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Astaxanthin Mitigates Streptozotocin Induced Diabetic Nephropathy, Targeting HMGB1, TLR4 and NF-κB

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ABSTRACT

Background: Diabetic nephropathy is a major health concern. Astaxanthin (ASX) has antioxidant and anti-inflammatory impacts. **Objective:** To illustrate the reno protective impact of ASX in STZ induced diabetic nephropathy and the underling mechanisms involved. **Material & methods:** Thirty Wister albino male rats divided into control, DN, DN+ASX groups. After 8 weeks serum levels of glucose, glycosylated Hb $A1_{c,}$ urea, creatinine, cystatin C in addition to creatinine clearance, urinary albumin, renal MDA, SOD TNF-α, IL-6, caspase-3, Renal genes HMGB1, TLR4 and NF-κB were assessed. Histopathological assessment of renal tissue was done.

Results: Serum levels of glucose, glycosylated Hb $A1_{c,}$ urea, creatinine, cystatin C in addition to urinary albumin, renal MDA, renal TNF-α, renal IL-6, renal caspase-3, renal HMGB1, TLR4 and NF-κB gene expression of DN group were significantly increased compared to control however renal SOD and creatinine clearance values of DN group were significantly decreased compared to control. ASX dramatically improved diabetic nephropathy induced changes.

Conclusion: ASX alleviated diabetic nephropathy induced changes by anti-oxidant, anti-inflammatory, anti-apoptotic mechanisms and modulating HMGB1, TLR4 and NF-κB renal genes expression.

Keywords: Astaxanthin, Caspase-3, Diabetic nephropathy, HMGB1, NF-κB, TLR4.

INTRODUCTION

About 20% to 40% of people with diabetes mellitus (DM) worldwide suffer from diabetic nephropathy (DN), a serious condition that is the primary cause of end-stage renal failure [1]. Even with the best care, diabetes patients' morbidity and death are still greatly increased by DN globally. A growing body of research indicates that hyperglycemia, or elevated glucose, causes renal damage in diabetic kidney disease (DN) through a variety of molecular pathways, including oxidative stress induction, proinflammatory cytokine release, fibroblast activation, the renin-angiotensin system, and apoptosis [2].

As of now, there are no effective treatments that can stop the condition from progressing; they can only stop it from happening again. When there is clear proteinuria, the illness progresses into an irreversible phase. DN is still becoming more commonplace nowadays, and this has been deemed a global problem. As a result, the development of innovative treatments for the illness is desperately needed [2].

One of the key chemokines generated by inflammatory cells, high-mobility group box 1 (HMGB 1), induces release of cytokines [3], via binding to AGE receptors (RAGE), the previously identified HMGB1 receptor binds Toll-like receptor (TLR)-4, which activates nuclear factor kappa B (NF-κB) and subsequent translocation into nucleus [4].

It has been claimed that the xanthophyll carotenoid astaxanthin (ASX), which is present in salmon, shrimp, crabs, and algae like Haematococcus pluvialis, can reduce insulin resistance by shielding myocytes from oxidative stress. When taken with meals, ASX is safe and has no negative effects. It is fat soluble, accumulates in rat, and doesn't appear to have any harmful consequences [5]. Additionally, ASX shields DM patients against oxidative stress, inflammatory responses, and apoptosis [6]. This study's goal was to illustrate the Reno protective impact of ASX on DN caused by streptozotocin (STZ) and the underlying mechanisms including the HMGB1, TLR4, and NF-κB pathway.

MATERIAL AND METHODS

Ethical approval:

The Menoufia University Faculty of Medicine's Animal Experimentation Ethics Committee requirements were followed in conducting this investigation with [IRB no: 10/2024Bio16/1].

Animals and Experimental Design

We utilized thirty mature male Wistar albino rats, 120 to 150 grams. The rats were kept in housing that ranged from 20 to 24 degrees Celsius.

DM induction and grouping

According to the preceding description, type 1 diabetes was generated by a single ip injection of streptozotocin (STZ, 65 mg/kg) in 0.2 ml citrate buffer (Sigma-Aldrich Chemical Co., USA) [7].

Diabetes was verified to have developed 48 hours after the STZ injection. The experiment was conducted on animals who were diagnosed as diabetics with fasting serum glucose >200 mg/dl.

Thirty rats were divided into three groups (n = 10);

1- Control group for eight weeks, rats were given a single ip injection of 10 mmol/L citrate buffer and a single oral gavage of 2 mL olive oil.
2- Diabetic Nephropathy (DN) group: diabetes was induced as previously mentioned. Rats received 2 mL olive oil by oral gavage once daily for 8 weeks.

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3- Astaxanthin-treated DN group (DN+ASX): Following the induction of diabetes, ASX (Sigma, St Louis, MO, USA) in a dose of 50 mg/kg every day for eight weeks [2]. The drug was produced and diluted in olive oil before to usage.

Experimental Procedures

Urine Collection. Urine samples were collected every day for 24 hours from rats housed in separate metabolic cages. Urine samples were measured for volume, and then centrifuged for 10 minutes at 1000 revolutions per minute (rpm) to measure creatinine and microalbuminuria. Measurements of Creatinine Clearance. The Microalbuminuria ELISA kit (Exocell Inc., Philadelphia, USA) was used to measure the amount of albumin in the urine. Urine creatinine concentration (mg/dL) multiplied by urine volume (mL/min) to plasma creatinine concentration (mg/dL) yielded the creatinine clearance (ml/min), with values reported in mL/min [8].

Blood Sampling: Blood samples were drawn from the retroorbital venous plexus under mild anesthesia after a 12-hour overnight fast, and they were split evenly between two tubes. The first tube was centrifuged at a speed of four thousand rpm. The collected serum was stored at -20 until it was required for further examination. The blood was drawn into the second tube, an EDTA tube, and the HbA1c was estimated using this proportion.

Renal homogenate preparation: Renal specimens were homogenized using a tissue homogenizer (MPW120, MPW Medical Instruments, China) at the conclusion of the research period. After centrifuging the crude tissue homogenate in an ice-cold centrifuge for 15 minutes at 10,000 rpm, the supernatant was collected and kept at -80°C for further biochemical testing.

Biochemical Analysis

Fasting blood glucose (Diamond Diagnostic, Egypt), serum HbA1c (Stanbio Glycohemoglobin, Egypt), were measured by colorimetric kits. Colorimetric kits (Spectrum Diagnostics, Egypt) were used to measure the concentrations of creatinine in plasma, urine, and urea. On the other hand, rat ELISA kits (TNF-α: ERT2010-1, Assaypro LLC, Saint Charles, Missouri, USA, cystatin C: ab201281, IL-6: ab100772, Abcam, Cambridge, UK) were used to measure the concentrations serum cystacin C, renal TNF-α and renal IL-6, as per the manufacturer's instructions. ELISA kits from Cusabio Technology Llc, Houston, TX, USA (CSB-E08857r) were used to investigate Caspase-3. Using colorimetric kits (Bio diagnostic Company, Dokki, Giza, Egypt) and following the manufacturer's recommendations, renal MDA and renal SOD were measured.

Quantitative assay of renal HMGB1, TLR4 and NF-κB genes expression using reverse transcriptase polymerase chain reaction technique (RT‑PCR).

Renal tissues were processed for total RNA isolation using Qiagen RN easy plus Universal Kit from the USA. RNA purity and quality were then guaranteed. RNA was kept at -80 °C until it was needed. Subsequently, the first stage involved synthesizing cDNA using the QuantiTect Reverse Transcription Kit, manufactured by Qiagen in the USA, in a single cycle on an Applied Biosystems 2720 heat cycler located in Singapore. GAPDH primers were employed as an RNA loading control in RT-PCR procedures. The second stage was cDNA amplification: cDNA was employed in SYBR green-based quantitative real-time PCR for Relative Quantification (RQ) of HMGB1, TLR4 and NF κB by SensiFASTTMSYBR Lo-ROX Kit, USA. The forward primer for HMGB 1 was *(TGAGGGACAAAAGCCACTC),* and the reverse primer was *(TTGGGAGGGCGGAGAATC*), The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC). The reverse primer was (GAGCAGTCATGTCCTTGGGT). The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT), and the Reverse was (GTCCTTGACCCACTGCAAGA)

Ultimately, data analysis was completed using the 2.0.1 edition of the Applied Biosystems 7500 software. Using a comparative ΔΔCt approach, which normalizes the quantity of target gene (HMGB1, TLR4, and NF κB) mRNA to reference gene (GAPDH) and compares it to a control, the RQ of HMGB1, TLR4, and NF κB gene expression was carried out.

Histological study

Was the 10% neutral formaldehyde used to preserve the renal tissues? The tissues then were processed for Paraffin slices of approximately 5μm thickness. Hematoxylin and eosin was used to stain the slices.

Statistical Analysis: SPSS version 16 was utilized to analyze the data. We used the mean± SD. The significance of the group differences was evaluated using ANOVA, and a Post-hoc Tukey test was performed. Dramatically significant was defined as a P value <0.05.

RESULTS

Serum levels of glucose, glycosylated Hb $A1_{c,}$ urea, creatinine, cystatin C in addition to urinary albumin, renal MDA, renal TNF-α, renal IL-6, renal caspase-3, Renal genes HMGB1, TLR4 and NF-κB of DN group were significantly increased compared to control however renal SOD and creatinine clearance values of DN group were substantially decreased compared to control. Serum levels of glucose, glycosylated Hb $A1_{c,}$ urea, creatinine, cystatin C in addition to urinary albumin, renal MDA, renal TNF-α, renal IL-6, renal caspase-3, renal HMGB1 TLR4 and NF-κB of DN+ASX group were substantially decreased compared to DN but still significantly increased compared to control, however renal SOD and creatinine clearance values of DN+ASX were significantly increased compared to DN but still substantially decreased compared to control (**Table 1).**

Table (1): The measured serum glucose, glycosylated Hb $A1_{c,}$ urea, creatinine, cystatin C, creatinine clearance, urinary albumin, renal TNF-α, renal IL-6, ranal caspase-3, ALT, AST, MDA, SOD, TNF-α, IL10, and caspase-3 Renal HMGB1 gene expression , Renal TLR4 gene expression and Renal NF-κB gene expression in all studied groups

	Control group	DN group	DN+ ASX group
Serum Glucose (mg/dl)	90.8±5.6	299.7±7.2 *	183±9.6 *#
Serum glycosylated Hb $A1_c$ (% of normal Hb)	4.3±.06	12.7±.2 *	8.3±0.26 *#
Serum Urea (mg/dl)	40.2±4.6	108.7±6.2 *	78±3.6 *#
Serum Creatinine (mg/dl)	0.49±.08	1.6±0.09 *	0.82±0.01 *#
Serum cystatin C (mg/L)	0.89±0.09	2.4±0.03 *	1.66±0.1 *#
Creatinine clearance (mL/min)	1.37±0.03	0.51±0.08 *	0.89±0.02 *#
urinary albumin (mg/day)	2024±4.15	180±7.8 *	110±7.5 *#
Renal MDA (nmol/ gm. Tissue)	14.1 ±2.9	39± 1.3*	23± 2.04*#
Renal SOD (U/gm. Tissue)	12.9 ±1.9	4.98±0.91*	9.18±0.77*#
Renal TNF-α (ng/ml)	23.8±0.37	49.8±1.91*	34.9±1.1*#
Renal IL-6 (pg/mL)	115±6.42	199±1.89*	148±2.1*#
Serum Caspase-3 (ng/ml)	39±2.42	128±4.89*	84±3.1*#
Renal HMGB1 gene expression	1	4.1±0.1*	2.5±0.09*#
Renal TLR4 gene expression	1	4.3±0.09*	2.8±0.1*#
Renal NF-κB gene expression	1	3.8±0.09*	2.1±0.07*#

* Significant compared with control, # Significant compared with DN.

Histopathological evaluation of renal tissue

Sections of male albino rats of kidney of control rats showed normal renal parenchyma (nrp) see arrow (Fig.1A). The diabetic group (DN) (second group) showed mesangial expansion and proliferation with an increase in the glomerular capillary thickening (Fig. 1B). Also, nodular glomerulosclerosis and hypercellularity were noticed (Fig. 1B) see arrows. Protected group (DN+ ASX) (third group) showed dramatic improvement of renal parenchyma (Fig. 1C).

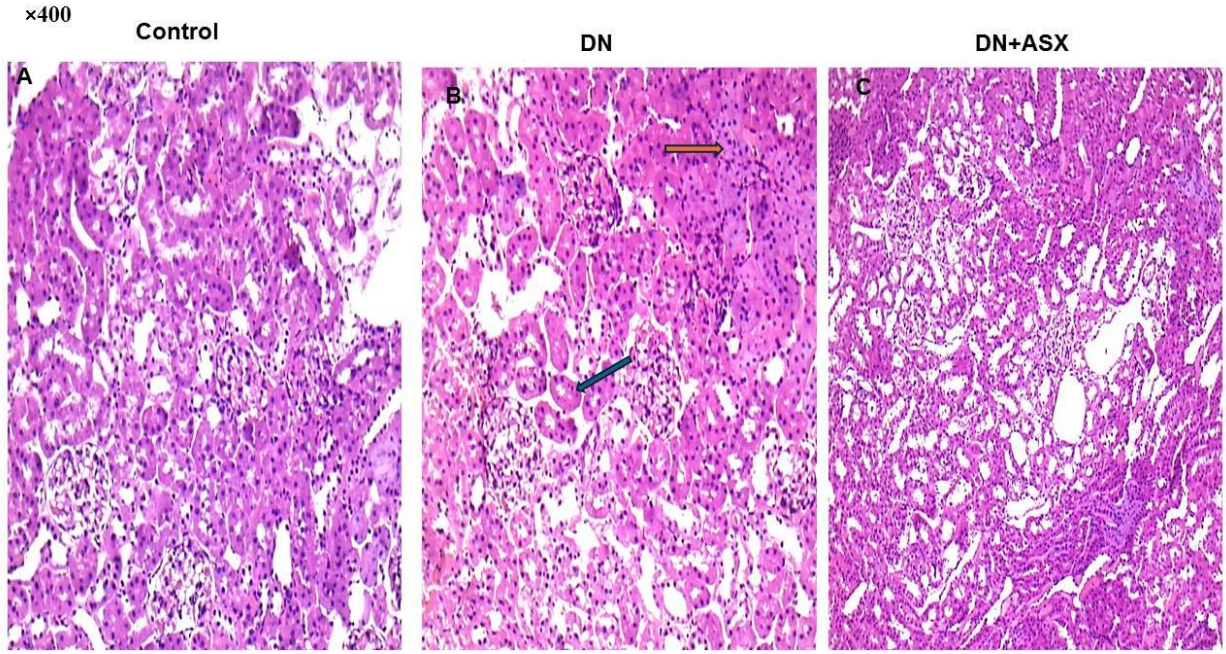

Fig. (1): Photomicrographs of kidney sections stained by H&E- in all studied groups with the same magnification (H&E X:400). (A) Photomicrographs of kidney sections of control group showed normal renal parenchyma. (B) Photomicrographs of kidney sections of diabetic group showed mesangial expansion and proliferation with increase in the glomerular capillary thickening (black arrow). Nodular glomerulosclerosis and hypercellularity were detected (orange arrow). (C) Photomicrographs of kidney sections of protected group showed marked improvements in the renal tissue. All photos at (X:400).

DISCUSSION

Our research offers proof that ASX can prevent kidney damage in a DN model generated by STZ. We demonstrate that new cell signaling pathways, such as HMGB1, TLR4, and NF-kB mediates the renoprotective effect of ASX against DN in rats. Our DN group's results showed an altered glycemic status, which was consistent with results from other studies [9].

As compared to the DN group, DN+ASX improved the glycemic status, which was consistent with a prior research [5]. In a NASH model, ASX's antioxidant action can lessen insulin resistance and glucose intolerance [10]. ASX's hypoglycemic impact is achieved by activation of PPARγ [11].

As a filtration marker, serum cystatin C is better than creatinine [12]. **Asleh *et al.*** [13] reported that podocytes were highly susceptible to the harmful effects of hyperglycemia and participated in DN with proteinuria due to changes in podocyte structure. The DN group's findings of impaired renal function than control group were consistent with these findings.

However DN+ASX dramatically improved renal function compared to DN group and this was in line with previous study [6].

Our findings are in line with prior research, which has shown that DN promotes oxidative stress, as evidenced by a significant increase in renal MDA levels and a decrease in SOD in the DN group [14].

Increased free radicals and a decrease in cellular antioxidants can result in hyperglycemia, which can further exacerbate oxidative stress and lipid peroxidation. Oxidative stress causes structural damage to proteins, lipids, DNA, and RNA, which leads in DN symptoms [15].

When compared to the DN group, DN+ASX significantly reduced the oxidative stress caused by DM, which was consistent with [16]. The microsomal membrane's peroxidation is effectively inhibited by ASX. The protective function of ASX in squelching free radicals and inhibiting lipid peroxidation to ensure the survival of pancreatic b-cells may be the cause of its antidiabetic action. Studies on the renal histology showed a correlation with the biochemical markers.

Renal inflammation has a role in nephropathy [17]. According to previously published data [14], the DN group exhibited a considerable rise in proinflammatory markers. By encouraging the production of chemokines, albuminuria and inflammatory substances generated in reaction to excessive hyperglycemia may all lead to more inflammation [18].

Because of its antioxidant properties, ASX has a potent anti-inflammatory impact and a better inflammatory state. It has been demonstrated that ASX has anti-inflammatory qualities through NF-Kβ pathway suppression. Previous research has connected the medicinal uses of ASX to its ability to suppress inflammatory genes and the generation of cytokines [19].

Renal tubular apoptosis and enhanced oxidative stress and inflammation were both facilitated by complicated pathway interaction in the pathophysiology of renal damage [20]. In the DN group, caspase 3 levels significantly rose, which was consistent with earlier research [21]. Furthermore, treatment with ASX decreased caspase-3 level compared to DN and this agrees with [19].

An essential inflammatory signal route in renal diseases is HMGB1/TLR4/NF-κB [22]. HMGB1 plays a significant role in maintaining renal inflammation. When blood sugar levels are high, HMGB1 is liberated and binds to its receptors on tubular cells, TLR4. This interaction causes an inflammatory response by turning on the NF-κB signaling cascade, which in turn causes the release of proinflammatory cytokines. As a result, a high concentration of cytokines promotes the recruitment of macrophages into renal tissue, which feeds back into the cycle of renal inflammation. Concurrently, a number of proinflammatory cytokines induce macrophages to actively release HMGB1, which, through positive feedback, exacerbates the kidney's chronic inflammation [23].

According to our research, DN exhibits a comparable up-regulation in the expression of the TLR4, HMGB1, and NF-kB genes when compared to control. These findings are consistent with earlier research [3]. Studies have documented the noteworthy effects of ASX on targeting several pathways [6, 19, 24]. AST substantially downregulated HMGB1/ TLR4/ NF-κB pathway, this agreed with previous study [25].

CONCLUSION

By suppressing HMGB1/ TLR4/ NF-κB pathway and utilizing anti-oxidant, anti-inflammatory, and anti-apoptotic processes, ASX significantly reduced the degree of DN caused by streptozotocin.

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REFERENCES

1. **Kaikini A, Muke S, Peshattiwar V *et al.* (2021):** Ethyl ferulate, a lipophilic phenylpropanoid, prevents diabetes-associated renal injury in rats by amelioration of hyperglycemia-induced oxidative stress via activation of nuclear factor erythroid 2-related factor 2. Journal of Food Biochemistry, 45: 1445. DOI 10.1111/jfbc.13607.
2. **Ma L, Liu S, Zhen X *et al.* (2022):** Astaxanthin delayed the pathogenesis of diabetic nephropathy in type 1 diabetic rats. Biocell, 46(8): 1911-1916.
3. **Awad A, Elshaer S, Gangaraju R *et al.* (2024):** Ameliorative effect of montelukast against STZ induced diabetic nephropathy: targeting HMGB1, TLR4, NF-κB, NLRP3 inflammasome, and autophagy pathways. Inflammopharmacology, 32(1): 495-508.

4. **Wu H, Chen Z, Xie J *et al.* (2016):** High mobility group box-1: a missing link between diabetes and its complications. Mediators Inflamm., 16:3896147. doi: 10.1155/2016/3896147
5. **Zhuge F, Ni Y, Wan C *et al.* (2021):** Anti-diabetic effects of astaxanthin on an STZ-induced diabetic model in rats. Endocrine Journal, 68(4): 451-459.
6. **Hong M, Nie Z, Chen Z *et al.* (2024):** Astaxanthin attenuates diabetic kidney injury through upregulation of autophagy in podocytes and pathological crosstalk with mesangial cells. Renal Failure, 46(2): 2378999. doi: 10.1080/0886022X.2024.2378999.
7. **Al-Awar A, Kupai K, Veszelka M *et al.* (2016):** Experimental diabetes mellitus in different animal models. Journal of Diabetes Research, 16: 9051426. doi: 10.1155/2016/9051426.
8. **Bazzano T, Restel T, Porfirio L *et al.* (2015):** Renal biomarkers of male and female Wistar rats (Rattus norvegicus) undergoing renal ischemia and reperfusion. Acta Cir Bras., 30(4): 277–288.
9. **Khodir S, Samaka R, Ameen O (2020):** Autophagy and mTOR pathways mediate the potential renoprotective effects of vitamin D on diabetic nephropathy. International Journal of Nephrology, 20(1): 7941861. doi: 10.1155/2020/7941861
10. **Ni Y, Nagashimada M, Zhuge F *et al.* (2015):** Astaxanthin prevents and reverses diet-induced insulin resistance and steatohepatitis in mice: a comparison with vitamin E. Sci Rep., 5: 17192. doi: 10.1038/srep17192.
11. **Inoue M, Tanabe H, Matsumoto A *et al.* (2012):** Astaxanthin functions differently as a selective peroxisome proliferator-activated receptor gamma modulator in adipocytes and macrophages. Biochem Pharmacol., 84: 692–700.
12. **Dharnidharka V, Kwon C, Stevens G (2002):** Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. American Journal of Kidney Diseases, 40(2): 221–226.
13. **Asleh R, Nakhoul F, Miller-Lotan R *et al.* (2012):** Poor lysosomal membrane integrity in proximal tubule cells of haptoglobin 2-2 genotype mice with diabetes mellitus. Free Radical Biology and Medicine, 53(4): 779–786.
14. **Mahmoud A, Ashour M, Abdel-Moneim A *et al.* (2012):** Hesperidin and naringin attenuate hyperglycemia- mediated oxidative stress and proinflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. Journal of Diabetes and its Complications, 26(6):483–490.
15. **Manda G, Checherita A, Comanescu M *et al.* (2015):** Redox signaling in diabetic nephropathy: hypertrophy versus death choices in mesangial cells and podocytes. Mediators of Inflammation, 15:604208. doi: 10.1155/2015/604208.
16. **Sila A, Ghlissi Z, Kamoun Z *et al.* (2015):** Astaxanthin from shrimp by-products ameliorates nephropathy in diabetic rats. European Journal of Nutrition, 54: 301-307.
17. **Landon R, Gueguen V, Petite H *et al.* (2022):** Impact of astaxanthin on diabetes pathogenesis and chronic complications. Marine Drugs, 18(7): 357. doi: 10.3390/md18070357
18. **Navarro-González F, Mora-Fernández C (2008):** Role of inflammatory cytokines in diabetic nephropathy. Journal of the American Society of Nephrology, 19(3): 433–442.
19. **Khodir S, Sweed E, Gadallah M *et al.* (2022):** Astaxanthin attenuates cardiovascular dysfunction associated with deoxycorticosterone acetate-salt-induced hypertension in rats. Clinical and Experimental Hypertension, 44(4): 382-395.
20. **Martınez-Castelao A, Hueso M, Sanz V *et al.* (1988):** Treatment of hypertension after renal transplantation: long-term efficacy of verapamil, enalapril, and doxazosin. Kidney Int., 68: 130–134
21. **Ahmed O, Ali T, Abdel Gaid M *et al.* (2019):** Effects of enalapril and paricalcitol treatment on diabetic nephropathy and renal expressions of TNF-α, p53, caspase-3 and Bcl-2 in STZ-induced diabetic rats. PloS One, 14(9): e0214349. doi: 10.1371/journal.pone.0214349
22. **Rabadi M, Ghaly T, Goligorksy M *et al.* (2012):** HMGB1 in renal ischemic injury. Am J Physiol Renal Physiol., 303: 873-885.
23. **Yuan Y, Liu Y, Sun M *et al.* (2022):** Aggravated renal fibrosis is positively associated with the activation of HMGB1-TLR2/4 signaling in STZ-induced diabetic mice. Open Life Sciences, 17(1): 1451-1461.
24. **Fakhri S, Abbaszadeh F, Dargahi L *et al.* (2018):** Astaxanthin: A mechanistic review on its biological activities and health benefits. Pharmacol Res., 136:1–20
25. **Abbaszadeh F, Jorjani M, Joghataei M *et al.* (2023):** Astaxanthin ameliorates spinal cord edema and astrocyte activation via suppression of HMGB1/TLR4/NF-κB signaling pathway in a rat model of spinal cord injury. Naunyn Schmiedebergs Arch Pharmacol., 396(11): 3075-3086.