A possible mechanistic approach of synthetic flavonoids in the management of pain

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Abstract: Flavonoids are phenolic compounds that have always attracted pharmaceutical researchers and food manufacturers. Nature has indirectly provided us flavones in our daily diet i.e. tea, fruits, juices and vegetables. Flavones have got special position in research field of natural and synthetic organic chemistry due to their biological capabilities. Three substituted flavone derivatives have been synthesized from substituted O-hydroxy acetophenones and 4-trifluoromethyl benzaldehyde in good yield. The structures have been established by different spectroscopic techniques like 1HNMR, 13CNMR, IR spectroscopy. The compounds were then screened for their enzyme inhibition potential and antinociceptive response in mice models with writhings induced by acetic acid, tail immersion and formalin-induced nociception assay procedures and structure activity relationship was established. The effects following pretreatment with naloxone were also studied to reveal the involvement of opioid receptors in the antinociceptive action. The flavone derivatives showed moderate to weak inhibition against LOX. Moreover, significant to moderate decrease in the number of abdominal constrictions, increase in paw-licking response time in both phases and a significant raise in latency time in nociception models. Moreover, the antinociceptive response was significantly attenuated by pretreatment with opioid receptor antagonist suggesting the involvement of opioiergic system in the analgesic action. The flavone derivatives showed analgesic response in all models of nociception suggesting the possible involvement of opioidergic system in the antinociceptive action.

Keywords: Flavone derivatives, LOX, analgesic, naloxone, opioid.

INTRODUCTION

Pain and Inflammation are common indications of many disorders. The opiates (centrally acting) and nonsteroidal anti-inflammatory (peripherally acting) drugs commonly known as NSAIDs have been used typically in these circumstances with general adverse reactions like respiratory depression, renal damage, gastrointestinal disturbances and possible dependency (Carlos et al., 2013; Domaj et al., 1999; Amir et al., 1999). Recently, interest in finding new analgesic and anti-inflammatory drugs with fewer adverse effects from natural and synthetic sources has been felt (Asie et al., 2015).

The endogenous pain mediators like serotonin 5-HT, histamine, prostaglandins and bradykinin etc have been reported responsible for the pain sensation (Collier et al., 1968; Ronaldo et al., 2000; Roger et al., 1980) and the analgesics exert its activity by inhibiting these mediators. Lipoxygenases LOX are the members of a class of non-haeme iron (Fe) containing dioxygenases that catalyze the first step in the arachidonic acid cascade that lead to formation of lipoxins and leukotrienes involved in the variety of inflammatory responses (Mohan et al., 2013).

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Synthetic as well as naturally occurring flavonoid derivatives have many interesting pharmacological activities and among these, the important action is its analgesic response. Natural or semi-synthetic compounds may be used to prevent or treat the development of analgesics (Josefina et al., 2013; Santanu et al., 2011). But the adverse effects of flavon derivatives in humans appear to be rare (Elliot et al., 2000). Generally the safety margin of flavones derivatives is better than the currently used drugs (Bent, 2002). Keeping in view the pharmacological and chemical importance of natural and synthetic flavonoid derivatives, herein we report synthesis, characterization, enzyme inhibition and analgesic potentials of three flavone derivatives. The synthesized compounds (crystals) were structurally characterized by X-ray crystallography and Infra-red spectroscopy.

MATERIALS AND METHODS

Chemicals and instruments

All chemicals and preparative work was handled in normal atmosphere. Substituted ketones o-hydroxy acetophenones and benzaldehyde 4-trifluoro- methyl benzaldehyde, quercetin, enzyme 15-lipoxigenase, used were purchased from Sigma Aldrich Chemical Company.
TLC plates were of Merck 60 F254, Darmstadt Germany. Solvents and chemicals like ethanol, n-hexane, ethyl acetate used were of extra pure analytical grade were purchased from E. Merck. Tween-80 was purchased from Daejung Chemicals, Korea. Diclofenac sodium, morphine sulphate naloxone and indomethacin were purchased from local market.

1H-NMR was recorded in deuterated chloroform DCD3 on Bruker SF spectrometers operating at 300 and 75 MHz frequencies respectively. Chemical shifts values are expressed in δ ppm downfield relative to TMS which was used as an internal standard. Infrared spectra were recorded on ThermoScientific USA Nicolet 6700, Infrared spectrometer on KBr disk method. All melting points were determined in open capillary tubes using Electrothermal 9100 apparatus Barnstead UK. Reaction extents and final products purities were checked on TLC plates Merck 60 F254, Darmstadt Germany and spots were visualized under UV Lamp 180-365 nm and with subsequent staining with iodine vapours.

**General procedure for the synthesis of flavone derivatives**

Three flavone derivatives were synthesized according to reported procedure (Susanti et al., 2012). Briefly, equimolar quantities 15 mili mol of 4-trifluoromethylation benzaldehyde and substituted o-hydroxy acetophenones (2’-hydroxyacetophenone for F1, 2’-hydroxy-4’-methoxyacetophenone for F2 and 2’-hydroxy-5’-bromoacetophenone for F3) were taken in 20ml ethanol and 5ml of 50% ethanolic KOH was added drop wise to this stirring solution. The reaction was kept on stirring for 16 hours and monitored with TLC. Upon completion of reaction the mixture was carefully poured into ice crushed cold water and neutralized with 1N HCl. Solid products were obtained, filtered and washed thoroughly with excess of water.

In next step, the chalcones were then cyclized to respective flavones derivatives in DMSO at 120°C 1 hour in the presence of Iodine. The final products were purified with column chromatography in EtOAc n-hexane 3:7 and recrystallized in chloroform ethyl acetate mixture.

**In-vitro lipoxygenase activity**

The lipoxygenase activity of synthesized flavones was determined by using spectrophotometer with slight modification (Shailasree et al., 2013). The in vitro inhibition was assessed by determining the loss in soybean 15-LOX activity 5µg with 0.2µM linoleic acid as the substrate prepared in borate buffer 0.2M, pH 9.0. The percent inhibition (triplicate) at various concentrations of synthetic flavones (12.5-200µg/ml was recorded at 234 nm using UV-Vis spectrophotometer. Standard inhibitors, quercetin and indomethacin were used as positive control, while methanol was used as negative control. IC50 indicating the concentration to 50% inhibition was also calculated (Shailasree et al., 2013).

**Analytical activity**

**Acute toxicity**

The synthesized compounds F1, F2 and F3 suspended in 2% tween-80 were subjected to acute toxicity study on mice. Briefly, mice n=6 were given different doses of flavones by i.p route to six groups each in two phases and were kept under observation for 30 minutes followed by

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**References**

2. Dongbing et al., 2013.
3. Susanti et al., 2012.
4. Lydia et al., 2012.
six hourly observation for 24 hours. The animals were kept for the next 14 days for any behavioral changes and manifestation of the synthetic flavonoids toxicity (Dietrich, 1983; Niaz et al., 2013).

**Writhing test**
Antinociceptive effects of the synthesized compounds were assessed by using mice as an animal model with slight modifications (Koster et al., 1959; Ali et al., 2013). The synthesized compounds at different dose concentrations in 2% tween-80 were dispensed to respective groups of animals n=6 by s.c. route and diclofenac sodium was administered intraperitonially. The control group only received 2% tween-80 in normal saline. After 30 minutes of time interval, 0.1ml of acetic acid at a concentration of 1% v/v was injected intraperitonially. Data was recorded in the form of number of writhes abdominal constriction, extension of hind legs and turning of trunk during 30 minutes of test period, starting 3 minutes after the administration of acetic acid (Koster et al., 1959; Ali et al., 2013).

**Formalin test**
This test was performed by the method of assessing the licking response of formalin-induced in paw of mice. 20 µl of 1% formalin prepared in 0.9% saline was administered by s.c. route into the dorsal region of hind paw and immediately placed in the transparent box for observing the licking response. The duration of reaction time (sec) paw licking or biting was determined between 0-5min first phase and 15-30 min second phase. Animals in different groups were treated s.c. route 30 min prior to administration of formalin with synthesized compounds 50mg/kg, indomethacin 10mg/kg (i.p.) and morphine 5 mg/kg (i.p.). An opioid receptor antagonist, naloxone 2 mg/kg (i.p.) was given 20min prior to treatment of animals with test compound and standard. The animals in control group received the vehicle 0.1ml/10gm. The reaction time (sec) of the animals in respective groups was compared to control group and expressed as percent inhibition (Subash et al., 2013).

**Tail immersion test**
Albino mice were used to assess the central antinociceptive response of the synthesized compounds. Briefly, animals in respective groups were treated with synthesized compounds 50mg/kg and vehicle 2% tween-80 by s.c. route 30 minutes and morphine and naloxone by i.p. route 15 minutes before the screening. The latency period time taken by the mice to deflect the tail was determined by immersing 1-2cm of mice tail in water that was kept warm at 53±1°C (Mohammad and Chandra, 2014; Mohammad and Mohammad, 2014).

**STATISTICAL ANALYSIS**
Data are presented as mean ± SEM. Analysis of variance and Dunnett's test is statistically manipulated with GraphPad prism 5 version 5.01 software.

**RESULTS**

**Spectroscopic analysis**
The physical parameters of the final synthesized flavones derivatives are given in table 1 while the 1H NMR, 13C NMR and IR study are given in experimental portion. The compounds F1, F2 and F3 showed characteristic peaks for methene proton at C-3 of the chromone ring at 6.88, 6.83 and 6.90 ppm respectively relative to TMS as singlets. This range is reported for many such flavonoid derivatives as well. Moreover, the methoxy group of F2 attached to C-7 of the chromone ring gave singlet of 3 protons at 3.97 ppm.

**In-vitro enzyme inhibition activity**
It is evident from the results given in table 2 that, F2 demonstrated inhibitory activity against LOX IC₅₀=83.53±1.48 µg/ml to a greater extent than F1 and F3. Moderate inhibition of LOX activity was demonstrated by F3 IC₅₀=88.35±1.75 µg/ml. A weak inhibitory action on LOX was observed by F1 IC₅₀=97.28±1.41 µg/ml. Whereas, the standard indomethacin and quercetin showed significant inhibition of LOX activity having IC₅₀ values of 53.66±1.38 µg/ml and 38.50±1.72 respectively.

**Analgesic activity**
Acute toxicity
In the in-vivo acute toxicity studies of the synthesized flavones derivatives, there were no observation of gross
Table 1: Physical parameters of flavone derivatives

<table>
<thead>
<tr>
<th>Flavone</th>
<th>Ketone</th>
<th>Aldehyde</th>
<th>Yeild</th>
<th>Appearance</th>
<th>R_f</th>
<th>m.p.ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td></td>
<td></td>
<td>85.3%</td>
<td>Monoclinic crystals</td>
<td>0.63</td>
<td>134-137</td>
</tr>
<tr>
<td>F2</td>
<td></td>
<td></td>
<td>83.4%</td>
<td>Triclinic crystals</td>
<td>0.57</td>
<td>167-170</td>
</tr>
<tr>
<td>F3</td>
<td></td>
<td></td>
<td>85.7%</td>
<td>Monoclinic crystals</td>
<td>0.71</td>
<td>171-173</td>
</tr>
</tbody>
</table>

Table 2: *In vitro* enzyme inhibition potentials of flavone derivatives

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>LOX IC_{50}µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>97.28±1.41</td>
</tr>
<tr>
<td>F2</td>
<td>83.53±1.48</td>
</tr>
<tr>
<td>F3</td>
<td>88.35±1.75</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>53.66±1.38</td>
</tr>
<tr>
<td>Quercetin</td>
<td>38.50±1.72</td>
</tr>
</tbody>
</table>

All the values were expressed as mean ±SEM n=6.

Table 3: Acetic acid induced analgesic activity data of the compounds.

<table>
<thead>
<tr>
<th>Treatment/Dose</th>
<th>Number of writhing</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 2% Tween 80</td>
<td>74.83±1.01</td>
<td>**</td>
</tr>
<tr>
<td>F1 50mg</td>
<td>31.13±2.08***</td>
<td>**P&lt;0.01</td>
</tr>
<tr>
<td>F2 50mg</td>
<td>26.07±1.81**</td>
<td>**P&lt;0.01</td>
</tr>
<tr>
<td>F3 50mg</td>
<td>27.93±1.36**</td>
<td>**P&lt;0.01</td>
</tr>
<tr>
<td>Diclofenac sodium 10 mg</td>
<td>10.16±0.70**</td>
<td>**P&lt;0.01</td>
</tr>
</tbody>
</table>

All the values were expressed as mean ±SEM n=6. **P<0.01, ***P<0.001 when compared to control group.

Since the compounds even at a dose of 1200 mg/kg didn’t show any mortality rate in mice so it is consider to be comparatively safe, during the study it was observed that the systemic administration of the compounds didn’t produced any sedation, alteration in locomotor activity or motor dysfunction in animals.

**Writhing test**

The synthesized compounds F1, F2 and F3 caused significant inhibition of the analgesic response induced by acetic acid with maximum effect of 57.30% P<0.01, n=6, 65.16% P<0.001, n=6 and 62.67% P<0.01, n=6 respectively at a dose of 50 mg/ kg as shown in table 3. The results were comparable to that of the standard drug diclofenac sodium that displayed 86.42% P<0.001, n=6 at a dose of 10mg/kg.

**Formalin test**

The administration of the compound F1 (50 mg/kg) significantly inhibited both phases to 45.83% P<0.01, n=6 and, 62.64% P<0.001, n=6 of formalin-induced paw licking response respectively. Similarly, the inhibition response of F2 was observed as 53.45% (P<0.001, n=6) and 67.20% (P<0.001, n=6). Maximum response was observed in F3 that was appeared to be 48.16% P<0.01, n=6 and 57.38% P<0.01, n=6. Results are shown in table 4 and fig. 1a and b.

Animals pretreated with morphine at a dose of 5 mg/kg significantly inhibited both phases to 86.86% (P<0.001, n=6) and 96.11% (P<0.001), n=6 of formalin-induced paw licking response respectively. Pre-treated animals with naloxone reversed the inhibitory effects of synthesized compounds.

It is also evident from the results that naloxone caused obvious reversal of the analgesic response of morphine in early and late phases of formalin test. Indomethacin (10 mg/kg) caused marked reduction in the paw-licking time to 74.37% (P<0.001, n=6) in the second phase while mild reduction in paw-licking time 18.43% in the first phase was observed.

**Tail immersion test**

Table 5 shows that the analgesic response of flavone derivatives F1, F2 and F3 at a dose of 50mg/kg was significant in tail immersion test. The maximum analgesic effect of F1 was observed at 75 min latency increased 51.13%, P<0.01. Similarly, the F2 showed maximum...
response of 59.11%, P<0.001 at 90 min and it was observed to be 61.82%, P<0.001 for F3. Whereas, morphine, a centrally acting opioid analgesic agent, exhibited powerful activity recorded at 60 min after treatment 84.80%, P<0.001. Animals treated with naloxone produced significant reduction in the analgesic activity of morphine and flavone derivatives table 5.

DISCUSSIONS

The writhing method is a minimal noxious stimulus and very sensitive method associated to visceral pain because of the release of free arachidonic acid from tissue phospholipid via cyclooxygenase, histamine, prostaglandins and serotonin which is an endogenous mediator and capable of accelerating the neurons sensitivity and even with which weak analgesic agents can be detected (Ali et al., 2013; Vaz et al., 1996; Hasan et al., 2010). The boost the level of prostaglandin raises the inflammatory pain and reduction in the number of twitching by agents like flavonoid derivatives will produce analgesic effect by blocking of prostaglandin synthesis pathway and ultimately peripheral pain is inhibited (Zakaria et al., 2008; Ari f et al., 2014).

For confirmation of the analgesic mechanism, the compounds were further screened for formalin induced paw licking response. As the formalin pain model is very useful for explaining the mechanism of analgesia (Tjolsen et al., 1992). This model involves two phases of pain, the first phase and the late or second phase (Hunskaar and Hole, 1987; Murray et al., 1988). The flavones derivatives which caused evident decrease in paw-licking/ biting response of mice during observations in both phases like morphine. It is clear that the NSAIDs, peripherally acting drugs inhibit the second phase and opioids, centrally acting drugs like morphine acts on both phases to reduce the paw-licking response (Santos et al., 1994; Shibata et
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...in formalin-stimulated nociception test. The ability to suppress both phases of the formalin-stimulated pain response by compounds showed that the analgesic effect is due to central mechanism of pain inhibition. An opioid antagonist like naloxone distinctly inhibits the effects of morphine and the compounds suggest that the analgesic effect is mediated by opioid receptors.

The writhing model and late phase formalin model represents the pain response of inflammatory origin (Tjolsen et al., 1992; Tjolsen and Hole, 1997). These findings indicated that the compounds can be potent anti-inflammatory agents. The Lipoxygenases enzymes (LOX) are associated with inflammatory and allergic reactions due to the formation of the leukotrienes LTs (Rakha et al., 2014). These results of LOX activity suggest that change in the position or additional moiety may increase or decrease the potency of the flavonoids. The results of this study concluded that anti-inflammatory activity of the compounds could be due to inhibition LOX enzymes and thus supports the use of flavonoids in inflammatory disorders.

The central analgesic effect of the compounds were further confirmed by tail immersion screening method which is consider more specific for such studies (Robert, 1965). The flavonoid derivatives have been reported to possess analgesic and anti-inflammatory properties (Selvaraj et al., 2014; Raquel et al., 2014; Qnais et al., 2014). Based on the above findings it is concluded that the flavonoids may be used an alternative to other synthetic analgesic and anti-inflammatory drugs. These finding needs further work to confirm the anti-inflammatory response and safety profile of the synthetic flavonoids derivatives.

CONCLUSION

This work is a part of our ongoing research on synthesis and pharmacological evaluation of flavonoid derivatives. In this study, three flavonoid derivatives are successfully synthesized and characterized by ^1^HNMR, ^1^CNMR, IR spectroscopy. The compounds were then screened for enzyme inhibition potential and antinociceptive response in mice models. The flavone derivatives showed moderate to weak inhibition against LOX. The flavone derivatives showed analgesic response in all models of nociception suggesting the possible involvement of opioidergic system in the antinociceptive action of synthetic flavonoids.

REFERENCES


