Journal of Experimental and Clinical Medicine
https://dergipark.org.tr/omujecm

Research Article

J Exp Clin Med
2022; 39(1): 216-220
doi: 10.52142/omujecm.39.1.42

Silymarin ameliorates cisplatin-induced nephrotoxicity by downregulating TNF-α and NF-kB and by upregulating IL-10

Fikret ALTINDAĞ*

Department of Histology and Embryology, Faculty of Medicine, Van Yüzüncü Yıl University, Van, Turkey

Received: 13.06.2021 • **Accepted/Published Online:** 01.07.2021 • **Final Version:** 01.01.2022

Abstract

Cisplatin (CP) is one of the antineoplastic agents used to treat many types of cancers. Besides these effects of CP, it causes various side effects such as nephrotoxicity. Inflammation is considered to be one of the main pathogeneses of CP-induced nephrotoxicity. Silymarin (SIL) is a flavonoid with beneficial pharmacological properties such as antioxidant and anti-inflammatory. The current study aimed to investigate the beneficial effects of Silymarin against CP-induced nephrotoxicity. Albino Wistar male rats were randomly divided into four groups (n=7): Control group was administered saline (0.5 ml) for 10 days intraperitoneally (IP), CP group was received CP (a single dose of 8 mg/kg, IP), CP+ SIL group was administered saline (0.5 ml) for 10 days and was received CP (a single dose of 8 mg/kg). To measure the inflammatory response, TNF-α, NF-kB and IL-10 expressions were performed. As a result, TNF-α and NF-kB expressions significantly increased while IL-10 expression decreased in the kidney of the CP group compared to the control group. However, SIL treatment significantly decreased TNF-α and NF-kB expressions and increased IL-10 expression in kidney CP-treated rats. These findings reveal that SIL may ameliorates the CP-induced nephrotoxicity in rats by inducing downregulation of TNF-α and NF-kB expressions and upregulation of IL-10 expression

Keywords: Cisplatin, IL-10, Nephrotoxicity, NF-kB, Silymarin, TNF-α

1. Introduction

Cisplatin (CP) is a platinum-derived anticancer agent used to treat many types of human cancers such as lung, bladder, ovary, testicular, leukemia, brain, kidney, head and neck tumors. It also plays a key role in the treatment of germ cell cancer (1). Despite its very strong effects in cancer treatment, it has side effects such as neurotoxicity and ototoxicity, especially neurotoxicity. More than 30% of patients receiving CP treatment suffered from renal dysfunction (2). Even though the toxic effects of CP have been proven, it is still a widely prescribed drug. Because it is a standard drug for some cancer treatments such as head and neck (3). Although the pathophysiology of acute kidney injury (AKI) caused by CP is complex, it mainly includes oxidative stress, apoptosis and inflammation (4). Inflammation is critical for understanding the pathogenesis of CP-induced nephrotoxicity (5). TNF-α is a major inflammatory factor that plays a crucial role in CP-induced nephrotoxicity. Production of TNF-α is mostly dependent on ROS and NF-kB activation (6). IL-10 is an anti-inflammatory cytokine that inhibits the production of proinflammatory cytokines mainly produced by T cells, macrophages and Dendritic cells (7). Since CP is both nephrotoxic and an indispensable drug in the treatment of many tumors, reducing or preventing kidney damage caused by it becomes an important problem to be solved.

Studies have reported that the administration of natural compounds may be a protective strategy against CP-induced nephrotoxicity (8, 9). Silymarin is a natural polyphenol compound extracted from the thistle (Silybum marianum) plant (10). It has been reported to have anti-inflammatory, antioxidant, antiviral, immunomodulatory, anti-proliferative properties (11, 12). Silymarin has been proven to have a hepatoprotective effect against liver toxicity induced by various hepatotoxic agents (13, 14, 15, 16). Recently, some studies have reported the protective effects of Silymarin to prevent CP-induced nephrotoxicity (17, 18). However, the effect mechanism of Silymarin in CP-caused nephrotoxicity has not been fully elucidated. Therefore, in our study, the beneficial effects of Silymarin were investigated by evaluating the expressions of TNF-α, NF-kB and IL-10 in the treatment of CP.

2. Material and Method

2.1. Experimental animals and drug treatment

Twenty-eight healthy *Albino Wistar* male rats weighing 180-200 g were randomly divided into four groups (n=7): Control (C) group was administered 0.5 ml saline for 10 days, CP group was received a single dose of 8 mg/kg cisplatin intraperitoneally (IP) on the 5th day of the study (19), Cisplatin + Silymarin group was received 50 mg/kg silymarin (Sigma

* Correspondence: altindagfikret@gmail.com

Aldrich Biotechnology, CAS Number: 65666-07-1) orally for 10 days, and a single dose 8 mg/kg cisplatin IP was administered on the 5th day of the study, Silymarin group was received 50 mg/kg silymarin orally for 10 days (20). Animals were obtained from Van Yüzüncü Yıl University Experimental Medicine Application and Research Center. Animals were maintained normal light and dark cycles (12h:12h light/dark), at temperature (21 ± 2 ºC) and humidity (50 % ± 10), their feed and water were given in accordance with the standards (*ad libitum*). All protocols of the experiment have been approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (approval number: 2021/05-01). At the end of the experiment, the rats were anesthetized with intraperitoneal ketamine (75mg/kg). The kidney was removed and was fixed in 10% neutral buffered formalin for immunohistochemical evaluation.

2.2. Immunohistochemical preparation

Kidney tissue was embedded in paraffin after undergoing routine histological processing stages. The sections of 4 µm thickness were taken from paraffin blocks. The taken sections were deparaffinized and dehydrated. After incubation with 3% Hydrogen peroxide (H_2O_2), the sections were incubated in citrate buffer (ph 6.1) by heating in a microwave oven. They were incubated in Ultra V Block. Antibodies of TNF-α (Santa Cruz Biotechnology, dilution:1/50), NF-κB (Santa Cruz Biotechnology, dilution:1/50) and IL-10 (Santa Cruz Biotechnology, dilution:1/50) were used as primary antibody. The sections were incubated with primary antibodies +4C overnight, then they were incubated in Biotinylated Goat Anti-Polyvalent, and Streptavidin–peroxidase conjugate respectively. Diaminobenzidine (DAB) was used as a chromogen, and then stained with Mayer's hematoxylin. TNF-α, NF-κB and IL-10 immunopositive cells were counted using cellSens Software imaging systems (Olympus, Japan) in a light microscope (Olympus BX53, Japan) and assessed by H-score.

3. Results

3.1. Immunohistochemical findings

According to the results of the immunohistochemical assay performed to measure the inflammatory response showed that CP significantly increased TNF-α and NF-kB expressions, while decreased IL-10 expression compared to the control group ($p<0.05$). However, treatment with silymarin reduced TNF-α and NF-kB expressions and increased IL-10 expression in CP-induced rat kidneys ($p<0.05$). Only a few TNF-α, NF-kB and IL-10 immunopositive cells were observed in the Control and Silymarin groups (Fig. 1 and 2).

4. Discussion

This study aimed to investigate the beneficial effects of Silymarin in CP-induced nephrotoxicity. The findings of our study showed that Silymarin has a nephroprotective effect by reducing kidney inflammation caused by CP treatment. The effect mechanism of silymarin is schematized in Fig. 3.

One of the most prominent complications of CP used to treat many solid tumors is acute kidney injury (AKI) (4). It has been reported that inflammation is one of the most main pathogeneses of CP-induced AKI. Inflammation is a complex biological response that occurs after tissue damage (21). Previous studies have proven the role of inflammatory mechanisms in AKI caused by toxic substances (22). After kidney injury, the balance between pro-and anti-inflammatory mediators in the kidney significantly influences the extent of tissue damage and repair (23). Since AKI is closely related to inflammation, it is important to clarify inflammation in determining the necessary treatment modalities to prevent or treat AKI (21). The cellular damage and its associated molecular products are thought to be key triggers for inflammation after acute tissue injury (24). Pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-1β, IL-23, IL-17 and anti-inflammatory cytokines such as IL-4, TGF-β, IL-10 are crucial factors in determining tissue damage and treatment strategies (25).

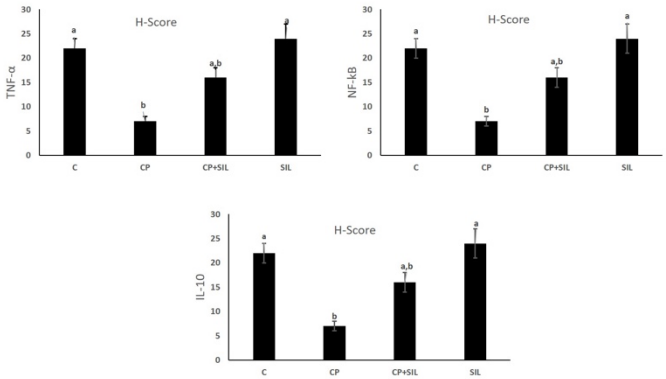

Fig. 1. The effect of silymarin on TNF-α, NF-kB and IL-10 expressions in kidney tissue of CP induced rats. H-Score

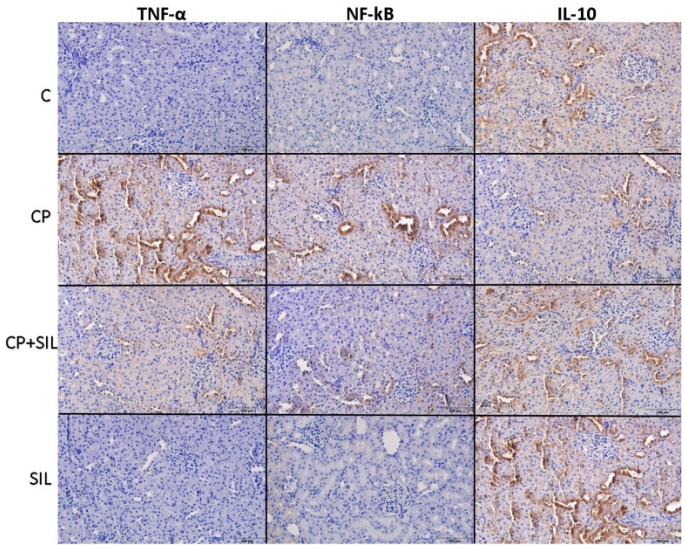

Fig. 2. Immunohistochemical figures of kidney tissues of rats. CP increased TNF-α and NF-kB expressions, while decreased IL-10 expression. However, treatment with silymarin reduced TNF-α and NF-kB expressions and increased IL-10 expression

NF-κB is an important factor that plays an active role in the activation of transcription of genes encoding proinflammatory

cytokines especially TNF-α, and thus mediates inflammation (26). TNF-α is a potent cytokine that mediates inflammatory tissue damage in the kidney, and specifically activates IL1-β, MCP-1, and IL-6 pro-inflammatory cytokines (28). Previous studies have reported that CP causes an inflammatory response by stimulating the production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, MCP-1 and NF-kB in the kidney (5). It has been demonstrated that CP administration activates NF-kB which stimulates the expression of other pro-inflammatory cytokines such as TNF-α in the kidney. It also inhibits the production of IL-10 which suppresses the expression of TNF-α. IL-10 has protective effects on tissue damage as well as inhibiting the production of inflammatory cytokines (28). In parallel to studying of Kim et al. (2010), the current study revealed that CP treatment-induced inflammation by increasing the expression of NF-kB and TNF-α in the kidney. On the other hand, it inhibited the anti-inflammatory mechanism by suppressing IL-10 expression.

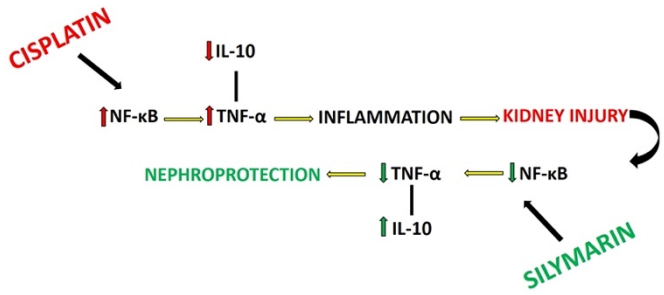

Fig. 3. Mechanism of action of silymarin in CP-induced nephrotoxicity. CP treatment upregulates TNF-α and NF-kB expression in the kidney, while it downregulates IL-10 expression. However, silymarin treatment exerts a renoprotective effect by suppressing TNF-α and NF-kB expressions and stimulating IL- expression in CP-induced kidney

Recently, it has been reported that antioxidants have a nephroprotective effect by suppressing oxidative stress and inflammation in CP-induced nephrotoxicity (29). Soetikno et al. (2018) reported that increased NF-κB and TNF-α expressions in CP-induced kidneys were significantly inhibited by curcumin treatment, and it also showed a nephroprotective effect by increasing IL-10 expression (30). Similarly, Sánchez-González et al. (2017) reported that CP-induced inflammatory markers such as TNF-α, iNOS and neutrophil infiltration, and Quercetin treatment improved these inflammatory markers (31). Similar to previous studies, in the current study, the increase in the expression of NF-kB and TNF-α caused by CP was significantly restored with Silymarin treatment. On the other hand, IL-10 expression suppressed by CP was significantly increased with Silymarin treatment (30, 31). These findings of our study reveal that Silymarin has an anti-inflammatory effect against inflammation caused by CP in the kidney of rats.

One of the most important ways of preventing CP-induced kidney damage is to inhibit increased TNF-α. It has been reported that TNF-α can directly damages the glomerular and tubular cells and activates internal and external apoptotic pathways (32). Gawad and Mohamed (2010) revealed that it was observed histopathological changes such as diffuse renal tubular necrosis, degeneration and mononuclear cellular infiltration in the kidney tissues of rats treated with CP (33). They also reported that Silymarin can be used as a nephroprotective agent by improving these structural changes induced by CP. Similarly, Abdelmeguid et al. (2010) reported that CP application caused deteriorations in the kidney such as glomerular atrophy, dilated filtration gap, loss of brush border of proximal collecting tubules, hypertrophied podocyte pedicels and tubular cell vacuolization, but Silymarin treatment ameliorated these changes (34). It is estimated that the inflammation caused by CP may be related to the histopathological findings of previous studies (13, 33). This study shows that increased TNF-α, which is an important marker of inflammation, can have a direct toxic effect on kidney cells (32). In addition, the current study reveals that silymarin suppresses the expression of TNF-α and increases the expression of IL-10, which is both an anti-inflammatory and tissue-protective cytokine, and it may be a protective agent for cells in kidney tissue. In this respect, our study is thought to support previous studies (13, 33).

The results of our study showed that silymarin may have a renoprotective effect by reducing the increased inflammation in the kidney induced. It is considered that the renoprotective effects of silymarin are mainly related to the downregulation of the expression of TNF-α and NF-kB and the upregulation of the expression of IL-10. Therefore, it is estimated that silymarin may be used as a protective agent against CP-induced nephrotoxicity.

Conflict of Interest

The authors declare that there are no conflicts of interest

Funding

This research did not receive any grant from funding agencies

Acknowledgments

This study was carried out with the resources provided by the Department of Histology and Embryology, Faculty of Medicine, Van Yüzüncü Yıl University.

References

1. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol. 2014 Oct 5;740:364-78. doi: 10.1016/j.ejphar.2014.07.025. Epub 2014 Jul 21. PMID: 25058905; PMCID: PMC4146684.
2. Chen X, Wei W, Li Y, Huang J, Ci X. Hesperetin relieves cisplatin-induced acute kidney injury by mitigating oxidative stress, inflammation and apoptosis. Chem Biol Interact. 2019 Aug 1;308:269-278. doi: 10.1016/j.cbi.2019.05.040. Epub 2019 May 31. PMID: 31153982.
3. Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel). 2010 Nov;2(11):2490-518. doi: 10.3390/toxins2112490. Epub 2010 Oct 26. PMID: 22069563; PMCID: PMC3153174.

4. Ozkok A, Edelstein CL. Pathophysiology of cisplatin-induced acute kidney injury. Biomed Res Int. 2014;2014:967826. doi: 10.1155/2014/967826. Epub 2014 Aug 6. PMID: 25165721; PMCID: PMC4140112.

5. Gao L, Liu MM, Zang HM, Ma QY, Yang Q, Jiang L, Ren GL, Li HD, Wu WF, Wang JN, Wei B, Liu XQ, Jiang C, Huang C, Li J, Meng XM. Restoration of E-cadherin by PPBICA protects against cisplatin-induced acute kidney injury by attenuating inflammation and programmed cell death. Lab Invest. 2018 Jul;98(7):911-923. doi: 10.1038/s41374-018-0052-5. Epub 2018 Mar 26. PMID: 29581579.

6. Dashti-Khavidaki S, Shahbazi F, Khalili H, Lessan-Pezeshki M. Potential renoprotective effects of silymarin against nephrotoxic drugs: a review of literature. J Pharm Pharm Sci. 2012;15(1):112-23. doi: 10.18433/j3f88s. PMID: 22365093.

7. Bamboat ZM, Ocuin LM, Balachandran VP, Obaid H, Plitas G, DeMatteo RP. Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion. J Clin Invest. 2010 Feb;120(2):559-69. doi: 10.1172/JCI40008. Epub 2010 Jan 19. PMID: 20093775; PMCID: PMC2810082.

8. Wang SW, Xu Y, Weng YY, Fan XY, Bai YF, Zheng XY, Lou LJ, Zhang F. Astilbin ameliorates cisplatin-induced nephrotoxicity through reducing oxidative stress and inflammation. Food Chem Toxicol. 2018 Apr;114:227-236. doi: 10.1016/j.fct.2018.02.041. Epub 2018 Feb 20. PMID: 29471006.

9. Güntürk I, Yazici C, Köse SK, Dağli F, Yücel B, Yay AH. The effect of N-acetylcysteine on inflammation and oxidative stress in cisplatin-induced nephrotoxicity: a rat model. Turk J Med Sci. 2019 Dec 16;49(6):1789-1799. doi: 10.3906/sag-1903-225. PMID: 31655538; PMCID: PMC7518668.

10. Ninsontia C, Pongjit K, Chaotham C, Chanvorachote P. Silymarin selectively protects human renal cells from cisplatin-induced cell death. Pharm Biol. 2011 Oct;49(10):1082-90. doi: 10.3109/13880209.2011.568506. Epub 2011 May 18. PMID: 21591838.

11. Vargas-Mendoza N, Madrigal-Santillán E, Morales-González A, Esquivel-Soto J, Esquivel-Chirino C, García-Luna Y González-Rubio M, Gayosso-de-Lucio JA, Morales-González JA. Hepatoprotective effect of silymarin. World J Hepatol. 2014 Mar 27;6(3):144-9. doi: 10.4254/wjh.v6.i3.144. PMID: 24672644; PMCID: PMC3959115.

12. Lamia SS, Emran T, Rikta JK, Chowdhury NI, Sarker M, Jain P, Islam T, Gias ZT, Shill MC, Reza HM. Coenzyme Q10 and Silymarin Reduce CCl_4-Induced Oxidative Stress and Liver and Kidney Injury in Ovariectomized Rats—Implications for Protective Therapy in Chronic Liver and Kidney Diseases. *Pathophysiology*. 2021; 28(1):50-63. https://doi.org/10.3390/pathophysiology28010005

13. Abdelmeguid NE, Chmaisse HN, Abou Zeinab NS. Silymarin ameliorates cisplatin-induced hepatotoxicity in rats: histopathological and ultrastructural studies. Pak J Biol Sci. 2010 May 15;13(10):463-79. doi: 10.3923/pjbs.2010.463.479. PMID: 21848071.

14. Chen IS, Chen YC, Chou CH, Chuang RF, Sheen LY, Chiu CH. Hepatoprotection of silymarin against thioacetamide-induced chronic liver fibrosis. J Sci Food Agric. 2012 May;92(7):1441-7. doi: 10.1002/jsfa.4723. Epub 2011 Nov 18. PMID: 22102319.

15. Zaulet M, Kevorkian SEM, Dinescu S, Cotoraci C, Suciu M, Herman H, Buburuzan L, Badulescu L, Ardelean A, Hermenean A. Protective effects of silymarin against bisphenol A-induced hepatotoxicity in mouse liver. Exp Ther Med. 2017 Mar;13(3):821-828. doi: 10.3892/etm.2017.4066. Epub 2017 Jan 20. PMID: 28450905; PMCID: PMC5403334.

16. Ramezannezhad P, Nouri A, Heidarian E. Silymarin mitigates diclofenac-induced liver toxicity through inhibition of inflammation and oxidative stress in male rats. J Herbmed Pharmacol. 2019;8(3): 231-237. doi: 10.15171/jhp.2019.34.

17. Turgut F, Bayrak O, Catal F, Bayrak R, Atmaca AF, Koc A, Akbas A, Akcay A, Unal D. Antioxidant and protective effects of silymarin on ischemia and reperfusion injury in the kidney tissues of rats. Int Urol Nephrol. 2008;40(2):453-60. doi: 10.1007/s11255-008-9365-4. PMID: 18368506.

18. Momeni A, Hajigholami A, Geshnizjani S, Kheiri S. Effect of silymarin in the prevention of Cisplatin nephrotoxicity, a clinical trial study. J Clin Diagn Res. 2015 Apr;9(4):OC11-3. doi: 10.7860/JCDR/2015/12776.5789. Epub 2015 Apr 1. PMID: 26046020; PMCID: PMC4437094.

19. Tomar A, Vasisth S, Khan SI, Malik S, Nag TC, Arya DS, Bhatia J. Galangin ameliorates cisplatin induced nephrotoxicity in vivo by modulation of oxidative stress, apoptosis and inflammation through interplay of MAPK signaling cascade. Phytomedicine. 2017 Oct 15;34:154-161. doi: 10.1016/j.phymed.2017.05.007. Epub 2017 Jun 15. PMID: 28899498.

20. Jahan S, Khan M, Imran S, Sair M. The hepatoprotective role of Silymarin in isoniazid induced liver damage of rabbits. J Pak Med Assoc. 2015 Jun;65(6):620-2. PMID: 26060158.

21. Rabb H, Griffin MD, McKay DB, Swaminathan S, Pickkers P, Rosner MH, Kellum JA, Ronco C; Acute Dialysis Quality Initiative Consensus XIII Work Group. Inflammation in AKI: Current Understanding, Key Questions, and Knowledge Gaps. J Am Soc Nephrol. 2016 Feb;27(2):371-9. doi: 10.1681/ASN.2015030261. Epub 2015 Nov 11. PMID: 26561643; PMCID: PMC4731128.

22. Ramesh G, Reeves WB. Inflammatory cytokines in acute renal failure. Kidney Int Suppl. 2004 Oct;(91):S56-61. doi: 10.1111/j.1523-1755.2004.09109.x. PMID: 15461705.

23. Ratliff BB, Rabadi MM, Vasko R, Yasuda K, Goligorsky MS. Messengers without borders: mediators of systemic inflammatory response in AKI. J Am Soc Nephrol. 2013 Mar;24(4):529-36. doi: 10.1681/ASN.2012060633. Epub 2013 Jan 24. PMID: 23349311.

24. Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010 Dec;10(12):826-37. doi: 10.1038/nri2873. Epub 2010 Nov 19. PMID: 21088683; PMCID: PMC3114424.

25. Kurts C, Panzer U, Anders HJ, Rees AJ. The immune system and kidney disease: basic concepts and clinical implications. Nat Rev Immunol. 2013 Oct;13(10):738-53. doi: 10.1038/nri3523. Epub 2013 Sep 16. PMID: 24037418.

26. Mercantepe F, Mercantepe T, Topcu A, Yılmaz A, Tumkaya L. Protective effects of amifostine, curcumin, and melatonin against cisplatin-induced acute kidney injury. Naunyn Schmiedebergs Arch Pharmacol. 2018 Sep;391(9):915-931. doi: 10.1007/s00210-018-1514-4. Epub 2018 Jun 2. Erratum in: Naunyn Schmiedebergs Arch Pharmacol. 2019 Mar 1;: PMID: 29860655.

27. Sharp CN, Doll MA, Dupre TV, Shah PP, Subathra M, Siow D, Arteel GE, Megyesi J, Beverly LJ, Siskind LJ. Repeated administration of low-dose cisplatin in mice induces fibrosis. Am J Physiol Renal Physiol. 2016 Mar 15;310(6):F560-8. doi: 10.1152/ajprenal.00512.2015. Epub 2016 Jan 6. PMID: 26739893; PMCID: PMC4796272.

28. Kim MG, Yang HN, Kim HW, Jo SK, Cho WY, Kim HK. IL-10 mediates rosiglitazone-induced kidney protection in cisplatin nephrotoxicity. J Korean Med Sci. 2010 Apr;25(4):557-63. doi: 10.3346/jkms.2010.25.4.557. Epub 2010 Mar 19. PMID: 20357998; PMCID: PMC2844593.

29. Gómez-Sierra T, Eugenio-Pérez D, Sánchez-Chinchillas A, Pedraza-Chaverri J. Role of food-derived antioxidants against cisplatin induced-nephrotoxicity. Food Chem Toxicol. 2018 Oct;120:230-242. doi: 10.1016/j.fct.2018.07.018. Epub 2018 Jul 7. PMID: 29990577.

30. Soetikno V, Sari SDP, Ul Maknun L, Sumbung NK, Rahmi DNI, Pandhita BAW, Louisa M, Estuningtyas A. Pre-Treatment with Curcumin Ameliorates Cisplatin-Induced Kidney Damage by Suppressing Kidney Inflammation and Apoptosis in Rats. Drug Res (Stuttg). 2019 Feb;69(2):75-82. doi: 10.1055/a-0641-5148. Epub 2018 Jun 26. PMID: 29945277.

31. Sánchez-González PD, López-Hernández FJ, Dueñas M, Prieto M, Sánchez-López E, Thomale J, Ruiz-Ortega M, López-Novoa JM, Morales AI. Differential effect of quercetin on cisplatin-induced toxicity in kidney and tumor tissues. Food Chem Toxicol. 2017 Sep;107(Pt A):226-236. doi: 10.1016/j.fct.2017.06.047. Epub 2017 Jun 30. PMID: 28669851.

32. Ramesh G, Reeves WB. Salicylate reduces cisplatin nephrotoxicity by inhibition of tumor necrosis factor-alpha. Kidney Int. 2004 Feb;65(2):490-9. doi: 10.1111/j.1523-1755.2004.00413.x. PMID: 14717919.

33. Gawad SHA, Mohamed AAK. Silymarin administration protects against cisplatin-induced nephrotoxicity in adult male albino rats. Egypt J Histol. 2010; 33(4), 683-691.

34. Abdelmeguid NE, Chmaisse HN, Abou Zeinab NS, Protective Effect of Silymarin on Cisplatin-induced Nephrotoxicity in Rats. Pak J Nutr. 2010;9(7),1680–5194.