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Phytochemical Compounds Attenuate Nonylphenol Induced Oxidative Stress, Liver injury and Estradiol Disruption: Experimental and Molecular Docking Insights

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Abstract

Nonylphenol (NP) is a synthetic xenoestrogen causes sex hormones imbalance and oxidative stress inducer. Resveratrol (RES) and Naringenin (NG) are phytochemicals possessing antioxidant properties and estrogenic activity. This study was conducted to study the toxicity of NP and the mitigating effects of RES and NG on NP toxic impacts in rats. Thirty male rats were classified into 5 groups as follows: 1- Normal control (NC) group, 2- Dimethyl sulfoxide (DMSO) group, 3- NP group, 4- NP+RES and 5- NP+NG. Results revealed that NP treatment significantly decreased the Glutathione (GSH) and sulfhydryl (R-SH) groups content compared to NC group. In addition, significant escalation was observed in the levels of liver functions parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), except total protein level was decreased compared to NC. Besides, lipid profile parameters, total cholesterol, low density lipoprotein (LDL), very low density lipoprotein (VLDL) and triglycerides exhibited significant increase compared to NC, but high density lipoprotein (HDL) level showed significant reduction. All the recorded effects induced by NP treatment were effectively countered by co-treatment with RES or NG. in addition, molecular docking studies were carried out to investigate the interactions between Estradiol, NP, RES, NG and estrogen receptor alpha which provide a possible mechanism for their potential estrogenic activity. Overall, our study contributes to a deeper understanding of the toxic effect of NP on antioxidant capacity, liver and kidney function, blood composition, regulated lipid levels and estradiol disruption. Besides, it underscores the potential therapeutic utility of RES and NG in alleviating these adverse effects.

Keywords: Nonylphenol; Xenoestrogen; liver function; Resveratrol; Naringenin; estrogen receptor; Molecular docking

1. Introduction

Nonylphenol (NP) is a ubiquitous synthetic xenoestrogen (compound that is somewhat similar to the estrogen hormone in structure and mimic its function, as it has the ability to bind to estrogen receptors) that is widely used in industrial and commercial applications **[1,2]**. NP consists of a phenol ring bearing a side chain of 9 carbon atoms. NP is a precursor of the important non-ionic surfactants nonylphenol ethoxylates (NPEOs), which are used in detergents, paints, personal care products, plastics, cosmetics, emulsifiers, resins, wetting and dispersing agents, lubricants, polystyrene tubes, paper, textile, herbicides and pesticide formulations in industry, agriculture, food and household sectors. NP also reacted to form tris(4-nonyl-phenyl) phosphite (TNPP) an antioxidant used as a stabilizer in plastic food packaging**[1,3,4]**. It is released and accumulated in the environment, commonly found in soil, water and air, and can also enter the food chain from the polluted environment. So, NP contamination has been found both in foods (including fish, meat and vegetables) and the plastics used in food processing and packing **[5,6].**Therefore, Human exposure to NP occurs through its use in pesticides, plastic food packaging, and its presence in household products such as detergents and cosmetics, human beings are inevitably exposed to NP via ingestion, inhalation and dermal routes throughout their whole lifetimes, with the food intake as a major route via the food chain from bioaccumulation in the polluted environment **[6,7,8].** It has gained medical attention due to its potential negative effects on human health and the environment. NP is well recognized for its ability to disrupt endocrine properties owing to its similarity to estrogen. Numerous health issues, such as developmental defects, reproductive diseases, liver and kidney impairments and carcinogenic effects, have been linked to exposure to NP **[9]**. We designed this study to examine the toxic effects of NP on the levels of reduced glutathione (GSH) as an essential cellular antioxidant and sulfhydryl (R-SH)

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groups as R-SH groups are essential for maintaining protein structure and function. Additionally, we investigated the possible protective effects against NP-induced oxidative damage offered by phytochemical substances such as naringenin (NG) and resveratrol (RES). NG and RES, both well characterized antioxidants, were tested with NP to evaluate their ability to alleviate NP induced oxidative stress **[10, 11, 12, 13].** Further, we evaluated the functional effects of NP exposure on liver function, lipid profile parameters. We extended our investigations to study NP binding with estrogen receptor alpha by molecular docking analysis to gain better insights into the molecular mechanisms behind NP-induced estradiol disruption and the possible protective effects of NG and RES. Overall, our work underscores the intricate impacts of NP exposure on oxidative stress, antioxidant defense mechanisms, liver function, and estradiol disruption and the significance of taking proactive measures to reduce NP-related health concerns.

Material and methods

2.1. Chemicals

All chemicalsused in this study were analytical grade and obtained from Piochem and Loba Chemie for laboratory chemicals and were purchased from the importing company Afak for chemicals and medical requisities (Cairo, Egypt).
Natural sources of Resveratrol (RES) are grapes (*Vitis vinifera*), blueberries (*Vaccinium corymbosum*), peanut (*Arachis hypogaea*) and pistachio nut (*Pistacia vera*).Natural sources of Naringenin (NG) are grapefruit (*Citrus paradisi*), sweet orange (*Citrus sinensis*), and tomato (*Lycopersicon esculentum*) and rosemary (*Rosmarinus officinalis*)

2.2. Biochemical parameters kits

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), Total protein (TP), Cholesterol, High density lipoprotein cholesterol (HDL-C), Triglycerides, Urea and hemoglobin kits were obtained from Bio diagnostic company, Dokki, Giza, Egypt.

2.3. Animals

Thirty (30) adult male albino rats of similar weights and ages about (120 g) were chosen as an animal model for the present study. Rats were obtained from Animal Health Institute, Dokki, Giza, Egypt. Rats were maintained on a balanced standard diet and water ad-libitum. Animals were kept for one week, fed on basal diet, as an adaptation period before starting the experiment.

2.4. Experimental design

After a housing-acclimatization period of one week as previously mentioned, Rats were randomly divided into 5 groups (6 rats each) with average weight (150±5 g). Experimental groups were as follows:
Group 1(NC): Normal Control group: only fed on normal diet. Group 2 (DMSO): animals were treated only with Dimethyl sulfoxide (DMSO) and served as DMSO control group. Group 3 (NP): Nonylphenol (NP) Group: animals were treated with NP. Group 4 (NP+RES): Nonylphenol and Resveratrol (RES) Group: animals were treated with NP and RES. Group 5 (NP+NG): Nonylphenol and Naringenin (NG) group: animals were treated with NP and NG.

2.5. Administration and doses of NP and phytochemicals:

Nonylphenol was administered to rats orally by gavage syringe in a dose of 5mg/kg body weight. Resveratrol and Naringenin were administered to rats orally by gavage syringe in a dose of 10 mg/kg body weight. The doses of naringenin and resveratrol were selected based on previously reported protective and antioxidant properties of this compound in rats **[14, 15, 16].** Nonylphenol, Resveratrol, Naringenin and DMSO were gavaged daily from first day to the end of the experiment (90 days). DMSO is used as a vehicle for NP, RES and NG, so a group for DMSO control was designed. Resveratrol and Naringenin treatments were gavaged to rats before NP.

2.6. Preparation of serum, plasma and tissues homogenate:

Blood samples were obtained by sacrificing rats, blood samples were collected from rats of each group at the end of the experiment (90 days). Rats were anaesthetized with ether by placing the rat in an anesthetic box fumed with ether vapor using a piece of cotton wetted with ether put it on the box base then animals were sacrificed by cervical decapitation. Blood samples were collected in non-heparinized and heparinized tubes. Non-heparinized blood was allowed to clot and centrifuged for serum separation. For plasma, the tubes contained heparin as anticoagulant. After 10 min of centrifugation at 3000 rpm, plasma and buffy coat were separated. The isolated red cells were washed three times with physiological saline (0.9 %). Plasma and serum were tightly kept in sealed Eppendorf tubes at -18ºC until it was processed for biochemical assays. The tissues (liver and kidneys) were dissected out and washed using ice cold saline solution. A known amount of tissue was weighed and homogenized in an appropriate buffer for the estimation of various biochemical parameters.

2.7. Biochemical assays:

Reduced glutathione (GSH) was determined according to the method described by Beutler et al., (1963) **[17]**. Total thiols groups (R-SH) were assayed according to the method of Sedlak and Lindsay, (1968); Koster et al., (1986) **[18, 19].** Levels of ALT, AST, ALP, LDH, Total cholesterol, HDL, triglycerides, Total protein, Hemoglobin and Urea, in samples were evaluated using kits purchased from (Bio diagnostic company, Dokki, Giza, Egypt) and the standard technique given along with the kits was followed.

2.8. Molecular Docking

2.8.1. Ligand Preparation:

Ligand structure was drawn using Chem Draw 21.0.0 (PerkinElmer, Waltham, MA, USA) and saved as SDF files. Ligand energy was minimized by MM2 calculation, logP was calculated, and the structures of the ligands were converted to pdb file format using Chem3D 21.0.0. Nonpolarhydrogen atoms were deleted, Gasteiger charges were calculated, torsion root was detected, and the structures were saved as pdbqt file format using AutoDockTools-1.5.6.

2.8.2. Target Protein Preparation

The target protein, estrogen receptor alpha (ERA) structure encoding 6CBZ **[20]**, was downloaded from Protein Data Bank "www.rcsb.org/. (accessed on 13, 20 November 2023)". The target protein structure was prepared by deleting water and solvent molecules and ligands, adding polar hydrogen atoms, calculating Kollman charges, and saving the structure of target protein as pdbqt using AutoDockTools-1.5.6 (The Scripps Research Institute, San Diego, CA, USA).

2.8.3. Molecular Docking Procedures

The molecular interactions between the ligand binding domain of Estrogen receptor alpha and Estrogen or NP or RES or NG, were determined using AutoDock Vina 1.2.0 (The Scripps Research Institute, San Diego, CA, USA)**[21]**.A random seed number was used and the exhaustiveness function was 8. A 14 × 5 × 35 Å grid box with 82 × 64 × 54 grid point spacing of 0.37 Å for ERA, was used for docking the ligand into the receptor binding domain.

2.8.4. Analysis and Visualization of Protein–Ligand Interactions

The conformers of each ligand were separated using vina_split command. The conformer with the highest affinity and in silico interactions between the ligands and ERA was analyzed and visualized using Discovery Studio-21 software (Dassault Systems BIOVIA San Diego, CA, USA).

2.9. Statistical analysis:

All results calculated were presented as means ± SD from six replicates and subjected to one way analysis of ANOVA test. The means of different treatments were compared using Duncan's multiple range test (L.S.D) at $p \leq 0.05$. Statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics, version 20) **[22]**.

3. Results and discussion:

3.1. Effects of NP, RES and NG on reduced glutathione (GSH) levels in plasma, liver and kidney tissues of the experimental rats

Toxic effects of NP on reduced glutathione (GSH) levels in plasma, liver and kidney tissues (Table 1) of experimental rats were evidently noted, as evidenced by significant reductions compared to the control and DMSO-treated groups. In the blood, NP exposure led to a profound decrease in GSH levels from 31.68±0.63 mg/dl in the control group to 17.43±0.52 mg/dl in the NP treated group ($P < 0.05$). Similarly, in the liver, GSH levels decreased significantly from 151.77±3.05 mg/100 mg tissue in the control group to 83.49±2.52 mg/100 mg tissue in the NP treated group ($P < 0.05$). In the kidney, NP exposure resulted in a significant decrease in GSH levels from 103.97±2.1 mg/100 mg tissue in the control group to 57.19±1.73 mg/100 mg tissue in the NP treated group ($P < 0.05$).The abovementioned results are in agreement with**[23].**

However, the co-administration of either RES or NG effectively reversed these effects, demonstrating GSH levels comparable to those of the control and DMSO-treated groups. In the liver, GSH levels in the NP + RES and NP + NG groups were 154.52±4.63 mg/100 mg tissue and 148.34±3.27 mg/100 mg tissue, respectively, while in the kidney, GSH levels were raised to 102.84±3.17 mg/100 mg tissue and 99.3±2.24 mg/100 mg tissue, respectively. The blood levels of GSH were enhanced to 32.25±0.96 in NP+RES treated group and 31.17±0.68 in NP + NG treated group as shown in table 1. Our results of RES and NG treatments that displayed a restoration of GSH levels to normal values are consistent with **[24, 25]**

Table 1. Effect of NP exposure on blood, liver and kidney GSH in rats treated with RES or NG

parameter	NC	DMSO	NP	NP+ RES	NP+ NG
Blood GSH (mg/dl)	**31.68 ± 0.63**	**30.67 ± 0.84**	**17.43* ± 0.52**	**32.25 ± 0.96**	**31.17 ± 0.68**
liver GSH (mg/100 mg tissue)	**151.77 ± 3.05**	**149.91 ± 4.04**	**83.49* ± 2.52**	**154.52 ± 4.63**	**148.34 ± 3.27**
kidney GSH (mg/100 mg tissue)	**103.97 ± 2.1**	**100.63 ± 2.76**	**57.19* ± 1.73**	**102.84 ± 3.17**	**99.3 ± 2.24**

Each value represents the mean ±SE.*Significant change between groups and control($P < 0.05$).

3.2. Effects of NP, RES and NG on sulfhydryl (thiol) (R-SH) groups in plasma and experimental rat tissues

Data in Table (2) showed that Nonylphenol exposure led to statistically significant reductions in sulfhydryl (thiol) (R-SH) groups across plasma (42% reduction level), liver (33.4% reduction level), and kidney tissues (25.76% reduction level) in experimental rats with ($P < 0.05$) respectively.

Table 2: Effect of NP exposure on blood, liver and kidney R-SH groups in rats treated with RES or NG

parameter	NC	DMSO	NP	NP+ RES	NP+ NG
plasma R-SH groups (µmol/L)	**479.9 ±11.35**	**481.62 ±12.65**	**297.39* ± 6.9**	**497.73 ± 10.1**	**490.28 ± 8.47**
liver R-SH (nmoles/mg tissue protein)	**101.58 ± 2.73**	**102.39 ± 2.93**	**67.97* ± 2.12**	**108.35 ± 3.57**	**105.28 ± 2.43**
kidney R-SH(nmoles/mg tissue protein)	**81.35 ± 1.57**	**81.65 ± 2.14**	**60.43* ± 1.32**	**84.38 ± 1.69**	**83.11 ± 2.97**

Each value represents the mean ±SE. * Significant change between groups and control ($P < 0.05$).

However, the co-administration of resveratrol or naringenin effectively reversed this effect, restoring R-SH levels to those comparable with control and DMSO treated groups, with no statistically significant differences detected among the tested tissues ($P < 0.05$) as displayed in table (2).

3.3. Effect of nonylphenol (NP) on liver function in experimental rats

The most common indices of liver function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) exhibited significant increases following exposure to NP ($p < 0.05$) respectively (Table 3). The levels of AST exhibited a marked increase following NP exposure, with values elevated from 58.25±1.1 U/L in the control group to 136.62±1.99 U/L in the NP exposed group. Similarly, the levels of ALT were significantly increased in NP treated rats, escalating from 27.74±0.89 U/L in the control to 98.45±2.38 U/L in the NP treated group. Besides, ALP levels showed a profound increase in response to NP treatment, rising from 75.54±1.27 U/L in the control to 181.77±2.23 U/L in the NP treated group. Furthermore, LDH levels displayed a remarkable change in response to NP treatment in experimental rats. LDH levels of 27.21±0.35 µKat/L were recorded in control group, the levels in the DMSO treated group remained comparable at 27±0.67 µKat/L. Conversely, the exposure to NP led to a statistically significant increase in LDH levels, escalating to 32.09±0.4 µKat/L ($P < 0.05$).

Conversely, total protein levels were notably decreased compared to control and DMSO-only treated groups (Table 3), with values dropping from 6.52±0.12 g/dl in the control to 5.41±0.19 g/dl in the NP exposed group. All together indicating disturbances in liver function. These findings align with several previous studies investigating the impact of NP on liver and

kidney function in animal models. Specifically, Shi et al. 2021 **[23]** demonstrated that NP exposure led to elevated levels of liver enzymes and kidney function parameters, including AST, ALT, ALP and urea, indicative of hepatocellular and nephrotoxic damage

Similarly, studies by Korkmaz et al. 2010 **[26]** reported significant increases in serum liver enzyme levels following NP exposure in rats, consistent with our observations.However, treatment with either RES or NG effectively reversed these effects, with values returning to near-baseline levels and displaying non-significant differences compared to the control or DMSO-treated groups ($P < 0.05$) as reported in table (3). These results are in coincided with many previous studies that reported a hepatoprotective effect of RES and NG**[30, 31].**

Table 3: Effect of NP exposure on AST, ALT, ALP, LDH and total protein in rats treated with RES or NG

parameter	NC	DMSO	NP	NP + RES	NP + NG
AST (U/L)	**58.25 ± 1.1**	**58.62 ± 1.14**	**136.62* ±1.99**	**62.52 ± 2.6**	**63.03 ±2.16**
ALT (U/L)	**27.74 ±0.89**	**28.33 ± 1.28**	**98.45* ± 2.38**	**28.55 ±1.27**	**27.99 ± .58**
ALP (U/L)	**75.54 ±1.27**	**76.32 ± 1.55**	**181.77* ± 2.23**	**77.55 ±1.94**	**79.76 ±1.87**
LDH (µKat/L)	**27.21 ±0.35**	**27 ± 0.67**	**32.09* ±0.4**	**26.83 ± 0.7**	**28.13 ±0.48**
Total protein (g/dl)	**6.52 ±0.12**	**6.51 ± 0.13**	**5.41* ± 0.19**	**6.43 ±0.25**	**6.64 ± 0.22**

Each value represents the mean ±SE. * Significant change between groups and control ($P < 0.05$).

3.4. Impact of NP, RES and NG on biochemical markers reflecting renal function and blood composition

The levels of serum urea used as one of the common metabolic indexes of kidney function in the clinical studies**[27]**. It was noticed that the content of serum urea was sharply increased by the exposure to NP rising from 40.5±0.82 mg/dl in the control group to 50.92±1.2 mg/dl in the NP-treated group, ($p < 0.05$). Administration of resveratrol (RES) or naringenin (NG) successfully counteracted these effects, leading to insignificantly different levels compared to those observed in the control or DMSO-treated groups ($p < 0.05$) as presented in Table (4).

On the contrary, hemoglobin, an essential marker of blood composition, exhibited a decrease following exposure to NP (Table 4), with values declining from 14.92±0.24 g/dl in the control group to 13.6±0.39 g/dl in the NP-treated group. Nevertheless, the alteration was not statistically significant.Regarding the Hb levels, Korkmaz et al., 2010 **[26]** investigations agree with our findings where NP administration leads to no-significant differences compared to the control group in the studied rats. Hb values were subsequently normalized upon co-treatment with either RES or NG (with means 14.23±0.48 g/dl and 13.9±0.47 respectively) as shown in table 4.

Table 4: Effect of NP exposure on serum urea and hemoglobin in rats treated with RES or NG

parameter	NC	DMSO	NP	NP + RES	NP + NG
Serum urea (mg/dl)	**40.5 ±0.82**	**40.27 ± 0.83**	**50.92* ± 1.2**	**39.13 ±0.73**	**38.38 ± 0.9**
Hemoglobin (g/dl)	**14.92 ±0.24**	**15.1 ± 0.32**	**13.6 ± 0.39**	**14.23 ±0.48**	**13.9 ±0.47**

Each value represents the mean ±SE. * Significant change between groups and control ($P < 0.05$).

3.5. Impact of NP, RES and NG on lipid profile parameters

Data in Table (5) displayed that All lipid profile parameters including total cholesterol (TC), LDL (low-density lipoprotein), VLDL (very low-density lipoprotein), and triglycerides exhibited significant elevation following NP treatment, except for HDL (high-density lipoprotein), which significantly reduced compared to both control and DMSO-treated groups.

Particularly, plasma total cholesterol levels exhibited a dramatic increase upon NP exposure, escalating from 83.92±2.02 mg/dl in the control group to 160.64±3.57 mg/dl in the NP-exposed rats ($P < 0.05$). LDL measurements displayed a remarkable increase in NP treated rats, rising from 31.32±0.91 mg/dl in the control to 98.90±2.26 mg/dl in the NP subjected group ($P < 0.05$). Besides, VLDL levels showed a marked escalation following NP drug administration, soaring from 14.83±0.32 mg/dl in the control to 36.04±0.88 mg/dl in the group received NP treatment ($P < 0.05$).

Table 5: Effect of NP exposure on TC, HDL-C, LDL-C, VLDL-C and triglycerides in rats treated with RES or NG

parameter	NC	DMSO	NP	NP + RES	NP + NG
TC (mg/dl)	**83.92 ±2.02**	**82.88 ± 2.42**	**160.64* ± 3.57**	**92.57 ±2.93**	**88.81 ±3.13**
HDL-C (mg/dl)	**37.76 ±1.16**	**36.87 ± 1.06**	**25.68* ± 0.91**	**38.47 ±1.31**	**39.82 ±1.47**
LDL-C (mg/dl)	**31.32 ± 1.0**	**30.93 ± 1.53**	**98.90* ± 3.47**	**38.93 ±1.65**	**33.1 ± 1.9**
VLDL-C (mg/dl)	**14.83 ±0.32**	**15.08 ± 0.39**	**36.04* ± 0.88**	**15.16 ±0.37**	**15.88 ±0.22**
Triglycerides (mg/dl)	**74.16 ±1.64**	**75.42 ±1.96**	**180.22* ± 4.44**	**75.82 ±1.87**	**79.41 ± 1.1**

Each value represents the mean ±SE. * Significant change between groups and control ($P < 0.05$).

Furthermore, triglycerides measurements displayed a statistically significant increase in response to NP treatment, with values escalated from 74.16±1.64 mg/dl in the control to 180.22±4.44 mg/dl in the group received NP treatment ($P < 0.05$). Regarding HDL levels, a substantial decrease was recorded in response to NP treatment, dropping from 37.76±1.16 mg/dl in the control to 25.68±0.91 mg/dl in the NP exposed rats ($P < 0.05$). Our results regarding the dysregulation of lipid profile parameters following NP exposure are supported by previous research. For example, Korkmaz et al. 2010 **[26]** reported elevated levels of total cholesterol and triglycerides levels, in rats exposed to NP.

All lipid profile parameters which showed significant increases with NP treatment, were effectively reversed by administration of either RES or NG. Conversely, HDL levels which exhibited a significant decrease with NP treatment, were effectively restored to normal levels by administration of RES or NG ($P < 0.05$) as found in Table (5).These results are in acceptance with **[28, 29].**

3.6. Interaction between estrogen receptor alpha and various ligands, including Estradiol (E2), Nonylphenol, Resveratrol and Naringenin

Regarding the interaction with estrogen receptor Alpha (Erα) (Table 6), Estradiol (E2) exhibits a binding affinity of -7.3 kcal/mole, forming hydrogen bonds with GLY 420 residue and Alkyl and Pi-alkyl bonds with residues such as ILE 424, LYS 520, and ARG 548 which is contributing by two interactions as shown in Table (6) and Figure (1).NP interacts with estrogen receptor alpha (Table 6 and figure 2) with a binding affinity of -6.8 kcal/mole, forming hydrogen bonds with LEY 346 residue, and Pi-Pi T shaped bond with PHE 404 residue. Additionally, it engages in Alkyl and Pi-alkyl bonds with residues including LEU 391, LEU 387, LEU 384, ALA 350, LEU 525, and LEU 346 as displayed in Table (6) and Figure (2).

Resveratrol has an affinity of -7.6 kcal/mole with estrogen receptor Alpha (Table 6). Four bonds shared in this interaction, hydrogen bond with HIS 524 and ARG 394 interacting residues, Pi-Sigma bond with LEU 387 residue, Pi-Pi T-shaped bond with LEU 387 residue, and Pi-alkyl bond with residues such as PHE 404, LEU 525, LEU 391, and ALA 350 as found Table (6) and Figure (3).

Naringenin interacts with estrogen receptor alpha (Table 6 and Figure 4) with -7.6 kcal/mole binding affinity. Three bonds share in this interaction namely, hydrogen bond with the GLU 523 residue contributing with three interactions, Pi-cation bond with LYS 520 interacting residue and Pi-sulfur bond with MET 427 interacting residue as shown in Table (6).

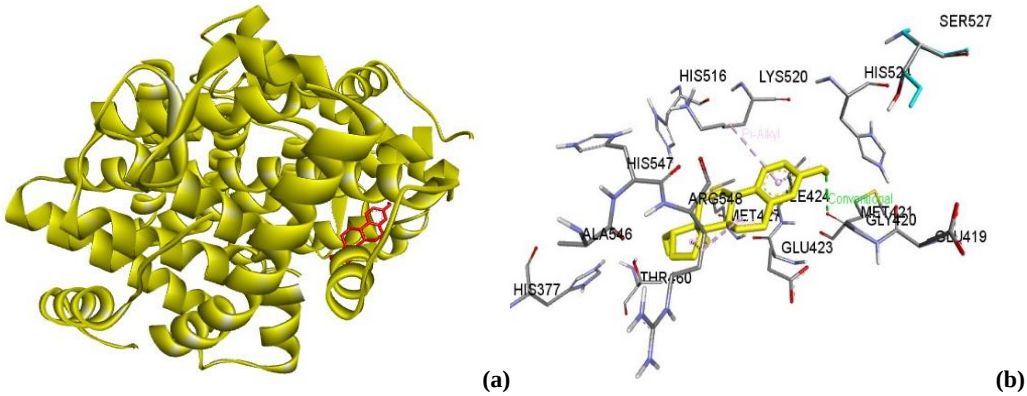


Fig. 1: Interaction between ERα and Estradiol, (a)The conformation of estradiol in the binding domain of ERα, the docked estradiol in red sticks and the ERα in cartoon; (b) 3D interactions between the docked estradiol and residues in binding domain of ERα. Hydrogen bonds in green dashes and other bonds in different colors as illustrated previously in Table (6), the interacted residues in gray sticks and estradiol in yellow sticks.

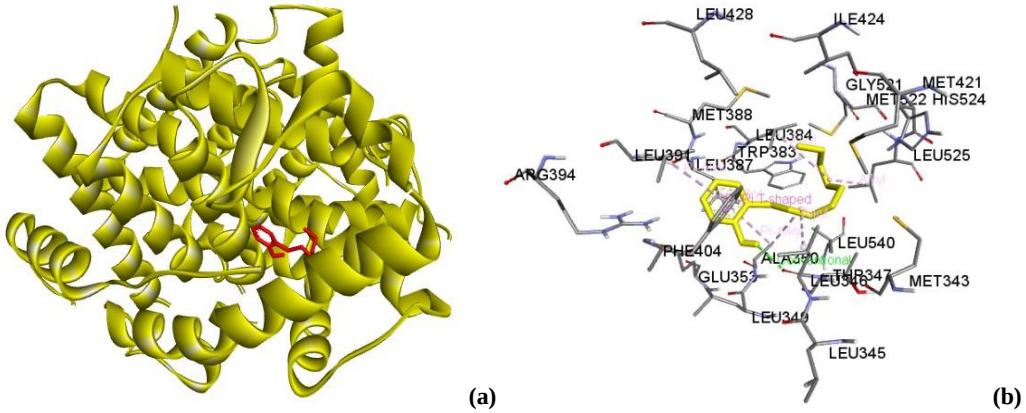


Fig. 2: Interaction between ERα and NP, (a)The conformation of NP in the binding domain of ERα, the docked NP in red sticks and the ERα in cartoon; (b) 3D interactions between the docked NP and residues in binding domain of ERα. Hydrogen bonds in green dashes and other bonds in different colors as illustrated previously in Table (6), the interacted residues in gray sticks and NP in yellow sticks.

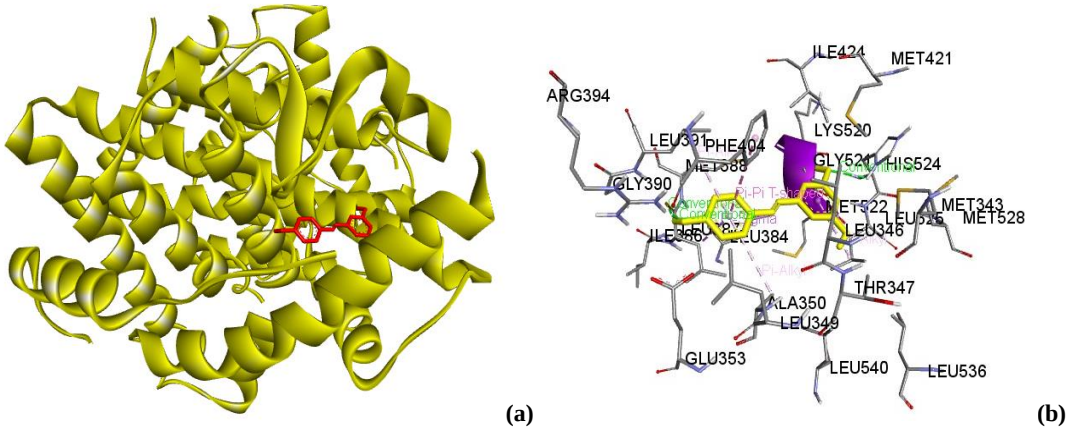


Fig. 3: Interaction between ERα and RES, (a) The conformation of RES in the binding domain of ERα, the docked RES in red sticks and the ERα in cartoon; (b) 3D interactions between the docked RES and residues in binding domain of ERα. Hydrogen bonds in green dashes and other bonds in different colors as illustrated previously in Table (6), the interacted residues in gray sticks and RES in yellow sticks.

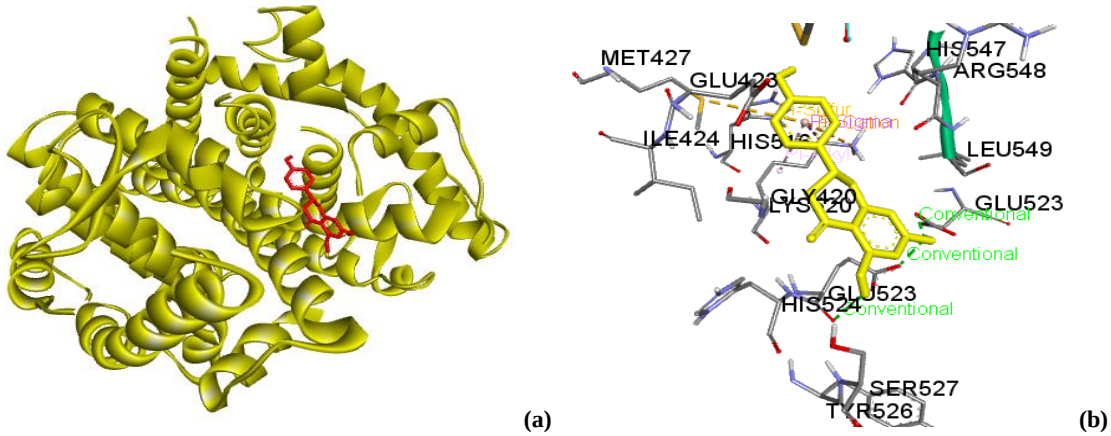
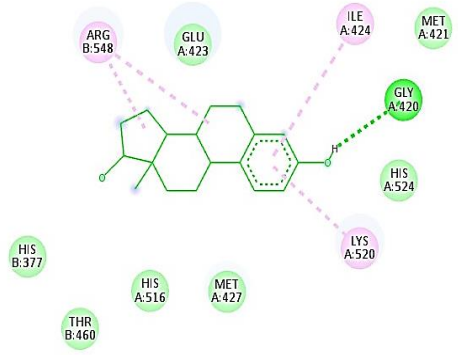
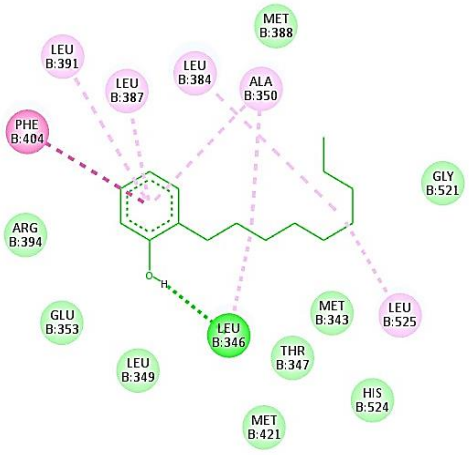
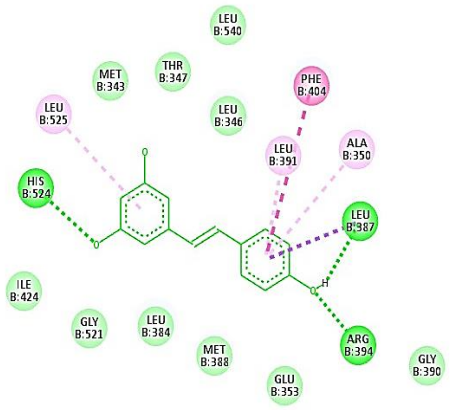
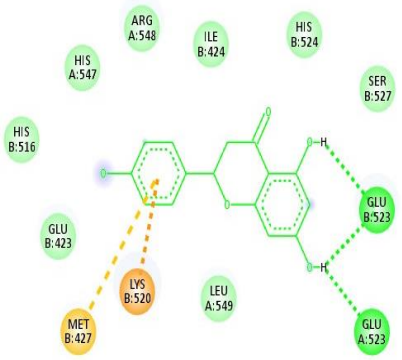
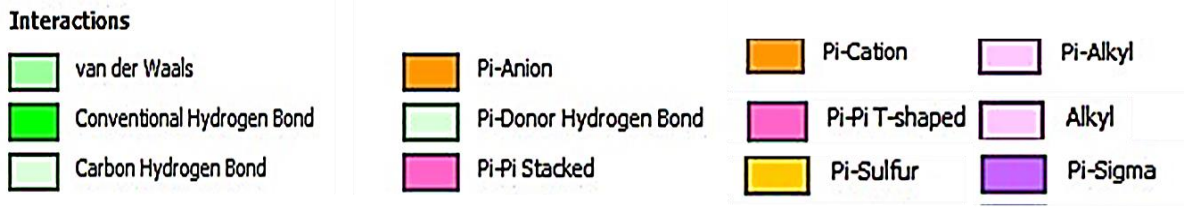


Fig. 4: Interaction between ERα and NG, (a)The conformation of NG in the binding domain of ERα, the docked NG in red sticks and the ERα in cartoon; (b) 3D interactions between the docked NG and residues in binding domain of ERα. Hydrogen bonds in green dashes and other bonds in different colors as illustrated previously in Table (6), the interacted residues in gray sticks and NG in yellow sticks.

Table 6: Interaction between estrogen receptor alpha and various ligands, including Estradiol (E2), NP, Resveratrol, and Naringenin.

Ligand	2D Interactions	Type of bonds	Interacted Residues	Affinity (Kcal/mol)
Estradiol (E2)		Hydrogen bond	GLY 420	-7.3
		Alkyl and Pi-Alkyl	ARG 548 (2) ILE 424 LYS 520	
Nonylphenol (NP)		Hydrogen bond	LEU 346	-6.8
		Pi-Pi T-shaped	PHE 404	
		Alkyl and Pi-Alkyl	LEU 391 LEU 387 LEU 384 ALA 350 LEU 525 LEU 346	

Resveratrol (RES)		Hydrogen bond Pi-Sigma Pi-Pi T-shaped Pi-Alkyl	HIS 524 ARG 394 LEU 387 LEU 387 PHE 404 LEU 525 LEU 391 ALA 350	-7.6
Naringenin (NG)		Hydrogen bond Pi-Cation Pi-Sulfur	GLU 523 (3) LYS 520 MET 427	-7.6



3.7. Discussion

This study investigated the toxic effects of Nonylphenol on the antioxidant capacity, cellular and estradiol disruption, underscoring the potential protective effects of phytochemical compounds such as resveratrol and naringenin. Findings revealed significant alterations in various parameters in response to NP exposure, indicating a significant impact on systemic, liver, and kidney oxidative status. Namely, our study demonstrated a significant decrease in reduced glutathione (GSH) levels in plasma, liver, and kidney tissues following NP exposure. Reduced glutathione is a crucial antioxidant that is involved in cellular detoxification and defense against oxidative stress. Similarly, we observed significant reductions in R-SH groups across plasma, liver, and kidney tissues following NP exposure, indicating enhanced oxidative stress as sulfhydryl groups are essential for maintaining protein structure and function, and their depletion indicates oxidative damage. However, co-administration of RES or NG effectively restored GSH levels to normal levels, mitigated the effect of NP, restoring R-SH groups levels to near-baseline levels, highlighting their protective effects against NP-induced oxidative stress. The liver function, a critical determinant of overall health, was significantly impacted by exposure to nonylphenol (NP) in our experimental rat model. We observed notable alterations in several key liver enzymes and biochemical markers indicative of liver health.

AST, ALP, ALT, and LDH, essential indicators of liver function, exhibited substantial increases following NP exposure compared to the control and DMSO-treated groups. Specifically, NP exposure led to marked elevations in AST and ALT levels, suggesting liver cell damage and leakage of these enzymes into the bloodstream. Similarly, ALP levels showed a profound increase,

indicating liver damage or potential bile duct obstruction. LDH levels also displayed a significant change in response to NP exposure, further reflecting liver cell damage. These findings collectively point towards NP-induced hepatotoxicity and disruption of liver function.Furthermore, NP exposure resulted in a notable decrease in total protein levels compared to control and DMSO-treated groups. This decline in total protein levels suggests impaired liver protein synthesis, further exacerbating the disturbances in liver function induced by NP. In addition to its effects on liver function, NP exposure also had significant implications for renal function and blood composition. Elevated serum urea levels, indicative of impaired kidney function, were observed following NP exposure, underscoring its nephrotoxic potential.

Also, NP exposure led to alterations in blood composition, as evidenced by changes in hemoglobin levels. Although not statistically significant, NP-treated rats exhibited decreased hemoglobin levels compared to control groups, suggesting potential hematological disturbances. Co-treatment with RES or NG normalized liver enzymes, urea and hemoglobin levels, indicating their protective effects against NP-induced hematological alterations. Moreover, NP exposure resulted in dysregulation of lipid profile parameters, including significant increases in total cholesterol, LDL, VLDL, and triglyceride levels, accompanied by a decrease in HDL levels. These alterations in lipid profile parameters signify disturbances in lipid metabolism and may predispose individuals to cardiovascular complications. Similarly, co-administration of RES or NG effectively reversed these effects, restoring lipid profile parameters to normal levels.These findings align with several previous studies investigating the impact of NP on liver and kidney function in animal models and in vitro systems. Specifically, Shi et al. 2021**[23]**) demonstrated that NP exposure led to elevated levels of liver enzymes and kidney function parameters, including AST, ALT, ALP and urea, indicative of hepatocellular and nephrotoxic damage. Similarly, studies by **Korkmaz et al. 2010[26]** reported significant increases in serum liver enzyme levels following NP exposure in rats, consistent with our observations. Regarding the Hb levels, **Korkmaz et al., 2010[26]** investigations agree with our findings where NP administration leads to no-significant differences compared to the control group in the studied rats.Moreover, our study adds to the existing literature by demonstrating the protective effects of resveratrol and naringenin against NP-induced hepatotoxicity, nephrotoxicityanddyslipidemia.

To our knowledge, no studies have specifically investigated the therapeutic potential of RES and NG in mitigating NP-induced damage. However, there is growing evidence supporting their hepatoprotective, lipid-lowering effects and favorable endocrine effect in various experimental models**[28-34].** Several studies investigated the effects of NP exposure on endocrine function in male rats and observed a significant decrease in serum testosterone levels and an increase in estradiollevels**[32- 35].** These findings are consistent with our results of molecular docking study which revealed that NP has the ability to bind with estrogen receptors specifically ER alpha with affinity degree -6.8 compared to -7.3 in estradiol binding with the same receptor, indicating that NP exposure induces hormonal imbalances favoring estrogenic activity over androgenic activity as reported in another study carried out by our team. Furthermore, our study demonstrates that the binding affinity of RES and NG with ERα was -7.6 compared to -7.3 in estradiol binding with the same receptor, which indicate to the high affinity of these compounds in binding with Erα. These findings may illustrate the potential therapeutic efficacy of resveratrol (RES) and naringenin (NG) in mitigating NP-induced endocrine and reproductive dysfunction and suggest that RES and NG have protective effects against NP-induced endocrine disruption and reproductive toxicity.

Toxicity mechanisms of NP:

Exposure to NP increase ROS synthesis more specifically superoxide anions and H2O2 levels due to a robust increase in NOX4 (a ROS-generating NADPH oxidase) at both mRNA and protein levels **[36]** and decrease the expression of phase 2 metabolizing enzymes (GST and UGT (uridine 5-diphospho-glucuronosyltransferase)) which play an important role in the detoxification of exogenous substances, carcinogens and oxidative stress products. So that NP induce disruption of redox homeostasis and elevate oxidative stress. Further, excessive ROS activate the Keap1-Nrf2 (Kelch- like ECH- associated protein 1- nuclear factor E2- related factor) signaling pathway, that is essential for resisting oxidative stress and is also an upstream pathway for phase II drug metabolizing enzymes**[37,38].** NP exposure enhanced Keap1 expression but reduced the expression of cytoplasmic and nuclear Nrf2 that responsible for the transcriptional activity of antioxidant enzymes, leading to increase in oxidative stress, cell apoptosis, and then decreasing cell viability as well as protein expression **[39, 40].** As a result of high oxidative stress state, NP induce an increase in lipid peroxidation (MDA level), indicating heightened membrane damage. In addition, NP cause an escalation of srebf1 (Gene encoding sterol regulatory elementbinding factor 1) expression (an upstream regulator of fatty acid synthase (fasn) transcription) that induce a robust increase in fasn, a multi-component enzyme that catalyses the synthesis of palmitate using acetyl CoA and malonyl CoA as the substrate. Congruent with an increase in transcript abundance of ampka (Adenosine-monophosphate-activated protein kinase alpha), NP exposure in vivo triggers a significant increase in SIRT1 (Sirtuin 1) expression. More importantly, NP exposure induces a significant attenuation in pgc1α (Peroxisome proliferator-activated receptor-gamma coactivator one alpha) expression, a prime regulator of fatty acid oxidation/lipolysis, collectively indicating altered lipid metabolism and homeostasis leading to lipid accumulation. Importantly, immunoblot data revealed that exposure to NP in vivo induces a sharp increase in Erα (estrogen receptor alpha) immunoreactive protein and abrogates hepatic Erβ expression. Moreover, an increase in p38 MAPK (p38 Mitogen activated

protein kinase) phosphorylation (activation) revealed elevated stress response and participation of ER subtypes in mediating the actions of NP in rats **[36].**

Potential Therapeutic mechanisms of RES and NG:

RES and NG are considered as nuclear factor E2- related factor (NRF2) activators (figures 5, 6 and 7), whereas enhancing NRF2 activity increases the expression of antioxidant enzymes and the defense against oxidative stress. NRF2 was found to be a transcription activator of NADPH Quinone Dehydrogenase 1 (NQO1 gene) that bound to the antioxidant response element (ARE) in the promoter. NRF2–ARE signalling has a central role in the regulation of antioxidant genes expression **[41, 42].**

NRF2–ARE signalling regulates the basal and inducible expression of more than 200 genes that encode proteins involved in antioxidant defence, detoxification, apoptosis, DNA repair, removal of oxidized protein by the proteasome, inflammation and other processes**[43, 44]**, deficiency of NRF2 signalling suppresses the induction of target antioxidant enzymes in response to oxidative stress, increases susceptibility to oxidative damage **[45]** and accelerates the inflammatory response **[46].** Under basal conditions, most NRF2 protein binds to Kelch- like ECH- associated protein 1 (KEAP1) and/or β- transducin repeat- containing protein (βTrCP) and is rapidly degraded by 26S proteasome after ubiquitylation. KEAP1 is an adaptor for Cullin 3- containing ubiquitin ligase E3 complex **[47]** and βTrCP is a substrate receptor for Cul1- based ubiquitin ligase **[48].** In response to oxidative stimuli, KEAP1 is oxidatively modified and loses the capacity to present NRF2 for degradation.

Simultaneously, oxidative inhibition of glycogen synthase kinase 3β (GSK3β)- mediated NRF2 phosphorylation at the Neh6domain stops the interaction of NRF2 and βTrCP. NRF2 can also be activated through p62- mediated autophagic degradation of KEAP1 **[49].** With the activation of these pathways, NRF2, both dissociated from KEAP1 and newly synthesized, escapes from degradation and is then translocated into the nucleus where it forms heterodimers with small Maf (musculoaponeurotic fibrosarcoma) or Jun family proteins (transcription factors), binds to EpRE (Electrophile responsive element) in the promoter and increases transcription of target genes. G. Bhattarai et al. 2016 **[50]** indicated that RES could potentiating the activity of Nrf2/HO-1, resulting in a decline in the ROS content. It was also found that RES or NG activates the SIRT1/AMPK and rasing the antioxidant defenses by the Nrf2 pathway. RES potentiates the expression of Nrf2 through dissociation of Nrf2-Keap1 binding and increases the translocation of Nrf2 into the nucleus. RES dissociates the bindings between Nrf2-Keap1 through increase the interaction between p62-Nrf2. RES also activates Nrf2/ARE via stimulation p38 MAPK and SIRT1/FOXO1 (forkhead box O transcription factors)signaling. RES elevates the Nrf2 expression through suppression of the inhibitory signaling**[42]**.

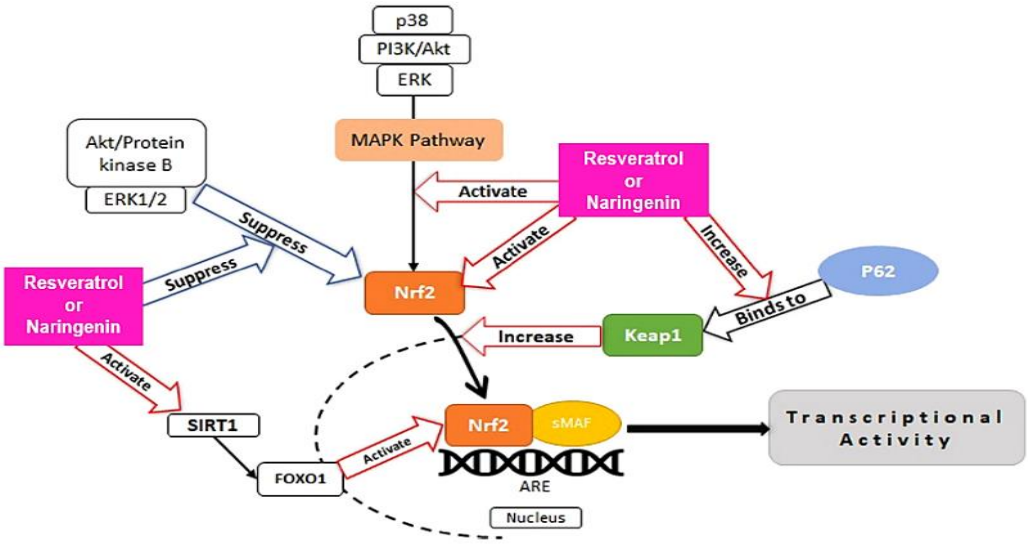


Fig. 5: Mechanisms by which RES and NG activates the transcriptional activity of Nrf2 **(Tahereh et al., 2020)[42]**.

As shown in Figure (5), the main mechanism by which Res or NG enhances the Nrf2 expression is the disruption of Nrf2-Keap1 binding and increases the translocation of Nrf2 into the nucleus. RES or NG also dissociates the bindings between Nrf2-Keap1 through increase the interaction between p62-Nrf2. Res or NG also activates Nrf2/ARE through stimulation p38 MAPK and SIRT1/FOXO1 pathways. Res or NG can increase the expression of Nrf2 through suppression of the inhibitory signaling pathway such as Akt/ERK1/2. (serine-threonine protein kinase (AKT1)/extracellular signal-regulated kinases (ERKs) (ERK2)**[42, 51, 52, 53]**

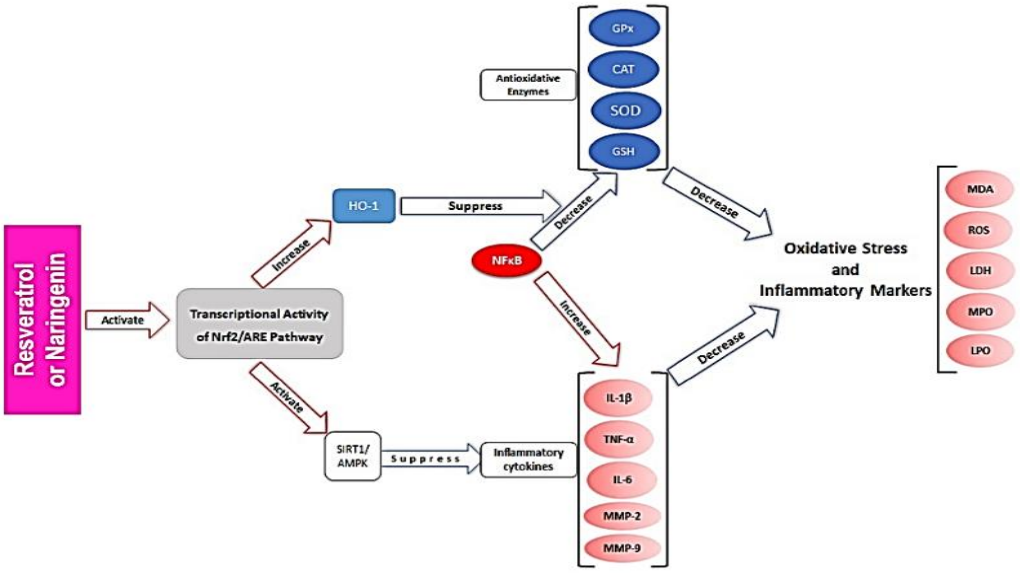


Fig. 6: RES or NG acts as the activator of the Nrf2/ARE pathway and consequent decreases oxidative stress and inflammation (Tahereh et al., 2020).

Activation of Nrf2/ARE by Res or NG stimulates the expression of HO-1 (Heme Oxygenase-1) and thereby suppresses the inhibitory effect of NF-Kβ (nuclear factor kappa light chain enhancer of activated B cells-transcription factor protein complexes) on antioxidants and also its stimulatory effect on inflammatory cytokines. Additionally, Nrf2/ARE activation increase the expression of SIRT1/AMPK leading to a decrease in the inflammatory cytokines [40, 49, 50, 51]. Altogether, these events increase the resistance of the cells to oxidative stress (Figure 6).NRF2 activators like RES and NG cause the activation of NRF2 (Figure 7), which regulates the transcription of the two subunits of glutamate–cysteine ligase(GCL), and alsoGSH synthetase (GS) that responsible for GSH synthesis, a crucial antioxidant that is involved in defence against oxidative stress.

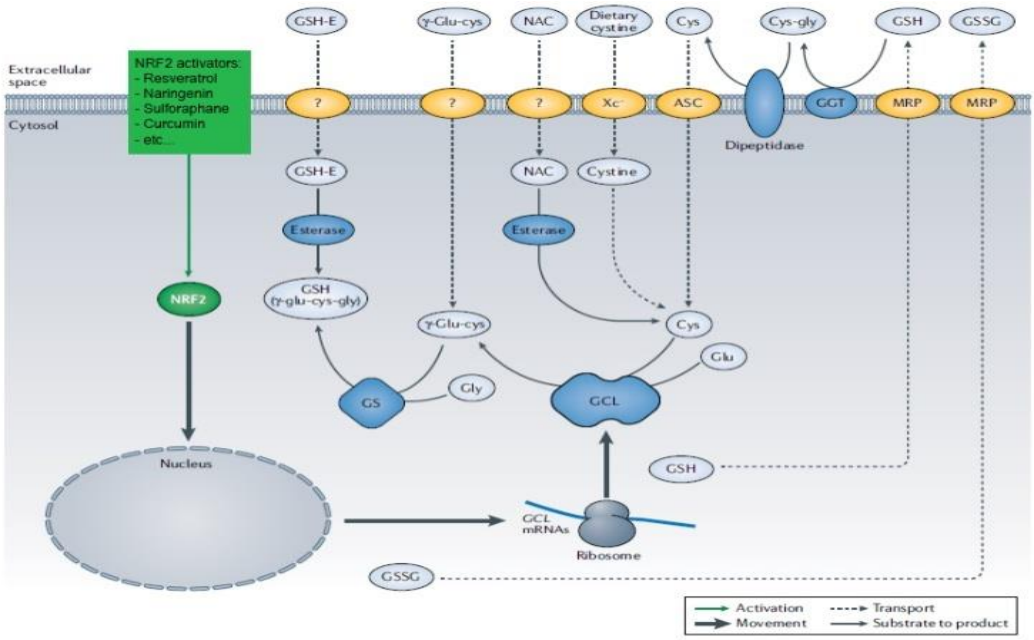


Fig. 7: RES and NG role as activators for NRF2 in Glutathione metabolism and strategies to increase glutathione. (Henry and Hongqiao., 2021) **[54].**

As shown in Figure (7) Glutathione (GSH) is synthesized through reactions catalysed by glutamate–cysteine ligase (GCL) and GSH synthetase (GS), with GCL as the rate-limiting enzyme and cysteine as the rate- limiting substrate. Both reduced GSH and glutathione disulfide (GSSG) are exported from cells through multidrug resistance protein (MRP), and extracellular GSH is

sequentially metabolized by membrane- bound γ- glutamyl transpeptidase (GGT) into cysteinylglycine and γ- glutamyl products, and dipeptidase hydrolyses cysteinylglycine to cysteine and glycine. The amino acids are transported back into cells and participate in GSH synthesis. N- acetylcysteine (NAC) is deacetylated by esterase action into cysteine, while GSH esters (GSH- E) are directly converted by esterase into GSH. γ- Glutamylcysteine (γ- glu- cys) can bypass GCL, the rate- limiting step for GSH synthesis.Some transporters have been identified: ASC, sodium dependent alanine- serine- cysteine transporter; Xc−, system cystine/ glutamate antiporter. Question marks denote the unidentified transporters/ channels for GSH- E, γ- glu- cys and NAC.

4. Conclusion

Overall, our study contributes to a deeper understanding of the toxic effect of NP on antioxidant capacity, liver and kidney function, blood composition, regulated lipid levels and estradiol disruption due to the binding between NP and ERα. Besides, it underscores the potential therapeutic utility of RES and NG in alleviating these adverse effects.

5. Conflicts of interest

There are no conflicts to declare

6. Formatting of funding sources

Not applicable

7. Acknowledgments

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