Original Article

Screening of ferulic acid related compounds as inhibitors of xanthine oxidase and cyclooxygenase-2 with anti-inflammatory activity

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A B S T R A C T

The ferulic and gallic acid related compounds from natural origin were studied against xanthine oxidase and cyclooxygenase-2 along with their anti-inflammatory activity. The compounds gallic acid, ferulic acid, caffeic acid and p-coumaric acid revealed promising anti-inflammatory activity (30–40% TNF-α and 60–75% IL-6 inhibitory activity at 10 μM). Bioavailability of compounds were checked by in vitro cytotoxicity using CCK-8 cell lines and confirmed to be nontoxic, but found toxic at higher concentration (50 μM). Gallic, ferulic, caffeic acid was demonstrated potential dual inhibition toward xanthine oxidase and cyclooxygenase-2 as calculated by IC50: 68, 70.2, and 65 μg/ml (xanthine oxidase) and 68.5, 65.2, and 62.5 μg/ml (cyclooxygenase-2), respectively. The structure activity relationship and in silico drug relevant properties (HBD, HBA, PSA, cLogP, ionization potential, molecular weight, E_homo and E_lumo) further confirmed that the compounds were potential candidates for future drug discovery study, which was expected for further rational drug design against xanthine oxidase and cyclooxygenase.

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Introduction

Gouty arthritis is a well known disease with an abrupt attack causing extreme pain in and around the joints due to activities occurred by xanthine oxidase (Mohapatra et al., 2015). Gout is nearly always associated with chronic hyperuricemia, a long-lasting abnormally high concentration of uric acid (hyperuricemia) in the blood. The higher levels of uric acid can reach a point where uric acid crystals (monosodium urate) are put differently and this hyperuricemia results in the deposition of crystals of sodium urate in tissues, especially in kidneys and joints (Bardin, 2004; Choi and Curhan, 2005). This process generates oxygen metabolites, which damage tissue, resulting in the release of lysosomal enzymes that induce an inflammatory response. This will lead to local decrease of pH which further causes more deposition of urate crystals (Nuki and Simkin, 2006). The diagnosis of gout is based on the presence of monosodium urate crystals in the synovial fluid (Martinon and Glimcher, 2006). This deposition will exacerbate leading to recurrent episodes of acute arthritis, the classic manifestation of gout. Meanwhile, controlling the uric acid level in the blood is still the main target particularly in the management of the chronic attacks (Mandell, 2002; Dincer et al., 2002). Xanthine oxidase (XO) has a major role in the uric acid production as XO is responsible for catalyzing the oxidation of hypoxanthine to form xanthine and finally to uric acid. Thus, this enzyme coordinates the reaction and produces uric acid from its precursors (Nile et al., 2013; Li et al., 2013). Similarly, certain enzymes such as cyclooxygenase-2 (COX-2) contributes its role in the gouty inflammation, though it heightened expressions in the presence of the accumulated MSU crystals, which in turn enhances the production of inflammatory prostaglandins leading to the increased production of IL-1β. Thus, COX-2 plays a major role in arousing the inflammatory responses and thus taking part in the advancement of the acute inflammation in the gouty arthritis patients (Pouliot et al., 1998; Mohapatra et al., 2015). There are certain measures taken in order to treat the disease by using non-steroidal anti-inflammatory drugs (NSAID), colchicine or glucoso corticoids. NSAID are the class of drugs which causes COX-2 inhibition whereas colchicine is an antimycotic alka loid that apart from disturbing the microtubule polymerization, it also inhibits the inflammation by preventing the IL-1β processing which was stimulated by the MSU crystals (Martinon et al., 2006; Ricciotti and FitzGerald, 2011). Examples of such drugs are allopurinol, probenecid and sulfipyrazone (Mandell, 2002; Aggarwal et al., 2011). Modern medicines from natural products have little to offer for alleviation of gout, oxidative and inflammatory activity. There is an urgent need to discover compounds with xanthine oxidase inhibitory activities but devoid of the undesirable effects of allopurinol. One potential source of such compounds is medicinal
materials of plant origin which is used to treat conditions similar to gouty arthritis (Nile and Park, 2014a,b). Phenolics and flavonoids are considered important components in the human diet because of their beneficial effects on human health. The phenolics and flavonoids are naturally occurring compounds that are constantly distributed in various foods, fruit juices and beverages from plant origin and display many bioactive and therapeutic properties, such as antioxidant, anticancer, antiviral, anti-inflammatory, and cardiac protective effects (Proestos et al., 2005; Nile and Park, 2014c).

Most of the therapeutic properties of phenolics and flavonoids have been demonstrated to have potent antioxidant, anti-inflammatory and enzyme inhibition properties. Several phenolics and flavonoids have been described as inhibitors of the xanthine oxidase (XO) enzyme, similar to allopurinol in the treatment of gout (Bandgar et al., 2009; Nile and Park, 2013), for this reason, research into ferulic acid seems promising. Thus, the aim of this study is to investigate the potency of naturally occurring ferulic acid related compounds as xanthine oxidase and cyclooxygenase-2 inhibitors along with the properties like anti-inflammatory activity, structure activity relationship (SAR) and in silico drug relevant properties were studied.

Materials and methods

Chemicals

Xanthine oxidase, xanthine, allopurinol, myricetin, and isoquercetin were purchased from Sigma Chemical (Seoul, Korea). Ferulic acid (2), gallic acid (6), and their esters were supplied by Hi-Media Laboratories, Mumbai, India. Caffeic acid (3), sinapic acid (4), p-coumaric acid (1) procured from Sigma–Aldrich, Mumbai, India. Cyclooxygenase fluorescent inhibitor screening assay kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Bovine milk xanthine oxidase procured from (grade 1, ammonium sulfate suspension) Sigma–Aldrich, Mumbai, India.

\[
\text{R}_1=\text{R}_2=\text{CH}_3 \\
\text{R}_1=\text{OCH}_3; \text{R}_2=\text{H} \\
\text{R}_1=\text{OH}; \text{R}_2=\text{H} \\
\text{R}_1=\text{R}_2=\text{OCH}_3
\]

\[
\text{H}_3\text{CO} \quad \text{CO}_2\text{H} \\
\text{HO} \quad \text{CO}_2\text{R} \quad \text{OH}
\]

Anti-inflammatory and cytotoxicity assay

Pro-inflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Bandgar et al. (2009). During assay, THP-1 cells were cultured with penicillin and streptomycin (100 U/ml) and inoculated with 10% fetal bovine serum (FBS, JRH) in RPMI 1640 culture medium (Gibco BRL, Paisley, UK). Cells were differentiated with phorbol myristate acetate (PMA, Sigma). Following cell plating, the test compounds (10 μM) in 0.5% DMSO was poured to each well and the plate were incubated for 30 min at 37 °C. Finally, LPS (Escherichia coli 0127:B8; Sigma Chemical Co., St. Louis, MO) was added, at a final concentration of 1 μg/ml in each well. Plates were further incubated at 37 °C for 24 h in 5% CO2. After incubation, supernatants were harvested, and assayed for TNF-α and IL-6 by ELISA as described by the manufacturer (BD Biosciences, India). The cells were simultaneously evaluated for cytotoxicity using CCK-8 from Dojindo Laboratories. Percent inhibition of cytokine release compared to the control was calculated. In this the cytotoxicity was checked at lower, optimum and higher concentration (10, 25 and 50 μM), 50% inhibitory concentration (IC50) values were calculated by a nonlinear regression method (Bandgar and Gawande, 2010; Nile and Khobragade, 2011).

In silico pharmacological property and SAR study

The pharmacological properties of the compounds, such as molecular weight, cLog P and quantum chemical descriptors such as E_HOMO (energy of highest occupied molecular orbital) and E_LUMO (energy of lowest unoccupied molecular orbital) of the synthesized compounds were calculated using a BioMed CaChe 6.1 (Fujitsu Ltd), a computer aided molecular design modeling tool for windows 98/20000/XP operating system. Other parameters such as HBA (hydrogen bond acceptor), HBD (hydrogen bond donor), molecular PSA (polar surface area), drug score and drug likeness of the compounds were also studied using online Osiris property explorer for drug bioavailability of chemical compounds. Since compounds are considered for oral delivery, they were also assessed for toxicity using in silico ADME prediction methods (Bandgar and Gawande, 2010; Nile and Khobragade, 2011).

XO inhibitory activity

Xanthine oxidase (XO) activity was assayed spectrophotometrically by measuring the uric acid formation at 290 nm using a UV-visible spectrophotometer at 25 °C. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 75 μM xanthine and 0.08 units of XO. Inhibition of XO activity of individual isolated phenolics from maize (1.5 ml, 2 mM), was measured by following the decrease in the uric acid formation at 293 nM at 25 °C. The enzyme was pre incubated for 5 min, with test compound, dissolved in DMSO (1%, v/v), and the reaction was started by the addition of xanthine. Final concentration of DMSO (1%, v/v) did not interfere with the enzyme activity. The XO kinetic study was carried out using screening of ten ferulic acid related compounds (10, 25, 50 and 100 μg/ml) comparing with allopurinol (10, 25, 50 and 100 μg/ml) as positive control. All the experiments were performed in triplicates and IC50 values were expressed as means of three experiments (Nile et al., 2013; Nile and Park, 2014a).

COX-2 inhibitory activity

Cayman’s COX fluorescent inhibitor screening assay provides a convenient fluorescent-based method for screening both ovine COX-1 and human recombinant COX-2 for isozyme-specific inhibitors. The assay utilizes the peroxidase component of COX. The reaction between prostaglandin- G2 and 10-acetyl-3, 7-dihydroxyphenoxazine produces the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analyzed with an excitation wavelength of 540 nm and an emission wavelength of
595 nm. The COX-2 assay consisted of a 200-μl reaction mixture containing 150 μl assay buffer, 10 μl heme, 10 μl COX-2, 10 μl fluorometric substrate, and 10 μl of ten ferulic acid related compounds (25, 50, 100 μg/ml). The reactions were initiated by quickly adding 10 μl AA, then incubating for 5 min at 37 °C temperature. Dup-697 was used as a positive control. All the experiments were performed in triplicates and IC_{50} values were expressed as means of three experiments (Li et al., 2013; Mohapatra et al., 2015).

**Statistical analysis**

All data are expressed as mean ± SD of triplicate experiments.

**Results and discussion**

**Anti-inflammatory and cytotoxicity assay**

All ten ferulic acid related compounds were evaluated for anti-inflammatory activity by TNF-α and IL-6 inhibition assays. Not all of the compounds showed a promising TNF-α inhibitory activity except gallic acid, ferulic acid, caffeic acid and p-coumaric acid up to 30–40% at 10 μM concentrations, also a promising IL-6 inhibitory activity was shown by same compounds that is by gallic acid, ferulic acid, caffeic acid and p-coumaric acid up to 60–75% inhibition at 10 μM concentration. As compared to the standard dexamethasone, the activity results are revealed to be comparable as summarized in Table 1. Most of the compounds did not show significant cytotoxicity at 10 μM concentration except lauryl gallate, methyl gallate and stearyl gallate at very negligible level but at higher concentrations many of the compounds revealed cell toxicity. The results presented for ferulic acid related compounds revealed excellent anti-inflammatory activities as compared to dexamethasone, a known anti-inflammatory agent. It is known that during inflammation and associated processes, there is an increased production of superoxide ions. It may be possible that the inhibition of superoxide generation in peritoneal macrophages is related to the anti-inflammatory activity of ferulic acid related compounds (Nil and Khobragade, 2011; Nile and Park, 2014d). Pro-inflammatory cytokines such as TNF-α, and IL-6 are produced and play critical roles in the inflammation processes. Among these pro-inflammatory cytokines, TNF-α has been highlighted recently as a main mediator in the inflammatory diseases mechanism (Maxiaid et al., 2011). High levels of pro-inflammatory cytokines, including TNF, have been detected in psoriatic skin lesions and joints of patients with the inflammatory disease (Mease, 2002). TNF-α may have a significant role in pathogenesis of several inflammatory diseases, such as psoriatic arthritis, juvenile rheumatoid arthritis, and Crohn’s disease. Decrease in the TNF-α and an IL-6 level by these compounds suggests that it may be useful in a variety of inflammatory conditions (Ellerin et al., 2003; Kuek et al., 2007).

**XO inhibition by ferulic acid related compounds**

The experimental evidence indicates that, all the ten ferulic acid related compounds under this study showed a good to excellent activity profile toward inhibition of xanthine oxidase inhibitory activity and formation of hydroperoxide. All of the ten ferulic acid related compounds demonstrated XO-inhibiting activity among which ferulic acid, gallic acid, caffeic acid, p-coumaric acid, alkyll gallate, and methyl gallate, showed an inhibition >50% at 100 μg/ml (Fig. 1). Flavonoids and phenolics are well known antioxidants and attract a significant curiosity and scientific demand by research scientist for utilization as possible therapeutic agents against various disorders and diseases mediated by active free radicals (Nile and Park, 2013, 2014b). Phenolics and flavonoids may acts as potent inhibitors against the metabolic enzymes such as cyclooxygenase, xanthine oxidase and lipoxygenase (Hoorn et al., 2002). Thus, the phenolic constituents may play an essential role in the inhibition of XO and these XO inhibitors and uricosuric agents are commonly used against the treatment and curing of inflammatory diseases and gouty arthritis. So the drug allopurinol is the drug of choice against XO activity; however it has serious side effects (Nile and Park, 2013, 2014b). Thus, new alternatives with increased therapeutic activity and lesser side effects are desired. This study suggested that a ferulic acid related compound imparts xanthine oxidase inhibition and significantly reduce formation of uric acid in human body, indirectly helping to reduce risk of gout by slowing the XO activity. Further in vivo experiments were needed to identify whether the active ferulic acid related compounds are potent inhibitors of XO or not, which are directly correlated to check the in progress and development of gout and related inflammatory disorders.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Inhibition at 10 μM</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
<td>IL-6</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>40</td>
<td>72</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>35</td>
<td>68</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>23</td>
<td>56</td>
</tr>
<tr>
<td>Allyl ferulates</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>48</td>
<td>75</td>
</tr>
<tr>
<td>Methyl gallate</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>Lauryl gallate</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Stearyl gallate</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>60</td>
<td>92</td>
</tr>
</tbody>
</table>

To qualify the compound as a drug candidate, it is analyzed by the parameters set by Lipinski’s rule of five using Osiris property explorer. The cLogP is the important physiochemical property indicating the lipophilicity and the ability of molecule to cross the various biological membranes. According to Lipinski’s rule of five the cLogP value below 5 is feasible for a compound to be a future drug. The all ten ferulic acid related compounds showed a marginal lipophilicity within the range of 4.0–5.0. The molecular weight property of the compound is related to its in vivo administration. All the ferulic acid related compounds have the molecular weight within the acceptable range, that is, 400–500. The compounds showed the HBA below 10 and HBD below 5, which is also within the limit set by Lipinski’s rule. The polar surface area (PSA) > 140 Å² is thought to have low oral bioavailability, which is also revealed within the range for these compounds (Table 2). Interestingly the compounds also presented a better drug likeness values. Overall, the compounds ferulic acid, gallic acid, caffeic acid and sinapic acid showed a good drug score, calculated by combining all parameters. Drug toxicity is a factor of great importance for a potential commercial drug, since a significant number of drugs are disapproved in clinical trials based on their high toxicity profile (Bandgar et al., 2009; Bandgar and Gawande, 2010). The toxicity of the compounds is calculated in terms of mutagenicity, tumorigenic, reproductively effective and irritant. All the compounds were confirmed as non-mutagenic and therefore were biologically safe for intake.
Table 2
In silico pharmacological parameters for bioavailability of ferulic acid related compounds.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular formula</th>
<th>Molecular weight (g/mol)</th>
<th>Melting point (°C)</th>
<th>E_HOMO</th>
<th>E_LUMO</th>
<th>HBD</th>
<th>HBA</th>
<th>Mol. PSA</th>
<th>cLogP</th>
<th>Solubility</th>
<th>Drug likeness</th>
<th>Drug score</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Coumaric acid</td>
<td>C_9H_6O_3</td>
<td>164</td>
<td>212</td>
<td>-4.28</td>
<td>-0.65</td>
<td>4</td>
<td>56.20</td>
<td>4.45</td>
<td>-4.20</td>
<td>0.53</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>C_10H_8O_4</td>
<td>194</td>
<td>170</td>
<td>-5.52</td>
<td>-0.61</td>
<td>5</td>
<td>56.20</td>
<td>4.12</td>
<td>-4.50</td>
<td>0.52</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>C_9H_8O_4</td>
<td>180</td>
<td>224</td>
<td>-4.25</td>
<td>-0.58</td>
<td>4</td>
<td>56.20</td>
<td>4.60</td>
<td>-4.20</td>
<td>0.41</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>C_13H_26O_5</td>
<td>224</td>
<td>204</td>
<td>-5.34</td>
<td>-0.70</td>
<td>4</td>
<td>65.5</td>
<td>5.40</td>
<td>-5.34</td>
<td>-0.22</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Alkyl ferulates</td>
<td>C_10H_16O_3</td>
<td>614</td>
<td>96</td>
<td>-9.55</td>
<td>-0.85</td>
<td>6</td>
<td>85.5</td>
<td>6.86</td>
<td>-8.50</td>
<td>0.85</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>C_7H_6O_4</td>
<td>170</td>
<td>260</td>
<td>-4.10</td>
<td>-0.60</td>
<td>4</td>
<td>55.40</td>
<td>4.11</td>
<td>-4.10</td>
<td>0.55</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Methyl gallate</td>
<td>C_8H_8O_3</td>
<td>184</td>
<td>202</td>
<td>-4.61</td>
<td>-0.56</td>
<td>4</td>
<td>55.20</td>
<td>4.20</td>
<td>-4.22</td>
<td>0.32</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>C_10H_17O_3</td>
<td>212</td>
<td>150</td>
<td>-5.45</td>
<td>-0.55</td>
<td>5</td>
<td>60.5</td>
<td>5.10</td>
<td>-4.40</td>
<td>-0.23</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Lauryl gallate</td>
<td>C_13H_23O_3</td>
<td>338</td>
<td>98</td>
<td>-6.28</td>
<td>-0.68</td>
<td>5</td>
<td>72.5</td>
<td>6.85</td>
<td>-5.30</td>
<td>-0.42</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Stearyl gallate</td>
<td>C_23H_45O_3</td>
<td>423</td>
<td>105</td>
<td>-6.85</td>
<td>-0.75</td>
<td>6</td>
<td>75.5</td>
<td>6.20</td>
<td>-6.10</td>
<td>0.65</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition of COX-2 by ferulic acid related compounds

Five ferulic acid related compounds demonstrated COX-2-inhibiting activity (Fig. 2), among which gallic acid, ferulic acid and caffeic acid showed an inhibitor >50% at 100 μg/ml. The mechanism of anti-inflammatory action is assumed to be mediated through the inhibition of COX-1 and COX-2. These enzymes catalyze the biosynthesis of prostaglandin H2 from the arachidonic acid substrate. The inhibition of COX-1 results in some undesirable side-effects, whereas COX-2 inhibition provides therapeutic effects in pain, inflammation, cancer, glaucoma, Alzheimer’s and Parkinson disease (Blobaum and Marnett, 2007). Therefore, we intended to examine the COX-1 and COX-2 inhibitory activity of ferulic acid related compounds demonstrated on purified enzymes as a mechanism of its topical anti-inflammatory action. Also it was found that many phenolic compounds such as chlorogenic acid, gallic acid,
Rosmarinic acid, catechin, hesperidin, hesperetin, luteolin, naringenin, and naringin have been demonstrated possessing inhibitory effect for COX-2 expression (Raso et al., 2001; Shen et al., 2008). Thus the result indicates that the ferulic acid related compounds may reduce the inflammatory activity by virtue of inhibition of COX-2 enzyme which provides pro-inflammatory activity.

The result indicates that the ferulic acid related compounds may reduce the inflammatory activity by virtue of inhibition of xanthine oxidase and COX-2 enzyme which provide pro-inflammatory activity. These compounds might be acts as good source of xanthine oxidase and COX-2 inhibitory agents, but further in vivo and clinical study should be required for their characterization as a drug against various diseases and disorders related to activity of xanthine oxidase and COX-2 enzymes. Furthermore the individual the ferulic acid related compounds can be subjected to optimization so as to design and develop a lead anti-inflammatory and antigout drug with an improved potency. For development of rational drug development against XO and cyclooxygenase-2 it is important to check the structure–activity relationships and other factors of related to ferulic acid compounds such as binding affinity, kinetic parameters and changes at active site of these studied enzymes were discussed, which is expected for future drug development and design.

**Authors’ contributions**

SHN performed the laboratory work including in vitro xanthine oxidase and cyclooxygenase-2 activity, also in silico study and data analysis. VB assisted in anti-inflammatory assays. DHK provided idea and project plan with subsequent editing and checking of manuscript data and SHN contributed to critical reading and writing of the manuscript.

**Ethical disclosures**

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.
Conflicts of interest
The authors declare no conflicts of interest.

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