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Original article

Oridonin supplementation in rabbits may help to improve the outcome of induced atherosclerosis via autophagy induction

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Abstract. *Background and aim:* Inflammation plays a crucial role in the development of atherosclerotic plague. Oridonin is the major active ingredient of the traditional Chinese medicinal herb *Rabdosia rubescens*. It is a natural terpenoids that is known as a strong anti-inflammatory supplement by acting as a potent inhibitor of the TXNIP/NLRP3 pathway. Hence, it can reduce the severity of inflammation and improve the outcome of atherosclerotic changes. This study aims to evaluate the anti-inflammatory effects of oridonin in the progression of atherosclerotic plague in rabbits. Methods: Sixty-three male rabbits were included. The rabbits were randomly assigned to one of the three study groups (21 rabbits in each group), normal control diet (NC) fed normal diet for 8 weeks, atherogenic control (AC) fed atherogenic diet (2% cholesterol-enriched diet) for 8 weeks, and oridonin treated group (OT) fed atherogenic diet (2% cholesterol-enriched diet) with oridonin (purity 94%, Sigma-Aldrich, USA) at 20 mg/kg orally daily for 8 weeks. After the end of the study, blood and tissue samples were collected for analysis of various markers of inflammation and atherosclerotic plaque progression. Results: Serum lipids showed a statistically significant improvement in terms of reduction in total cholesterol and low-density lipoprotein (LDL) in the OT group compared to the AC group. This was associated with a significant reduction in serum F2-isoprostane (marker of inflammation) and LC3B (marker of tissue autophagy) between the OT group compared to the AC group. There was also a significant reduction in NLRP3 inflammasome RNA expression in OT group, $P<0.001$. Conclusions: In animal model, with atherogenic diet, oridonin supplementation can significantly improve the outcome of atherosclerosis by its strong anti-inflammatory action. (www.actabiomedica.it)

Key words: Oridonin; Atherosclerotic plaque; anti-inflammation; LC3B; Lipid profile; Rabbits

Introduction

Natural supplements have been widely used for treating symptoms of chronic disease (such as type 2 diabetes and rheumatoid arthritis) in developing countries for their believed powerful anti-inflammatory effects (1). However, there is no clear understanding about their scientific background, mechanism of actions, and their bioavailability after *in vivo* use (1). Oridonin is one such compound that

has been promoted as a natural anti-cancer therapeutic agent (1, 2). Oridonin is a kaurene-type diterpenoid compound, isolated from *Rabdosia rubescens* and is widely used in traditional Chinese medicine (2). There have been several data to show the anti-inflammatory, antibacterial and immune regulatory effects of oridonin, however, the mechanism of action, and bioavailability of this compound is still under investigation (2).

Previous studies have reported that oridonin can reduce the activation of proinflammatory cytokines by inhibiting NF-κB or MAPK activation and reduce the production of tumor necrosis factor (TNF)-α and interleukins (1). In addition, oridonin can be used as a protective agent in bacterial infection such as colitis, sepsis, and neuroinflammation (1). Still, the underlying mechanism of actions and target organ are yet to be determined (3, 4).

The NLRP3 inflammasome can be defined as a complex protein unit complex that consists of immune sensor NLRP3, and caspase-1 (3). This complex protein can activate the caspase-1 pathway leading to activation of a series of inflammatory cytokines such as interleukin 1β and interleukin-18, which plays a key role in modulating inflammation (3-5).

The precise mechanism of NLRP3 activation is still unclear. In addition, NLRP3 inflammasome activation contributes to the progress of several chronic illnesses, including type 2 diabetes, and atherosclerosis (3, 5). The latter is a chronic inflammatory condition characterized by a significant increase in the levels of inflammatory cytokines that may cause damage to blood vessels and play a crucial role in the progress of cardiovascular disease (CVD) (6). It is associated with building up of cholesterol in the interstitial space and may cause a significant rise of serum lipids. As part of the pathophysiology of atherosclerosis, the low-density lipoprotein cholesterol (LDL) particles enter the blood vessels wall and are exposed to chemical modification prior to being phagocytized by macrophages thereby, increasing the severity of the condition (6). This study aims to evaluate the beneficial effects of oridonin in improving the outcome of atherosclerosis in rabbits treated with an atherogenic diet.

Material and methods

Preparation of animals

This study including a total of sixty-three male rabbits with an average weight of 1.75 kg. This study was reviewed and approved by Animal Research Ethics, College of Medicine, University of Kufa, Iraq (Approval No. 15779, on 14th December 2020). During the study period, all animals were kept at 25° C, with fifty: fifty day: night cycle. Rabbits were kept for the first 2 weeks to acclimatize to the environment and were provided with a normal chow diet and tap water ad libitum before initiation of the intervention study.

Study design

Following the first two weeks, animals were randomly divided into one of the three study groups (21 animals in each study group):

Normal Control group (NC): in this group, rabbits received standard chow diet and water ad libitum for 8 weeks.

Atherogenic control group (AC): In this group, rabbits were given an atherogenic diet (consisting of 2% cholesterol-enriched diet) and water ad libitum for 8 weeks period.

Oridonin Treated group (OT): In this group, rabbits received an atherogenic diet, water ad libitum and oridonin (purity 94%, Sigma-Aldrich, USA) supplements at 20 mg/kg orally daily for 8 weeks.

Induction of atherosclerosis

To induce atherosclerotic changes, animals were provided with 2% cholesterol, BDH Chemicals Ltd Poole England, prod 43011, in their diet to develop atherosclerotic changes in the aorta following 8 weeks supplementation (7).

At the end of the 8 weeks intervention period, animals were kept fasting for overnight, before being anesthetized using ketamine (66 mg/kg) and xylazine (6 mg/kg) intramuscular injection (8). Following anaesthesia, thoracotomy was performed to expose the

heart and collect the blood. The aortic arch was dissected and collected as well. Blood and tissue samples were collected to measure the following:

- Serum lipid profile (total cholesterol - TC, triglyceride - TG, low density lipoprotein cholesterol – LDL, high density lipoprotein cholesterol – HDL, and very low-density lipoprotein cholesterol – VLDL.
- Serum F2-isoprostane to assess the degree of lipid peroxidation. This can be measured colormetrically via ELISA (Abcam, USA, ab175819). In brief, combine 1 ml of serum (adjusted with approximately 12 µL of acetic acid to pH 4) and 1 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Collect the upper organic phase and follow the instructions provided in the kit to measure the se-rum levels of F2-isoprostane.
- Histopathological examination of the aorta looking for atherosclerotic changes. Following thoracotomy and dissection of the aorta a section from the aortic arch was isolated and cleared from the neighbouring tissue and fat. The section was then divided into 3 subsections, one part was immediately placed in a fixative solution (10% formaldehyde solution) for 24 hours for histopathology examination, one part was homogenized in TRIzol® for measuring Caspase1 via reverse transcriptase – polymerase chain reaction (RT-PCR) technique. And the third part was frozen in liquid nitrogen and stored at -80°C for measurement of tissue autophagy marker LC3B by fluorometric assay.
- Tissue LC3B as a marker of tissue autophagy marker using fluorometric assay (Sigma Aldrich, USA MAK138-1KT). This The Autophagy Assay kit provides a simple and direct procedure for measuring autophagy in a variety of cell types using a proprietary fluorescent autophagosome marker (λex = 333/λem = 518 nm). In summary, following the autophagy induction, prepare a working solution of the Autophagosome Detection Reagent by diluting 20 µL of the 500× solution with 10 mL of the Stain Buffer. This is sufficient volume for one 96 well plate. This volume can be scaled down accordingly if fewer wells will be used. Remove the medium from the cells and add 100 µL of the autophagosome detection reagent working solution to each well (samples and controls). If working with suspension cells, spin down cells prior to removing medium and gently resuspend pellet in the autophagosome detection reagent working solution. Incubate the cells at 37 ˚C with 5% CO2 for 15 minutes to 1 hour. Wash the cells with the Wash Buffer 3–4 times by gently adding 100 µL of Wash Buffer to each well. If working with suspension cells, spin down cells and resuspend pellet in wash solution. Remove carefully to prevent dislodging the cells. Measure the fluorescence intensity (λex = 360/λem = 520 nm) using a fluorescence microscope or microplate reader.
- NLRP3 inflammasome was measured using reverse transcription polymerase chain reaction (RT-PCR), using (a TRIzol® reagent kit, Thermo Fisher, catalogue Number 12183555). Total RNA was extracted from heart tissue samples. 100mg heart tissue sample was homogenized by adding 750 µl of TRIzol® reagent. Add 200 µl chloroform, stirred for 15 seconds, then place on ice for 5 minutes before centrifugation. Transfer 500 ul into a new tube and add 500 ul isopropanol and incubate in a fridge for 10 minutes, before centrifugation. Discard the supernatant, add 1 ml of ethanol and repeat the mixing and centrifugation as above. Discard the supernatant and leave the RNA pellet until dry out, then add 100 ul of free nuclease H_2O to dissolve before RNA extraction.

Statistical analysis

Means and standard error of means (SEM) were measured using statistical package for social sciences (SPSS) version 23, IBM, USA. Results were analyzed using one-way ANOVA for multiple comparisons and LSD post-hoc test. *P* value of < 0.05 was considered significant. The degree of atherosclerotic lesions was measured and described by median and interquartile range and analyzed using Kruskal-Wallis H test.

Results

Effect of an atherogenic diet and treatment on the lipid profile and atherogenic index

There was a statistically significant increase in the mean serum levels of total cholesterol, TG, LDL and VLDL in the AC study group as compared to the NC group, (P<0.001). However, significant reduction was observed in the mean serum levels of TC and LDL in the OT group as compared to the AC group, (P<0.001), (Figure 1, and Table 1). No significant difference was found in serum levels of TG and VLDL.

In addition, mean serum HDL levels were significantly reduced in the AC group compared to the NC group and then improved significantly following oridonin treatment, (P<0.001), (Figure 1, and Table 1).

Effect of an atherogenic diet and treatment on the oxidative stress maker F2-isoprostane

Similarly, the mean plasma levels of F2-isoprostane increased significantly following the ingestion of an atherogenic diet for 8 weeks (AC group) compared to the normal control group, (P<0.001). Oridonin ingestion exerted a protective effect in terms of significantly reducing plasma

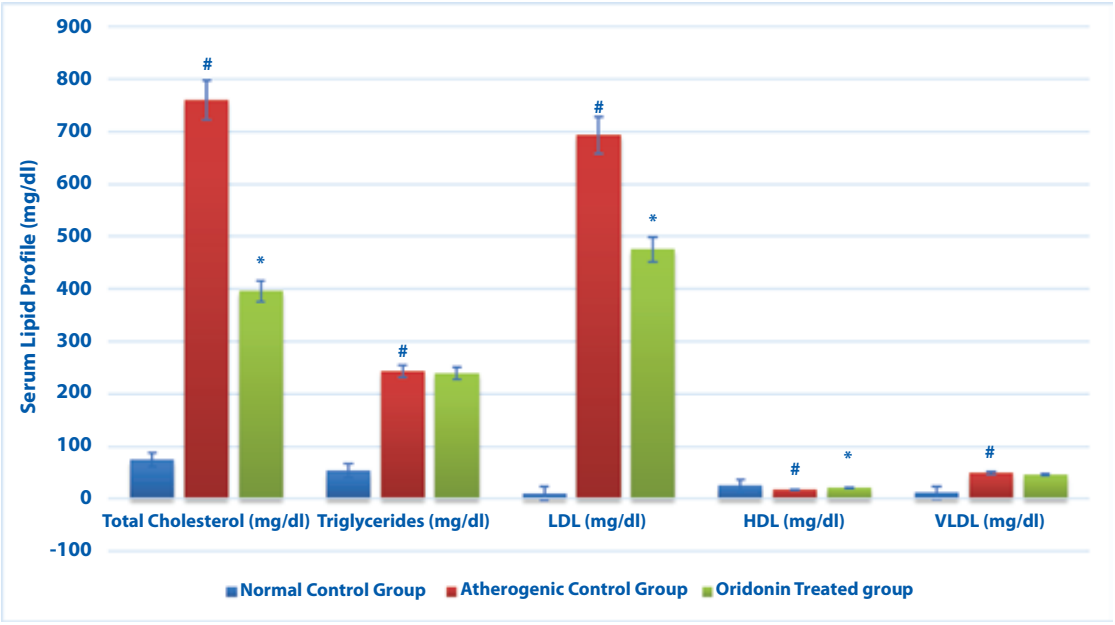

Figure 1. Serum lipids profile among the three study groups, (P<0.001).

Table 1. Serum levels of lipid profile among the three study groups.

	Normal Control Group	Atherogenic control group	Oridonin Treated group
Total Cholesterol (mg/dl)	74.6±1.3	759.3±23.4#	395.6±7.0*
Triglycerides (mg/dl)	53.5±1.7	243±24.6#	239.5±36.2
LDL (mg/dl)	10.5±1.1	693.4±36.4#	475.6±26.4*
HDL (mg/dl)	24.5±3.5	17.5±1.7#	21.3±1.2*
VLDL (mg/dl)	1011.3±0.58	49.3±2.6#	46.4±1.9
F2-Isoprostane (pg/ml)	166.9±5.2	792.4±47.9	612.2±59.7

#: Significant P<0.001 difference between the atherogenic group and the normal control group.
**: Significant P<0.001 difference between the oridonin group and the atherogenic group.*

levels of F2-isoprostane in the OT group compared to the AC group, (P<0.005), (Table 1, and Figure 2).

Effect of an atherogenic diet and treatment on the aortic atherosclerotic lesion degree

Our study showed that the median of the atherosclerotic lesion degree was highest (25) in the atherogenic control group, and it was at its lowest level (4) in the normal control group. Treatment with oridonin appears to exert a protective effect on the aortic wall by reducing the median atherosclerotic lesion to 14. Using the Mann-Whitney test to find for the significance across the three study groups, we identified significant differences between the three study groups, (P<0.001), Figure 3.

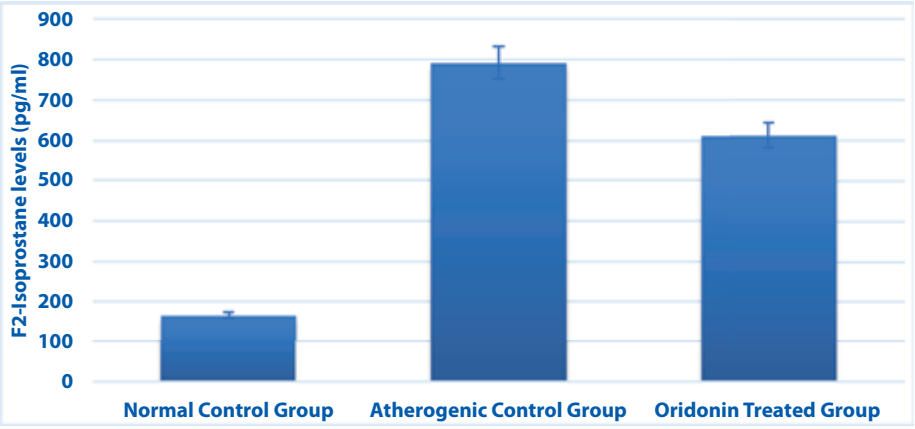

Figure 2. Plasma F2-isoprostane levels among the three study groups, (P<0.005).

#: Significant P<0.001 difference between the atherogenic group and the normal control group.
*: Significant P<0.001 difference between the oridonin group and the atherogenic group.

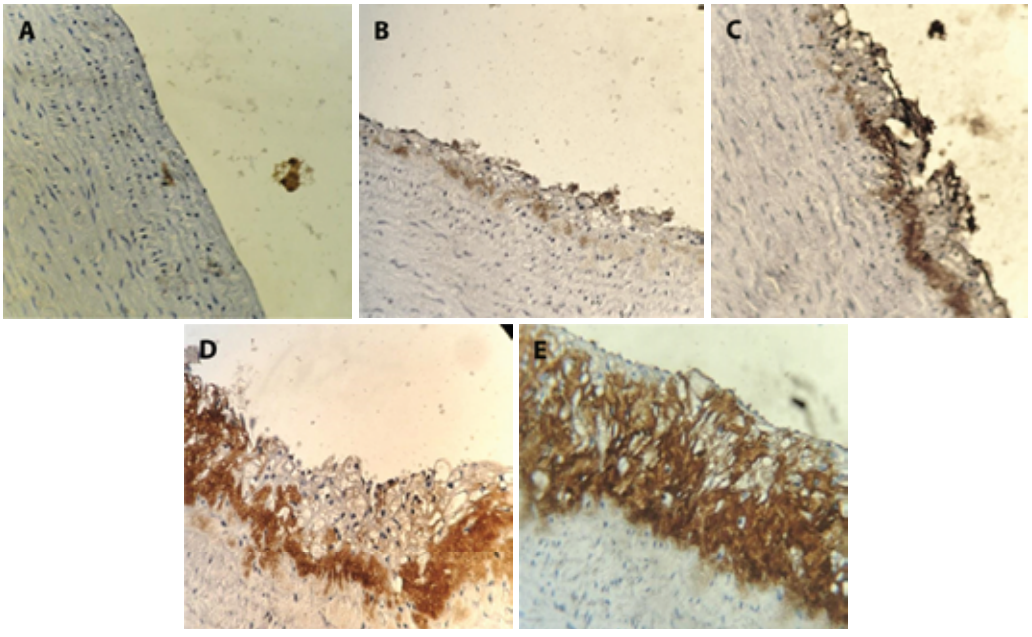

Figure 3. A cross section of aortic arch from hypercholesteremic rabbits represented atherosclerosis progression staining for TNFR1 expression (x40). **A:** negative, **B:** weak stain intensity, **C:** moderate stain intensity, **D:** strong stain intensity, and **E:** very strong stain intensity.

The assessment of atherosclerotic changes was performed according to the American Heart

Association classification of atherosclerosis; Type I (initial lesions), Type II lesions (fatty streak lesions), Type III lesions (intermediate lesions), Type IV lesions (atheroma), Type V lesions (advance lesion) and Type VI (complicated lesion). We have used the scoring methodology to interpret the lesions, Table 2.

In addition, there was a significant reduction in LCB3 tissue levels in the atherogenic control group compared to the normal control group, (P<0.001). Oridonin treatment exerted a beneficial effect on improving LCB3 tissue levels compared to the atherogenic control group, (P<0.001), (Figure 4).

Furthermore, the mean NLRP3 Inflammasome index showed a significant increase in the atherogenic control group compared to the normal control group, (P<0.001). Then it significantly reduced following oridonin treatment, (P<0.001), (Figure 4).

Discussion

The primary objective of this study is to illustrate the beneficial effects of oridonin in reducing inflammation and improving the outcome of atherosclerosis. Following 8 weeks of oridonin supplementation in rabbits fed with atherogenic diet, oridonin significantly improved serum lipids in terms of significantly reducing TC and LDL and significantly increasing HDL levels. There was no significant reduction in the level of TG although a recent study showed that TG can be improved following oridonin supplementation (9). The beneficial effects of oridonin on serum lipids could be due to its ability to inhibit NLRP3 pathway and thereby suppress activation of pro-inflammatory molecules leading to less production of interleukins. The latter have been identified to play a key role in regulating lipid metabolism (10).

Besides, its lipid lowering effects, oridonin supplementation showed to be effective in reducing the severity of lipid peroxidation as per the significant reduction in plasma levels of F2-isoprostane. This finding agrees with previous studies that provide the lipid protective effects of oridonin in animals (9, 11). These benefits could be explained through the protective mechanism of oridonin in mitochondria through its anti-inflammatory effects by reducing the generation of hydrogen and hydroxyl radical that can cause lysosomal damage, in addition to its radical scavenging properties to eliminate the reactive oxygen species and suppresses further damage to mitochondrial membrane (9, 11).

Furthermore, the finding of our study showed the beneficial effects of oridonin supplementation on the levels of the NRLP3 pathway. This is a key step in inducing cellular autophagy by increasing the levels of

Table 2. Difference in the median tissue TNFR1 immunostaining intensity between the 3 study groups.

	Normal control group N (%)	**Atherogenic group N (%)**	**Oridonin treated group N (%)**	***P* (Kruskal-Wallis)**
Immunohistochemistry (TNFR1)				<0.001
Negative	7 (100)	0 (0)	0 (0)	
+	0 (0)	0 (0)	3 (42.9)	
++	0 (0)	0 (0)	4 (57.1)	
+++	0 (0)	2 (28.6)	0 (0)	
++++	0 (0)	5 (71.4)	0 (0)	
Total	7 (100)	7 (100)	7 (100)	
Median	Negative	++++	++	
Mean rank	4	25	14.5	

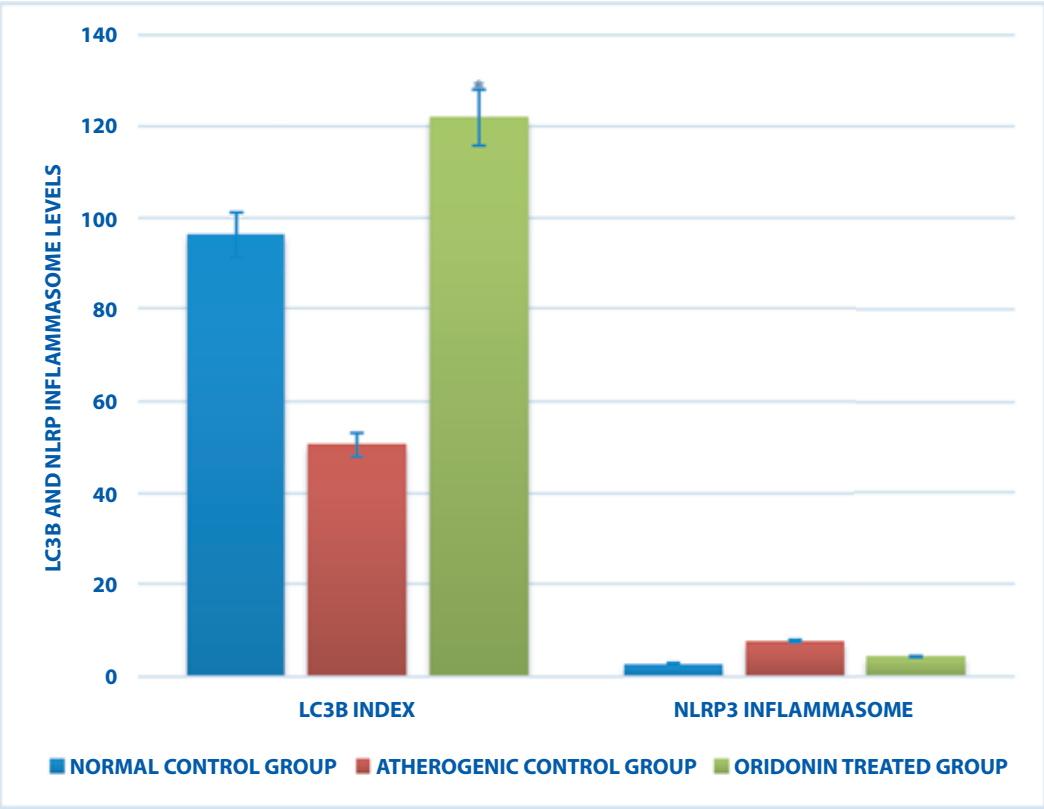

Figure 4. Markers of atherosclerotic changes (LC3B and NLRP3 Inflammasome) in aortic tissue following the end of the study, (P<0.005).

#: Significant P<0.001 difference between the atherogenic group and the normal control group.
*: Significant P<0.001 difference between the oridonin group and the atherogenic group.

LC3B in major blood vessels such as the aorta (12). These inflammasomes are large complex protein units that play a role in NLRP3 pathway and induce the synthesis and release of pro-inflammatory molecules such as IL-1B and IL-6 (13).Consequently, the use of NLRP3 inhibitors, such as oridonin, could exert potential benefits in reducing the synthesis of these inflammasomes leading to less activation of the inflammatory cytokines and an overall reduction in the degree of inflammation and oxidative stress (13). It is also worth mentioning the multiple actions of oridonin on the NLRP3 pathway, which could directly cause inhibition of the pathway through its anti-inflammatory effects. It can also induce alkalinization of the critical cysteine residues in the caspase subunit leading to reduction in the activity of ATPas enzymes in NLRP3-dependant pathways (14).

A few studies have reported some unwanted side effects for the use of oridonin. Xiang Li, et al in 2021, have mentioned that oridonin supplementation can cause suicidal erythrocyte death, induce the expression and activation of CYP2C and CYP3A family, and interfere with the early embryonic development of zebrafish (3). However, we could not identify any unwanted effects in the current study.

Conclusion

We conclude that oridonin supplementation induces a significant reduction in the degree of oxidative stress, lipid peroxidation, and inflammation which may ultimately help reduce the severity of atherosclerotic lesion in an animal model with atherogenic diet. This

could be explained through the oridonin inhibitory effects on the caspase-1 pathway.

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Ethic Committee: All the procedures were performed according to the guidelines approved by the National Institutes of Health. All animal experiments were approved by the Animal Research Ethics, College of Medicine, University of Kufa, Iraq (Approval No. 15779, on 14th December 2020).

Conflict of Interest. Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Author Contributions: The authors declare that all data were generated in-house and that no paper mill was used. The authors responsibilities were as follows: Author Contributions: "Conceptualization, A.H., B.M. and N.H.; methodology, A.H., B.M. A.P., and HA; software, D.J., A.S. and A.A.; validation, B.M., H.A. and N.H.; formal analysis, A.S.; investigation, D.J., A.P., and A.A.; resources, A.S. and N.H.; writing—original draft preparation, A.S., B.M. and N.H.; writing—review and editing, All authors.; supervision, B.M., N.H.; project administration, A.S. H.A. All authors have read and agreed to the published version of the manuscript."

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