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In Vitro Regulation of Enzymes of the Renin-angiotensin-aldosterone System by Isoquercitrin, Phloridzin and their Long Chain Fatty Acid Derivatives

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ABSTRACT

Background: Hypertension is a crucial risk factor for development of cardiovascular and neurological diseases. Flavonoids exhibit a wide range of biological effects and have had increased interest as a dietary approach for the prevention or possible treatment of hypertension. However, continuous efforts have been made to structurally modify natural flavonoids with the hope of improving their biological activities. One of the methods used for the possible enhancement of flavonoid efficacy is enzymatic esterification of flavonoids with fatty acids.

Objective: The current study is designed to investigate the antihypertensive activity of isoquercitrin (quercetin-3-*O*-glucoside, Q3G) and phloridzin (PZ) in comparison to their twelve long chain fatty acid derivatives via enzymatic inhibition of renin angiotensin aldosterone system (RAAS) enzymes.

Methods: The novel flavonoid esters were synthesized by the acylation of isoquercitrin and phloridzin with long chain unsaturated and saturated fatty acids (C_{18}–C_{22}). These acylated products were then tested for their *in vitro* angiotensin converting enzyme (ACE), renin and aldosterone synthase activities.

Results: The linoleic and α-linolenic acid esters of PZ were the strongest (IC_{50} 69.9-70.9 µM) while Q3G and PZ (IC_{50} >200 µM) were the weakest renin inhibitors *in vitro* ($p \leq 0.05$). The eicosapentaenoic acid ester of PZ (IC_{50} 16.0 µM) was the strongest inhibitor of ACE, while PZ (IC_{50} 124.0 µM) was the weakest inhibitor ($p \leq 0.05$) among all tested compounds. However, all investigated compounds had low (5.0-11.9%) or no effect on aldosterone synthase inhibition ($p \leq 0.05$). The parent compound Q3G and the eicosapentaenoic acid ester of PZ emerged as the strongest ACE inhibitors.

Conclusions: The structural modification of Q3G and PZ significantly improved their antihypertensive activities. The potential use of PZ derivatives as natural health products to treat hypertension needs to be further evaluated.

Keywords: hypertension, phloridzin, isoquercitrin, flavonoids, ACE, renin, RAAS, acylation, fatty acids

INTRODUCTION:

Hypertension is the most common risk factor for cardiovascular diseases including myocardial infarction, stroke and kidney disease. The renin angiotensin aldosterone system (RAAS) is a physiological mechanism which regulates the blood pressure through the modulation of renal vasomotor function, salt and water balance [1]. Renin enzyme converts angiotensinogen into angiotensin I (Ang I) while Angiotensin Converting Enzyme (ACE) produced by endothelial lung cells further hydrolysis decapeptide Ang I into octapeptide angiotensin II (Ang II), triggering aldosterone secretion, which maintains normal blood pressure and salt balance under normal physiological conditions [1, 2]. However, the overproduction of the principal biochemical components of the RAAS system, including renin, ACE and aldosterone synthase, disturbs the RAAS equilibrium and triggers the pathophysiology of hypertension. Many drugs target inhibition of these three enzymes of RAAS (renin, ACE and aldosterone synthase) for attenuation of hypertension and other cardiac disorders [3]. In recent years, there has been an interest in identifying natural compounds and their derivatives for their antihypertensive properties based on RAAS inhibition. One particular category of natural compound that has received recent attention for their antihypertensive action is the flavonoids [4, 5].

Flavonoids are a class of polyphenolic plant secondary metabolites with diverse structures and functions [6]. There are over 9,000 characterized flavonoids that exhibit pharmacological properties such as antioxidants, anticarcinogens and immunomodulators [7, 8]. The vast spectrum of *in vitro* properties of flavonoids is questionedbecause the low bioavailability of flavonoids, due to poor absorption and extensive metabolism [9]. To enhance bioavailability and biological activities of flavonoids, various techniques, such as microencapsulation [10] and enzymatic esterification of flavonoids with fatty acids [11], have been employed. In a previous study, 12 novel acylated derivatives of isoquercitrin and phloridzin were synthesized and examined for selected biological properties [12]. In the current study, both precursor flavonoids isoquercitrin and phloridzin along with 12 fatty acid derivatives were studied for their renin, ACE and aldosterone synthase inhibitory properties *in vitro*.

METHODS:

Materials

The ACE, Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg (renin substrate), dimethyl sulfoxide (DMSO), ethyl acetate, HCl, histidine-leucine (His-Leu), histidine-L-hippuryl-L-leucine-chloride (HHL), isoquercitrin, lipase B from *Candida antarctica* (with an activity of 10,000 propyl laurate units), NaOH, *o*-pthaldehyde, phloridzin, renin (human recombinant) and Tris buffer were purchased from Sigma Aldrich Canada Ltd.,

Oakville, ON, Canada. Kidney tissue lysate was obtained from GeneTex, INC, Irvine, CA, USA. [4-^{14}C]-11-Deoxycorticosterone was obtained from Phenomenex Torrance, CA, USA. Dulbecco's modified eagle medium **(**DMEM**)** media and cell culture grade DMSO were obtained from Cederlane Labs, Burlington, ON, Canada. Round bottom 96-well clear and black microplates were obtained from BD International, Mississauga, ON, Canada and Corning Incorporated, Edison, NY, USA, respectively.

Synthesis of novel esterified flavonoids: The synthesis of novel esterified flavonoids has been carried out as previously described [12]. Briefly, the acylation of isoquercitrin and phloridzin was performed at a temperature between 45–50 °C in acetone with lipase B (Novozym 435®) from *Candida antarctica* with long chain unsaturated and saturated fatty acids (C_{18}–C_{22}). In order to achieve high enzyme efficiency, the substrate was dried for a period of 18–20 h over P_2O_5 before initiating the synthesis reaction. The progress and products of reaction were monitored and detected using thin-layer chromatography (TLC). The structures of acylated flavonoids were determined by ^{1}H NMR and ^{13}C NMR spectroscopic analysis using Bruker AVANCE 300 MHz spectrometer (Bruker Corp., Billerica, MA, USA). The resynthesized compounds were stored at -80°C. The compounds were dissolved separately in DMSO to make a stock solution of 1 mM, which was then diluted for further analysis.

Renin inhibition assay: The renin inhibitory potential of parent flavonoids and their esterified derivatives was determined with accordance to previously described methodology, allowing for some modifications [13]. Then, 50 µl of renin was dissolved in the assay buffer consisting of 50 mM Tris-HCl buffer (pH 8.0) and 100 mM NaCl (assay buffer). The activated enzyme was stored at -80°C, awaiting further analysis. Renin substrate (Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg) was diluted with DMSO to achieve a concentration of 500 µM. The assay was performed using 96-well black polypropylene plates. The background controls contained 20 µl of substrate along with 160 µl of assay buffer and 10 µl of DMSO (solvent control). The positive control was comprised of 20 µl of substrate, 150 µl of assay buffer and 10 µl of DMSO. The test compounds were analyzed using 20 µl of substrate, 150 µl of assay buffer and 10 µl of the test compound at the desired concentrations. The reaction was catalyzed by adding 10 µl of renin solution to the positive control and test compounds. The reaction mixture was incubated at 37°C for 45 minutes and the fluorescence signal produced from the cleavage of fluorophore-EDANS to peptide-EDANS by renin was measured at the excitation wavelength of 340 nm and the emission wavelength of 490 nm using the FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The renin inhibition was expressed at percentage inhibition using the following formula:

$$\%\ \textbf{Inhibition} = \left\{\frac{\textbf{Fluorescence (control)} - \textbf{Fluorescence (test compound)}}{\textbf{Fluorescence (control)}}\right\} \textbf{x 100}$$

ACE inhibition assay: The ACE inhibition potential of two flavonoids along with its esterified derivatives was tested according to a previously reported methodology [5]. The analysis was

carried out using test compounds at different concentrations, 2.5 mU ACE, 0.80 mM HHL and 2.5 mM sodium borate buffer (pH 8.3). The evaluation of ACE inhibitory potential of test compounds was dependent on His-Leu formation by the cleavage of HHL in the presence of ACE. This assay was performed using 96-well clear polystyrene plates (BD International, Mississauga, ON, Canada). The formation of His-Leu was measured using the florescence method, using the FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) at excitation and emission wavelengths of 360 nm and 500 nm, respectively. Calculations were performed, as previously outlined [5]. The results were expressed as the percentage inhibition of ACE inhibition and the IC_{50} values were calculated by linear interpolation.

Aldosterone synthase inhibition assay: The assay was conducted according to the method outlined in a previous study [14], with some modification. This assay is based on the principle that the potential aldosterone inhibitor can reduce the conversion rate of substrate, [4-^{14}C]-deoxycorticosterone to [4-^{14}C]-aldosterone. One hundred microliters of human kidney lysate was transferred onto a 24-well plate containing 700 µl of DMEM media per well. Test compounds were added, at the final test concentrations of 100 µM, along with DMSO for assay control. The compounds were incubated for 20 minutes and the assay substrate 500 nM [4-^{14}C]-deoxycorticosterone was added. The assay mixture was allowed to incubate for 6 h and the steroids were removed using ethyl acetate. The steroid mixture was dried and then re-dissolved in 20 µl chloroform and analyzed with respect to a positive control using the FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) at the wavelength of 490 nm.

Statistical and correlational analysis: Completely randomized design was used to perform the experiments, where each compound was assayed in triplicates (n=3). Statistical analysis was carried out using SAS 9.3 for Windows (SAS Institute Inc, Cary, NC, USA) and Minitab 16 (State College PA, USA). One way ANOVA was performed and the resultant values at $p \leq 0.05$ were considered significant. The Pearson coefficient obtained from Minitab 16 (State College PA, USA) was used for correlational analysis.

RESULTS:

Renin inhibition: Renin is an important enzyme in RAAS, as its pathological overproduction increases the level of peptide angiotensin I in the blood, leading to higher blood pressure. Natural compounds [15] and plant extracts [16] have shown strong renin inhibitory properties. Renin inhibition assay *in vitro* was conducted to assess the renin inhibitory properties of novel flavonoid derivatives (Table 1). For the series of Q3G derivatives, the renin assay resulted in significantly stronger renin inhibitory potential for the Q3G derivatives than the parent flavonoid ($p \leq 0.05$).

The IC_{50} value for Q3G was >200 µM, however the IC_{50} values for Q3G derivatives ranged between 73.03 to 93.09 µM. Among the Q3G derivatives, the oleic acid derivative (IC_{50} 73.0 µM) showed the strongest renin inhibition, while the eicosapentaenoic acid (EPA) ester of Q3G (IC_{50} 93.1 µM) was the weakest renin inhibitor ($p \leq 0.05$). Similar to Q3G, all the phloridzin series esters showed the higher antihypertensive potential than its parent compound ($p \leq 0.05$).

Table 1 Renin inhibition by novel fatty acid esters of isoquercitrin (Q3G) and phloridzin *in vitro*.

Concentration (μM)[x]	*In vitro* Percentage inhibition of Renin [§]						
	Q3G[∞]	Q3G-Stearic	Q3G-Oleic	Q3G-Linoleic	Q3G- α Linoleic	Q3G-EPA	Q3G-DHA
1	N.D[y]	N.D	N.D	N.D	N.D	N.D	N.D
10	17.03[c]	37.82[c]	51.69[b]	46.80[c]	47.74[c]	29.78[c]	48.69[c]
100	25.22[b]	52.31[b]	53.40[b]	61.19[b]	63.31[b]	49.73[b]	59.56[b]
1000	31.19[a]	67.59[a]	69.14[a]	73.93[a]	70.40[a]	68.64[a]	62.28[a]
IC_{50}[z] (μM)	>200[h]	89.34[f]	73.03[b]	78.69[d]	76.37[c]	93.09[g]	81.49[e]
	PZ	PZ-Stearic	PZ-Oleic	PZ-Linoleic	PZ -α Linoleic	PZ-EPA	PZ-DHA
1	N.D	N.D	N.D	N.D	N.D	N.D	N.D
10	5.73[c]	58.07[c]	40.6[c]	44.98[c]	30.41[c]	33.60[c]	26.02[c]
100	26.64[b]	63.31[b]	65.94[b]	69.83[b]	68.65[b]	62.70[b]	48.77[b]
1000	40.59[a]	66.65[a]	73.59[a]	90.66[a]	80.24[a]	94.22[a]	82.14[a]
IC_{50} (μM)	>200[h]	76.45[c]	72.89[ab]	69.94[a]	70.86[a]	79.19[d]	95.61[g]

[§]Data are presented as mean of three replicates. Mean with various subscripts in each column represent levels of significance ($p \leq 0.05$);

[x] Micro molar (μM)

[y] Not detected (ND)

[z] Inhibitory Concentration 50 percent (IC_{50})

[∞] Isoquercitrin (Q3G); Stearic acid ester of Q3G (Q3G-Stearic); Oleic acid ester of Q3G (Q3G- Oleic); Linoleic acid ester of Q3G (Q3G- Linoleic); α-linolenic acid ester of Q3G (Q3G- α-linolenic); Eicosapentaenoic acid ester of Q3G (Q3G-EPA); Phloridzin (PZ); Stearic acid ester of PZ (PZ-Stearic); Oleic acid ester of PZ (PZ- Oleic); Linoleic acid ester of PZ (PZ- Linoleic); α-linolenic acid ester of PZ (PZ- α-Linolenic); Eicosapentaenoic acid ester of PZ (PZ-EPA); Docosahexaenoic acid ester of PZ (PZ-DHA)

Phloridzin exhibited lower renin inhibition (IC_{50} value > 200 μM) than that of its esters. The IC_{50} values of phloridzin esters ranged between 69.9 to 95.6 μM. The strongest renin inhibitory potential among the compounds was that of linoleic ester of phloridzin with IC_{50} value of 69.9 μM ($p \leq 0.05$). Linoleic ester of phloridzin was also accompanied by its α-linolenic acid ester (IC_{50} 70.9 μM) as the strongest renin inhibitor. However, the EPA ester of phloridzin exhibited the weakest renin inhibition *in vitro* ($p \leq 0.05$). The results showed that presence of a longer carbon chain with multiple double bonds negatively impacted the renin inhibitory potential of the lipophilized flavonoids. However, the short chained α-linolenic acid and linoleic acids when attached to a parent flavonoid elevated its antihypertensive abilities.

ACE inhibition: The overproduction of ACE catalyzes uncontrolled conversion of angiotensin I to angiotensin II, leading to the constriction of blood vessels through bradykinin degradation and, subsequently, hypertension. Polyphenolic compounds are potent antihypertensive agents [17] with strong ACE inhibitory properties [4, 5]. Many flavonoid-rich extracts and powder have

also shown to exhibit antihypertensive properties [4, 5, 18]. The present study investigated the ACE inhibitory potential of acylated derivatives of phloridzin and Q3G (Table 2).

Table 2 ACE inhibition by novel fatty acid esters of isoquercitrin (Q3G) and phloridzin *in vitro*.

Concentration (μM)[x]	*In vitro* Percentage inhibition of Angiotensin Converting Enzyme (ACE) [§]						
	Q3G[∞]	**Q3G-Stearic**	**Q3G-Oleic**	**Q3G-Linoleic**	**Q3G- α Linoleic**	**Q3G-EPA**	**Q3G-DHA**
1	15.07[d]	3.42[d]	5.60[d]	N.D[y]	N.D	5.61[d]	12.30[d]
10	30.88[c]	8.54[c]	8.65[c]	9.93[c]	8.39[c]	29.25[c]	28.86[c]
100	75.17[b]	54.42[b]	52.55[b]	60.18[b]	63.24[b]	56.67[b]	55.68[b]
1000	91.24[a]	89.27[a]	84.89[a]	84.70[a]	76.22[a]	76.63[a]	73.48[a]
IC_{50}[z] (μM)	24.28[b]	72.96[g]	84.77[h]	73.69[g]	90.92[i]	68.03[f]	70.80[f]
	PZ	**PZ-Stearic**	**PZ-Oleic**	**PZ-Linoleic**	**PZ-α-Linoleic**	**PZ-EPA**	**PZ-DHA**
1	3.04[d]	9.54[d]	12.52d	8.54[c]	5.46[c]	7.62[c]	9.98[c]
10	9.57[c]	16.50[c]	23.78[c]	38.86[b]	34.57[b]	49.30[b]	27.93[b]
100	43.20[b]	61.89[b]	71.07[b]	79.91[a]	86.14[a]	87.08[a]	81.24[a]
1000	80.57[a]	85.71[a]	85.06[a]	80.24[a]	88.40[a]	89.35[a]	86.23[a]
IC_{50} (μM)	123.96[j]	54.59[e]	37.27[d]	26.57[c]	23.91[b]	16.03[a]	28.21[c]

[§]Data are presented as mean of three replicates. Mean with various subscripts in each column represent levels of significance ($p \leq 0.05$);

[x] Micro molar (μM)

[y] Not detected (ND)

[z] Inhibitory Concentration 50 percent (IC_{50})

[∞] Isoquercitrin (Q3G); Stearic acid ester of Q3G (Q3G-Stearic); Oleic acid ester of Q3G (Q3G- Oleic); Linoleic acid ester of Q3G (Q3G- Linoleic); α-linolenic acid ester of Q3G (Q3G-α-linolenic); Eicosapentaenoic acid ester of Q3G (Q3G-EPA); Phloridzin (PZ); Stearic acid ester of PZ (PZ-Stearic); Oleic acid ester of PZ (PZ- Oleic); Linoleic acid ester of PZ (PZ- Linoleic); α-linolenic acid ester of PZ (PZ- α-Linolenic); Eicosapentaenoic acid ester of PZ (PZ-EPA); Docosahexaenoic acid ester of PZ (PZ-DHA)

In contrast to inhibition of renin, the parent flavonoids showed active inhibition of ACE *in vitro*. For the series of Q3G derivatives, the ACE assay has resulted in significantly stronger ACE inhibitory potential for the parent compound Q3G than its lipophilized derivatives ($p \leq 0.05$). The IC_{50} value for Q3G was 24.3 μM while the IC_{50} values for Q3G derivatives ranged between 68.0 to 90.9 μM. The EPA (IC_{50} 68.0 μM) and DHA (IC_{50} 70.8 μM) esters of Q3G showed the strongest ACE inhibition among all the Q3G derivatives. Opposite to the renin inhibitory analysis, the oleic and α-linoleic esters of isoquercitrin exhibited weak ACE inhibitory

potential ($p \leq 0.05$). The weakest ACE inhibitor *in vitro* in Q3G series was its α-linoleic ester with IC_{50} value of 90.9 μM ($p \leq 0.05$). The ACE inhibitory activity of phloridzin and its derivatives followed its trend of renin inhibitory analysis. All of the phloridzin series esters showed the stronger antihypertensive potential ($p \leq 0.05$) than their parent compound phloridzin, which represented the weakest ACE inhibition with IC_{50} value of 123.9 μM ($p \leq 0.05$). The IC_{50} values of phloridzin esters ranged between 16.03 and 54.6 μM. The strongest ACE inhibitory potential among the compounds was that of EPA ester of phloridzin (IC_{50} 16.03 μM) with approximately eight times lower IC_{50} value than phloridzin. It was closely followed by its α-linolenic acid ester (IC_{50} 23.9 μM) as strong ACE inhibitor ($p \leq 0.05$). Unlike ACE inhibition by oleic and α-linoleic esters of isoquercitrin, their phloridzin counterparts were potent ACE inhibitors ($p \leq 0.05$). The weakest ACE inhibition in phloridzin series esters was exhibited by its stearic acid ester (IC_{50} 54.59 μM) with approximately two times weaker ACE inhibition than phloridzin. Overall, the long chain EPA and docosahexaenoic acid (DHA) esters of phloridzin emerged as the potent ACE inhibitors *in vitro*.

Aldosterone synthase inhibition: Aldosterone, produced by aldosterone synthase, is a principal mineralocorticoid in humans that plays a part in fluid regulation and hypertension. The blockage of its mineralocorticoid receptor is accompanied by the alternative approach of aldosterone synthase inhibition for treating hypertension [19]. Polyphenols have been found to attenuate hypertension and its pathological manifestations in rodent model of hyperaldosteronism [20]. Our study was focused on possible aldosterone synthase inhibition by two flavonoids and their derivatives *in vitro* (Figure 1).

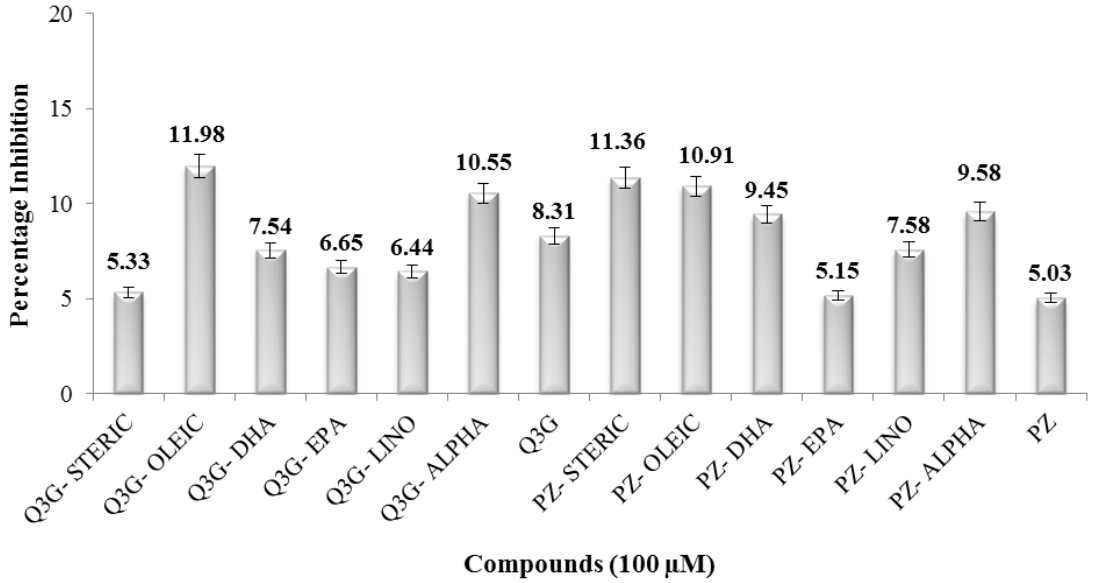


Figure 1. Aldosterone synthase inhibition by novel fatty acid esters of Isoquercitrin (Q3G) and Phloridzin (PZ) in vitro. Data are presented as percentage inhibition. Phloridzin (PZ); Stearic acid ester of PZ (PZ-Steric); Oleic acid ester of PZ (PZ- Oleic); Linoleic acid ester of PZ (PZ- Lino); α-linolenic acid ester of PZ (PZ- alpha); Eicosapentaenoic acid ester of PZ (PZ-EPA); Docosahexaenoic acid ester of PZ (PZ-DHA); Isoquercitrin (Q3G); Stearic acid ester of Q3G (Q3G-Steric); Oleic acid ester of Q3G (Q3G- Oleic); Linoleic acid ester of Q3G (Q3G- Lino); α-linolenic acid ester of Q3G (Q3G- alpha); Eicosapentaenoic acid ester of Q3G (Q3G-EPA).

The results showed very weak inhibition of aldosterone synthase by assayed compounds. The overall inhibition percentage of aldosterone synthase by assayed compounds ranged from 5.03% to 11.98%. In the Q3G series, some of the fatty acid derivatives exhibited higher aldosterone synthase inhibition, compared to the parent compound (Q3G). Q3G displayed 8.3% inhibition of aldosterone synthase *in vitro*. The highest amount of inhibition was shown by oleic acid ester of Q3G (12.0%) while its stearic acid ester exhibited the lowest inhibition (5.3%) at 100 µM. The phloridzin series also exhibited very weak inhibition of the enzyme as the parent compound exhibited only 5.0% inhibition while its stearic acid derivative showed 11.3% inhibition *in vitro*. However, there was no significant difference among the Q3G and phloridzin series compounds assessed ($p \leq 0.05$) for their aldosterone synthase inhibition.

Correlation Analysis: Correlation analysis was conducted to see the interrelation between selected biological profiles (ACE and renin inhibition) of the flavonoids and their structural derivatives (Table 3). The Pearson coefficient was obtained using Minitab 16 (State College PA, USA). Among the Q3G series, there was a strong direct relationship between ACE and renin inhibition in stearic acid, EPA and DHA esters. The parent compound Q3G exhibited a strong inverse relationship between ACE and renin inhibition *in vitro*. However, due to small sample sizes (n=3) the Pearson coefficient obtained from Q3G series was not statistically significant ($p \leq 0.05$). Similar to Q3G series, EPA and stearic acid showed a strong direct relationship between ACE and renin inhibition. Similarly, strong inverse relationship was also seen in second parent compound, phloridzin.

Table 3 Correlation between extent of ACE and renin inhibition.

Compound no.	Compound	ACE Inhibition (IC_{50}[∞] value)	Renin Inhibition (IC_{50} value)	Pearson correlation	p-value
1	Phloridzin (PZ)	123.96	>200	-0.961	0.124
2	Stearic acid ester of PZ	54.59	76.45	0.673	0.530
3	Oleic acid ester of PZ	37.27	72.89	0.314	0.739
4	Linoleic acid ester of PZ	23.91	70.86	0.346	0.779
5	α-Linolenic acid ester of PZ	26.57	69.94	0.571	0.439
6	EPA ester of PZ	16.03	79.19	0.789	0.342
7	DHA ester of PZ	28.21	95.61	-0.535	0.641
8	Isoquercitrin (Q3G)	24.28	>200	-0.725	0.215
9	Stearic acid ester of Q3G	72.96	89.34	0.836	0.370
10	Oleic acid ester of Q3G	84.77	73.03	-0.142	0.909
11	Linoleic acid ester of Q3G	90.92	76.37	0.693	0.513
12	α-Linolenic acid ester of Q3G	73.69	78.69	-0.019	0.988
13	EPA ester of Q3G	68.03	93.09	0.765	0.446
14	DHA ester of Q3G	70.80	81.49	0.815	0.619

[∞]Inhibitory Concentration 50 percent (IC_{50})

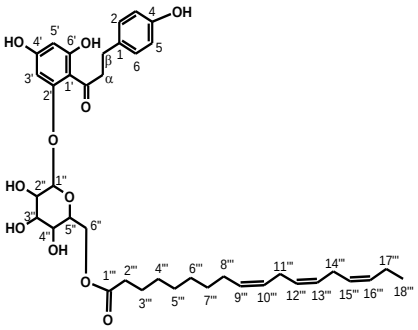
The common feature of both series was a strong inverse relationship between ACE and renin inhibition by parent flavonoids, along with a direct relationship between EPA and stearic acid esters of parent compounds.

DISCUSSION:

The current study was conducted to investigate and compare the antihypertensive activity of flavonoids and their derivatives *in vitro*. The enzyme inhibition assays were conducted to examine the RAAS inhibitory potential of two flavonoids and their derivatives. The isoquercitrin emerged as a weak inhibitor of renin *in vitro* like other large polyphenol molecules, including (-)-epigallocatechin gallate (IC_{50} = 45 µM), (-)-epicatechin gallate (IC_{50} = 619 µM) and (-)-epigallocatechin (IC_{50} = 2175 µM) [21]. We found that in Q3G series of compounds, the strongest renin inhibitory potential was exhibited by oleic acid ester of Q3G. Oleic acid, a monounsaturated omega-9 fatty acid has diverse biological properties including antihypertensive activity. A previously reported rodent model study [22] also confirmed improved renin inhibition using emulsion formulation containing oleic acid. Similarly, another study also confirmed the strong renin inhibitory properties of oleic acid [23]. The renin inhibitory activity of Q3G series shows that enzymatic esterification significantly improved the renin inhibition *in vitro*. The presence of double bonds in fatty acids of the Q3G derivatives may be associated with the improved enzyme inhibition. Although phloridzin in current study emerged as a weak renin inhibitor but its linoleic (18:2 ω-6) and α-linolenic acid (18:3 ω-3) esters emerged as most potent renin inhibitors *in vitro*. The results from the current study are similar to earlier investigations [23, 24] confirming the independent renin inhibition by linoleic acid. Earlier reports also suggested that linoleic acid modulates renin *in vivo* [24]. The linoleic acid derivative of phloridzin holds strong clinical relevance as linoleic acid also exhibits strong renin inhibition in human plasma [25]. While a large body of scientific data confirms independent renin inhibitory activity of linoleic acid [23-25] but there is no literature that provides strong support for the current results, showing α-linolenic acid ester as renin inhibitor. When compared to a clinical renin inhibitor, aliskiren (IC_{50} 0.6 nM), with bioavailability 2.6% [26] these esterified compounds may exhibit higher bioavailability leading to renin inhibition *in vivo*. Overall, the IC_{50} values indicated a common feature in both series of compounds, i.e., the stronger renin inhibitory potential of esterified flavonoids. Based on the current results, it can be concluded that esterification of flavonoids improved their renin inhibitory potential. These results encourage the use of these novel compounds as nutraceuticals or supplements for possible direct renin inhibition.

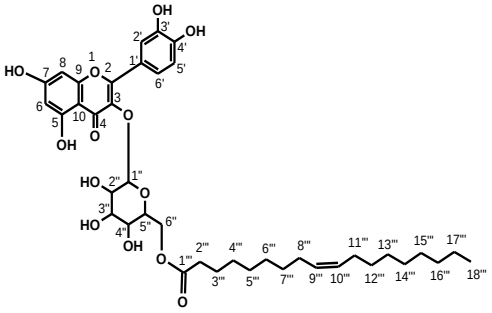
ACE is responsible for conversion of Angiotensin I (Asp-Arg-Val-Tyr-IIe-His-Pro-Phe-His-Leu) to Angiotensin II (Asp-Arg-Val-Tyr-IIe-His-Pro-Phe) and this conversion step in RAAS pathway serves as a drug target for antihypertensive therapy. An *in vitro* assay aimed at ACE inhibition was conducted in order to assess the antihypertensive action of flavonoids and their esterified derivatives. In Q3G series, the parent compound emerged as the strongest ACE inhibitor *in vitro* compared to its derivatives. However, the EPA (20:5 ω-3) and DHA (20:6 ω-3) esters of Q3G were the most active ACE inhibitors among Q3G series derivatives. An earlier report [27] also supported the antihypertensive role of polyunsaturated fatty acids (PUFA), i.e.,

EPA and DHA. The increasing number of double bonds in derivatives was found to be a related factor for stronger ACE inhibition (Figure 2).



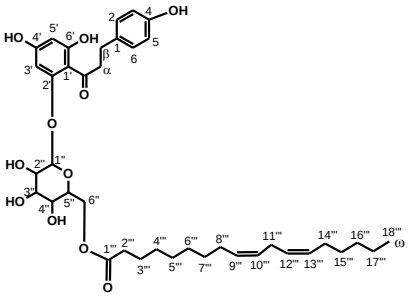
α-Linolenic acid Ester of Phloridzin; 18:3 (n-3)

Molecular Formula = $C_{39}H_{52}O_{11}$
Formula Weight = 696.82358
Composition = C(67.22%) H(7.52%) O(25.26%)



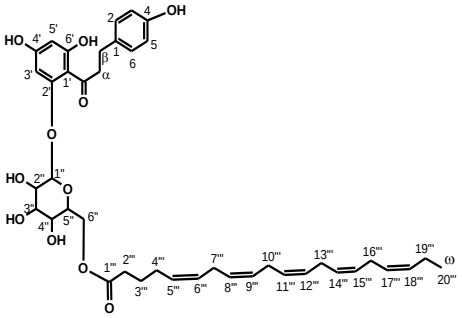
Oleic Acid Ester of Isoquercitrin;18:1 (n-9)

Molecular Formula = $C_{39}H_{52}O_{13}$
Formula Weight = 728.82238
Composition = C(64.27%) H(7.19%) O(28.54%)



Linoleic acid Ester of Phloridzin;18:2 (n-9)

Molecular Formula = $C_{39}H_{54}O_{11}$
Formula Weight = 698.83946
Composition = C(67.03%) H(7.79%) O(25.18%)



Eicosapentaenoic Acid Ester Phloridzin; 20:5 (n-3)

Molecular Formula = $C_{41}H_{52}O_{11}$
Formula Weight = 720.84498
Composition = C(68.31%) H(7.27%) O(24.41%)

Figure 2. Structures of selected novel *in vitro* antihypertensive fatty acid esters of Isoquercitrin and Phloridzin

However, the comparative analysis between Q3G and its derivatives showed that the addition of long chain fatty acids to Q3G didn't exhibit an impact on the ACE inhibition. The loss of the hydroxyl group from sugar moiety could be a contributory factor resulting in lower ACE inhibition by Q3G derivatives compared to their parent compound. In the phloridzin ester series, the EPA (20:5 ω-3) ester emerged as the strongest ACE inhibitor. EPA ester of phloridzin with polyene saturation and a higher number of double bonds exhibited a stronger interaction with ACE enzyme *in vitro*. The α-linolenic acid ester (18:3 ω-3) of phloridzin closely followed the EPA ester of phloridzin in ACE inhibition. The findings are supported by earlier evidence which suggests the nontoxic and strong antihypertensive potential of α-linolenic acid *in vivo* in humans [28]. The weakest ACE inhibitor among phloridzin esters, i.e., stearic acid derivative of phloridzin, (IC_{50} 54.6 μM) exhibited approximately two times weaker ACE inhibitory activity than the parent compound. The stearic acid (18:0) esters in both series of compounds exhibited weak ACE inhibition. The absence of double bonds and saturated status could be a contributory

factor towards weak ACE inhibition by stearic acid esters of flavonoids. Interestingly, the loss of hydroxyl group in phloridzin esters did not result in lower ACE inhibitory potential. The improved ACE inhibitory activity of the phloridzin esters could be due to flexible phloridzin backbone, i.e., more conformational isomers resulting in its free rotation [12]. With a higher number of conformational isomers, it is possible that phloridzin might interact better with the ACE enzyme. The strong ACE inhibitory potential of precursor flavonoids esterified with EPA was a common feature of the analysis. The IC_{50} value of EPA ester of phloridzin was at par with active natural ACE inhibitors including epicatechin – tetramer (IC_{50} 4 µM), epicatechin – hexamer (IC_{50} 8 µM), quercetin-3-*O*-glucuronic acid (IC_{50} 27 µM) and captopril (IC_{50} 1 µM) [4]. Based on the results, it can be concluded that fatty acid esters of phloridzin are stronger ACE inhibitors compared to the Q3G derivatives. The EPA and α-linolenic acid esters of phloridzin emerged as promising ACE inhibitors *in vitro*. The results suggest that these flavonoid derivatives can be explored as potential candidates for ACE inhibition *in vivo.*

The final assay focused on the aldosterone synthase inhibitory activity of the compounds. All compounds exhibited very weak inhibitory activity against aldosterone synthase. The oleic (18:1 ω-9), α-linoleic acid (18:3 ω-3) and steric acid (18:0) esters of flavonoids exhibited strong aldosterone synthase inhibition. Among all of the compounds assayed, the oleic acid (18:1 ω-9) ester of Q3G was the strongest aldosterone synthase inhibitor. The findings of this study are similar to an earlier report [29], which showed that oleic acid was most potent inhibitor of aldosterone synthesis among fatty acids and also inhibited the aldosterone synthesis from exogenous 18-hydroxycorticosterone. Similarly, another study further confirmed aldosterone inhibition by oleic acid [30]. Among the phloridzin series, all compounds exhibited higher aldosterone synthase inhibition than the parent compound, phloridzin. The oleic acid ester of phloridzin showed similar activity and almost equal enzyme inhibition compared to the oleic acid derivative of Q3G. These results are in accordance with a previous report which suggested that fatty acids may regulate aldosterone secretion and mediate blood pressure [31]. However, in both Q3G and PZ series, there was no significant difference among all the compounds assessed ($p \leq 0.05$) for their aldosterone synthase inhibition. The current study revealed that lipase assisted esterification of flavonoids significantly improved their antihypertensive activities as they emerged as active dual RAAS inhibitors *in vitro*.

CONCLUSION:

The fatty acid esters of Q3G and phloridzin were found to be potential antihypertensive agents based on the inhibition of renin, ACE and aldosterone synthase *in vitro*. The novel compounds emerged as promising renin inhibitors *in vitro* as both series of derivatives exhibited stronger renin inhibition compared to their parent compounds. The linoleic acid and α-linolenic acid esters of phloridzin were the most potent renin inhibitor amongst all of the compounds assayed, thus promising therapeutic attenuation of hypertension. The EPA ester of phloridzin was the most effective ACE inhibitor identified among both series of flavonoids and their derivatives. Overall, the phloridzin esters were stronger ACE inhibitors *in vitro* compared to their Q3G counterparts. Following the trend, all phloridzin esters also exhibited stronger potency in inhibiting aldosterone synthesis compared to their parent compound. In the series of both flavonoids (Q3G and PZ), the oleic acid derivatives were strong inhibitors of aldosterone

synthase *in vitro*. The improved biological activity of the phloridzin esters could be related to flexible phloridzin backbone and addition of biologically active fatty acids as it increased the lipophilicity of the compounds, which can enhance hydrophobic interactions with enzyme targets. The results suggest that novel fatty acid derivatives of flavonoids have strong potential as dual RAAS modulators through enzymatic inhibition. However, studies *in vivo* using animal models and clinical studies are required to confirm the *in vitro* findings.

Abbreviations: renin-angiotensin-aldesterone system (RAAS), Angiotensin converting enzyme (ACE), Quercetin-3-*O*-glucoside, Q3G), Phloridzin (PZ)

Competing interest: The authors have no conflicts of interest.

Authors' contributions Dr. Ziaullah contributed by the bio-catalytic synthesis and chemical characterization of the new compounds Mr. Khushwant Singh Bhullar completed all the biological assays and contributed to writing the manuscript .Dr. H.P.V Rupasinghe, the principle investigator, designed this study and co-authored the manuscript

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