

Design, Synthesis and Evaluation of Quercetin-Meclofenamic acid Conjugate: A Mutual Pro-drug for Safer NSAIDs

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used medications worldwide for the treatment of pain, fever and acute, chronic inflammatory diseases like arthritis. Although they are effective in the treatment of pain and inflammation, their routine and long-term administration is limited due to their gastrointestinal and renal side effects. Most of the natural constituents, polyphenolic compounds and flavonoids, possess potent anti-inflammatory effect and fewer side effects. They may be used as the potent anti-inflammatory agents; however, they suffer from the bioavailability problems. The present study attempted to synthesize a conjugate of quercetin and meclofenamic acid, on the basis of the merits and demerits of both of these compounds. The conjugate and the intermediates were characterized by determining melting point, R_f value by Thin Layer Chromatography, UV Spectrophotometry, Infrared Spectra, MASS by L.C.M.S. The anti-inflammatory activity of the conjugate was compared with quercetin and meclofenamic acid by animal study. The probable mechanism of action for the synthesis was discussed. The in vivo activity of the conjugate quercetin-meclofenamic acid significantly inhibited paw edema in the first and second phase, suggesting an inhibitory effect on the release of histamine, serotonin, kinins and prostaglandins. The improved pharmacological activity may be contributed to aqueous solubility and comparatively lesser molecular weight of the conjugate. In conclusion, a mutual prodrug quercetin-meclofenamic acid, associated with anti-inflammatory activity, was successfully synthesized and characterized.

Keywords: Mutual prodrug, Quercetin, Meclofenamic acid, Anti-inflammatory, Natural Compounds

INTRODUCTION

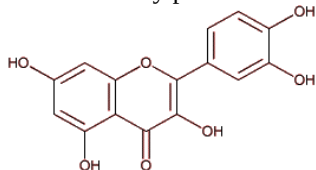
Nonsteroidal anti-inflammatory drugs take their name from the class of compounds which act as anti-inflammatory and analgesic (painkiller). Nonsteroidal anti-inflammatory drugs (NSAIDs) also reduce fever. A steroid is a compound that occurs naturally or produced by synthesis. Steroids are defined as chemical compounds that possess 17 carbon atoms, arranged in a series of four rings. The endogenous cortisone is also anti-inflammatory steroid. NSAIDs are used therapeutically to reduce the pain and swelling associated with the joint irritation, often due to overuse of the joint. NSAIDs function by neutralizing the cyclooxygenase (COX) enzyme that is produced at the site of a musculoskeletal injury. COX enzymes are present in the body in two forms, COX-1 and COX-2. COX-1 is present in cells throughout the human body. It plays an important role in the regulation of the protection of the stomach lining, the regulation of salt and fluid balances, and the flow of blood to the kidneys. COX-2 is found primarily in the immune cells and the cells of the central nervous system (CNS). Each produces prostaglandins, the actual cause of inflammation and pain. NSAIDs function by inhibiting these enzymes those otherwise produce prostaglandins. (Vane et al., 1998) In

the early 1990s, radiograph crystallography clarified the COX3-dimensional structure, showing a long narrow channel, ending in a hairpin bend (Kalgutkar et al., 2000; Arrault, 2004).

NSAIDs are associated with gastrointestinal ulcers, serious cardiovascular events, hypertension, acute renal failure, and worsening of preexisting heart failure, although the adverse effects may be reduced by limiting NSAID dosage and duration (Vonkeman et al., 2010). The unwanted side effects of NSAIDs are due to the inhibition of housekeeping enzyme COX-1 and the beneficial anti-inflammatory effect is due to inhibition COX-2 (Vane et al 1998) If this "COX-1 inhibition is good, COX-2 inhibition is bad" hypothesis is true, then a COX-2-selective NSAID would be an ideal drug, with analgesic, antipyretic, and anti-inflammatory benefits without gastric or other side effects (Kalgutkar et al., 2000; Arrault, 2004).

Natural compounds are being used in the treatment of various diseases for centuries. The emergence of today's Pharmaceutical Industry, in large part, has been based on natural products. Most of the natural constituents, polyphenolic compounds and flavonoids, possess potent anti-inflammatory effect due to their anti-

oxidant activity. They are advantageous over NSAIDs since they are associated with reduced adverse effects (Vonkeman et al., 2010; Brooks, 1998). They may be used as the potent anti-inflammatory agents; however, they suffer from the bioavailability problems.

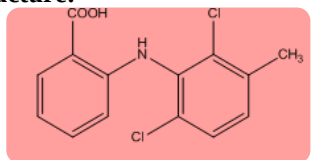


1.1 Quercetin: The naturally occurring flavonoid such as Quercetin is widely distributed in the plant kingdom. It is found in many often-consumed foods, including apple, onion, tea, berries, and brassica vegetables, as well as many seeds, nuts, flowers, barks, and leaves.

Description: IUPAC Name: 2-(3, 4-dihydroxyphenyl) - 3, 5, 7-trihydroxy-4H- chromen- 4-one, Chemical Formula: $C_{15}H_{10}O_7$, Melting Point: 316°C . Flavonoids are present in food as glycosides that means a combination of sugar molecule (rhamnose, glucose, galactose, etc.) attached to the center C ring. Quercetin is the aglycon, that means non sugar moiety, of a number of flavonoids, including rutin, quercetrin, isoquercetin, and hyperoside.

Quercetin's anti-inflammatory activity appears to be due to its antioxidant and inhibitory effects on inflammation producing enzymes (cyclooxygenase, lipoxygenase) (Formica et al., 1995; Comalada et al., 2005). The mechanism of action of quercetin involves release of quercetin that inhibits inflammation through down regulation of NF-KB path way (Comalada et al., 2005) Quercetin is effective in preventing, delaying or slowing the onset or progression of hypertension (Jalili, 2008).

Chemical Structure:



IUPAC name :	2-[(2,6-dichloro-3-methylphenyl) Am amino benzoic acid
Chemical Formula :	$C_{14}H_{10}Cl_2N NaO_2H_2O$
Chemical Name	Anthranilic acid, N-(2, 6-dichloro-m-tolyl)-, monosodium salt, monohydrate.
Melting Point	257°C

1.2 Meclofenamic Acid: Description: A non-steroidal anti-inflammatory agent with antipyretic and antiggranulation activities. It also inhibits prostaglandin biosynthesis. The fenamate NSAIDs such as meclofenamic, flufenamic, mefenamic and niflumic acids are

derivatives of N-phenylanthranilic acid. It is a well-known fact that the fenamate inhibits the cyclooxygenase enzymes, which catalyzes the biosynthesis of prostaglandins (Winder et al., 1965).

1.3 Pro-drug and Mutual Pro-drug: Pro-drugs are pharmacologically inactive molecules of an active drug molecule that, prior to exerting a pharmacological effect, require an enzymatic and/or chemical transformation to release the active parent drug *in vivo* (Han et al., 2000). Pro-drugs and the soft drugs are used to overcome several undesirable properties in order to achieve the best clinical drug application. (Stańczak et al., 2006).

Mutual pro-drug is a type of carrier-linked pro-drug, where the carrier used is another biologically active drug instead of some inert molecule. A mutual pro-drug consists of two pharmacologically active agents coupled together so that each acts as a promoiety for the other agent and *vice versa*. Mutual pro-drug design is no different from the general drug discovery process. It includes identification of a unique substance having desirable pharmacological effects, and modification of its properties, thus leading to the better drug. It is a very fruitful area of research, and its introduction in human therapy has given successful results in improving the clinical and therapeutic effectiveness of drugs, suffering from some undesirable properties, that otherwise hinder their clinical usefulness. (Bhosle et al., 2006). The mutual pro-drugs of NSAIDs are designed with an objective of retention of the anti-inflammatory activity with less ulcerogenic side-effects. Diclofenac- antioxidant mutual pro-drug has the potential of safer NSAID due to its mutual pro-drug nature.¹² The pro-drug of quercetin i.e. quercetin penta benzenesulfonate possesses better physical properties such as solubility, lipid / water partition coefficient, log P & hydrolysis kinetic than original form (Kalgutkar et al., 2000; Arrault, 2004). Quercetin-glutamic acid conjugate shows remarkable increase in water solubility, stability, and cell permeability compared to quercetin (Kim et al., 2009). The objective of the study was to design a mutual pro-drug from a naturally occurring flavonoid quercetin, and the NSAID meclofenamic acid. The present study describes the synthesis of quercetin-meclofenamic acid conjugate and its physico-chemical characterization. The quercetin-meclofenamic acid conjugate was studied for anti-inflammatory activity by rat paw method.

MATERIALS AND METHODS

2.1 Materials: Meclofenamic acid was purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. Quercetin and carrageenan were purchased from Himedia Laboratories, Mumbai. All the chemicals and reagents, used in the synthesis, were of analytical grade and were purchased from S.D. Fine Chemicals, Indore.

2.2 Synthesis of Quercetin-Meclofenamic Acid Conjugate

STEP 1: Conversion of Meclofenamic Acid Sodium to Meclofenamic Acid Chloride: The meclofenamic acid sodium powder (360 mg.) was added to thionyl chloride (0.6ml 0.0072ml) and toluene (3.5ml). The mixture was refluxed for 2 hours until the evolution of hydrogen chloride. The off white precipitate was checked by TLC to confirm the conversion of meclofenamic acid sodium to meclofenamic acid chloride. Yield = 89%.

STEP 2: Preparation of 3, 3', 4', 5, 7-Pentaacetoxyflavone: A drop of concentrated sulphuric acid was added to an ice cold suspension of quercetin dihydrate (5 g, 0.15 mol) in acetic anhydride (30 ml). An immediate color change from yellow to orange was observed. The reaction mixture was heated to 90° C for 25 min, and then cooled on an ice bath. A heavy, off-white precipitate was formed and collected by filtration, washed with water and dried in vacuum over phosphorus pentoxide at room temperature until no water could be detected by Karl-Fischer titration. Yield = 70%.

STEP 3: Preparation of 5-(3, 5, 7-trisbenzyloxy)-4-oxo-4H-Chromen-2-yl)-2-(benzyloxy) phenylacetate):

3,3',4',5,7-pentaacetoxyflavone (3 g, 0.11 mol), potassium iodide (0.243 g, 0.026 mol), potassium carbonate (7 g, 0.92 mol) and benzyl chloride (7 ml) were heated at reflux in butanone (44 ml) which had been dried over boric anhydride. After 48 hour, the reaction mixture was allowed to cool to room temperature and filtered. The residue was washed with acetone (3×200 ml) and the combined washings and filtrate were evaporated in vacuum. The evaporation residue was re-crystallized twice from ethyl acetate to obtain a yellowish white solid. Yield = 44%.

STEP 4: Preparation of 3,5,7 tris (benzyloxy) – 2-(4-(benzyloxy) -3- hydroxyphenyl) -4H –chromen-4-one): Aqueous sodium hydroxide solution (12.34 ml of a 10% w/v solution) was added to a solution of 3'-acetoxy-3,4',5,7-tetrabenzyloxyflavone (4 g, 0.088 mol) at reflux in methanol/ acetone (50.4 ml of a 2:5 v/v solution). After 1 hour, the reaction mixture was cooled to ambient temperature, diluted with water (31 ml) and acidified to pH 1 with hydrochloric acid (17 ml of a 2M solution). A yellow precipitate was isolated by filtration, washed with water (3×20 ml), dried in vacuum and re-crystallized from ethyl acetate. Yield = 45%.

STEP 5: Coupling of 5-(3,5,7,-tris (benzyloxy)-4-oxo-4 H-Chromen-2-yl)-2-(benzyl oxy) phenyl 2-(2,6-dichloro-3-methylphenyl amino) benzoate and 2-(2,6-dichloro-3 methyl phenyl amino) benzoic acid: Compound **VI** (600 mg) was dissolved in tetrahydrofuran (12ml) at 0° C and 1 gm of sodium hydride was added to it. The reaction mixture was stirred for 30 min. at the end of stirring; meclofenamic acid chloride (300 mg) was added

to the reaction mixture and stirred for 4 hours at 0° C. After confirming by the TLC, the mixture was neutralized with ammonium chloride and the resulting solution was extracted with dichloromethane and dried over magnesium sulphate and filtered. Yield = 63%.

STEP 6: Hydrogenation of 5-(3,5,7,-tris (benzyloxy)-4-oxo-4 H-Chromen-2-yl)-2-(benzyl oxy) phenyl 2-(2,6-dichloro-3-methylphenyl amino) benzoate: Hydrogenation was carried out in deoxygenated absolute ethanol at 50° C and 65 psi pressure for 12 hours. The reaction products were studied by LCMS and IR spectroscopy.

2.3 Structural Elucidation of the Compounds 1-8

Structure of all the compounds 1-6 has been established on the basis of their consistent TLC, FT-IR, LCMS. The solubility, melting point, UV spectra of the compounds 1-6 was studied. (Table 1, 2)

2.4 Evaluation of Anti-Inflammatory Activity

The synthesized compounds were screened for anti-inflammatory potential and compared with meclofenamic acid and quercetin.

Albino rats of wistar strain (weighing 150-200 g) of both sexes were used in the study. The animals were housed in standard polypropylene cages under standard laboratory conditions (12:12 hour light/dark cycle at 25±2°C). They were allowed free access to standard commercial diet and water. The animals were fasted for 12 hours, before the experiment. The synthesized conjugate was tested for in vivo anti-inflammatory activity using carrageenan induced rat paw edema assay.

The animals were weighed individually and numbered. The right hind paw of each animal was marked just beyond the tibio-tarsal junction, so that every time the paw was dipped in the mercury column up to the fixed mark to ensure constant paw volume. The initial paw volume of each rat was noted by mercury displacement method. The rats were divided into four groups namely G₁, G₂, G₃, and control each comprising of three rats. G₁, G₂, G₃ group was given quercetin –meclofenamic acid conjugate, quercetin and meclofenamic acid respectively while the control group received only the vehicle. All the test compounds were suspended in 2% carboxymethylcellulose aqueous solution and administered orally to each animal by using a gastric gavage needle. The test compounds quercetin –meclofenamic acid conjugate were given at a dose of 20 mg/kg body weight whereas the standard drugs quercetin and meclofenamic acid were administered as 10 mg/kg body weight. After 30 min of administration, 0.1 ml of carrageenan (1% w/v in normal saline) was administered in the plantar region of the right hind paw of each animal. The paw volume was noted at 0, 1, 2 and 3hr after carrageenan challenge. Then mean change in paw volume

was calculated for all the four groups and was compared. The edema was expressed as an increase in the paw volume and the percent inhibition of edema (% anti-inflammatory activity) was calculated by using following formula.

$$\% \text{Anti-inflammatory Activity} = \left(1 - \frac{V_t}{V_c} \right) \times 100$$

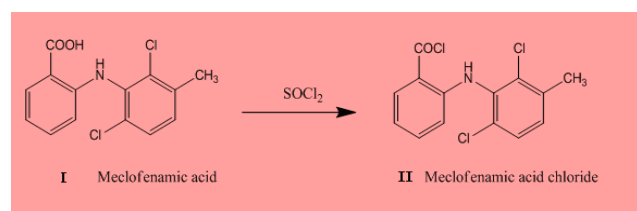
V_t = Mean change in paw volume of the test group, V_c = Mean change in paw volume of the control group

RESULTS

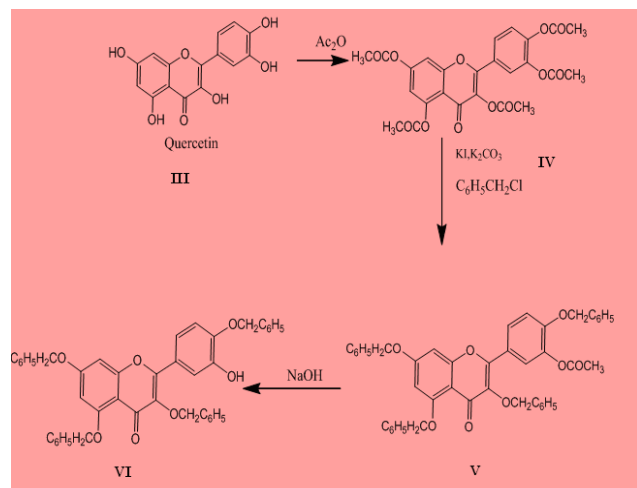
Quercetin has wide activities due to potent antioxidant nature, combating the destructive "free radical" molecules that play a part in many diseases, including inflammation. Considering the beneficial antioxidant and anti-inflammatory effect of quercetin, a conjugate of quercetin-meclofenamic acid was synthesized.

3.1 Reaction Mechanism: The reaction mechanism, involved in the synthesis of quercetin -meclofenamic acid conjugate, is described as follows.

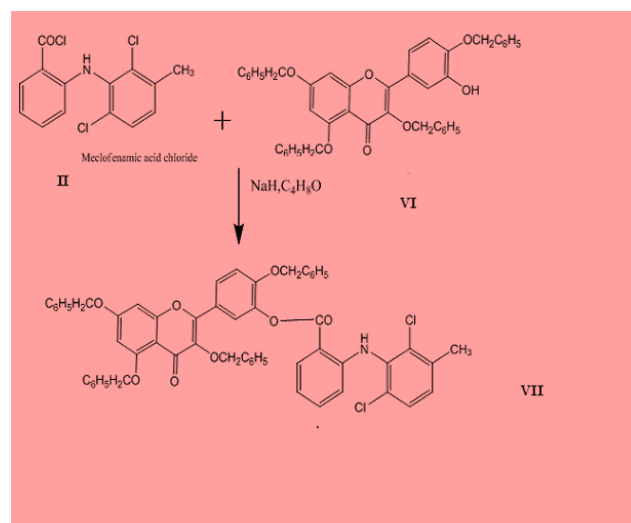
Step 1: Conversion of Meclofenamic acid to Meclofenamic acid chloride.



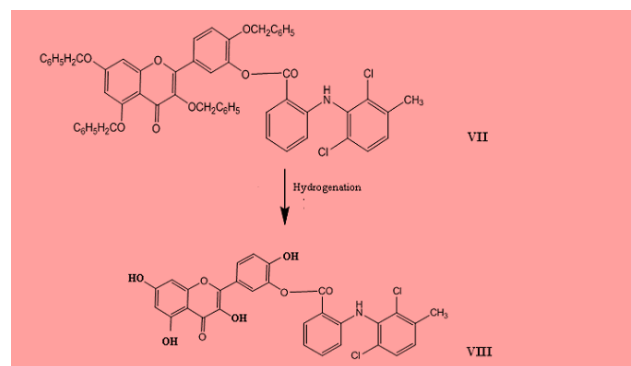
Step 2: Conversion of 3, 5, 7 trihydroxy-2-(3, 4-dihydroxy phenyl)-4H-Chromen-4-one (III) to 3, 5 7-tris (benzyloxy)-2-(4-benzyloxy)-3-hydroxy phenyl)-4H-chromen-4-one (VI).



Step 3: Coupling of 2-(2, 6-dichloro-3 methylphenyl amino) benzoic acid (II) with y 3, 5, 7 tris (benzyloxy)-2-(4-benzyloxy)-3-hydroxyphenyl)-4H-chromen-4-one (VI).



Step 4: Hydrogenation of 5-(3, 5, 7,-tris (benzyloxy)-4-oxo-4H-Chromen-2-yl)-2-(benzyloxy) phenyl 2-(2, 6-dichloro-3-methylphenyl amino) benzoate. (VII)



3.2 Structural Elucidation of the Compounds 1-8: The final conjugate showed improved solubility in water/alcohol, due to its lower melting point as compared to standard drug. The IR studies confirmed the OH groups which also contributed the product more soluble. Due to the presence of quercetin, the NSAID meclofenamic acid showed improved anti-inflammatory activity.

3.3 Evaluation of Anti-Inflammatory Activity: Anti-inflammatory activity of the synthesized compound was determined in terms of percentage inhibition of edema in right hind paw of albino rats. The edema was introduced by injecting 0.1 ml of carrageenan solution in right hind paw and comparing the decrease in edema due to test compound as well as standard drugs quercetin and meclofenamic acid, using plethysmometer. Carrageenan induced hind paw edema is the standard experimental

model of acute inflammation. Moreover, the experimental model exhibits high degree of reproducibility. Synthesized mutual pro-drug of quercetin and meclofenamic acid inhibited the paw edema by 50% after 1 hour, 80% after 2 hours of carrageenan administration and % protection of paw edema, after 3 hours of carrageenan administration, was found to be 80%. (Table 3). In case of quercetin, the percentage protection of paw edema was found to be 80% after 1 hour, 60% after 2 hour and 60% after 3 hour. For meclofenamic acid, the percentage protection of paw

edema was found to be 15% after 1 hour, 40% after 2 hour and 60% after 3 hours. (Table 3). Carrageenan induced Inflammation involves three distinct phases of the release of mediators, including serotonin and histamine in the first phase (0-2 hours), kinins in the second phase. The results of our study indicated that the quercetin-meclofenamic acid conjugate, a mutual prodrug, significantly inhibited paw edema induced by carrageenan in the first and second phase, suggesting an inhibitory effect on the release of histamine, serotonin, kinins and prostaglandins.

Table 1. Structural elucidation of the synthesized compounds I to IV

Specifications	Meclofenamic acid sodium (I)	Meclofenamic acid Chloride (II)	Quercetin(III)	Compound IV
Solubility	Water/Alcohol/Toulene/ Ethyl Acetate	Water/Alcohol/Toulene/ Ethyl Acetate	Toulene/ Ethyl Acetate	Toulene/ Ethyl Acetate
Mol. Formula	C ₁₄ C ₁₁ Cl ₂ NO ₂ Na	C ₁₄ H ₁₀ Cl ₃ NO	C ₁₅ H ₁₀ O ₇	C ₂₅ H ₂₀ O ₁₂
Mol. Weight	318.13	336.63	338.47	512.42
M.P. (°C)	257-257	252	316	148-153
R _f value	0.40	0.51	0.38	0.25
%Yield	-	89		70
FT-IR (cm ⁻¹)	2815 (-CO-O-Ar)	3049 - CH	3074-OH group	1765.71(-CO-O-Ar')
		1790 C=O {Raised due to Electro negative Cl-atom }		1353.72 (-CH ₃ -CO-)
		1747 Fermi resonance C=O stretching		1371.2 (-CH ₃)
				1238 (-C(=O)-O)
LCMS				534 [M] ⁺ + Na
UV λ _{max} nm	282	282	255.8	254

Table 2. Structural elucidation of the synthesized compounds V –VIII

Specifications	Compound V	Compound VI	Compound VII	Final Compound VIII
Solubility	Toulene/ Ethyl Acetate	Toulene/ Ethyl Acetate	Toulene/ Ethyl Acetate	Water/ alcohol
Mol. Formula	C ₄₅ H ₃₆ O ₈	C ₄₃ H ₃₄ O ₇	C ₅₇ H ₄₃ Cl ₂ NO ₈	C ₂₉ H ₁₉ Cl ₂ NO ₈
Mol. Weight	704	662	933.0	580.37
M.P. (°C)	104-110	107-115	103-108	125-130
R _f value	0.28	0.37	0.45	0.39
Yield	44%	45%	63%	55%
FT-IR (cm ⁻¹)	807.15(O-CH ₂ -)	3027(-OH)	1280 Ester Absorption{C-O}	2921.96 (-OH)
			1183.25 Ester {C=O in C-O}	
	1361.65 (-CH ₃ -CO-)	807.15 -O-CH ₂ -		
	1247 (-C(=O)-O)			
LCMS	705 [M+H]	664 [M+ 2H]	930 [M+ 3H]	580
UV λ _{max} nm	259.6	259	258	258.80

Table 3. Evaluation of Anti-inflammatory activity

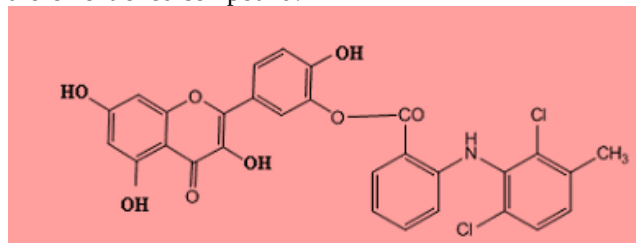
Treatments	Average change in paw volume	Inhibition of paw edema	Average change in paw volume	Inhibition of paw edema	Average change in paw volume	Inhibition of paw edema
	after 1 hour	after 1 hour	after 2 hours	after 2 hours	after 3 hours	after 3 hours
	Mean±SE	(Mean)	Mean±SE	(Mean)	Mean±SE	(Mean)
G4	0.2003±0.044	---	0.3333±0.044	---	0.3333±0.044	--
G2	0.033±0.033**	83 %	0.133±0.033*	60%	0.133±0.033*	60%
G3	0.167±0.0183*	17%	0.2±0.0183*	40%	0.1367±0.0183*	59%
G1	0.1±0.011*	50%	0.067±0.011**	80%	0.067±0.011**	80%

n=3 (n = Number of animals in each group) *P<0.01, **P<0.001

DISCUSSION

Considering the merits and demerits of quercetin and meclofenamic acid, a mutual pro-drug was synthesized. The resultant compound and the intermediates were characterized for the physicochemical properties. The anti-inflammatory activity of the resultant compound was compared with quercetin and meclofenamic acid. The compound quercetin-meclofenamic acid conjugate showed improved anti-inflammatory activity. The compound is soluble in water and ethanol, the molecular weight is less, yet the compound appears to be more lipids soluble. The bioavailability of the compound may be improved although it requires extensive study. The compound has following structure and is derivative.

Further toxicity studies, pharmacokinetic studies would prove the potential therapeutic efficacy of aforementioned compound:



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