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Original article

Design, synthesis and evaluation of mutual prodrug of 4-biphenylacetic acid and quercetin tetramethyl ether (BPA–QTME) as gastrosparing NSAID

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ABSTRACT

A novel mutual prodrug consisting of 4-biphenylacetic acid (BPA) and quercetin tetramethyl ether (QTME) has been synthesized as a gastrosparing NSAID, devoid of ulcerogenic side effects. The physicochemical properties, including aqueous solubility, partition coefficient, chemical stability and enzymatic hydrolysis of synthesized derivative have been studied to assess its prodrug potential. Its antiinflammatory, antiulcer and analgesic activities were also evaluated. The results indicated that BPA–QTME derivative is chemically stable, biolabile and possesses optimum lipophilicity. The synthesized compound also exhibited retention of antiinflammatory activity with reduced ulcerogenicity. Based on these observations, the therapeutic potential of this mutual prodrug is discussed.

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1. Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs) belong to one of the most commonly used therapeutically group of agents worldwide for the treatment of pain, fever and inflammation. However, usefulness of these agents is limited due to higher incidence of gastrointestinal (GI) damage including gastric ulceration, perforation and their associated complications. These side effects are related to the intrinsic mechanism responsible for their desired activity [1,2]. NSAIDs exert their therapeutic effects by inhibiting the activity of the enzyme cyclooxygenase (COX), resulting in the prevention of prostaglandins synthesis [3]. Now, it is known that COX exists in two isoforms, namely COX-1 and COX-2 [4,5]. COX-1 is constitutive and provides cytoprotection in the GI tract, whereas COX-2 is inducible and mediates inflammation. The mucosal integrity in normal GI tract is primarily maintained by the prostaglandins that are derived from COX-1 and therefore, inhibition of COX-1 rather than COX-2 by NSAIDs is responsible for their ulcerogenic side effect [6–8]. Based on these observations, it has been suggested that selective COX-2 inhibitors may act as safer NSAIDs, devoid of their ulcerogenic side effects and a number of such selective COX-2 inhibitors have been developed and introduced in the market for clinical use [9,10]. However, long term use of these agents has shown some potential limitation, including ulcer exacerbation in high risk patients, delayed GI ulcer healing, kidney toxicity, as well as, cardiovascular side effects [11]. These observations indicated that safety of these agents is questionable on their long term use and some of these agents have been withdrawn from the market [12]. Thus, the initial enthusiasm of developing selective COX-2 inhibitors has faded away and need for design and development of safer NSAIDs still remains.

During recent years, it has been well established that generation of reactive oxygen species (ROS) plays a significant role in the formation of gastric mucosal lesions associated with NSAIDs therapy [13,14]. Based on these observations, it has been suggested that coadministration of antioxidants and NSAIDs in pharmaceutical dosage forms may possibly decrease the risk of NSAIDs induced GI ulcerogenicity [15,16]. However, there are potential advantages in giving such agents with complementary pharmacological activities in the form of a single chemical entity. Such agents are named as mutual prodrugs or codrugs that are designed with improved physicochemical properties and release the parent drugs at the site of action [17–19]. We have reported the synthesis of a number of mutual prodrugs of 4-biphenylacetic acid (BPA) and different phytophenolics having single hydroxyl groups [20]. In the present study, quercetin, a naturally occurring polyphenolic flavonoid was selected to conjugate with BPA to obtain BPA-Antioxidant mutual prodrug. Quercetin is well known for antiulcerogenic activity due to its antioxidant properties [21,22]. Although, linking of quercetin with BPA in 1:1 ratio is difficult due to the presence of a number of hydroxyl groups, we have been able to conjugate this agent in the form of its derivative, quercetin tetramethyl ether

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(QTME) with BPA. In this paper, design, synthesis and evaluation of BPA–QTME mutual prodrug as safer NSAID are reported.

2. Chemistry

To conjugate polyphenolic flavonoid, quercetin (**1**) with carboxyl group containing NSAIDs in 1:1 ratio, an alternative strategy was developed. For this purpose, rutin (**2**), the glycoside of **1** was treated with dimethyl sulfate (**3**) in dry acetone in the presence of potassium carbonate. The methylated glycoside (**4**) was obtained as semisolid, which was not purified and subjected to hydrolysis by refluxing in ethanolic sulfuric acid to obtain the quercetin derivative, quercetin tetramethyl ether (QTME, **5**). The free hydroxyl group generated at 3-position in this derivative was used as a synthetic handle for conjugation with the BPA (**6**).

BPA (**6**) was treated with thionyl chloride (**7**) by stirring at room temperature and after removing excess of thionyl chloride, biphenylacetyl chloride (**8**) was obtained as yellow amorphous powder in 84% yield. Quercetin derivative, QTME (**5**) was treated with **8**, triethylamine and 4-dimethylaminopyridine using dichloromethane as solvent and the desired derivative, BPA–QTME (**9**) was obtained as light yellow solid. Sequence of various steps involved in the reaction is shown in Scheme 1.

3. Results and discussion

3.1. Solubility and partition coefficient

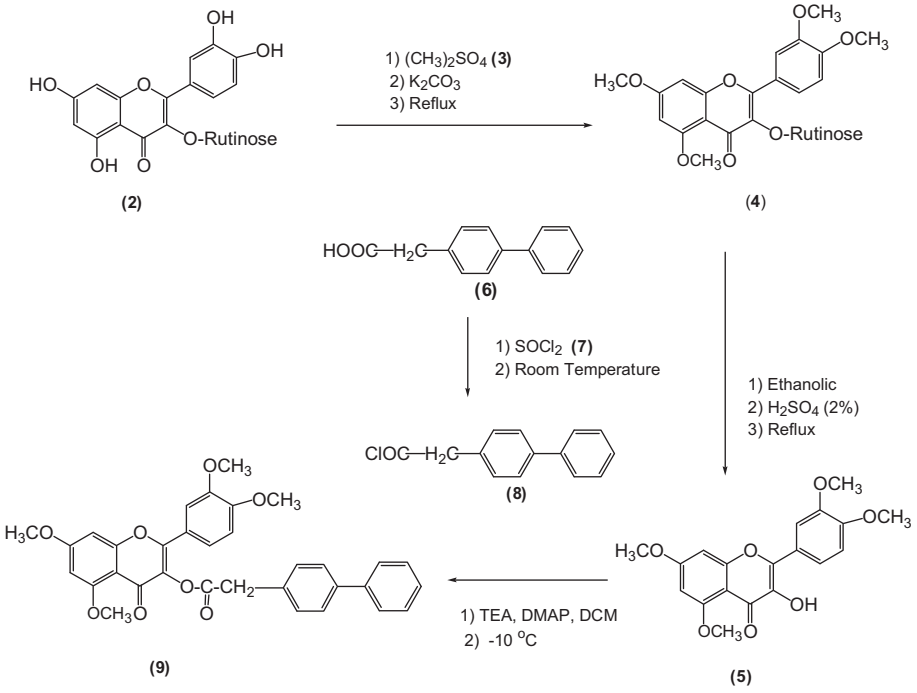
Lipophilicity is an important factor controlling the interaction of drugs with biological membranes. It is generally accepted that good absorption of an orally administered drug could be obtained when apparent coefficient partition (Log *P*) value is more than 2 and the aqueous solubility is more than 10 μg/ml [23]. The evaluation of solubility and apparent partition coefficient (Log *P*) of **9** was performed by saturation shake flask method [24–26]. For the quantification of **6** and **9**, standard plots were constructed using UV spectrophotometer and quantification was carried out accordingly. To assess the lipophilicity, the Log *P* of BPA–QTME (**9**) was determined in n-octonol and buffer layers and calculated by correlating the absorbance with the concentration in standard plot. The obtained values of Log *P* and solubility at pH 7.4 were found to be 3.95 and 14.5 μg/ml respectively, indicating that the synthesized derivative **9** meets the requirement for gastrointestinal absorption.

3.2. Chemical stability

A prodrug should be chemically stable so that it can be formulated in an appropriate pharmaceutical dosage form with optimum half life. At the same time, it should be biolabile to regenerate the parent drug molecule(s) to exhibit therapeutic activity. Therefore, the BPA–QTME (**9**) was assayed *in vitro* to evaluate its chemical stability [27]. The kinetics of chemical hydrolysis was studied at 37 °C using buffer solutions of pH 2 and 7.4. The reactivity to chemical hydrolysis was evaluated by pseudo first order rate constants obtained from slopes of semi logarithmic plots of **9** concentrations against time. The rate constants at pH 7.4 and 2 were found to be 0.0180 and 0.0066 h^{-1} respectively, indicating the stability in buffer solutions. The derivative showed considerable chemical stability at pH 2 ($t½ > 100$ h) which implies that it passed unhydrolysed through the stomach after oral administration. At pH 7.4, the compound showed enough stability ($t½ > 38$ h) to be absorbed intact from the intestine.

3.3. Enzymatic hydrolysis

In 80% human plasma (human plasma containing 20%, 0.02 m phosphate buffer, pH 7.4), BPA–QTME ester bond was found to be cleaved, forming the parent molecules, BPA (**6**) and QTME (**5**). The degradation process was found to correlated with pseudo first order kinetics for several half lives. The rate of hydrolysis of **9** was found to be 2.09 h^{-1} and half life ($t½$) being 19.8 min respectively, indicating that **9** is readily hydrolyzed in plasma to release the parent drug molecules. The rapid rate of hydrolysis observed in



Scheme 1. Sequence of steps involved in the synthesis of BPA–QTME (**9**) mutual prodrug.

plasma and more stability in the absence of plasma under similar conditions in buffers (pH 2 and 7.4) is important and implies that enzymatic reactivity of **9** is independent of their intrinsic ester reactivity.

3.4. Biochemical evaluation

The effects of BPA (**6**), BPA–QTME (**9**) and BPA + QTME physical mixture were studied on various peripheral markers of oxidative stress including lipid peroxidation (MDA levels), myeloperoxidase activity (MPO levels), superoxide dismutase activity (SOD), catalase activity and shown in Table 1.

MDA levels were found to be significantly increased ($P < 0.01$) in BPA treated group (22.57 ± 2.34) in comparison to control group (4.61 ± 1.28), indicating gastric damage. However, BPA–QTME derivative **9** showed MDA level (6.42 ± 0.55) close to the control group (4.61 ± 1.28) and significantly reduced as compared with the BPA + QTME physical mixture (13.36 ± 1.92) and BPA (22.57 ± 2.34). These results suggested that BPA–QTME may exhibit protective effect against gastric damage as compared to BPA + QTME physical mixture which may be due to the inhibition of lipid peroxidation and cell damage (Table 1).

Similarly, myeloperoxidase activity (%MPO) was significantly increased in BPA treated group (238.47 ± 19.39) as compared to control. BPA–QTME (**9**) significantly reduced myeloperoxidase activity (118.93 ± 5.55) as compared to BPA + QTME physical mixture treated group (227.33 ± 13.32), which is close to control group (100 ± 0). These results indicated lesser neutrophil adherence in BPA–QTME mutual prodrug treated group, which may result in reduction of ulcerogenic side effects (Table 1).

SOD is an endogenous antioxidant enzyme that scavenges superoxide radical ($O_2^{\bullet-}$) by catalyzing its dismutation to H_2O_2 and O_2. Decreased level of SOD indicates increased generation of $O_2^{\bullet-}$ and increased gastric mucosal injury. SOD activity was significantly decreased in BPA treated group (26.23 ± 6.31) as compared to the control group (325.40 ± 69.0). BPA–QTME treated group showed significantly increased levels of SOD (223.46 ± 24) as compared with BPA + QTME physical mixture treated group (60.41 ± 13.28) (Table 1).

Catalase is endogenous antioxidant enzyme that scavenges hydroxyl radical ($^{\bullet}OH$) by converting H_2O_2 to water. Decreased level of catalase indicates increased gastric damage. Catalase activity was significantly decreased in BPA treated group (3.52 ± 0.33) as compared to the control group (28.38 ± 2.85). BPA–QTME treated group showed maintenance levels of catalase (22.41 ± 1.68) close to control as compared with BPA + QTME physical mixture treated group (8.69 ± 0.30).

The results of studies on lipid peroxidation (MDA levels), myeloperoxidase activity (MPO levels), superoxide dismutase activity (SOD) and catalase activity suggested that BPA–QTME conjugate **9** exhibited significant activity as compared to BPA + QTME physical mixture. Therefore, this conjugate may exhibit antiinflammatory activity without/or reduced gastric damage. On the basis of these observations, this agent was also evaluated for various pharmacological activities.

3.5. Pharmacological evaluation

BPA and BPA–QTME mutual prodrug were evaluated for their antiinflammatory activity by carrageenan induced paw edema methods [28]. In this method, carrageenan (1% w/v) was used to produce paw edema in control group. BPA (15 mg/kg, 70 mmol, p.o.) significantly decreased the carrageenan induced increase in paw volume as compared to control. BPA–QTME at equivalent dose (39 mg/kg, 70 mmol) significantly inhibited carrageenan induced inflammation and showed antiinflammatory activity comparable to parent drug, BPA (Table 2).

These agents were studied for acute gastric damage evaluation [29]. For this purpose, BPA (54 mg/kg, 254 mmol, p.o.) was administered to produce a significant increase in ulcer index (6.2 ± 0.41) as compared to control group (0.2 ± 0.12). Equivalent dose of BPA–QTME **9** (140 mg/ml, 254 mmol, p.o.) showed significantly reduced gastric damage (ulcer index 0.45 ± 0.27). BPA + QTME physical mixture in equivalent doses (54 mg/kg, 254 mmol) + (86 mg/kg, 254 mmol, p.o.) also showed reduction in gastric damage (ulcer index 3.4 ± 0.57) but the inhibition of gastric damage by BPA–QTME **9** was to a greater extend which may be due to combined effect of improved physicochemical properties of the conjugate, resulting in improved absorption and antioxidant activity of the released QTME (Table 2).

For the evaluation of analgesic activity, the abdominal writhing assay method was followed [30]. In this method, vehicle treated control mice were given 1% acetic acid and exhibited writhing response (86 ± 3.1). BPA (9.9 mg/kg, 46 mmol, p.o.) significantly reversed the incidents of writhing (68.6 ± 3.8). BPA–QTME prodrug at equivalent dose (25.5 mg/kg, 46 mmol, p.o.) also significantly reduced the writhing response (62.4 ± 4.0). The BPA–QTME **9** showed analgesic activity comparable to the parent drug (Table 2).

The results listed in Table 2 showed that BPA–QTME **9** lack gastrointestinal ulcerogenic side effects with retention of antiinflammatory and analgesic activity.

4. Conclusion

In the present study, BPA–QTME (**9**) mutual prodrug has been designed, synthesized and evaluated as safer NSAID in which it has been possible to conjugate BPA (**6**) and QTME (**5**) in 1:1 ratio. The derivative has been found to be chemically stable and biolabile. It exhibited retention in antiinflammatory with significant reduced ulcerogenicity as compared to the BPA + QTME physical mixture. This may be due to improved physicochemical properties required for enhanced bioavailability. On the basis of these observations, it can be concluded that there is advantage of giving BPA and QTME in the form of a single molecule i.e. BPA–QTME mutual prodrug.

Table 1
Effect of BPA, BPA–QTME and BPA + QTME (physical mixture) on superoxide dismutase activity, catalase activity, lipid peroxidation (MDA levels) and myeloperoxidase activity.

Treatment	SOD activity (U/mg protein)	Catalase activity (U/mg protein)	nmoles MDA/mg protein	%MPO activity
Control	325.40 ± 69.00	28.38 ± 2.85	4.61 ± 1.28	100 ± 0
BPA–QTME	223.46 ± 24.01^{a}	22.41 ± 1.68^{a}	6.42 ± 0.55^{b}	118.93 ± 5.55^{b}
BPA + QTME	60.41 ± 13.26^{c}	8.69 ± 0.30^{a}	13.36 ± 1.92^{c}	227.33 ± 13.32
BPA	$26.23 \pm 6.31^{**}$	$3.52 \pm 0.33^{*}$	$22.57 \pm 2.34^{**}$	$238.47 \pm 19.39^{**}$

Data are expressed as mean ± SE of five experiments; (*) $p < 0.0001$ extremely significant as compared to control; (**) $p < 0.01$ very significant as compared to control; [a] $p < 0.0001$ extremely significant as compared to BPA; [b] $p < 0.001$ very significant as compared to BPA; [c] $p < 0.05$ significant as compared to BPA.

Table 2
Antiinflammatory, analgesic and ulcerogenic activities of BPA, BPA–QTME mutual prodrug; and BPA + QTME (physical mixture).

Treatment	% Increase in paw edema				%Inhibition in writhing	Ulcer index
	2 h	3 h	4 h	5 h		
Control	68.8 ± 2.06	81.1 ± 2.43	78.4 ± 2.35	65.2 ± 1.96	–	0.2 ± 0.12
BPA–QTME	34.2 ± 1.03[a]	37.5 ± 0.83[a]	31.6 ± 0.95[a]	33.1 ± 0.99[c]	62.4 ± 4.0	0.45 ± 0.27[a]
BPA	26.9 ± 0.81*	26.5 ± 0.80*	25.2 ± 0.76*	29.1 ± 0.87*	68.6 ± 3.8	6.2 ± 0.41*
BPA + QTME	–	–	–	–	62.6 ± 2.4	3.4 ± 0.57[b]

Data are expressed as mean ± SE of five experiments; – not determined; (*) $p < 0.0001$ extremely significant as compared to control; [a]$p < 0.0001$ extremely significant as compared to BPA; [b]$p < 0.001$ very significant as compared to BPA; [c]$p < 0.05$ significant as compared to BPA.

5. Experimental protocols

5.1. Chemistry

Melting points (mp) were determined on a Veego melting point apparatus and are uncorrected. For TLC, glass plates coated with silica gel (E. Merck) were used. The TLC plates were activated at 110 °C for 30 min and visualized by exposure to iodine vapors. Glass columns of appropriate sizes were used. Silica gel (60–120 mesh, BDH) was used as adsorbent. $UV_{(max)}$ spectra were recorded on Perkin Elmer Lambda 15 UV/VIS spectrometer using ethanol as solvent. IR spectra were recorded on Perkin Elmer 882 spectrometer using potassium bromide pellets. ^{1}HNMR and ^{13}CNMR spectra were recorded with Bruker AC 300F, 300 MHz spectrometer using $CDCl_3$ or DMSO-d_6 as solvents and tetramethylsilane as internal standard. Mass spectra were obtained with Vg-11-250J 70s mass spectrometer at 70 ev using electron ionization (EI) sources. The synthetic reactions were monitored by TLC. The identity of all new compounds was confirmed by ^{1}HNMR, ^{13}CNMR, IR data, UV (λ_{max}) data and mass spectrometer; homogeneity was confirmed by TLC. Solutions were routinely dried over anhydrous sodium sulfate prior to evaporation. Rutin trihydrate was purchased from Lancaster. Nitroblue tetrazolium, ethylenediaminetetracetic acid (EDTA), thiobarbituric acid, trichloroacetic acid were obtained from SD Fine Chem. *p*-Nitrosodimethyl aniline was purchased from LOBA chem. Hydrogen peroxide was purchased from Qualigens fine Chem. Pvt. Ltd. All other reagents and solvents were of AR grade.

5.1.1. Quercetin 5,7,3′, 4′-tetramethyl ether (QTME, **5**)

To a fine suspension of rutin trihydrate (**2**) (2 g, 0.003 mol) in dry acetone (200 ml), anhydrous potassium carbonate (8 g, 0.056 mol) and dimethyl sulfate (**3**) (8 ml, 0.059 mol) were added and the reaction mixture was refluxed for 60 h. The solution was filtered and insoluble potassium salts were washed with acetone. The washings were combined with the filtrate and solvent was removed under reduced pressure to obtain methylated glycoside (**4**) as semisolid residue.

The product was refluxed with ethanolic sulfuric acid (2%, 50 ml) for 2 h. The solvent was removed under reduced pressure and the residue obtained was recrystallized from ethanol to give QTME (**5**) (0.8 g, 85%), mp 195–197 °C, R_f 0.3 (dichloromethane:ethyl acetate: 9.5:0.5), UV (λ_{max}) nm: EtOH: 360, 250.8; $AlCl_3$: 419.0, 258.6; $AlCl_3$ + HCl: 219.0, 255.4, IR (KBr): 3338 (Ar O–H st), 2975 (Ar C–H st), 2861 (C–O–CH_3 st and C–H st), 1616 (Ar C=O st), 1518 (Ar C=C st), 1461 (C–H bend), 1375, 1220 (C=C–O–C ether st), 1156.4, 1086 (C–O–C st) cm^{-1}, ^{1}HNMR ($CDCl_3$) δ: 3.4, 3.6 (12H, overlapping s, 4 × OC**H**$_3$), 6.36 (1H, d, $J = 2.02$ Hz, C-6-**H**), 6.56 (1H, d, $J = 2.02$ Hz, C-8-**H**), 7.29 (2H, ABq, $J = 9$ Hz, C-5′-**H**, C-6′-**H**), 7.3 (1H, s, C-3-O**H**), 7.79 (1H, d, $J = 2.0$ Hz, C-2′-**H**), ^{13}CNMR ($CDCl_3$) δ: 171.6 (**C**-4), 164.3 (**C**-9), 160.5 (**C**-7), 156.6 (**C**-2), 150.3 (**C**-5), 146.6 (**C**-4′), 142.0 (**C**-3′), 137.5 (**C**-3), 123.7 (**C**-1′), 120.6 (**C**-6′), 110.9 (**C**-2′), 110.4 (**C**-5′), 106.1 (**C**-10), 96.2 (**C**-6), 95.6 (**C**-8), 56.3, 55.9 (2C), 55.6 (4 × O**C**H$_3$). MS: *m*/*z* 358 (M^+, 100%), 343 ($M^+ - CH_3$), 328 ($M^+ - 2 \times CH_3$), 312, and 297.

5.1.2. Biphenylacetyl chloride (**8**)

BPA (**6**) (2.12 g, 0.01 mmol) was added to thionyl chloride (**7**) (1.44 g, 0.96 ml, 0.012 mol) and stirred at room temperature for 18 h. The excess of thionyl chloride was removed under reduced pressure to give biphenylacetyl chloride (**8**) as yellow amorphous solid (2.057 g, 84%), mp 49 °C, R_f 0.72 (toluene), IR (KBr): 2940 (Ar C–H st), 2810 (aliphatic C–H st), 1690 (carboxylic C=O st), 1480 (Ar C=C st), 1255 (C–O st) cm^{-1}, ^{1}HNMR ($CDCl_3$) δ: 3.75 (2H, s, Ar-C**H**$_2$), 7.37 (4H, ABq, $J = 9.02$ Hz, *p-substituted* Ar-**H** *overalapping* 1H, m, Ar-**H**), 7.30–7.45 (4H, m, Ar-H).

5.1.3. BPA–QTME mutual prodrug (**9**)

QTME (1.74 g, 0.005 mol) was dissolved in dichloromethane (20 ml) containing triethylamine (5 drops) and 4-dimethylaminopyridine (1 pinch). The reaction mixture was cooled to −10 °C and biphenylacetyl chloride (**7**) (2.057 g, 0.01 mol) dissolved in dichloromethane (30 ml) was added dropwise over a period of 1 h. The reaction mixture was stirred overnight and the solvent was removed under reduced pressure. The solid product obtained was chromatographed on silica gel column using dichloromethane:ethyl acetate (99:1) as eluent and the solvent was removed under reduced pressure to obtain BPA–QTME (**9**); yield (0.7 g, 25.4%), mp 118–120 °C, R_f 0.85(dichloromethane), UV (λ_{max}) nm: EtOH: 332, 265; $E^{1\%}_{1cm}$: 300, IR (KBr): 3123 (Ar C–H st), 2358 (C–O–CH_3 st and C–H st), 1604 (Ar C=O st), 1510 (Ar C=C st), 1463 (C–H bend), 1310.5, 1251.1 (C=C–O–C ether st), 1159.3, 1086 (C–O–C st)cm^{-1}. ^{1}HNMR($CDCl_3$) δ: 3.65, 3.77 (12H, overlapping s, 4 × OC**H**$_3$), 4.0 (2H, s, Ar-C**H**$_2$), 6.36 (1H, d, $J = 2.0$ Hz, C-6-**H**), 6.51 (1H, d, $J = 2.0$ Hz, C-8-**H**), 6.96 (2H, ABq, $J = 9$ Hz, C-5′-**H**, C-6′-**H**), 7.22 (1H, d, $J = 2.0$ Hz, C-2′-**H**), 7.35 (1H, m, Ar-**H**), 7.51 (4H, ABq, $J = 9.02$ Hz, *p-substituted* Ar-**H** *overlapping* 4H, m, Ar-**H**), ^{13}CNMR ($CDCl_3$) δ: 171.6 (**C**-4), 168.4 (ester C=O), 164.4 (**C**-7), 161.4 (**C**-5), 159.7 (**C**-9), 149.2 (**C**-2), 140.7 (**C**-4′), 140.2 (C-3′), 135.0 (**C**-3), 132.4, 130.0, 128.7, 127.2, 126.9 (12C, Ar **C** of biphenyl), 122.3 (**C**-1′), 121.9 (**C**-6′), 111.7 (**C**-2′), 111.4 (**C**-5′), 108.0 (**C**-10), 96.3 (**C**-6), 93.0 (**C**-8), 56.17 (4 × O**C**H$_3$), 40.7 (**C**H$_2$), MS: *m*/*z* 552 (M^+), 358 ($M^+ - C_{14}H_{11}O$, 100%), 343 ($M^+ - C_{14}H_{11}O-CH_3$), 328 ($M^+ - C_{14}H_{11}O - 2 \times CH_3$), 314, 300 and 167.

5.2. Physicochemical properties

5.2.1. Solubility studies

Solutions of BPA were prepared in methanol. The λ_{max} was determined by scanning solution containing 20–50 μg/ml of BPA between 200 and 400 nm using UV–visible spectrophotometer. The λ_{max} of BPA was found to be 253 nm. Similarly, λ_{max} of BPA–QTME was determined and found to be 332 nm.

The standard plot for BPA was constructed in methanol. The stock solution containing 1 mg/ml of BPA was diluted to obtain solutions of concentration in the range of 2–10 μg/ml. The spectrophotometeric absorbances were recorded at 253 nm using methanol as blank. A highly linear calibration curve was obtained with slope as 0.0979 and $E^{1\%}_{1cm}$ 979. Similarly, calibration plot of BPA–QTME was established in chloroform at 332 nm and $E^{1\%}_{1cm}$ for BPA–QTME was found to be 300.

The solubility studies of BPA and BPA–QTME were carried out in phosphate buffer (pH 7.4). Excess amount of each compound was added to 10 ml of buffer and shaken for 24 h at 25 ± 2 °C using water bath shaker. Solutions were filtered and analyzed spectrophotometrically for determining the amount of compounds.

5.2.2. Partition coefficient determination

Partition coefficients of BPA and BPA–QTME were determined in octonol/phosphate buffer (pH 7.4) system using shake flask method. Saturated solutions of the compounds were prepared in n-octonol (10 ml) and equal volumes of phosphate buffer were added to the solutions in conical flasks. The sealed flasks were kept for shaking in a water bath shaker maintained at 25 ± 20 °C for 24 h and then allowed to stand for 30 min for the phases to fully separate. Thereafter, the respective phases were analyzed spectrophotometrically.

5.2.3. Kinetics of hydrolysis in aqueous solution

The rate of chemical hydrolysis of BPA–QTME was determined in isotonic phosphate buffer (pH 7.4) and HCl buffer (pH 2) at 37 °C. An appropriate amount of BPA–QTME codrug was dissolved in preheated buffer, and solution was placed in a thermostatically controlled water bath at 37 °C. At appropriate time intervals, samples were taken and analyzed for the appearance of BPA at 253 nm spectrophotometrically. Pseudo first order half time ($t½$) for the hydrolysis of BPA–QTME was calculated from the slope of the linear portion of the plotted logarithm of BPA concentration vs time.

5.2.4. Kinetics of hydrolysis in human plasma

Plasma from human was obtained by centrifugation of blood samples containing 0.3% citric acid at 3000 *g* for 15–20 min. Human plasma fractions (4 ml) were diluted with 1 ml of isotonic phosphate buffer (pH 7.4) to give a final volume of 5 ml (80% plasma). Incubation was performed at 37 ± 0.5 °C using shaking water bath. The reactions were initiated by adding 100 μl of stock solution of (1 mg/ml) to 5 ml of preheated plasma. At appropriate time intervals, samples were taken and diluted with phosphate buffer and analyzed spectrophotometrically at 253 nm for the appearance of BPA. The value of rate constants (K) and half lives ($t½$) for the hydrolysis of BPA–QTME was calculated from the linear portion of the plotted logarithm of BPA concentration vs time.

5.3. Pharmacology

5.3.1. Animals

Sprague-Dawley (sd) rats (weighing 150–200 g) of both sex and LACCA mice (male, 25–35 g) procured from central animal house, Panjab University, Chandigarh, India were used. The animals were housed in plastic cages (five rats/cage) under standard laboratory conditions and maintained on rat chow and water.

5.3.2. Antiinflammatory activity

Antiinflammatory activity was assessed by the carrageenan induced rat paw edema model. Rats were divided into 3 groups; i) vehicle (control); ii) BPA (standard); and iii) BPA–QTME. The rats were fasted for 12 h prior to test. The test compounds were suspended in carboxymethylcellulose (0.5%, CMC) and administered orally. Control animals were given the corresponding amount of vehicle (0.5%, CMC). The compounds were administered on molar equivalent basis of BPA.

5.3.3. Antiulcer activity

For the study of antiulcer activity of BPA–QTME, rats were fasted overnight and divided into 4 groups; i) vehicle (control); ii) BPA (standard); iii) BPA–QTME; and iv) BPA + QTME (physical mixture). Animals were treated with BPA (54 mg/kg, p.o.) and equimolar doses of BPA–QTME and BPA + QTME (physical mixture). Animals were sacrificed 4 h after the treatment. The stomach was removed, opened along greater curvature, washed with saline and observed for ulcers. The ulcers were scored as: 0 = no observable damage; 1 = punctiform lesion <1 mm, 3 = filiform lesion <5 mm, 4 = punctiform lesion >1 mm or filiform lesion >5 mm.

5.3.4. Analgesic activity

Analgesic activity was determined by using abdominal writhing assay. Mice were divided into 4 groups; i) vehicle (control); ii) BPA (standard); iii) BPA–QTME; and iv) BPA + QTME (physical mixture). The animals were food deprived overnight prior to the experiments. The samples were suspended in carboxymethylcellulose (0.5%, CMC), and administered orally (0.1 ml/10 g of body weight) at 1 h before the administration of freshly prepared acetic acid solution (1%, 10 ml/kg, i.p.) intraperitoneal. The number of writhes (constriction of abdomen, turning of trunk and extension of hind limbs) for each animal was recorded during 20 min period, beginning 3 min after the administration of acetic acid. The average number of writhes in each group of drug treated mice was compared with that of control group and degree of analgesia was expressed as % inhibition as follows:-

$$\%\ \text{Inhibition} = \left(1 - \frac{N_t}{Nc}\right) \times 100$$

Where, N_c = number of writhes in control, and
N_t = number of writhes in drug treated mice.

5.4. Biochemistry

5.4.1. Antioxidant activity (in vivo)

Preparation of tissue homogenate: The glandular parts of excised stomachs from ulcer studies were homogenized in ice cold phosphate buffer (pH 7.4) with a Potter-Elvehjerr glass homogenizer for 30 s. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was again centrifuged at 12,000 *g* for 15 min and the obtained post mitochondrial fraction (PMF) was used for following estimations.

5.4.1.1. Catalase assay. To 0.05 ml of PMF, freshly prepared hydrogen peroxide (3 ml, 30 mM in 50 mM phosphate buffer, pH 7) was added and change in absorbance was measured at 240 nm for 2 min at 30 s intervals. Catalase activity was estimated using the following equation:

$$\text{Catalase} = \frac{2.3}{\Delta t} \log \frac{A_1}{A_2}$$

A_1 = initial absorbance, and
A_2 = final absorbance, when $\Delta t = 1$ min.

Catalase activity was expressed as units/mg protein. One unit catalase is defined as the amount of enzyme required for decomposing 1 μmol of H_2O_2/min at 25 °C at pH 7.0.

5.4.1.2. Superoxide dismutase (SOD) activity. To 0.1 ml of PMF, 2 ml of nitroblue tetrazolium (96 mM, freshly prepared in 50 mM Na_2CO_3 solution containing 0.1 mM EDTA) and 0.5 ml of hydroxylamine hydrochloride (20 mM) were added and the change in absorbance was measured at 560 nm for 2 min at 30 s intervals. SOD activity was calculated from the following equation:

$$\text{SOD Activity} = \left(\frac{A_{\text{non-enz.}} - A_{\text{enz.}}}{A_{\text{enz.}}}\right) \times 100$$

$A_{\text{non-enz.}}$ – Absorbance of control (without homogenate)
$A_{\text{enz.}}$ – Absorbance of test (with homogenate).

5.4.1.3. Myeloperoxidase (MPO) activity. The stomach homogenate (prepared in 0.01 M phosphate buffer) was centrifuged at 10,000 rpm at 10 °C for 10 min. Supernatant was collected and mixed with *o*-phenylenediamine (660 μg/ml in phosphate buffer) and hydrogen peroxide (300 mM) was added to initiate the reaction. Absorbance was recorded at 492 nm at intervals of 30 s for 5 min. Change in optical density per min was calculated and results were expressed as % MPO activity, considering 100% MPO activity in control group.

5.4.1.4. Lipid peroxidation (LPO) activity. To 0.5 ml of PMF (phosphate buffer, pH 7.4), 0.5 ml of tris hydrochloric acid buffer (0.1 M, pH 7.4) was added. The mixture was incubated at 37 ± 2 °C for 2 h. To this mixture, ice cold trichloroacetic acid (1 ml, 10%) was added. The turbid solution was centrifuged at 1000 rpm for 10 min. To 1 ml of the supernatant obtained, thiobarbituric acid (1 ml, 0.67% w/v) was added. The mixture was boiled for exactly 10 min and cooled. To this solution, 1 ml distilled water was added and absorbance was recorded at 532 nm.

The concentration of malondialdelhyde (MDA) was calculated as:

$$\text{LPO} = 3 \times \frac{\text{Absorbance}}{\text{mg protein} \times 1.56 \times 10^{-5}} (\text{nmoles MDA/mg protein})$$

5.4.1.5. Protein estimation. To 0.1 ml of homogenate, 0.9 ml of distilled water and 5 ml of working alkaline copper sulfate solution were added. The mixture was incubated at room temperature for 10 min. To each test tube, 0.5 ml Folin's reagent was added and vortexed for 30 s. The test tubes were incubated for 30 min. The absorbance was measured at 750 nm against reagent blank. Protein contents were estimated from standard plot constructed from bovine serum albumin and expressed as mg protein/ml. The working alkaline copper sulfate solution (Lowry's reagent) was prepared by mixing 48 ml of sodium carbonate (2% w/v, in 0.1 M sodium hydroxide), 1 ml of copper sulfate (1% w/v) and 1 ml of potassium sodium tartarate (2% w/v).

5.5. Statisical analysis

Statistical analysis was carried out on *in vivo* studies data. The ulcer index data was subjected to student *t*-test (unpaired), analysis of variance (ANOVA) test, followed by Dunnett's test for determining the levels of significance in antioxidant studies. *P* values <0.05 were considered statistically significant.

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