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Article

Analysis of Anti-Arrhythmic Impacts of Crocin through Estimation of Expression of Cx43 in Myocardial Infarction Using a Rat Animal Model

Huan Li, Jian Li,* Juanli Wang, Obaid Afzal, Abdulmalik S. A. Altamimi, Shehla Nasar Mir Najib Ullah, Sireen Abdul Rahim Shilbayeh, Alnada Abdalla Ibrahim, and Shahanavaj Khan

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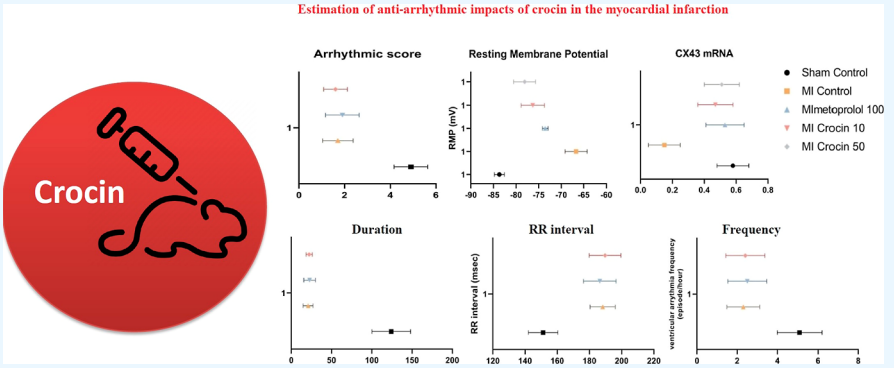

ABSTRACT: Arrhythmia is an important cause of death after myocardial infarction (MI). Different substances have been evaluated for their anti-arrhythmic effect in MI. This study was performed to evaluate the anti-arrhythmic impacts of crocin in an MI animal model (rat) by estimation of the expression of connexin 43 (Cx43). Fifty male Sprague−Dawley rats were grouped into 5 groups, each composed of 10 rats. The first group was regarded as the normal control group and the second one was considered as the MI group, which was caused by ligation of the left anterior descending artery. The other three groups received crocin 50 or 10 mg/kg/day or metoprolol 100 mg/kg/day for 1 week, following ligation of the left anterior descending artery. Evaluated outcomes were cardiac Cx43 expression, arrhythmia incidence, histological findings, and myocyte resting potential. Crocin-treated MI groups showed a significantly lower arrhythmia score than the non-treated MI group, 10 mg/kg/day (1.85 ± 0.55, $p < 0.01$) and 50 mg/kg/day (1.70 ± 0.33, $p < 0.01$). Groups that received crocin 10 mg/kg/day (66.30 ± 2.59, $p < 0.01$), crocin 50 mg/kg/day (68.10 ± 2.43, $p < 0.01$), and metoprolol 100 mg/kg/day (-63.54 ± 0.63 mV, $p < 0.01$) significantly prevented depolarization in comparison with the non-treated MI group. Expression of Cx43 mRNA in crocin 10 mg/kg/day (1.54 ± 0.24, $p < 0.01$), crocin 50 mg/kg/day (1.73 ± 0.09, $p < 0.01$), and metoprolol 100 mg/kg/day (1.75 ± 0.14, $p < 0.01$) treatment groups was significantly higher in comparison with the non-treated MI group. Crocin showed a preventive effect on the arrhythmogenic impact of MI in an experimental model of ischemic injury through an increase in expression of Cx43.

INTRODUCTION

One of the main mortality causes in the world is cardiovascular diseases (CVDs).[1] The World Health Organization (WHO) estimated that 17.9 million people died due to CVDs in 2017, which represents 31% of all deaths worldwide, out of which 6.7 million died from stroke and 7.4 million died because of coronary heart diseases.[2] CVDs are still a significant challenge in the age of nanotechnology, proteomics, and genomics, and researchers have attempted to develop a viable evidence-based approach for preventing CVD onset.[3]

It has been reported that there are some compounds with biological actions that can lead to the reduction of CVDs.[4] As an example, flavonoids can improve cardiovascular health via different mechanisms, such as antidiabetic and hypocholesterolemic activities, antioxidant effect, vasorelaxation effect, and anti-atherosclerotic characteristics.[5] Likewise, carotenoids, like zeaxanthin, lutein, and crocin, exert biological actions that can provide various cardiovascular advantages.[6]

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One of the most well-known spices is saffron (*Crocus sativus* L.), which has medicinal properties.[7] Commonly, it is utilized in European regions, including Spain, Greece, and Italy, and also in regions of Asia, like India and Iran.[8] Traditionally, it is known to have therapeutic effects on palpitation according to Persian, Ayurvedic, and Chinese medicine.[9]

The major constituent of saffron is crocin, which gives its red color and has various cardiovascular effects.[10] Crocin has been reported as a suppressor of the renin-angiotensin system.[11] Some studies have indicated that crocin has an impact on blood pressure.[12] According to the reports, in a dose-dependent manner, crocin lessens hypertension in rats caused by desoxycorticosterone acetate (DOCA) salt. In addition, cardiovascular toxicity induced by diazinon (DZN) in rats was improved by crocin through oxidative stress reduction.[12] Multiple studies have demonstrated that crocin has a protective effect against cardiac arrhythmias induced by reperfusion.[13]

The development of effective and experiential diagnostic techniques for the management and treatment of cardiac arrhythmias has exceeded the speed from the last decade. The detection of main candidate genes for monogenic arrhythmia syndromes demonstrates that to bring the fundamental biology to the clinic is the potent approach. Different biological pathways are suggested to be involved in the induction of cardiac ischemic arrhythmia.[14] For example, integrin-linked kinase induces multiple biological pathways such as glycogen synthase kinase 3β, protein kinase B, extracellular signal-regulated kinases, Rac1 pathways, and myosin light chain. It is known that deletion of the integrin-linked kinase can induce arrhythmia and cardiac death in an animal model of ischemic heart insult.[15] A recent study supported the hypothesis that inhibition of connexin 43 (Cx43) is involved in the anti-arrhythmic effect of the integrin-linked kinase pathway.[15] Different diagnostic modalities have been used in myocardial infarction (MI). Electrocardiography, serum markers (such as troponin, myoglobin, and creatine kinase), echocardiography, and angiography are the most popular modalities used in the diagnosis of MI.[16] Various prognostic models are also applied for MI.[17]

Although crocin's cardiovascular effects have been evaluated by several research works, and anti-arrhythmic effects have been reported for it, no study has investigated the underlying mechanism. The present research aims at examining the expression of Cx43 as the possible pathway for crocin's anti-arrhythmic effects by use of an experimental model of arrhythmia in MI infarction in rats.

RESULTS

Arrhythmia Score, Interval, Frequency, and Duration. There was no arrhythmia observed in the normal control group, which led to zero scores. The MI group showed different types of arrhythmias with an arrhythmia score of 4.2 $\pm$ 0.65(p < 0.01 in comparison with the control group). Treated MI groups, crocin 10 mg/kg/day (1.85 $\pm$ 0.55, p < 0.01), crocin 50 mg/kg/day (1.70 $\pm$ 0.33, p < 0.01), and metoprolol 100 mg/kg/day (1.850 $\pm$ 0.74, p < 0.01), showed a significantly lower arrhythmia score than the non-treated MI group. In Bonferroni's corrected multiple comparison test (p = 1.00), no significant differences were observed among the treated groups (Figure 1).

The RR interval was significantly longer, and the mean frequency and duration of ventricular arrhythmias were

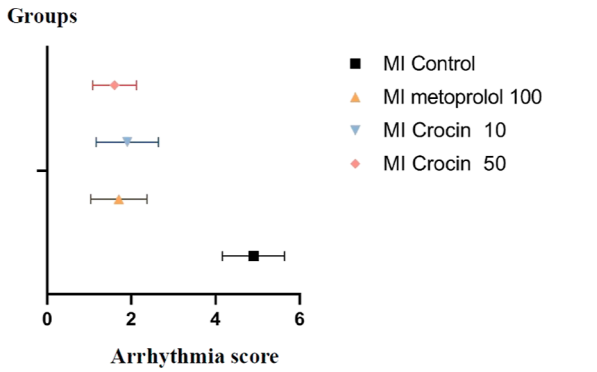

Figure 1. Arrhythmia scores in the study groups (MI control, MI metoprolol 100 mg/kg/day, MI crocin 10 mg/kg/day, and MI crocin 50 mg/kg/day). MI: myocardial infarction.

significantly lower in groups treated with crocin and metoprolol compared with the untreated MI group (p < 0.01) (Figure 2).

Resting Membrane Potential. The MI group showed a significantly depolarized resting membrane potential of cardiac myocytes from −83.71 $\pm$ 1.11 mV in the sham control to −66.70 $\pm$ 2.45 mV in the MI control group (p < 0.01). Groups

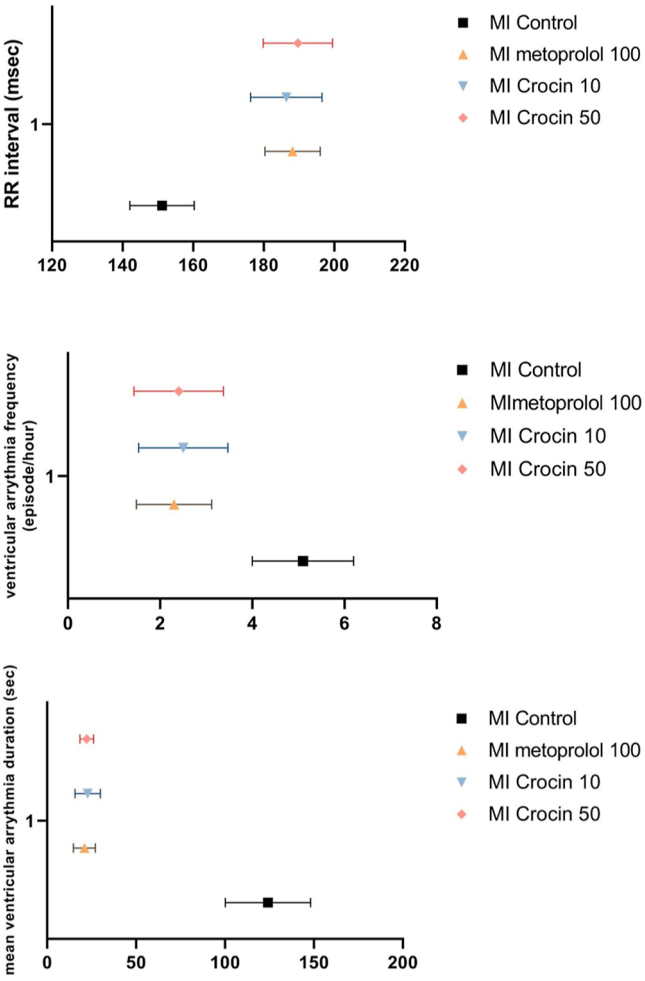

Figure 2. RR intervals, mean frequency, and duration of ventricular arrhythmia in the study groups (MI control, MI metoprolol 100 mg/kg/day, MI crocin 10 mg/kg/day, and MI crocin 50 mg/kg/day). MI: myocardial infarction.

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treated with crocin 10 mg/kg/day (−76.30 ± 2.59, $p < 0.01$), crocin 50 mg/kg/day (−78.10 ± 2.43, $p < 0.01$), and metoprolol 100 mg/kg/day (−73.54 ± 0.63 mV, $p < 0.01$) significantly prevented this depolarization in comparison with the non-treated MI group (Figure 3).

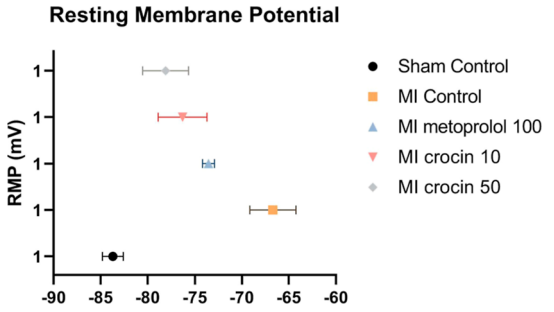

Figure 3. Resting membrane potential in the study groups (sham control, MI control, MI metoprolol 100 mg/kg/day, MI crocin 10 mg/kg/day, and MI crocin 50 mg/kg/day). MI: myocardial infarction.

Histology. Interstitial hemorrhage and extensive edema were observed in the hearts' anterior area in MI rats. Nevertheless, there was a significantly lower pathologic change in the rats' hearts in the metoprolol and crocin groups.

Western Blot. The western blot method was used for evaluating the Cx43 protein expression. According to the results of this method, Cx43 protein was significantly lower in the infarcted zone in the MI group (0.73 ± 0.24, $p < 0.01$) than in the normal control (2.12 ± 0.09). In crocin 10 mg/kg/day (1.54 ± 0.24, $p < 0.01$), crocin 50 mg/kg/day (1.73 ± 0.09, $p < 0.01$), and metoprolol 100 mg/kg/day (1.75 ± 0.14, $p < 0.01$) groups, this reduction decrease was significantly hindered in comparison with the non-treated MI group (Figure 4).

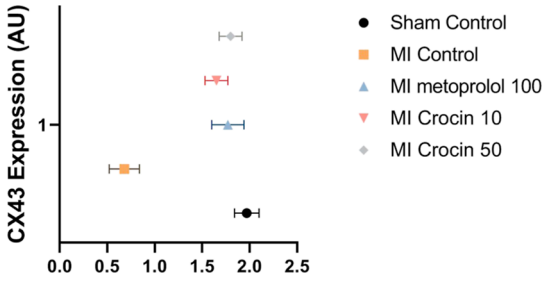

Figure 4. Western blot of Cx43 protein expression levels in the study groups (sham control, MI control, MI metoprolol 100 mg/kg/day, MI crocin 10 mg/kg/day, and MI crocin 50 mg/kg/day). MI: myocardial infarction.

Real-Time PCR Analysis. Real-time PCR (RT-PCR) analysis was used for evaluating the expression of the Cx43 mRNA, results of which indicated a lower expression of Cx43 mRNA in the infarcted zone in the MI group (0.14 ± 0.08, $p < 0.01$) in comparison with the normal control (0.61 ± 0.09). In crocin 10 mg/kg/day (0.39 ± 0.08, $p < 0.01$), crocin 50 mg/kg/day (0.62 ± 0.12, $p < 0.01$), and metoprolol 100 mg/kg/day (0.49 ± 0.11, $p < 0.01$) groups, this reduction was significantly hindered in comparison with the non-treated MI group (Figure 5).

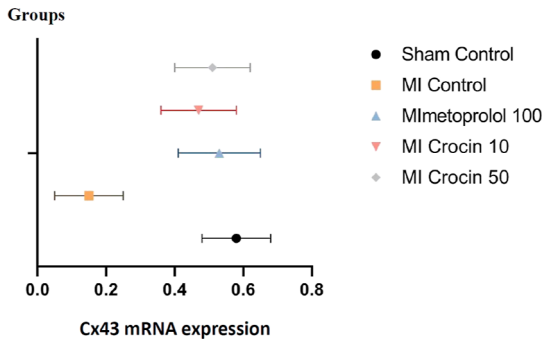

Figure 5. RT-PCR analysis of Cx43 mRNA expression in the study groups (sham control, MI control, MI metoprolol 100 mg/kg/day, MI crocin 10 mg/kg/day, and MI crocin 50 mg/kg/day). MI: myocardial infarction.

DISCUSSION

As shown by the results of the experiment, there are anti-arrhythmic effects observed for crocin, similar to metoprolol, which was applied dose dependently in experimental models of MI. In this regard, reduction in Cx43 expression in MI is prevented.

As previously demonstrated, different rhythm disturbances, such as ventricular fibrillation (VF), premature ventricular contraction (PVC), and ventricular tachycardia (VT), are induced by experimental myocardial ischemia in animals.[20] It is considered that these arrhythmias are attributed to delay following depolarizations in surviving Purkinje fibers, causing increased automaticity in the ischemic area in these fibers.[21]

As a beta-receptor blocker, metoprolol is a member of class II anti-arrhythmic drugs. It was shown that beta-blockers are preventers of sudden cardiac death resulting from malicious ventricular arrhythmias in MI. According to available evidence, metoprolol's preventive impact on the degradation of Cx43 serves as a significant pathway in its anti-arrhythmic effect in MI. By this pathway, gap junction communication can be recovered, which results in enhanced conduction velocity. Ultimately, these changes decrease vulnerability to ventricular arrhythmias. As supported by the current research findings, the crocin's anti-arrhythmic impact might be via mechanisms similar to metoprolol.

There is considerable evidence supporting the crocin's cardioprotective impact. As a result of pretreatment with crocin, protective effects are produced through the reduction of creatine phosphokinase (CPK) activities, the redox status restoration, and apoptosis suppression in cardiotoxicity caused by patulin in rats.[22] Additionally, the protective effect was produced by crocin through the restoration of activity of CK-MB and MDA levels in the heart, as well as improvement of histopathological changes, such as hemorrhages, cardiac muscle cell necrosis, hypertrophy, and infiltration of inflammatory cells in cardiotoxicity induced by DZN[23] Besides, crocin caused improved activity in CPK, CK-MB, and LDH in coronary effluent, cardiac tissue oxidative stress biomarker (MDA), antioxidant enzymes (SOD and catalase), as well as total antioxidant capacity in an animal model (rat) having cardiac ischemia−reperfusion injury.[24] Myocardial CK-MB activity was recovered and the elevated MDA level in the heart was reduced in isoproterenol-induced MI by crocin. Also, it caused improvement of changes in the heart tissue, like edema, myocardial necrosis, and leukocyte infiltration.[25] Additionally, in an ischemia-reperfusion model of separated heart tissues, the

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ST segment increase was recovered by crocin, and crocin enhanced cardiac dysfunction and decreased the infarct size.[26] A cardioprotective effect was produced by crocin in a rat heart ischemia/reperfusion model by enhancing the mechanical function and regulating the production of nitric oxide.[27] Moreover, it was indicated that crocin protects against cardiac arrhythmias induced by reperfusion, supposedly because of the anti-oxidant characteristics of crocin.[13] As found in our results, in addition to these effects, reduction in Cx43 expression is a significant pathway in the crocin effect in MI.

Potential anti-arrhythmic mechanisms of crocin were introduced in our results. Cx43, a critical protein in the cardiac gap junction structure, plays a vital role in cell coupling in electrical signal conduction. There is an association between the Cx43 expression changes and different kinds of arrhythmias in MI. As shown by the experiment in our study, crocin has a preventive effect in a reduction in protein levels and Cx43 messenger RNA in MI. Thus, our findings indicate that crocin has a potential anti-arrhythmia mechanism that might be like metoprolol in preventing degradation of Cx43. Nevertheless, future surveys are required for further evaluation of crocin's preventive mechanism in the degradation of Cx43.

CONCLUSIONS

In conclusion, crocin was demonstrated to be able to reduce the incidence of arrhythmias in an experimental model of MI. This effect was dose-dependent. Based on the observed results, Cx43 expression seems to be a potential pathway in crocin's anti-arrhythmic mechanisms.

METHODS

Animals. 50 male Sprague−Dawley rats (weighed 200−250 g) were kept at 23 ± 2 °C with a humidity of 60 ± 5%, under a 12 h light/dark cycle. The care and use of animals were followed for housing animals and conducting experimental procedures. The local Animal Ethics Committee of Shandong University approved all experimental protocols and procedures. First, animals were sorted into 5 groups composed of 10 rats each. The first group was regarded as the normal control group and the second one was considered as MI group, MI being caused by ligation of the left anterior descending artery. The other three groups were treated groups, which received crocin 50 or 10 mg/kg/day or metoprolol 100 mg/kg/day for 1 week following ligation of the left anterior descending artery.

Induction of MI. MI was induced via ligation of the left anterior descending artery as previously explained in detail.[18] For induction of MI, anesthesia was applied using sodium thiopental (60 mg/kg body weight, i.p). Then, the animals experienced tracheal intubation and then were ventilated at a tidal volume of 1.5 cm^3/kg and 60−70 breaths/min. This was followed by making an incision on the chest's left fourth intercostal site. The pericardium was gradually torn, and a 0.6 silk thread was cautiously passed around the left anterior descending artery and fastened. Following 30 min of ischemia, the LAD suture was removed and the ischemic myocardium was reperfused for 120 min. Surface electrocardiography was used for ischemic injury confirmation as previously described.[19] After an identical procedure, a sham group was made, but the actual tying of the suture was absent.

Recording the Electrocardiogram and Scoring of Arrhythmia. We recorded the limb lead II electrocardiogram (ECG) in rats. Assessment of the arrhythmias was done according to the previously described process. Using arrhythmia scores, the arrhythmia duration and incidence were quantified. To this end, as seen in Table 1, a grade was given to the animals. The Kubios HRV ECG analysis software was used for the analysis of duration and mean frequency ventricular arrhythmia and mean RR interval in each group.

Table 1. Scoring of Arrhythmia[a]

score	definition
0	no arrhythmia
1	<10 s PVC and/or VT
2	11−30 s PVC and/or VT
3	31−90 s PVC and/or VT
4	90−180 s PVC and/or VT, <10 s reversible VF
5	>180 s PVC and/or VT, >10 s reversible VF
6	irrversible VF

[a]Ventricular tachycardia (VT), ventricular fibrillation (VF), and premature ventricular contraction (PVC).

Isolation of Myocytes. According to a previously explained procedure, separation of myocytes from rat hearts was done. Shortly, following 7 days of treatment and recording of the ECG, rats were sacrificed, and their hearts were eliminated for retrograde perfusion through the coronary circulation. Using Krebs−Ringer solution with pH 7.4, 95% O_2 perfusion was conducted with a flow rate of 13 mL/min, maintaining the temperature of the heart at 37 °C. After mincing the peri-ischemic heart tissue in the storage solution, we centrifuged separated myocytes selected for recording electrophysiology. The electrophysiological recording was performed using the whole cell patch-clamp technique.

Evaluation of the Pathologic Changes. For pathologic investigation, three rats were selected and used from each group. To better visualize the infarcted area, we used a transjugular injection of 2,3,5-triphenyl tetrazolium chloride. In pathologic investigation, sections with full thickness from the myocardium were studied. Using formalin 10%, fixation was done with common laboratory pathologic sample processing.

RT-PCR and Cardiac Cx43 Western Blotting. After perfusion experiments, the heart's ventricular tissue was separated and instantly frozen. On the same day that ventricular sarcolemma was to be prepared, the kept ventricle was homogenized in the hypotonic membrane buffer. This buffer was composed of 1 mM iodoacetamide, 1,10-phenanthroline (1 mM), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM pepstatin A with an ultrasonic homogenizer. The cardiac Cx43 content was determined using the western blotting method.

RT-PCR, which is an assay of Cx43, was conducted. The reagent was used for RNA extraction from the cardiac tissue and transcribed into complementary DNA. The RT-PCR reaction solution included reverse and forward primers for an ultimate reaction, besides complementary DNA. Testing of genomic DNA was done using GAPDH as a negative control.

Statistical Analysis. Results of the analysis were presented as mean ± SD. Outcomes of groups were compared by a one-way ANOVA test. Using the Bonferroni test, the multiple comparisons test was performed for preventing the incorrect appearance of data as statistically significant. Data with P values below 0.05 were considered statistically significant. SPSS 25.0 software was used for data analysis. Graphs were created using Graph-Pad Prism 8.

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AUTHOR INFORMATION

Corresponding Author

Jian Li − *Department of Cardiovascular, Xi'an Children's Hospital, Xi'an 710003, China*; Phone: 0086 29 87692527; Email: lj3688856@sina.com; Fax: 0086 29 87692000

Authors

Huan Li − *Department of Cardiovascular, The First People's Hospital of Xianyang, Xianyang 710003, China*

Juanli Wang − *Department of Cardiovascular, Xi'an Children's Hospital, Xi'an 710003, China*

Obaid Afzal − *Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia*

Abdulmalik S. A. Altamimi − *Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia*

Shehla Nasar Mir Najib Ullah − *Department of Pharmacognosy, Faculty of Pharmacy, King Khalid University, Abha 61421, Saudi Arabia*

Sireen Abdul Rahim Shilbayeh − *Department of Pharmacy Practice, College of Pharmacy, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia*

Alnada Abdalla Ibrahim − *Department of Pharmacy Practice, College of Pharmacy, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia*

Shahanavaj Khan − *Department of Medical Lab Technology, Indian Institute of Health and Technology (IIHT), Saharanpur, Uttar Pradesh 247554, India; Department of Health Sciences, Novel Global Community Educational Foundation, Hebersham 2770 NSW, Australia;* orcid.org/0000-0002-4049-2244

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c03158

Author Contributions

Conceptualization, Data analysis, and Writing of draft: H.L., J.L., and J.W.; Investigation and manuscript review: O.A.; A.S.A.A., S.N.M.N.U., S.K., and S.V.; Project administration, supervision, and writing of draft: S.A.R.S., A.A.I., and S.K.

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Notes

The authors declare no competing financial interest.

Ethics Statement: The animal study in the present work was reviewed and approved by the Ethics Board of Shandong University.

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