2.5, 9.0 Hz)	3.48 (dd, 13.5, 17.5 Hz, 1H); 2.81 (dd, 3.0, 17.0 Hz, 1H)	3.78 (s, 3H)	7.95 (t, 6.0 Hz, 1H), 7.72 (t, 6.5 Hz, 1H),3.07-3.02 (m, 4H), 1.46-1.41 (m, 4H), 1.36-1.28 (m, 4H), 0.91-0.86 (m, 6H)	11.94 (s, 1H)

*NMR experimental conditions: 500 MHz, DMSO-d_6.

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Table 4. Chemical shifts (*ppm*) of the carbon atoms in the ^{13}C NMR spectra of O-alkyl and O-acyl flavonoid derivatives. *

Sub.	flavonoid moiety															O-alkyl or O-acyl			
	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'	R_1	R_2	R_3	R_4
1a	155.57	140.32	172.11	160.25	95.71	163.95	92.90	158.07	108.44	122.52	111.04	148.40	122.52	111.41	121.30	OMe: 59.18, 55.96, 55.87, 55.57, 55.54			
1b	155.46	136.77	178.01	160.89	98.17	163.98	93.10	156.14	105.14	122.24	113.11	150.48	147.33	113.73	122.23	72.48, 69.21, 68.92, 68.79 (OCH_2); 133.63, 133.52, 133.27, 132.72 (CH=); 118.14, 118.05, 117.65, 117.51 ($=CH_2$)			
1c	150.16	133.84	169.89	154.12	113.76	156.64	108.89	153.54	114.53	127.49	123.65	142.03	144.24	123.79	126.25	$OCOCH_3$: 169.10, 167.71, 167.64 (CO); 20.95, 20.86, 20.46, 20.33 (OCH_3)			
1d	149.69	133.22	169.13	154.46	114.74	156.37	109.97	153.31	114.12	126.64	123.46	142.18	144.47	123.64	127.11	$OCOCH_2CH_3$: 172.04, 171.75, 171.45, 171.28, 171.09 (CO); 26.96, 26.83, 26.66, 26.50 (CH_2); 8.82, 8.69, 8.63, 8.60 (CH_3)			
1e	155.55	131.60	176.89	155.61	104.56	160.30	101.05	157.15	107.42	126.44	123.86	146.22	143.56	124.35	125.81	153.22, 152.99, 152.88, 152.67 (CO); 39.50, 39.33, 39.17, 39.00 (CH_2NH); 32.55, 31.67, 31.29, 31.25 (CH_2); 19.44, 19.41, 19.26, 19.16 (CH_2); 13.63, 13.60, 13.54, 13.20 (CH_3).			
2a	160.20	107.03	175.64	159.65	96.16	163.59	93.38	159.11	108.26	123.11	109.11	148.99	151.55	111.64	119.27	-	OCH_3: 55.99, 55.92, 55.83, 55.65 (CH_3)		
2b	159.48	107.09	175.53	158.97	97.59	162.28	94.31	158.94	108.54	123.31	111.19	147.97	150.67	113.46	119.48	-	$OCH_2CH=CH_2$: 133.68, 133.33, 132.89, 132.81 (CH=);118.09, 117.55, 117.47, 116.94 ($=CH_2$); 69.19, 68.92, 68.84 (CH_2)		
2c	156.99	114.23	175.33	153.91	109.79	160.15	108.30	149.37	114.36	129.09	121.87	142.50	144.77	124.48	124.89	-	$COCH_3$: 168.72, 168.33, 168.09, 167.90 (CO); 20.81, 20.78, 20.33, 20.25 (CH_3)		
3a	159.60	107.05	175.59	159.00	97.63	162.32	94.34	158.98	108.56	123.07	111.98	147.76	151.86	110.80	119.60	-	55.74	$OCH_2CH=CH_2$: 133.66, 132.91, 132.84 (=CH); 118.11, 117.76, 116.98 ($=CH_2$); 69.24, 68.96, 68.87 (OCH_2)	
3b	156.94	113.20	175.22	153.92	109.73	160.86	106.84	153.75	114.07	125.64	121.07	139.61	149.33	114.31	122.80	-	56.23	$COCH_3$: 168.72, 168.35 (CO); 20.80, 20.30 (CH_3)	
4b	78.36	43.79	189.35	155.81	110.91	162.82	109.35	151.35	111.62	130.72	112.87	139.34	150.82	121.64	125.79	-	55.07	$COCH_3$: 168.83, 168.69, 168.48 (CO); 20.94, 20.88, 20.45(CH_3)	
4c	78.09	42.06	197.62	158.96	101.76	152.73	100.71	151.97	105.02	130.38	112.60	139.85	153.92	121.99	124.75	-	55.82	$CONH(CH_2)_3CH_3$: 162.17, 162.01 (CO); 40.12, 40.10 31.32, 31.08, 19.33,19.29(CH_2); 13.59, 13.55(CH_3)	

*NMR experimental conditions: 125 MHz, DMSO-d_6

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Anti-inflammatory activity

***In vitro* biochemical screening**

The *in vitro* anti-inflammatory activity was carried out using inhibition of bovine serum albumin denaturation method using diclofenac sodium as a reference standard. All the compounds were screened for *in vitro* anti-inflammatory activity depicted in **table 5**. Eight compounds **1b**, **1c**, **1d**, **2c**, **3**, **3b**, **4b** and **4c** showed good anti-inflammatory activity. Among them, the flavonoids **3**, **3b** and **4c** revealed excellent activity over standard diclofenac sodium for all tested concentrations. However, all the other compounds which were not selected to study *in vivo* anti-inflammatory activity, showed weak inhibitory activity of bovine serum albumin denaturation.

***In vivo* biological evaluation**

Compounds showing significant inhibition of bovine serum albumin denaturation were selected for further anti-inflammatory evaluation by carrageenan-induced mouse paw edema method using ketoprofen as a reference standard. The results of all the tested compounds are showed in **table 6**. The results revealed that among the tested flavonoids, derivatives **3**, **3b**, **4b** and **4c** inhibited the edema formation significantly ($p < 0.01$). In particular, the derivative **4b** (**Fig. 2**) showed highest inhibition of paw edema formation after only 1 day while the other compounds exhibited greater anti-inflammatory activity than ketoprofen on fourth day after the carrageenan injection.

Antiproliferative effect of flavonoids

The *in vitro* anti-proliferative activity of all flavonoid derivatives was tested in the breast cancer cell line MCF-7 according to the sulphorhodamine (SRB) assay method. [44] The pharmacological results are summarized in **table 7**. Although the IC50 values of the flavonoids are higher than that of the standard (campthotecin), compounds **1e**, **2c**, **3a** and **4b** displayed anti-proliferative activity against MCF-7 cells. Among them, derivative **4b** reveals the highest anti-proliferative activity (**fig. 3**). Therefore, **4b** has been chosen for the study on inducing degradation of DNA in MCF-7 cells.

Induction of apoptosis by 4b treatment.

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Biochemically, apoptosis is characterized by fragmentation of chromosomal DNA. Due to significant inhibition of proliferation, compound **4b** is used for further study on degradation of DNA in MCF-7 cells. As expected, Agarose gel electrophoresis of **4b** treated chromosomal DNA revealed a ladder-like pattern of DNA fragments consisting of multiples of approximately 180-200 bp (**fig. 4**). This suggests that **4b** is as a potential agent for the further studies and promotion of the clinical use.

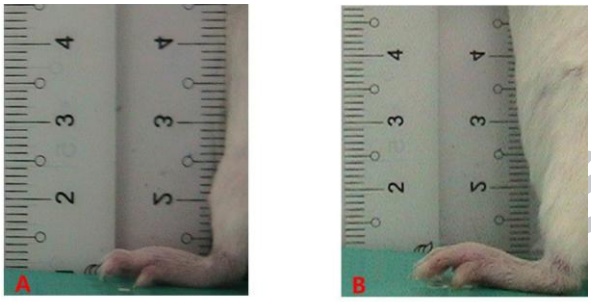

Fig. 2. Photographs of mouse paw at 6th day after the carrageenan injection: Control (**A**) and **4b** (B).

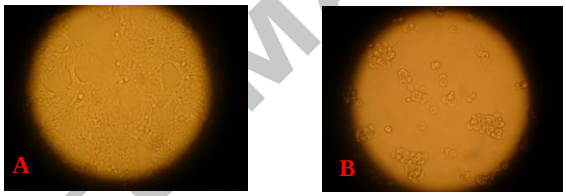

Fig. 3. Control (A) and MCF-7 cells treated with **4b** for 48h (B)

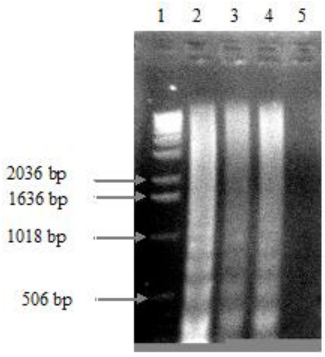

Fig. 4. Agarose gel electrophoresis pattern of DNA isolated from **4b** treated MCF-7 cells. Lane 1, marker. Lane 2, treated MCF-7 in 36 h. Lane 3, in 48h. Lane 4, campthotecin-treated MCF-7 in 48h. Lane 5, untreated MCF-7.

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Table 5. *In vitro* serum albumin inhibitory activity of flavonoids at various concentrations.

Sub.	Inhibition (%)		
	25 (μg/mL)	50 (μg/mL)	100 (μg/mL)
1	0.67	0.67	2.00
1a	7.33	10.00	10.00
1b	23.33	25.33	43.33
1c	9.33	39.33	36.67
1d	10.00	35.33	30.00
1e	7.33	12.00	12.67
2	14.67	15.33	14.67
2a	8.00	14.00	14.67
2b	20.67	21.33	24.67
2c	38.67	38.67	34.67
3	92.00	93.33	94.00
3a	0	0	0
3b	79.33	98.67	97.33
4	0	0	0
4a	23.33	26.67	26.00
4b	72.67	83.33	84.67
4c	96.67	96.67	98.67
Diclofenac sodium	73.33	90.00	94.67

Table 6. Anti-inflammatory activity of flavonoids against carrageenan-induced mouse paw edema with different time intervals.

Sub.	% Edema						
	3h	1 day	2 days	3 days	4 days	5 days	6 days
Control	79.84 ± 3.33	90.0 ± 5.50	79.7 ± 4.45	68.2 ± 4.90	62.23 ± 4.07	50. ± 6.83	45.9 ± 6.06
Standard#	76.28 ± 2.75	77.99 ± 2.88	65.4 ± 3.33 *	56.68 ± 3.47*	46.1 ± 4.42*	22.3 ± 4.77**	19.00 ±4.21**
1b	77.54 ± 6.96	72.26 ± 9.21	67.6 ± 10.9	59.07 ± 7.38	51.0 ± 5.90	43.82 ± 6.31	30.81 ± 6.13
1c	77.65 ± 4.88	77.8 ± 13.3	75.8 ± 11.3	54.7 ± 10.5	63.47 ± 7.48	46.18 ± 6.48	34.68 ± 6.72
1d	77.84 ± 4.60	75.6 ± 5.3	67.2 ± 15.2	50.3 ± 10.5	46.24 ± 7.67	38.96 ± 8.85	27.96 ± 9.17
2c	75.58 ± 5.36	85.53 ± 9.94	85.8 ± 13.7	69.9 ± 11.2	54.4 ± 10.0	51.67 ± 9.07	41.19 ± 9.58
3	85.12 ± 4.49	87.94 ± 6.18	70.02 ± 6.14	58.90 ± 4.50	47.22 ± 6.70*	6.11 ± 2.73**	0.854 ± 0.85**
3b	86.28 ± 3.83	88.32 ± 5.00	70.78 ± 2.23	46.53 ± 3.96*	33.52 ±6.56**	16.41 ± 5.72**	5.15 ± 2.23**
4b	68.25 ± 5.68	54.2 ± 10.5**	46.34±7.21**	48.15 ± 5.36*	36.41 ± 9.59*	17.64 ± 6.35**	19.67 ± 4.46**
4c	85.37 ± 4.91	77.04 ± 4.54	66.46 ± 4.67	56.76 ± 4.93	41.71 ± 8.63*	20.43 ± 5.24*	8.90 ± 3.77**

#Standard: Ketoprofen cream (2.5 %), each value is the mean ± SEM for six mice.
*p<0.05, **p<0.01 when compared with control group

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Table 7. Anti-proliferative activity of flavonoids against MCF-7 Cells.

Sub.	IC50 (μmol/mL)
1	0.128 ± 0.016
1a	0.208 ± 0.014
1b*	-
1c	0.084 ± 0.004
1d	0.049 ± 0.003
1e	0.036 ± 0.011
2	0.060 ± 0.002
2a*	-
2b*	-
2c	0.036 ± 0.006
3*	-
3a	0.035 ± 0.002
3b	0.190 ± 0.007
4	0.125 ± 0.007
4a	-
4b	0.020 ± 0.001
4c	0.067 ± 0.001
Camptothecin	0.001± 0.00002

*compounds get low solubility

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Experimental

Chemistry

All of the materials were purchased from Merck (Germany) or Aldrich. The other solvents were purchased from Fluka and used without further purification. Column chromatography was performed with Merck Kieselgel 60. Melting points were measured with an Electrothermal Model 9200 (England). IR spectra were recorded on a BRUKER EQUINOX 55 IR spectrophotometer. The ESI-MS were performed on a VG Zab Spec (70 eV) instrument. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR were recorded on a BRUKER AVANCE 500 NMR spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in δ relative to TMS.

General procedure for Alkylation of flavonoids

To a stirred solution of flavonoid (1 mmol), K_2CO_3 (552 mg, 4 mmol) in acetone (50 mL) was added dimethyl sulfate (5.5 mmol) or allyl bromide (5.5 mmol). The mixture was stirred and refluxed for 10 h, monitoring by TLC. After removing potassium carbonate, the solvent was evaporated in reduced pressure. The residue was purified by column chromatography on silicagel.

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General procedure for Acylation of flavonoids

To a stirred solution of flavonoid (1 mmol) in pyridine (2 mL) was added acyl chloride (6 mmol) or n-butyl isocyanate (6 mmol) in nitrogen atmosphere. The mixture was stirred for 24 h at room temperature. The reaction solution was poured into 100 mL of water with ice. Solid was filtered, washed with water and dried. The residue was purified by column chromatography or crystallization from mixture of methanol and acetone.

***In vitro* Anti-inflammatory activity**

All compounds were screened for *in-vitro* anti-inflammatory activity by inhibition of bovine serum albumin denaturation method according to Saso et al.[17] The test compounds were dissolved in DMSO and diluted with acetate buffer (pH 5.5). The test solution (2 mL) containing different concentrations of drug was mixed with 3 mL albumin solution (0.2 %, w/v) in acetate buffer and incubated at $37^o \pm 1^o$ C for 30 min. Denaturation was induced by keeping the reaction mixture at $67^o \pm 1^o$ C in a water bath for 3 min. After cooling the turbidity was measured at 660 nm. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and the average was taken. The standard solution was also prepared as similar to that of the test solution. Diclofenac sodium was used as a standard. The percentage of inhibition was calculated from the following formula.

$$\%\ Inhibition = 100\ x\ (1 - \frac{V_t}{V_c})$$

Where, V_t = drug absorbance of triplicate average, V_c = control absorbance of triplicate average.

***In vivo* Anti-inflammatory activity**

Mice weighing 22 ± 2g of either sex were kept in appropriate cages at a temperature controlled room (25 ± 2^oC) under a 12h light/dark cycle. The animals were assigned into several groups randomly, each group consisting of six mice. Before the experiment, all the animals were acclimatized at least two days with food and water *ad libitum* in accordance with the World Health Organization's International Guiding Principles for Animal Research. [45] The methods used for test compounds were Carrageenan-induced mice paw edema. [46] The flavonoids were prepared as cream at 5 % of the dose and ketoprofen cream

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(2.5 %) was used as a reference standard. The mice were divided into various groups (n = 5). Different groups administered with various test compounds and ketoprofen (2.5 %) cream. One group of mice served as a control. A solution of carrageenan (0.025 mL, 1%) was injected subcutaneously into the subplanter region of hind paw. The thickness of the paw was measured before and 3 h after carrageenan injection using a plethysmometer (Ugo Basile 7140, Italy). The mice having the paw edema volume over 50 % of normal paw volume were chosen into the experiments. The mice were creamed twice each day, in the morning after measurement the paw edema and in the afternoon, during six days. The average paw edema was calculated according to the following formula

$$\% \, Edema = \frac{V_t - V_o}{V_o} x \, 100$$

Where V_0 = the paw volume before induction of inflammation and V_t = the inflamed paw volume.

Cell Culture

MCF-7 cell line was purchased from National Cancer Institute (Frederick, MD, U.S.A). Cells were cultured in E'MEM medium supplemented with L-glutamine (200 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (1M), amphotericin B 0.1 % (v/v), 100 U/mL of penicillin, 100 mg/L of streptomycin and 10 % (v/v) foetal bovine serum (FBS) at 37^{o}C in 5% (v/v) CO_2 incubator. For synchronization, cells at confluence of 70-80% were incubated for 32 h in E'MEM medium without FBS.

Antiproliferative Activity Assay

Cell viability was determined using SRB assay.[44, 47] In brief, cells were harvested and plated in a 96-well plate. After a 24 h culture, cells were incubated for 48 h with varying concentrations of test specimens. A solution of TCA 50% and SRB 0.2% was added into each well. The level of coloured was then analyzed with a Multiskan Ascent reader (Thermo Electron Corporation) at 492 nm. Concentration inducing a 50% inhibition of cell growth (IC_{50}) was determined graphically using the curve fitting algorithm. Values were means ± STD from three independent experiments.

Analysis of DNA Fragmentation

Briefly, 5 x 10^6 cells were lysed in a 600 µl buffer containing 10 mM Tris-HCl pH 8.0, 5 mM EDTA,

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100 mM NaCl, 0.2 % Triton X-100 for 10 min in ice. The lysate was then centrifuged at 14,000 rpm for 10 min. The supernatant was incubated with 1 mg/mL RNase A at 37^{o}C for 2h. Samples were extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) and once with chloroform: isoamyl alcohol (24:1, v/v). After ethanol precipitation, DNA was analyzed by 2 % agarose gel electrophoresis at 50 V for 1h.

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Conclusions

We herein report synthesis, spectral studies and biological evaluation of a series of O-alkyl and O-acyl flavonoid derivatives including novel compounds **2b**, **4a** and **4c**. Alkylation of 5-OH group depends on the alkylated reagent and structure of ring C of flavonoids. All compounds were evaluated for *in vitro* inhibition of bovine serum albumin denaturation and *in vivo* Anti-inflammatory activity. Some compounds which showed significant inhibition of albumin denaturation were subjected to anti-inflammatory study. The present study revealed that the flavonoids **3**, **3b**, **4b** and **4c** were effective against the carrageenan-induced paw edema. It is clear that acyl derivatives of diosmetin and hesperetin constitute an interesting template for the evaluation of new anti-inflammatory agents and may be helpful for design of new therapeutic tools against inflammation. In the other hand, the flavonoids **2**, **2c**, **3a** and **4b** displayed anti-proliferative activity against breast cancer cells MCF-7. Particularly, triacetyl derivative of hesperetin (**4b)** inducing degradation of DNA in MCF-7 cells was observed. This suggests that **4b** is as a potential agent for the further studies on a new anticancer drug.

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Scheme and Figure Captions

Fig. 1. Structures of four important flavonoids: quercetin (**1**), luteolin (**2**), diosmetin (**3**), and hesperetin (**4**)

Scheme 1. Reagents. (i) Dimethyl sulfate or allyl bromide, K_2CO_3, acetone, reflux; (ii) CH_3COCl or C_2H_5COCl or $CH_3(CH_2)_3NCO$, Py

Fig. 2. Photographs of mouse paw at 6th day after the carrageenan injection: Control (**A**) and **4b** (B).

Fig. 3. Control (A) and MCF-7 cells treated with **4b** for 48h (B)

Fig. 4. Agarose gel electrophoresis pattern of DNA isolated from **4b** treated MCF-7 cells. Lane 1, marker. Lane 2, treated MCF-7 in 36 h. Lane 3, in 48h. Lane 4, campthotecin-treated MCF-7 in 48h. Lane 5, untreated MCF-7.

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Table Captions

Table 1 ^{1}H, ^{13}C NMR, DEPT spectral data and HSQC and HMBC correlations in compound **4a**

Table 2. physical data, IR and HR-MS spectra of O-alkyl and O-acyl flavonoid derivatives.

Table 3. Chemical shifts (*ppm*) of the protons in the ^{1}H NMR spectra of O-alkyl and O-acyl flavonoid derivatives.

Table 4. Chemical shifts (*ppm*) of the carbon atoms in the ^{13}C NMR spectra of O-alkyl and O-acyl flavonoid derivatives.

Table 5. *In vitro* serum albumin inhibitory activity of flavonoids at various concentrations.

Table 6. Anti-inflammatory activity of flavonoids against carrageenan-induced mouse paw edema with different time intervals.

Table 7. Anti-proliferative activity of flavonoids against MCF-7 Cells.

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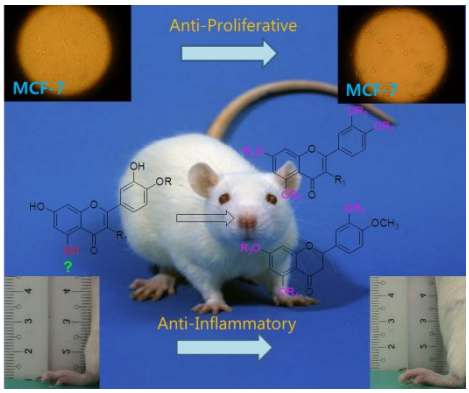
Graphical abstract



Highlights

- Six O-alkyl flavonoid derivatives including two novel compounds were synthesized with two alkylation reagents.
- Seven O-acyl flavonoid derivatives including a novel compound were synthesized with two acylation reagents
- Effect of structure of flavonoid on hydroxyl group reactivity at 5-position was showed.
- Anti-inflammatory activity of all flavonoids was examined by *in vitro* and *in vivo*.
- Anti-proliferative activity of all flavonoids was examined by SRB assay and apoptosis.

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