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PRE-CLINICAL *IN-VIVO* ACUTE TOXICITY AND ANTI-INFLAMMATORY EVALUATION OF AURONE DERIVATIVES

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Abstract

This study investigates the acute toxicity and anti-inflammatory potential of aurone derivatives (**C-A, M-A** and **DM-A)** using in vivo models. Male Swiss albino BALB/c mice were employed, and ethical clearance was obtained from Abdul Wali Khan University, Mardan. Acute toxicity test was conducted in two Phases. Phase-l for lower dose and Phase-ll for higher doses. For the anti-inflammatory assessment, a carrageenan-induced paw edema and xylene-induced ear edema were performed.

Acute toxicity assay of stated aurones on mice models revealed that these compounds were well-tolerated up to 500 mg/kg, with no observable symptoms of toxicity in terms of mortality and morbidity, thus confirming their safety profile. The aurone derivatives, showed dose dependent potential, particularly **DM-A**, demonstrated significant anti-inflammatory activity at a dose of 20 mg/kg showing the highest inhibition of paw edema (60.89%) after 3 hours post-administration as compared to control (3.81±0.07) and standard 1.6±0.15 (58%) in carrageenan assay and reduced significantly (p=0.05) the mice ear edema from 0.301±0.008 to 0.24±0.004 after 40 min of the treatment via xylene induced ear edema assay. This suggests that aurone derivatives may effectively modulate the inflammatory response, likely through the inhibition of pro-inflammatory cytokines or stabilization of cell membranes.

The findings suggest that aurone derivatives possess significant therapeutic potential as anti-inflammatory agents, with a favorable safety profile. These results warrant further investigation into their development as safer alternatives to conventional therapies.

Keywords: Aurone derivatives, anti-inflammatory, Carrageenan, Xylene, Paw edema, Pro-inflammatory mediators

1. Introduction

A disease, is "an impairment of normal body function which has a negative impact on Public health, causing mortality and morbidity (Organization, 2020), imposed substantial economic burden (Nugent, 2008), straining of the healthcare system (Brooks et al., 2020), disruption of daily life (Smedley et al., 2003) and health inequities (Davis III and Matteson, 2012). There are numerous diseases, causing acute to chronic complications. Among them, up to somehow inflammation is frequently linked to these complications.

Inflammation is the biological body's natural immune response initiated by damaged cells (due to injury, trauma, cut), infection (due to pathogens) and irritation (due to toxic materials)". This involves several cellular and molecular episodes that work together to enhance tissues repairing by eliminating the source of damaged tissues or cell debris (Chen et al., 2018). Various factors, including the primary cause, duration, and immune response, are used to categorize inflammation into a wide range of groups which comes under *acute* or *chronic* inflammation. *Acute Inflammation* has an abrupt start (minutes or hours) and vanishes in a few days due to influx of neutrophils and causing erythema, which occurs as increased circulation to a specific location as a result of blood vesicles dilation (Mitchell, 2006). The body's defence system carries white blood cells, cytokines, and various other chemicals to the region of damaged tissues or infection in order to discard the source (pathogens) and begin the healing (resolving) process (Gardemann et al., 2013). *Chronic Inflammation* has persistent beginning of inflammatory response normally takes a few days and can last for years, with fewer signs and symptoms, infiltration of macrophages, monocytes, and lymphocytes, chronic exposure to hazardous components and environmental chemicals such as cigarette smoke also contribute to chronic inflammation. Chronic inflammation can cause tissue damage and is linked to many disorders like autoimmune diseases such as rheumatoid arthritis, cancer and atherosclerosis (Stawicki et al., 2020). It happens when the body releases chemical substances that cause an immune reaction to fight off contamination or heal damaged tissue. Bacterial infections, tissue necrosis, autoimmune disease, allergens, stress, unhealthy diet, gut flora imbalance and various chemical irritants are the most common causes of inflammation (Asija et al., 2014) along with cardinal symptoms like redness (rubor), heat (calor), swelling (tumor), pain (dolor), loss of function (functiolaesa) and fever, fatigue and malaise.

Pathophysiology of inflammation describes that harmful stimulus in the form of damaged molecular patterns (DMPs) or pathogenic molecular patterns (PMPs) is recognized via immune cells (macrophages or dendritic) by the binding of patterns recognition receptors (PRRs) to them (DMPs or PMPs) and initiate inflammatory response (Ahmed, 2011). Immune cells release chemicals (inflammatory mediators) like prostaglandins, histamines, bradykinins, cytokines and interleukins causing widening of the blood vessels walls and also enhancing the permeable nature of the walls of the vessels which permit antibodies, immune cells and some other molecules to the nearby tissues (Wilhelm and Mason, 1960, Williams and Peck, 1977). Inflammatory mediators, chemotactic factors from damaged cells and extravasation increase and promote the transport of immune cells (monocytes, neutrophils etc) into the site of inflamed tissues (Hayashi et al., 1984, Johnson et al., 2006). Immune cells in the inflamed area engulf and parish the pathogens and damaged cells, also release more inflammatory mediators (chemokines, cytokines) to recruit more immune cells to ignite inflammatory response and trigger tissue repairing by releasing growth factors and fibroblasts to produce collagen to rebuild new blood vessels to restore blood supply and repair the damaged tissues and resolve the inflammation by releasing anti-inflammatory mediators (like lipoxins and interleukin-10) (Upadhyay, 2015, Lawrence et al., 2002).

Chronic inflammation involves continuous activation of immune system to release inflammatory mediators such as cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), tumournecroting factor-alpha (TNF-a) which produce a long term inflammatory response in the form of a disease (Medzhitov, 2008). Persistent inflammatory response via immune cells in chronic inflammation sometimes generates reactive species (free radicals) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause tissues damages and necrosis which is the main cause of many chronic diseases such as diabetes, cancer and neurodegenerative disorders (Gallucci and Matzinger, 2001,

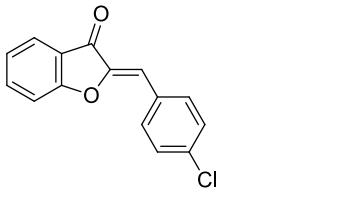
Valko et al., 2007). Acute inflammation can cause regulation of resolution pathway by producing anti-inflammatory mediators or resolving mediators to cause repairing of damaged tissues while in case of dysregulation of chronic inflammation causes the impairment of resolution pathway and leading to prolong inflammatory responses (Serhan and Levy, 2018). In some cases, alteration in gene expression (epigenetic modifications) is caused by chronic inflammation which produce an increased number of inflammatory mediators rather than anti-inflammatory mediators (Shanmugam and Sethi, 2012, Harb and Renz, 2015) which can lead to many chronic diseases.

Nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, disease-modified antirheumatic drugs (DMARDs) and corticosteroids are used against inflammation but sometimes they are associated with severe adverse effects such as GIT bleeding, ulcer, cardiovascular problems, hepatotoxic, nephrotoxic and erythrosclerotic (Vonkeman and van de Laar, 2010). To address these unwanted severe effects, a safe and effective drug is compulsory to explore.

Flavonoid is a polyphenolic compound, mostly obtained from plant source and responsible for many therapeutic purposes. It is categorized into many subgroups flavonols, flavanones, flavones, flavanols, isoflavones and anthocyanines (Ross and Kasum, 2002). *Flavones* consist of luteolin, epigenin and many others having pharmacological effects against inflammation, cancer, diabetes, bacterial infection and oxidative stress (López-Lázaro, 2009).

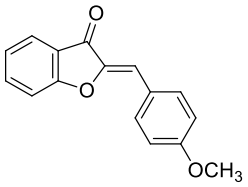
The exploration of the therapeutic potential of aurones and their derivatives, as evidenced by investigations into their analgesic and anti-inflammatory activities (Bandgar et al., 2010, Shin et al., 2011), underscores the broader need for comprehensive toxicological evaluation. Before any aurone-based compound can be considered for clinical application as an analgesic or anti-inflammatory agent, a thorough understanding of its safety profile is paramount. This necessitates rigorous acute toxicity studies to characterize the spectrum of adverse health effects that may arise from short-term exposure. Such studies, as previously described, are crucial for determining key parameters like the LD50 and the therapeutic index (Yadav and Rohane, 2021). These data are essential for establishing safe dosage ranges and predicting potential risks associated with the administration of these compounds. Therefore, while research highlights the promising pharmacological activities of aurone substitutes and derivatives, a parallel and equally critical focus on assessing their acute toxicity, utilizing contemporary methodologies that prioritize animal welfare and comprehensive dose-response characterization (Chinedu et al., 2013) is indispensable for their safe and effective development.

The current study is designed to test three (3) aurone derivatives such as chloro-aurone **(C-A)**, methoxy-aurone **(M-A)** and Dimethoxy-aurone **(DM-A)** in animal models for their acute toxicity and anti-inflammatory potential.



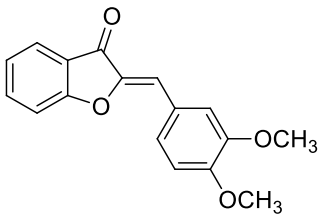
(*Z*)-2-(4-chlorobenzylidene)benzofuran-3(2*H*)-one

C-A



(*Z*)-2-(4-methoxybenzylidene)benzofuran-3(2*H*)-one

M-A



(*Z*)-2-(3,4-dimethoxybenzylidene)benzofuran-3(2*H*)-one

DM-A

Figure-1: Structures, IUPAC names and Codes for Aurone Derivatives

2. METHODOLOGY

2.1 Drugs

Already synthesized (Ikram et al., 2024) drugs (Z)-2-(4-chlorobenzylidene)benzofuran-3(2H)-one **(C-A)**, (Z)-2-(4-methoxybenzylidene)benzofuran-3(2H)-one **(M-A)** and (Z)-2-(3,4-dimethoxybenzylidene)benzofuran-3(2H)-one **(DM-A)** were collected from Pharmacognosy Lab, Department of Pharmacy, Abdul Wali Khan University Mardan, KP, Pakistan.

2.2 Animals and Ethical Approval

Male Swiss albino BALB/c mice weighing 20±2g were obtained from the Veterinary Research Institute (VRI) in Peshawar, Khyber Pakhtunkhwa (KP), Pakistan. They were housed in standard experimental conditions within the experimental animal house at the Department of Pharmacy, Abdul Wali Khan University in Mardan, KP, Pakistan. The mice were provided with standard feed and water *ad libitum* and subjected to a 12-hour light-dark cycle. Prior to the experiment, they were fasted for six hours. Fresh animals were used for each experiment. Ethical approval was approved from University Ethical Committee and Dean Faculty of Life Sciences, Abdul Wali Khan University, Mardan under ethical approval No. EC/AWKUM/2022/04/26. All ethical guidelines established for laboratory animals in 1979 were strictly followed during the study (Sulaiman et al., 2010).

2.3 Acute Toxicicity

The experimental animals in this study were Swiss albino mice of both sexes weighing between 25 and 40 grams each. The research was carried out in two phases. In Phase I, mice were given increasing dosages of each aurone derivative orally at concentrations of 10, 25, and 50 mg/kg, respectively. Phase II included the oral administration of greater doses of the same fraction, specifically 100, 250, and 500 mg/kg. Mortality was measured during a 24-hour period after injection in both phases. Additionally, a morbidity score system was used to evaluate probable side effects (Ali et al., 2020).

2.3 Mice grouping and dosing

Table 1: Grouping and dosing of mice in anti-inflammatory and antidepressant activity

Groups	Dosing
I	Normal saline 10ml/kg (p.o)
II-XV	Test samples (**C-A, M-A** and **DM-A**) 10mg/kg and 20mg/kg (p.o)
XVI	Standard; Indomethacin 10mg/kg (p.o) in carageenan induced paw edema and Xylene induced ear edema assays for anti-inflammatory activity

2.4 Anti-inflammatory Activity

2.4.1. Carrageenan-induced paw edema Assay

Test samples (**C-A, M-A** and **DM-A**)10mg/kg and 20mg/kg were given orally. After 30 min of test samples, carrageenan (Sigma Ald, Pakistan) 0.05ml of 1% was administered at the right hind paw to each Balb/c mouse. Readings were taken via digital vernier caliper (Premier Digital Vernier Caliper with 0.001 accuracy; Eisco Labs) in millimeter at hour 1, 2, 3 and 4 (Ikram et al., 2024).

2.4.2. Xylene-induced Ear Edema

This study utilized Swiss albino mice, which were randomly allocated into three distinct groups: a control group, a xylene-treated group, and a test group. The test substance or vehicle was topically applied to the right ear of each mouse, followed by the application of xylene to induce auricular edema. The left ear served as an internal control. Ear thickness measurements were taken at predetermined time points post-xylene application to quantify the extent of edema. The percentage inhibition of edema in the treated groups was calculated relative to the xylene-treated group, allowing for a comparative analysis of the anti-edematous effects (Singsai et al., 2020).

2.5 Statistical Analysis

All the results obtained were expressed as mean±SEM (standard error mean) of eight animals (n=8) in each group, percent inhibition was also carried out for results where it was necessary. Statistical analysis in the form ANOVA was implemented followed by post hoc Dunnet's test for multiple comparisons between and among groups. Effect of results were considered to significant at level of $P<0.05$.

3. Results

3.1 Acute Toxicity Test

The aurone derivatives (**C-A, M-A** and **DM-A**) were determined to be safe at both phases as shown in table 2. In phase –l, all the test compound at doses of 10, 25 and 50 mg/kg were safe while observing them for 24 hours. All the animals remained in a normal state and exhibited no signs of toxicity throughout the 24-hour assessment period. While At elevated dosages, the administered fraction induced a constellation of observable pharmacological effects in the animal model, including sedation, ptosis, tachypnea, piloerection, Straub's tail, and shivering (Table 3). Despite these dose-dependent physiological and behavioral alterations indicative of central and autonomic nervous system involvement, the experimental data revealed that the fraction demonstrated an acceptable safety profile up to a dosage of 500 mg/kg. This upper limit of safety suggests that no overt signs of severe toxicity or mortality were evident below this concentration in the tested population. Consequently, the fraction exhibits a relatively high tolerance threshold in the acute experimental setting.

Table 2: Acute toxicity assay results of Aurone derivatives in general

Phases	mg/kg	Mortality %	Survival%
Phase-l	10	0	100
	25	0	100
	50	0	100
Phase-ll	100	0	100
	250	0	100
	500	0	100

Six groups, n=8 mice

Table 2: Results of aurone derivatives on reaction of mice of different organs via acute toxicity assay

Reactions	10mg	25mg	50mg	100mg	250mg	500mg
Convulsion	-	-	-	-	-	-
Tremors	-	-	-	-	-	-
Ataxia	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-
Catatonia	-	-	-	-	-	-
Sedation	-	-	-	-+	+	+
Hypnosis	-	-	-	-	+	+
Algesia	-	-	-	-	-	-
Analgesia	-	-+	+	+	+++	+++
Diarrhea	-	-	-	-	-	-
Fever	-	-	-	-	-	-
Shivering	-	-	-	-	-	-
Skin Flushing	-	-	-	-	-	-
Cyanosis	-	-	-	-	-	-
St. Reaction	-	-	-+	+	++	+++

(-): no stated sign, (-+) showing stated sign some time, (+) showing weak sign, (++) showing a bit stronger sign and so on

3.2 Anti-inflammatory Assay

3.2.1. Carrageenan induced edema in paw)

Overall, the highest anti-inflammatory results were almost shown by all test samples at Hour 3 and all the results were significant ($p < 0.05$) as indicated in Table 3. Inhibition of inflammation was dose dependant and comparatively increased inhibition was exhibited by 20 mg/Kg b.w. Among the three test drugs, the highest significant anti-inflammatory effect was described by DM-4 at higher dose and the inhibition 1.49±0.06 (60.89%) was highest at hour 3 as compared to Control 3.81±0.07 and Indomethacin as standard 1.6±0.15 (58%). While the runner drug with high potency 1.55±0.10 (50.39%) was **C-A** at a dose of 20 mg/Kg. At 10mg of the dose, DM-A also possessed comparatively high anti-inflammatory effect among test drugs as shown in the table 4.

Table 4: Mean inhibition of inflammation of aurones after induction of inflammation in mice hind paw via carrageenan

Treatment: mg/Kg		**Carrageenan Induced Paw Edema (mm)**				**At 1 hr** %Inh.	**At 2 hr** %Inh.	**At 3 hr** %Inh	**At 4 hr** %Inh
		1hr	2 hr	3 hr	4 hr				
Control		3.92±0.03	3.95±0.02	3.81±0.07	3.68±0.11	----	----	----	----
C-A	10	2.67±0.11***	2.41±0.07***	2.36±0.05***	2.09±0.02***	31.88	38.98	38.05	36.41
	20	2.58±0.19***	2.19±0.06***	1.89±0.08***	1.52±0.06***	34.18	44.81	50.39	58.69
M-A	10	3.47±0.08	3.20±0.06**	2.89±0.04***	2.64±0.04***	11.47	18.98	24.14	28.26
	20	3.13±0.07*	2.43±0.09***	2.36±0.04***	2.25±0.02***	20.15	38.48	38.05	38.85
DM-A	10	2.87±0.17***	1.78±0.20***	1.75±0.18***	1.75±0.18***	26.78	54.93	54.04	58.15
	20	1.68±0.04***	1.67±0.04***	1.49±0.06***	1.47±0.06***	57.14	57.72	60.89	58.96
Indomethacin	10	2.34±0.07***	2.27±0.05***	1.6±0.16***	1.33±0.12***	40.30	47.59	58	63.85

Followed by Dunnett,s multiple comparison test vs control. Values are expressed as mean ± S.E.M. (n = 8 mice), ANOVA analysis *p<0.01, **p<0.001 and ***p<0.0001

3.2.2. Xylene-Induced Ear Edema Assay

The present study investigated the anti-inflammatory potential of aurone derivative using the xylene-induced ear edema assay in albino mice. Our findings demonstrated a significant dose dependant reduction in ear edema at 40 min as shown in table 5. The highest significant anti-inflammatory effect (mean±SEM) was shown by DM-A (0.240±0.004) at dose (20mg) just after 40min compared to the control (0.301±0.008) group that received only the xylene irritant as shown the table 5. This observation strongly suggests that these aurones possessed significant anti-inflammatory properties.

Table 5: Aurone Derivatives and their Effects on Xylene Induced Ear Edema in Albino Mice

Treatment: mg/Kg		**Xylene Induced Ear Edema (mm)**			
		20 min	40 min	60 min	120 min
N/Saline		0.309±0.003	0.301±0.008	0.299±0.007	0.291±0.009
C-A	10	0.267±0.005	0.260±0.007**	0.256±0.005	0.251±0.02
	20	0.261±0.009	0.255±0.006***	0.265±0.008	0.251±0.015
M-A	10	0.273±0.008	0.270±0.006*	0.267±0.004	0.259±0.005
	20	0.265±0.007	0.259±0.009**	0.250±0.004	0.0251±0.002
DM-A	10	0.261±0.17	0.255±0.002***	0.250±0.018	0.0249±0.010
	20	0.259±0.003	0.240±0.004***	0.24±0.006	0.24±0.006
Indomethacin	10	0.256±0.007	0.249±0.009***	0.240±0.001	0.240±0.003

Followed by Dunnett,s multiple comparison test vs control. Values are expressed as mean ± S.E.M. (n = 8 mice), ANOVA analysis *p<0.01, **p<0.001 and ***p<0.0001

3. Discussion

The present study investigates the anti-inflammatory and antidepressant potential of seven aurone derivatives using in vivo models. The findings provide significant insights into the therapeutic potential of these compounds in managing inflammation and depression.

Inflammation is a complex biological response to harmful stimuli, including pathogens, damaged cells, or irritants. This study employed the carrageenan-induced paw edema model to evaluate the anti-inflammatory activity of aurone derivatives. Carrageenan induces inflammation through the release of inflammatory mediators, which causes increased vascular permeability and leukocyte infiltration. The observed reduction in paw edema with the aurone derivatives, particularly **DM-A** suggests that this compounds possess substantial anti-inflammatory properties.

Aurone derivative DM-A demonstrated the highest anti-inflammatory activity. DM-A at 20 mg/kg exhibited a significant reduction in edema (60.89% inhibition at 3 hours) compared to the control and indomethacin. These results suggest that aurones can effectively modulate the inflammatory response, potentially by inhibiting the production or action of pro-inflammatory cytokines or by stabilizing cell membranes to prevent inflammatory mediator release.

The dose-dependence observed in the anti-inflammatory activity of aurone derivatives is consistent with the literature indicating that increased doses can enhance therapeutic efficacy. This finding aligns with previous studies on flavonoids, which have shown dose-dependent anti-inflammatory effects (Venkateswarlu et al., 2004; Bandgar et al., 2010). The significant reduction in inflammation observed with aurone derivatives highlights their potential as therapeutic agents in managing inflammatory conditions.

The xylene-induced ear edema model is a well-established and widely used in vivo assay for evaluating the acute inflammatory response. Xylene, a topical irritant, triggers a cascade of events including the release of inflammatory mediators such as histamine, serotonin, bradykinin, and prostaglandins, leading to increased vascular permeability, vasodilation, and subsequent edema formation in the ear tissue (Young et al., 1983). The significant reduction in ear thickness observed in the DM-A treated group indicates that the drug effectively interfered with one or more of these key processes involved in the inflammatory cascade.

The observed anti-inflammatory effect of aurones could be attributed to several potential mechanisms. Inhibition of inflammatory mediator release: these aurones might be acting by inhibiting the release or synthesis of key inflammatory mediators like histamine and serotonin from mast cells, which are known to play a crucial role in the early phase of xylene-induced edema (Tubaro et al., 1985). Suppression of prostaglandin synthesis: The later phase of xylene-induced edema is largely mediated by prostaglandins, particularly PGE2, synthesized via the cyclooxygenase (COX) pathway (Carlson et al., 1985). These derivatives could be exerting its anti-inflammatory effect by inhibiting COX-1 and/or COX-2 enzymes, thereby reducing prostaglandin production. Further studies using specific COX inhibitors could help elucidate this mechanism (Ikram et al., 2024).

The dose-dependent effect observed in our study further supports the specific pharmacological action of these aurones The higher doses generally exhibited a greater reduction in edema, indicating a direct relationship between drug concentration and its anti-inflammatory efficacy.

Comparison with existing anti-inflammatory drug: Indomethacin 10mg, DM-A showed even high anti-inflammatory potential after 40 min For instance, the mean inhibition of edema observed with this drug at its optimal dose was 0.240±0.004, which is comparable to or even greater than that reported for Indomethacin (0.249±0.009). This suggests that DM-A holds promising potential as an anti-inflammatory agent.

Conclusion

In conclusion, the findings of this study provide compelling evidence that aurone derivatives are safe with no toxic effect and possess significant anti-inflammatory activity, as demonstrated by its ability to effectively reduce Carrageenan and xylene-induced edema in albino mice model. These results warrant further investigation into the underlying mechanisms of action and the potential therapeutic application of these aurones in the management of inflammatory disorders.

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