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 ^{1}HNMR ^{13}CNMR, 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 opioidergic 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 flavone 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 indomethacine were purchased from local market.

^{1}H-NMR was recorded in deutrated chloroform CDCl3 on Bruker SF spectrometers operating at 300 and 75 megahertz 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 Nickolet 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-trifluoro-mehtyl benzaldehyde and substituted o-hydroxy acetophenones (2′-hydroxyacetophenone for F1, 2′-hydroxy-4′ methoxya-cetophenone for F2 and 2′-hydroxy-5′ bromoacetophen-one 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 recrystalized in chloroform ethyl acetate mixture.

2-4-trifluoromethylphenyl-4H-chromen-4-one (F1)

^{1}H NMR 300 MHz, Chloroform-*d* δ 8.25 dd, *J*=8.0, 1.7 Hz, 1H, 8.06 d, *J*=8.2 Hz, 2H, 7.85-7.69m, 3H, 7.61 dd, *J* =8.4, 1.1 Hz, 1H, 7.47m, 1H, 6.88 s, 1H. ^{13}C NMR 75 MHz, $CDCl_3$ δ 178.14, 161.57, 156.17, 135.16, 134.10, 133.77, 132.90, 126.62, 126.02, 125.77, 125.54, 123.91, 118.11, 108.71. IR KBr *υ, cm^{-1}*, 3073.3 =C-H, 1641.8 C=O, 1316.8 C-F, 1165.3 C-O, 849.8 C-F. ESI: m/z $C_{16}H_9F_3O_2$ H+: calculated, 291.0627, found: 291.0629 (Lydia *et al.,* 2012; Dongbing *et al.,* 2013).

2-4-trifluoromethylphenyl-7-methoxy-4H-chromen-4-one (F2)

^{1}H NMR 300 MHz, Chloroform-*d* δ 8.16 d, *J*=8.7 Hz, 1H, 8.10-8.00m, 2H, 7.80 d, *J*=8.3 Hz, 2H, 7.09-6.97m, 2H, 6.83 s, 1H, 3.97 s, 3H.^{13}C NMR 75 MHz, $CDCl_3$ δ 177.56, 164.45, 161.23, 157.98, 135.27, 132.77, 127.18, 126.51, 126.08, 125.98, 125.93, 117.82, 114.76, 108.76, 55.91. IR KBr *υ, cm^{-1}*, 3130.4 =C-H, 2815.3 C-H, 1650.4 C=O, 1381.4 C-F, 1111.7 C-O, 836.4 C-F. HRMS ES$^+$ m/z $C_{34}H_{22}F_6O_6Na$ requires 663.1213; Found 663.1234 (Sherif *et al.*, 2013).

6-bromo-2-4-trifluoromethylphenyl-4H-chromen-4-one (F3)

^{1}H NMR 300 MHz, Chloroform-*d* δ 8.38 d, *J*=2.5 Hz, 1H, 8.06 d, *J*=8.1 Hz, 2H, 7.88-7.78 m, 3H, 7.58-7.45m, 1H, 6.90 s, 1H. ^{13}C NMR 75 MHz, $CDCl_3$ δ 176.88, 161.93, 154.95, 137.13, 134.13 128.47, 126.72, 126.18, 126.13, 125.21, 124.32, 120.13, 119.07, 108.73. IR KBr *υ, cm^{-1}*, 3090.4 =C-H, 1633.7 C=O, 1316.3 C-F, 1173.2, 828.4 C-F, 632.1 C-Br. HRMS ES$^+$ m/z C_{32} H_{16} $Br_2F_6O_4Na$ requires 760.9212; found 760.9164 (Sherif *et al.,* 2013).

Pharmacological activities

Animals

Swiss Albino mice of either sex weighing 18-25gm were purchased from National Institute of Health NIH Islamabad. The animals were housed in individual cages at the animal house of University of Malakand with free access to water and standard diet and starved for 12-18 hours before experimentation. Ethical Committee of the Department of Pharmacy, University of Malakand approved the experimental protocols and ensured its compliance with provisions of the "Animal Bye-Laws 2008, Scientific Procedures Issue-I of the University of Malakand".

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. IC_{50} indicating the concentration to 50% inhibition was also calculated (Shailasree *et al.,* 2013).

Analgesic 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

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±1ºC (Mohammad and Chandra, 2014; Mohammad and Mohammad, 2014).

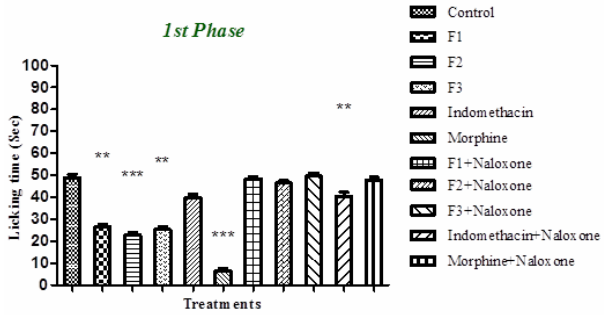
STATISTICAL ANALYSIS

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

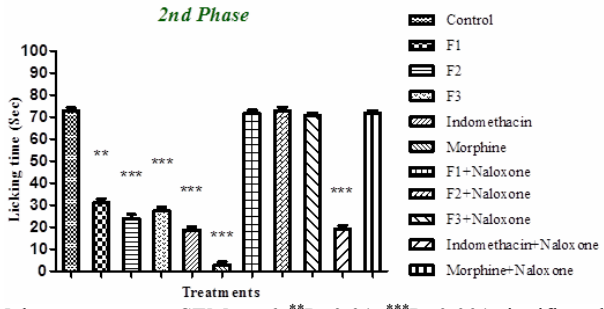
Spectroscopic analysis

The physical parameters of the final synthesized flavones derivatives are given in table 1 while the ^{1}H NMR, ^{13}C 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.



Values are mean ± SEM, n=6, $^{**}P<0.01$, $^{***}P<0.001$ significantly different compared with control.

Fig. 1a: Formalin induced licking response in first phase of the test.



Values are mean ± SEM, n=6, $^{**}P<0.01$, $^{***}P<0.001$ significantly different compared with control.

Fig. 1b: Formalin induced licking response in second phase of the test.

In-vitro enzyme inhibition activity

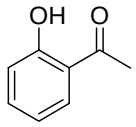
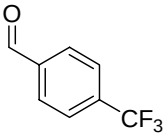
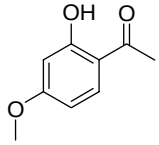
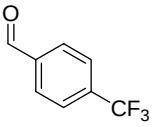
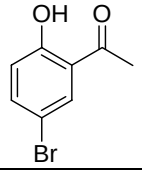
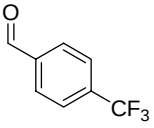
It is evident from the results given in table 2 that, F2 demonstrated inhibitory activity against LOX IC_{50}=83.53±1.48 µg/ml to a greater extent than F1 and F3. Moderate inhibition of LOX activity was demonstrated by F3 IC_{50}=88.35±1.75 µg/ml. A weak inhibitory action on LOX was observed by F1 IC_{50}=97.28±1.41µg/ml. Whereas, the standard indomethacin and quercetin showed significant inhibition of LOX activity having IC_{50} 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

Flavone	Ketone	Aldehyde	Yeild	Appearance	R_f	m.p ºC
F1			85.3%	Monoclinic crystals	0.63	134-137
F2			83.4%	Triclinic crystals	0.57	167-170
F3			85.7%	Monoclinic crystals	0.71	171-173

physical and behavioral changes for 24 hours and no mortality occurred within the observation period of 14 days.

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

Test Sample	LOX IC_{50} *µg/ml*
F1	97.28±1.41
F2	83.53±1.48
F3	88.35±1.75
Indomethacin	53.66±1.38
Quercetin	38.50±1.72

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

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

Treatment/Dose	Number of writhing	% inhibition
Control 2% Tween 80	74.83±1.01	---
F1 50mg	31.13±2.08**	57.30
F2 50mg	26.07±1.81***	65.16
F3 50mg	27.93±1.36**	62.67
Diclofenac sodium 10 mg	10.16±0.70***	86.42

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

Table 4: Formalin-induced paw-licking response.

Treatment/Dose	Licking time Sec		Inhibition %	
	1st Phase	2nd Phase	1st Phase	2nd Phase
Control 2% Tween 80	48.83±1.627	72.83±1.405	----	----
F1 50mg	26.45±1.268**	27.21±1.799***	45.83	62.64
F2 50mg	22.73±1.394***	23.89±1.805***	53.45	67.20
F3 50mg	25.31±1.454**	31.04±1.744**	48.16	57.38
Indomethacin 10mg	39.83±1.541	18.66±1.498***	18.43	74.37
Morphine 5mg	6.416±1.165***	2.83±1.260***	86.86	96.11
F1 50mg + Naloxone 2mg	48.24±1.268	71.57±1.455	1.20	1.73
F2 50mg + Naloxone 2mg	46.51±1.258	72.94±1.639	4.75	-0.15
F3 50mg + Naloxone 2mg	49.71±1.429	70.66±1.173	-1.80	2.97
Indomethacin 10mg + Naloxone 2mg	40.50±1.828**	19.16±1.429***	17.05	73.69
Morphine 5mg + Naloxone 2mg	47.66±1.520	71.83±1.142	2.39	1.37

Table 5: Analgesic activity Tail flick method data of the compounds.

Treatment/Dose	Tail Flick (sec) / Response (%)					
	15 min	30 min	45 min	60 min	75 min	90 min
Control 2% Tween 80	0.78±0.301	0.88±0.421	0.98±0.521	0.93±0.384	0.86±0.251	0.92±0.417
F1 50mg	0.92±0.312 (15.21%)	1.14±0.352* (22.80%)	1.53±0.641** (29.41%)	1.64±0.311** (43.29%)	1.76±0.313** (51.13%)	1.87±0.403** (50.80%)
F2 50mg	1.03±0.325* (24.27%)	1.28±0.418* (31.25%)	1.63±0.216** (39.87%)	1.94±0.421** (52.06%)	2.08±0.262*** (58.65%)	2.25±0.318*** (59.11%)
F3 50mg	1.01±0.209* (22.77%)	1.30±0.216* (32.30%)	1.84±0.242** (46.73%)	2.06±0.319*** (54.85%)	2.19±0.413*** (60.73%)	2.41±0.408*** (61.82%)
Standard Morphine 5mg	1.54±0.214** (49.35%)	2.11±0.166** (58.29%)	4.52±0.328*** (78.31%)	6.12±0.354*** (84.80%)	4.98±0.501*** (82.70%)	4.74±0.274*** (80.59%)
F1 50mg + Naloxone 2mg	0.81±0.237	0.83±0.265	0.96±0.419	0.92±0.238	0.97±0.258	0.82±0.424
F2 50mg + Naloxone 2mg	0.91±0.412	0.96±0.336	0.82±0.431	0.89±0.547	0.91±0.338	0.84±0.156
F3 50mg + Naloxone 2mg	0.88±0.186	0.96±0.531	1.02±0.416	0.93±0.325	0.95±0.514	0.86±0.238
Morphine 5mg + Naloxone 2mg	0.72±0.422	0.88±0.346	0.92±0.460	0.97±0.348	0.91±0.309	0.95±0.426

All the values were expressed as mean ±SEM. *P<0.05, **P<0.01 and ***P<0.001 when compared to control group one way ANOVA followed by Dunnetts: compare all vs control test.

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 ultimaletly 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*

al., 1989) 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 (Rekha *et al.,* 2014). These results of LOX activity suggest that change in the position or additional moiety may increase or decrease the potency of individual flavones. 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 flavones 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, ^{13}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 flavones.

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