Current Research in Pharmacology and Drug Discovery 7 (2024) 100199

Contents lists available at ScienceDirect

Current Research in Pharmacology and Drug Discovery

journal homepage: www.journals.elsevier.com/current-research-in-pharmacology-and-drug-discovery

Hepatoprotective effect of Nobiletin against 5-fluorouracil induce hepatotoxicity

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ARTICLE INFO

Keywords:
Caspase-3
Corn oil
HMOX-1
Hepatotoxicity
Nobiletin effect
5-FU

ABSTRACT

5-florouracil is a widely used anticancer/anti-metabolite drug used to treat solid tumor like colon cancer, head and neck, rectum, stomach, pancreas and breast cancer; but, it can cause hepatotoxicity by induction of apoptosis through activation of caspases enzymes and oxidative stress. Nobiletin is a citrus fruit-derived flavonoid that possess significant biological activity, including anticancer, and anti-inflammatory. This study was design to investigate the effects of nobiletin against 5-florouracil-indcued hepatotoxicity in male rats through the measurement of selected -inflammatory, -apoptosis, and -oxidative stress markers. By use male Albino rats weighing 150-250gm around 28 animals; giving them tap water ad libitum and fed commercial pellets; and randomized into four groups (7animals/group) as following arrangement: Group I oral administered only corn oil for rats 1 ml for each kilogram for day by using of oral gavage for rat for 14 days. Group II: oral administered Nobiletin at dose 10 mg for each kilogram for each day (dissolved in corn oil) via oral gavage for 14 days. Group III: oral administered corn oil via oral gavage for 14 days after that single IP injection of 5-FU (150 mg/kg) on the day fourteenth (14). Group VI: Rats oral administered nobiletin dissolved in corn oil daily by oral gavage at a dose 10 mg/kg for each day for 14 days and a single IP injection of (150 mg/kg) 5-florouracil was given on day 14. All groups, seven animals of each group were sacrificed at day fifteenth (15); and, serum was collected to measure inflammatory and anti-inflammatory markers (interlukin-6 and interlukin-10) and liver function tests(ALT, LDH and AST); furthermore, liver tissue samples were collected to measure level of caspase-3, malondialdehyde and reduced form of glutathione, assessment of Hemeoxygenase-1 and NADPH quinone dehydrogenase-1 enzymes. In addition, histopathological study of the liver tissue of rats was perform to detect difference between architecture of liver cells in all rats' groups. The protective effect of Nobiletin noted by decrease in apoptosis of hepatocytes by decreasing of caspase-3 and reduction on free radical through reduce in malondialdehyde level, also increase in Hemeoxygenase-1gene expression. Increase in NADPH quinone dehydrogenase-1 dehydrogenase enzyme. On histopath reduce in congestion and some inflammatory infiltration by using of nobiletin prior to give 5-florouracil.

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
5-FU	5-florouracil
NOB	Nobiletin
NQO-1	NADPH quinone dehydrogenase1
HMOX-1	Hemeoxygenase-1
LDH	L-lactate dehydrogenase
GSH	Reduced form of glutathione
MDA	Malondialdehyde
Cas-3	Caspase-3

1. Introduction

The 5-fluorouracil (5-FU) is an anticancer drug that is intravenously-used to treat many types of solid tumors like: Gastrointestinal (GIT)-, colorectal-, pancreatic-, head and neck-, breast-cancers (Zhang et al., 2008); also it can be used-topically as a cream in some types of skin condition like actinic keratosis, which can develop to skin cancer (Grem, 2000). Concerning the mechanisms of action of 5-FU, researchers mentioned that, after injection or topical application, such drug enters cells by sach carrier-mediated transporter system [organic anion

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https://doi.org/10.1016/j.crphar.2024.100199
Received 15 June 2024; Received in revised form 2 August 2024; Accepted 2 September 2024
Available online 12 September 2024
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transporter system-2 (OAT-2)]. Inside the cells converted to (FdUMP) 5-fluorodeoxyuridin monophosphate, which form complex with specific type of enzyme what is called thymidylate synthase (TS), the formation of this complex has ability to inhibiting the production of the very important compound which is responsible for DNA replication and repair [deoxythymidine monophosphate (dTMP)] (Michael Oman, 2021) (main mechanism). Diminution of (dTMP) compound cause an irregularity in intracellularly nucleotides leading to breaking done in double-stranded DNA by the enzyme endonuclease (Zhang et al., 2008) (RI, 2012 Apr).

Additionally, "5-FU also serves as a pyrimidine analog by mis-incorporation into RNA and DNA in place of uracil or thymine; and the overwhelming damage of DNA repair machinery caused by these mechanisms ultimately-results in cell death of rapidly proliferating cells" (Yokogawa et al., 2021). Concerning the pharmacokinetics of the 5-FU, around 80% of such drug is metabolized by the liver, which has large amount of dihydropyrimidine dehydrogenase (DPD) enzyme this enzyme which is responsible for transformation of 5-FU to inactive metabolite dihydrofluorouracil (Verma et al., 2022). Any defect in this enzyme, can cause either -decrease or -accumulation of 5-FU in liver and cause toxicity (Amstutz et al., 2011). Most dangerous toxicities can appear with use of 5-FU are myelosuppression, neurotoxicity, cardiotoxicity and hepatotoxicity. Where, the later organ toxicity caused by 5-FU can be due to -increased in the free radical and cytochrome C elaboration from mitochondria, -decrease in the antioxidant glutathione (GSH) level, and -the increase in the inflammatory -markers and -cells infiltration with the resultant of hepatocyte damage (da Silva et al., 2023). Nobiletin is a flavonoid compound belong to *Rutaceae* family; and it is isolated from tangerines (Citrus reticulate) and it is a polymethoxy-flavanoid (Cheigh et al., 2012). Moreover, such compound plays important roles as anti-inflammatory, anti-oxidant, anti-atherogenic effects (Huang et al., 2016). Furthermore, nobiletin inhibited colitis-associated colon carcinogenesis in azoxymethane/dextran sulfate sodium (DSS)-treated mice via down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression. Furthermore, other researchers mentioned that, nobiletin plays important role in suppression of tumor cells through different mechanisms by -triggering of tumor suppressor genes, -inhibition of angiogenesis, -anti-apoptotic and invasive mechanisms, -decrease in resistance of multi-drug produced by tumor cells, -induction of hypoxia and reactive oxygen species (ROS) inside cancerous cells (Moazamiyanfar et al., 2023). In this sitting study, aim to inquiring the potential protective effect done by nobiletin to the liver after induction of acute hepatotoxicity by using anti-cancer drug 5-florouracil.

2. Materials and methods

2.1. Ethics Approval

Present study was endorsement by the ethical committee of "University of Baghdad/College of pharmacy/department of Pharmacology and Toxicology". Protocol number was 6862 on 2023/12/21.

2.2. Animals

By using healthy male Albino rat Twenty-eight (28) one weighing 150-250gm; fedding on commercial pellets and tap water ad libitum were obtained from the Animal House of the University of Baghdad/College of pharmacy and maintained in an controlled temperature at 20 ± 5 °C with a controlled dark and light for 12:12 h. Moreover, the animals were naturalize for 3 days before starting of treatment.

2.3. Chemicals and supplies

Nobiletin (NOB) with corn oil were purchase from MACKLIN, China. 5-Florouracil from Onko ilac San, Turkey. Furthermore, biochemical kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes were obtain from Linear Chemicals SLU, Spain. L-lactate dehydrogenase (LDH) was obtain from Cloud-Clone Corp, USA. Moreover, Elisa kits for inflammatory and anti-inflammatory markers (IL-6 and IL-10), oxidative stress (OS) indicators like [malondialdehyde (MDA), and reduced form of glutathione (GSH)] and apoptotic enzyme caspase-3 were obtain from Cloud-Clone Corp, USA. Besides, the Western blot detection kit (E-IR-R304A) was provide by Elabscience (Houston, USA). The antibodies against beta actin (E-AB-40338) and NQO1 polyclonal antibody (E-AB-70139) were provide by Elabscience (Houston, USA). Additionally, the triazole reagent for RNA extraction was purchased from TransZol Up Plus RNA kit (ER501), China. The Entilink 1st Strand Cdna synthesis kit was obtain from ELK biotechnology, USA. PCR primers for heme oxygenase (HMOX-1) were synthesized and purchased from Integrated DNA Technologies (IDT, Iowa, USA).

2.4. Experimental protocol

Rats were divided in to 4 groups each contained seven (7) rats: Group (I) control which rats oral received 1 ml for each kilogram for each day corn oil by using of rats' oral gavage for 14 days, in this study using of corn oil as solvent like other studies (Gokulan et al., 2021) for dissolved nobiletin. Group (II) Nobiletin (NOB) only group, rats were oral administered NOB 10 mg/kg/day, this dose of NOB selected depend on other study find in reference (Güvenç et al., 2020) (dissolved in corn oil) via rats' oral gavage for 14 days. Group (III) 5-florouracil only group, rats were orally-administered 1 ml for each kilogram for each day of corn oil for 14 days via rats' oral gavage, and then at day 14th, the animals were intraperitoneal (IP)-injected with 5-FU 150 mg/kg (Khalaf et al., 2022). Group (IV) NOB + 5-florouracil, rats were oral administered 10 mg/kg/day NOB (after dissolving it in corn oil) via rats' oral gavage for 14 days and prior to giving 5-FU on day 14th, where the animals had IP-injected with 150 mg/kg 5-florouracil as single dose. Twenty-four (24) hours after the end experimental duration (for 15 days). Each rats' group was euthanized by diethyl ether, and then sacrificed by cervical dislocation; and following intra-cardiac puncture, 0.5–1 ml of blood was obtained and collected in a specialized serum separator collection tube (gel tube). Allow to clot; then, the blood was centrifuged for 20min at approximately 1000*g to obtain serum, which was stored it in −20 °C aliquot place which was utilized for the biochemical tests, and -determination of selected interleukin parameters. Additionally, liver tissue samples were obtained and utilized for the -determination of selected OS parameters levels, -caspase-3 level, NOQ-1 dehydrogenase gene protein level and HMOX-1 gene expression and – histopathology.

2.5. Biochemical tests

The principle of the biochemical tests for the determination of serum levels of ALT, AST and LDH of each rat utilized in this study is based on ELISA by the utilization of a monoclonal anti-ALT, anti-AST, and anti-LDH antibody (Ab) and the ALT-, AST-, and LDH-HRP conjugate, respectively.

The sample and buffer were incubated together with ALT-, AST-, LDH-horseradish peroxidase (HRP) conjugate, respectively in pre-coated plate for 60 min period. After that, the wells were poured and washed 5 times; then the wells were incubated with a substrate for HRP enzyme. The product of such reaction forms a blue colored complex. Lastly, an acidic solution was added to stop the reaction which turn the color of the complex solution to yellow. Then, the intensity of color was spectrophotometrically-measured at 450 nm by means of a microplate reader. The color's intensity is inversely-proportional to each of [ALT], [AST], and [LDH], respectively. A standard curve was plot to relate the intensity of the color (O.D.) to the [standard]. The activity of serum ALT, AST, and LDH was each expressed as U/L.

2.6. Determination of serums' interleukins levels

According to the procedure of ELISA kit from Cloud-Clone Corp, USA. the serum concentration of IL-6 and IL-10 was measured; where, the sample, standards, and blank were each added to the pre-coated Ab plate and incubated at 37 °C for 1h; then, specific Ab reagent, enzyme conjugate, chromogenic substrate, were added after color changing to blue then stop solutions were sequentially-add to stop reaction and the color change from blue to yellow; and then reading at optical density of 450 nm. The IL-6, and IL-10 level was each expressed in serum as pg/ml.

2.7. Determination of selected levels of oxidative stress (OS) parameters and caspase-3 in rats' liver tissue

After scarification of each animal/Group by cervical dislocation, the liver of each rat was extracted and washed with ice-cold PBS with a pH of (7.0 ± 0.05) to get rid of any blood or debris, then the liver tissues weighed, and then was minced to small pieces [1g of liver was put in a tube containing 9 ml of PBS (pH 7.0 ± 0.05)]. Then, the liver tissue was homogenized on ice condition by homogenizer for 15 min at 1500×*g* (or 5000 rpm); and then centrifuged for 5 min and the supernatant layer was collected and stored in −20 C. Then it is utilized for estimation of -MDA, -GSH, and -caspase-3 levels according to the ELISA kit procedure; where, samples, standards, and blank were each added to the pre-coated Ab plate and incubated at 37 °C for 1 h. Then a specific Ab reagents, enzyme conjugate, chromogenic substrate for each parameter was added; after color changing to blue then stop solutions were sequentially-add to stop reaction and color change from blue to yellow; and, then reading at optical density at 450 nm. The contents of MDA, GSH, and caspase-3 in the liver homogenate was expressed as nmol/ml.

2.8. Quantitative real-time polymerase chain reaction (RT-PCR) to determination hemeoxegenase-1 (HMOX-1) gene expression

After scarification of each experimental rat by cervical dislocation, 50 mg of liver tissue were taken and put in the TRIzol reagent then centrifuged at 12000×*g* for 5 min at 4 °C with the resultant aqueous colorless supernatant (containing the RNA); and, after extraction of the RNA from all liver tissue sample by several addition and washing steps, the concentration of RNA by nano-drop-once apparatus and all concentration of samples above 2 ng/μl was measured. Conversion of RNA to cDNA and storage it in −20 C for sub-sequence reaction steps. The RNA amplification reaction was performed by Rotoe-Gene Q 96 System Software (Version 2.1,Qiagen). The quantity of mRNA was normalize with the expression of beta actin, and all comparison data were calculated using the 2- ΔCt. HMOX-1 gene primer sequence for real-time PCE for forward primer (5′-3′) is (CTTTCAGAAGGGTCAGGTGTC). Reverse primer (5′–3′) is (TGCTTGTTTCGCTCTATCTCC).

2.9. Western blot (WB) for determination of NOQ-1 dehydrogenase protein level

After scarification of each rat by cervical dislocation, the liver was obtained and then washed with PBS [PH (7.00 ± 0.05)]. Then weight approximately 50 g of liver for protein extraction from samples add "RIPA lysis buffer was added with phenylmethylsulfonyl fluoride PMSF and sodium orthovanadate Na3VO4 in the following ratio: (0.3 g liver:1 ml RIPA lysis buffer: 10 μL of both PMSF and Na3VO4)". So, for 50 g of liver tissue add 333 μl of RIPA lysis buffer and 3.3 μl from both PMSF and Na3VO5. Homogenized thoroughly to ensure that all protein extracted from tissue, centrifuge the sample and collect supernatant layer. Calculate protein concentration by use BCA Protein Assay Kit (Elabscience, USA) and use Mobi-apparatus (Bio-Rad Laboratories, USA) to measured absorbance for each sample by wave length 563 nm. Conversion of absorbance to concentration by using regression equation. Then 10 μL of sample after normalized protein concentration and conversion to SDS-loading samples added per lane were separate by SDS PAGE gel and gels were transfer into polyvinylidene difluoride (PVDF) membranes. The membrane was block by using milk fat free at 5% concentration at room temperature for 1 h, then leave the membrane to incubated over night at 4 °C with antibodies directed against NOQ-1 dehydrogenase and beta-actin, then wash membrane by TBST for 3 times. After that, membranes were incubate with coated anti-rabbit antibody for 1 h and wash with TBST for 3 times. Finally, by using ECL-A and ECL-B detection reagents from Elabscience, USA which used to detect protein expression signals and take picture for PVDF membrane by ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Densitometry scanning of band intensities was quantified by Image-J (NIH). Use beta actin as an internal control in the analysis of the samples.

2.10. Histopathological study

For histopathological study, the liver of each animal was carefully-removed and washed with PBS solution [pH (7.00 ± 0.05)]; then, firmed with 10% formaldehyde solution for histological analysis according to the method of (Junqueira and Carneiro, 1995) using the paraffin sections technique. After that, the fixative tissue was dehydrated by the utilization of ascending [ethanol] (70%, 90% and 100%), then cleaned by xylene to eliminate alcohol and to provide the liver with some degree of transparency; then the tissues were saturated with paraffin wax, heated, and blocked by pouring in embedded models; then cut by microtome into 5 μm thick sections, and washed in a water bath, and then stay in the oven to complete dewaxing process; after that, it stained by Haematoxylin and Eosin (H&E) dye; finally, it examined by the utilization of a light microscope by the specialist histopathologist.

2.11. Statistical analysis

The data were analyzed with GraphPad Prism 8 (San Diego, CA, USA). Mathematical data was provided as the mean ± SD; and the statistical significance of the differences between various groups will be explored by one-way analysis of variance (ANOVA) Tukey multiple comparison tests were performed. Differences will statistically-regard significant for (P-value) less than (0.05). Use imageJ to calculated intensity of Western blot bands.

3. Results

3.1. Effects of nobiletin (NOB) on rats' liver function tests markers

Fig. 1 A, B, and C, respectively showed that the blood ALT, LDH, and AST levels were there not significantly different ($P > 0.05$) in II Group rats which oral administer NOB alone in comparison to such blood levels in control (I Group). Furthermore, there were significant increase ($P < 0.05$) in blood levels of ALT, LDA, and AST in III Group rats which IP injected with 5-FU comparing to the congruent blood levels in control I group. Fig. 1A, B, and C, respectively. In contrast, there were significantly decrease ($P < 0.05$) in blood levels of ALT, LDA, and AST in IV Group rats which given NOB+5-FU compared to corresponding blood levels of liver function tests markers (ALT, LDH, and AST) in Group III/ rats IP injected with 5-FU.

3.2. Inflammatory and anti-inflammatory markers in rats

Fig. 2A showed that there was not significantly different ($P > 0.05$) in serum inflammatory marker/IL-6 in II Group rats oral administered NOB alone comparing to such serum level in rats of control (I group). Moreover, Fig. 2A also showed that there was a significantly increased ($P < 0.05$) in the inflammatory marker IL-6 in rat of group III IP injected with 5-FU comparing to the congruent serum level in group I. In contrast serum level of IL-6 was significantly reduction ($P < 0.05$) in group IV rats which given NOB+5-FU match to the congruent serum

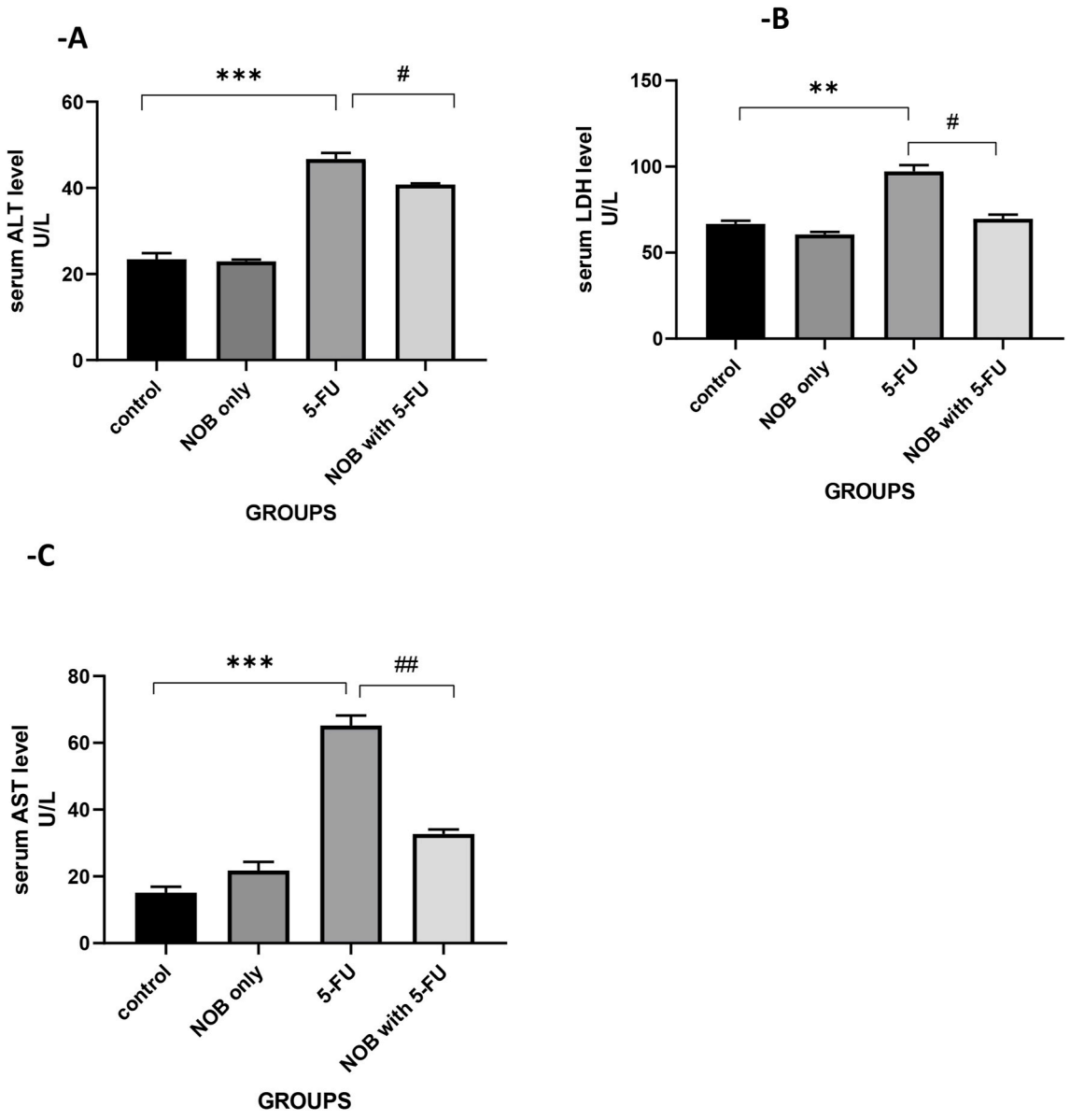

Fig. 1. Effect of nobiletin (NOB) on Levels of liver function test parameters (A)ALT,(B)LDH, (C)AST. Data are the mean ± SD (N = 7). P < 0.05 # represent significant differences compared with 5-FU group p < 0.05 *represent significant differences compared with control p < 0.05.

inflammatory marker in group III which given rats only IP injection of 5-FU. Fig. 2A.

Concerning the serum level of the anti-inflammatory marker, IL-10, Fig. 2B showed that there was a not significantly difference ($P > 0.05$) in serum anti-inflammatory marker/IL-10 in group II which oral given NOB alone compared to such serum level in control (I group). Moreover, Fig. 2B showed there was a significantly reduction ($P < 0.05$) in the anti-inflammatory marker/IL-10 in group III which given IP injected with 5-FU comparing to the congruent serum level in control I group. In contrast serum level of IL-10 was significantly improvement ($P < 0.05$) in group IV rats which given NOB+5-FU in matched to the congruent to serum anti-inflammatory marker in group III which given 5-FU only.

3.3. Effects of nobiletin (NOB) on selected oxidative stress (OS) parameters/oxidant, malondialdehyde (MDA), and the antioxidant, reduced glutathione (GSH) in rats

Fig. 3A showed there was not significantly difference ($P > 0.05$) in the MDA level in liver tissue homogenate in group II which oral administered NOB alone comparing to such tissue level in control group I. Furthermore, Fig. 3A also showed there was a significance elevation ($P < 0.05$) in the MDA in rats' liver tissue in group III rats IP injected with 5-FU comparing to the congruent serum level of group I. In contrast liver tissue level of MDA was significantly reduction ($P < 0.05$) in group IV which given NOB+5-FU in comparison to corresponding oxidant marker in group III which given only 5-FU. Fig. 3A. Concerning the liver tissue level of the anti-oxidant marker, GSH, Fig. 3B showed there was a not significantly difference ($P > 0.05$) in such reduced glutathione level in group II which given NOB alone comparing to such tissue level in group I. Furthermore, Fig. 3B showed that there was a significant decrease ($P < 0.05$) in the anti-oxidant marker (GSH) in group III which given IP injection of 5-FU comparing to the corresponding tissue level in group I. In contrast, liver tissue level of GSH was markedly improved ($P < 0.05$) in group IV which given NOB+5-FU in comparison to corresponding tissue anti-oxidant marker in group III which only given IP injection of 5-FU.

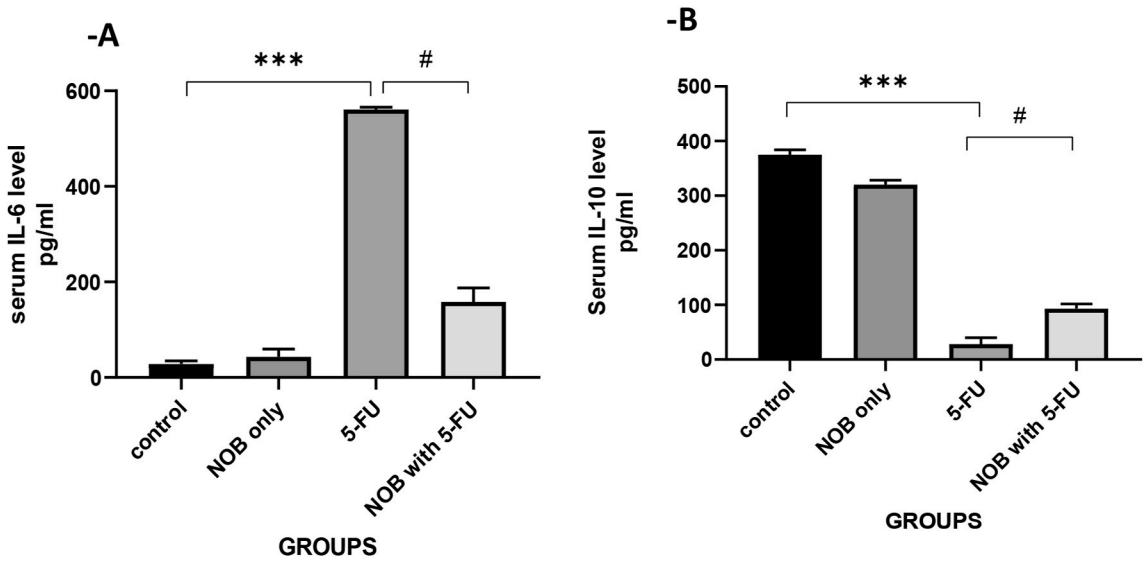

Fig. 2. Effect of nobiletin on level of serum interleukins level. (A) Inflammatory biomarker IL-6,(B) ant inflammatory biomarker IL-10. Data are the mean ± SD (N = 7). P < 0.05 # represent significant differences compared with 5-FU group p < 0.05 *represent significant differences compared with control p < 0.05.

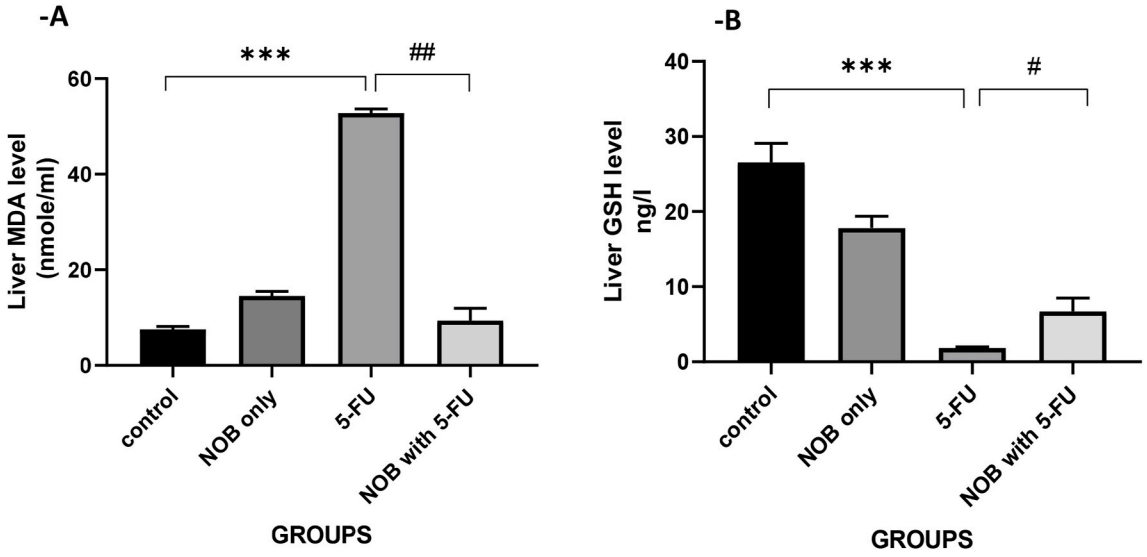

Fig. 3. Effect of nobiletin on Level of liver tissue oxidative stress. (A) MDA oxidative stress level, (B) GSH antioxidant level. Data are the mean ± SD (N = 7). P < 0.05 #represent significant differences compared with 5-FU group p < 0.05 *represent significant differences compared with control p < 0.05.

3.4. Effects of nobiletin (NOB) on the apoptotic marker (Caspase-3) in rats

Fig. 4 showed there was not significantly difference (P > 0.05) in the caspase-3 level in liver tissue homogenate in group II which given NOB alone comparing to such tissue level in group I. Moreover, Fig. 4 also showed there was markedly elevation (P < 0.05) in the caspase-3 in rats' liver tissue in group III which IP injected with 5-FU comparing to the congruent tissue level in control group I. In contrast, liver tissue level of caspase-3 was significantly decreased (P < 0.05) in group IV rats NOB+5-FU comparing to the congruent apoptotic marker in group III which given only IP injection of 5-FU. Fig. 4.

3.5. Effect of nobiletin on the Hemeoxygenase-1 gene expression (HMOX-1) in rate

Fig. 5 showed there was a significant elevation in the level of hemeoxygenase-1 (HMOX-1) gene expression in liver tissue homogenate in group II oral administered NOB alone in comparison to such tissue level in group I. Group III which only given IP injection of 5-FU there is significant increase in liver tissue HMOX-1 gene fold compare with control group. Group IV NOB with 5-FU there is significant improvement in HMOX-1 gene fold compare with Group III.

3.6. Effect of nobiletin (NOB) on the NAD(P)H quinone-1(NQO-1) dehydrogenase protein expression in rats

Fig. 6A: group I is control group there is very low concentration of NQO-1 protein. In group II, NOB only there is very increase in NQO-1 protein level compare with control group. In group III, 5-FU group there is significant increase in NQO-1 dehydrogenase protein level compare with control group. In group IV, NOB with 5-FU group there is significant increase in protein level from 5-FU group. Fig. 6B: indicated for intensity of protein NQO-1 in liver tissue by use PVDE membrane in Western blot test.

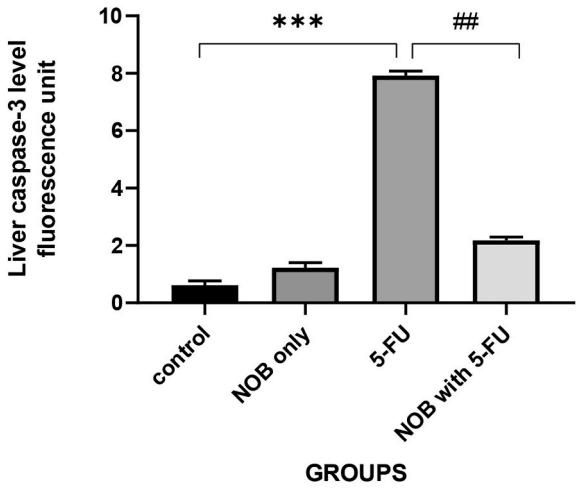

Fig. 4. Effect of nobiletin on Programed cell death mediator caspase-3 level in liver tissue. Data are the mean ± SD (N = 6). P < 0.05 #represent significant differences compared with 5-FU group p < 0.05 *represent significant differences compared with control p < 0.05.

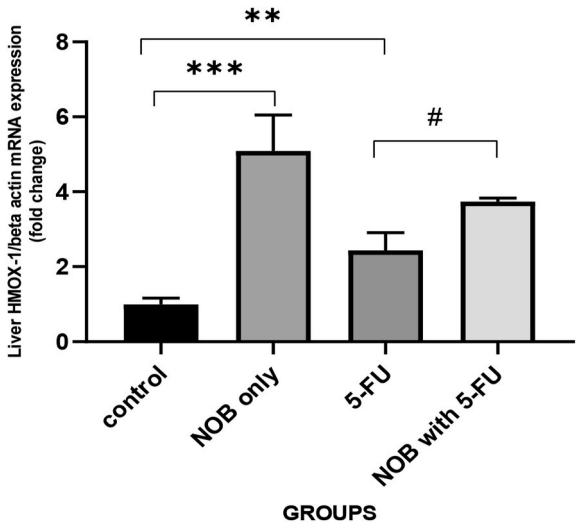

Fig. 5. Effect of nobiletin on Level of hemeoxygenase-1 gene expression in liver tissue. Data are the mean ± SD (N = 7). P < 0.05 #represent significant differences compared with 5-FU group p < 0.05 # represent no significant differences compared with control p < 0.05.

3.7. Histological examination of rats' liver tissue

Rats orally-administered 1 ml/kg/day corn oil by used of rats 'oral gavage for 14 days for group I (Control), there were normal hepatocytes with normal sinusoids, no any congestion or dilatation on central veins, no any infiltration for inflammatory cells like in Fig. 7A. The liver section of group III which given only IP injection of 150 mg/kg 5-FU showed very dilation in central veins of hepatic tissue in approximately 95%, endothelial cells of central veins detached and perivascular fatty change Fig. 7B. The liver section of Group IV rats/NOB prior to 5-FU showed there is no dilatation in central veins, no endothelial cells detached and less fatty change Fig. 7C.

4. Discussion

The 5-FU/a uracil analogue is the widely used anti-metabolite agent for the treatment of various cancers' types, including breast, colorectal, and head and neck cancer (Grem, 2000) (Rutman et al., 1954). However, such drug can induce many toxicities including hepatotoxicity such as other anti-neoplastic medications like cyclophosphamide; on this situation, 5-FU can induce hepatotoxicity through the inhibition of thymidylate synthase (TS) enzyme; additionally, such chemotherapeutic drug can be extensively-metabolized in the liver via the microsomal enzyme system with the production of a toxic intermediate that can trigger liver injury (Bruni et al., 2002; Chernyshev et al., 2007; Zhang et al., 2008). In the current study as show in Fig. 8, IP injection of a single toxic dose (150 mg/kg) of 5-FU (Induction/Group II rats) resulted in significant elevation in serum levels of liver function test parameters (ALT/the cytosolic enzyme, AST/the mitochondrial enzyme, and the LDH/the enzyme help with cellular respiration) (Fig. 1A, B, and 1C) (Orsolic, 2010). Respectively in rapprochement with each of such serum levels in control male rats (Group I). This can be due to sever oxidative production and the accumulation of its products in the liver that can damage the biological membranes and the endothelial lining of the liver; and this in turn cause elevation in the blood or serum levels of previously-mentioned biological markers/indicators of cellular damage and toxicity (Zeashan et al., 2009; MacNamara and Goldberg, 1985). Moreover in the present study, and in group of rats oral administer NOB for 14 days before 5-FU injection given group IV resulted in significant reduction (P < 0.05) (Fig. 1A, B, and 1C) in each of ALT, AST, and LDH serum enzymes levels comparing to each serum level of enzymes in group III which given only 5-FU IP injection. In this study, (in Group III) rats IP injected with a single toxic dose 150 mg/kg 5-FU, such drug caused a significant increase (P < 0.05) in the hepatic tissue level of MDA in rapprochement to such tissue level in group I (Fig. 2A); and such results are consistent with that of malondialdehyde (MDA) is one of the last products of polyunsaturated fatty acids (PUFAs) peroxidation in many cells (Ali, 2012; Abdul-Wahab and Al-Shawi, 2020; Zelber-Sagi et al., 2020). In the liver, it is an OS parameter that can stimulate the hepatic stellate cells/HSCs, which comprise a cell population in the liver that are primarily-known for their activation upon liver injury and for producing the collagen-rich extracellular matrix which cause fibrosis in the hepatic tissue. Any imbalance in production and degradation of reactive oxygen species (ROS) can cause increase in level of MDA and cause depletion in reduced form of glutathione level (GSH) (Schnug et al., 2022). Moreover, this study also showed that, in group IV which given oral administered NOB for 14 days before IP injection of toxic dose (150 mg/kg) 5-FU, there is a significantly decrease (P < 0.05) in the level of MDA in hepatic tissue comparison to such tissue level in the induction group II which only given IP injection of 5-FU (Fig. 2A). There are no previous studies concerning the effects of NOB administered prior to 5-FU-induced hepatotoxicity which concerning hepatic tissue level of MDA. Besides, the present study also showed that in group of rats IP injected with a single toxic dose of 5-FU (150 mg/kg)/Induction group III, there is a significant decrease (P < 0.05) in the liver tissue homogenate level of GSH compared to such tissue level in control group I (Fig. 2B). The results of this study are consistent with those obtained by others (Gelen et al., 2018). There is previous study shows that effect of NOB on GSH level (Nakajima and Ohizumi, 2019). Conversely, this study showed that in rats oral administered NOB prior to 5-FU group IV, there is significantly elevation IP injection of 5-FU (Fig. 2B); and this can indicate that NOB has antioxidant activity and can preserved the GSH level from depletion. Again, there are no previous studied concerning the effect of NOB against 5-FU-induced reduction in hepatic tissue level of GSH; thus, we did not have the chance to compare our results with others.

The reduced glutathione/GSH is the most abundant endogenous antioxidant that play important roles as hepatic protective antioxidant; where, it decreases deleterious effect of any toxic endogenous or xenobiotic that caused liver damage; and it possess has role in post translation regulation for protein and regulation of hepatocyte cell death(Abd Al-Zahra et al., 2017; Vairetti et al., 2021). Concerning the parameter of the apoptotic process/caspase-3, in the this study, there was an increase in the cellular hepatic level of caspase-3 of male rats IP with a single

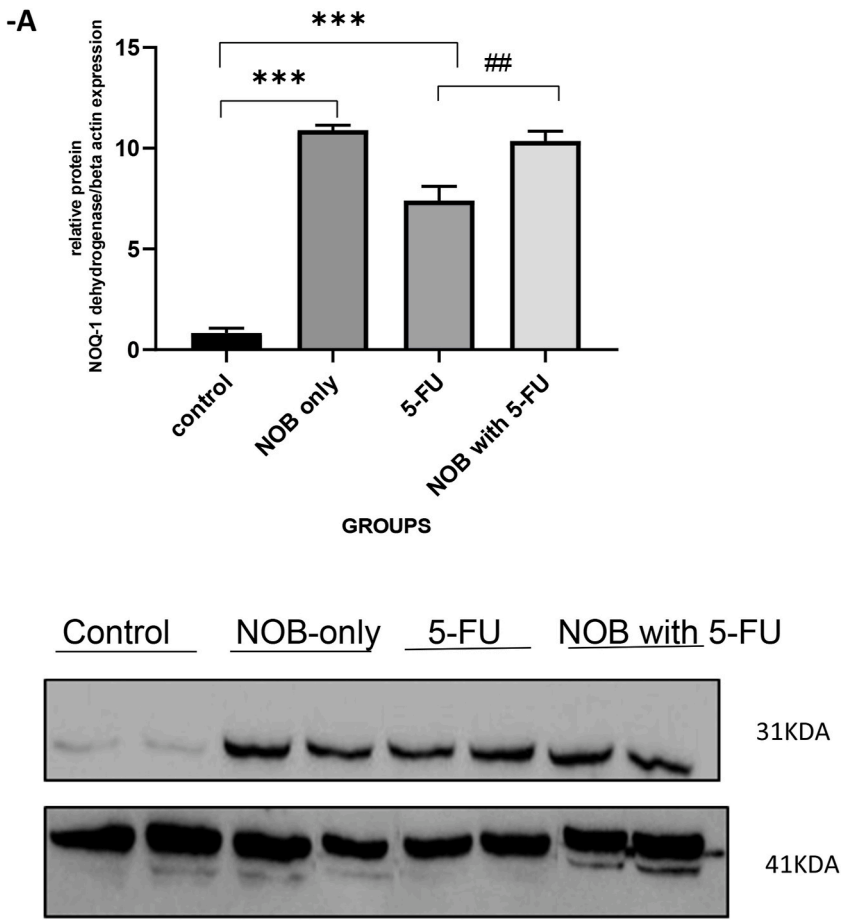

Fig. 6. Effect of nobiletin on level of NOQ-1 dehydrogenase protein in 5-FU induce hepatotoxicity.(A) relative protein expression NOQ-1 dehydrogenase.(B) Western blot images of NOQ-1 dehydrogenase. Data were expressed as mean ± SD #P < 0.05 compared with 5-FU group; *P < 0.05 compared with the control group.

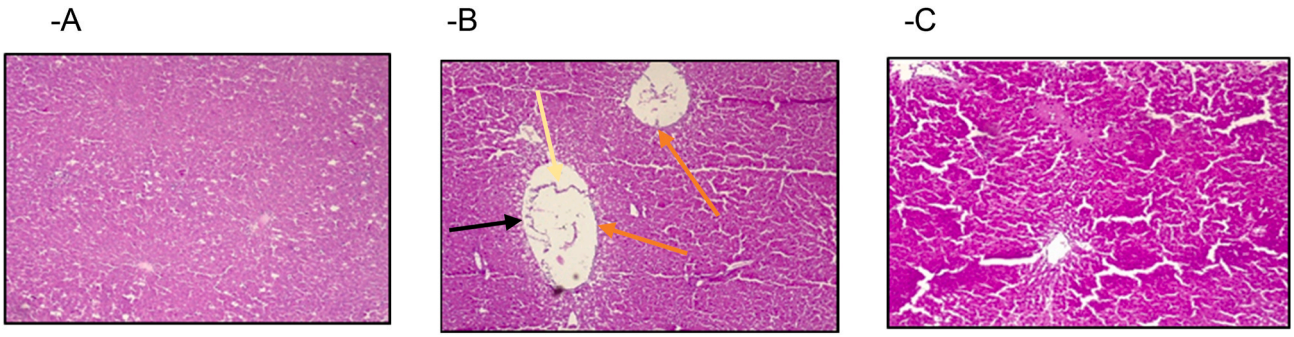

Fig. 7. The effect of nobiletin on histological changes of liver tissue in the 5-FU induce hepatotoxicity in rats. Image of H and E stained liver sections (10×magnification) at 24 h after 5-FU administration was shown. (A) Control group: no histological changes was observed (B) 5-FU group: hepatic vein was dilated marked by a red arrow, and the endothelial cells were detached marked by a black arrow and necrotic hepatocytes marked by a yellow arrow (C) Nobiletin treated group: histological structure there is less damage than 5-FU group.

toxic dose (150 mg/kg) of 5-FU (group III) comparing to such hepatic tissue level in control group I (Fig. 3); suggesting that apoptosis/or the process of programmed cell death can play a role in the pathogenesis of 5-FU-induced hepatotoxicity. These results are in accordance with those of other researchers (Khalaf et al., 2022).

Oxidative stress in tissues cannot calculated as it is, but by using other parameters like malondialdyhed considered as indicator for level of oxidative stress in tissue, any increasing in its level indicated for increase in OS level. However, reduced form of glutathione also deemed as parameter for OS so any decrease in its level indicated for increase in OS level. Oxidative stress mean there is defect in harmony between antioxidant found naturally in tissues like GSH and reactive oxygen species (ROS) (Elsayed et al., 2021). Any triggering for generation of ROS by any internal or external sources cause activation for lipid-peroxidation which cause damaging for cell membrane, mitochondrial membrane, peroxisome membrane and other organelles these degradation activate apoptosis of the cells. In this recent study 5-FU act as external drug cause oxidative stress inside hepatocytes, which cause activation of apoptotic pathway and cell lysis (Sadasivam et al., 2022).

Caspase-3 is the peculiar marker that activates the apoptotic pathway by catalyzing the cleavage of many essential cellular proteins (Porter and Jänicke, 1999). Additionally, researchers mentioned that,

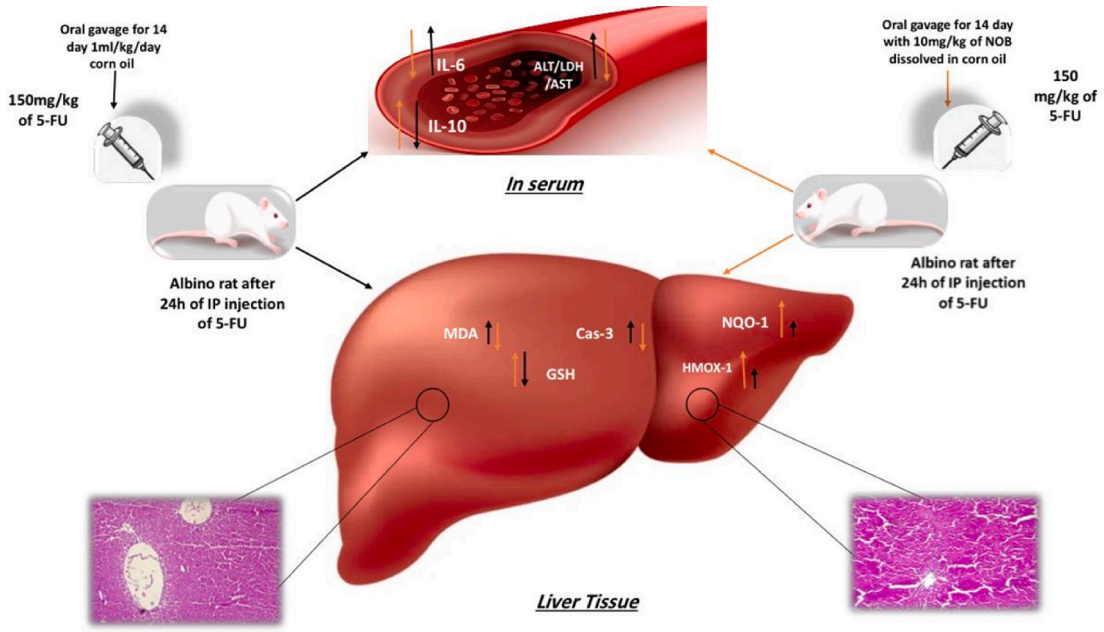

Fig. 8. Ability of NOB to lowering MDA and Cas-3 in liver tissue, elevating GSH, HMOX-1 and NQO-1 in liver tissue. Reduction of liver function enzymes and IL-6 in serum also elevated IL-10 in serum (all of them represented by orang arrow).

OS could activate caspase-3 and increase in incidence for apoptosis of hepatocytes (Cao et al., 2016). Furthermore, this study also showed that in rats of group IV which oral administered of NOB for 14 days before a single IP injection of toxic dose (150 mg/kg) of 5-FU, there is a significant reduced ($P < 0.05$) in the level of caspase-3 in liver tissue homogenate of rats comparing to such tissue level in group III (5-FU) rats (Fig. 3). There is no previous study concerning the effects of NOB against 5-FU-induced hepatotoxicity and the role of caspase-3 in rats; but, Li et al. (2020) showed that NOB can control the ratio of Bcl2 (anti--apoptotic)/Bax (apoptotic) proteins and thus, it possessed a protective effects against acute liver injury and apoptosis induced by the lectin/-concanavalin A on mice model (Li et al., 2020).

Concerning the process of inflammation, the present study showed that in rats of group III induction by IP injection with a single toxic dose of 5-FU (150 mg/kg), such agent causes a significantly increase ($P < 0.05$) in the serum IL-6 level comparing to such serum pro-inflammatory level in the control group I (Fig. 4A). The result of this study is consistent with those of (Alhoshani et al., 2022). Other researchers mentioned that the secretion of cytokine is the mediator of inflammation and can contribute to the pathogenesis of tissue injury (Laverty et al., 2010; Lacour et al., 2005). Where, there are increasing in levels of pro-inflammatory cytokines including IL-6 following 5-FU treatment in rats (Chang et al., 2017; Al-Geam and Al-Shawi, 2018). Besides, the present study also showed that in rats orally-administered the polymethoxyflavone/NOB for 14 days prior to a single toxic dose (150 mg/kg) 5-FU (Group IV), there is reduction in the serum IL-6 level compared with such serum level in 5-FU/Group II (Fig. 4A). Once more, there are no previous studies concerning the effects of NOB-orally-administered prior to 5-FU-induced hepatotoxicity in rats with respect to IL-6 serum levels but there is other study said that; NOB had ability to increase IL-6 level (Suzuki et al., 2022). Other study show opposite effect of NOB and said that, there is attenuation effect of NOB on generation of IL-6 (Yarim et al., 2022) after do this experiment NOB cause decrease in IL-6 level like noted in group IV.

Additionally, the current study showed that, there is a significant decrease ($P < 0.05$) in the anti-inflammatory marker/IL-10 serum level in Group II rats (5-FU) (Fig. 4B) compared to such serum level in control/Group I rats (Hoda Mohamed El-Emshaty, 2015; Abdul-Azeez and Mutlag, 2024). The anti-inflammatory cytokine (IL-10) is released from macrophage T-helper2 (Th2) and B lymphocyte; and it possesses anti-fibrotic property; and, its main function in the liver is the suppression of hepatic stellate cells (HSC), which is responsible for secretion of collagenous material that in turn can induce fibrosis and increase in HSCs apoptosis; thus 5-FU can induce fibrosis. In contrast, the use of NOB for 14 days prior to toxic dose (150 mg/kg) 5-FU group IV in this study, there is significant increase in serum IL-10 level (Fig. 4B); and this can indicate the inhibition in HSCs activation and possible reduction in fibrotic consequences of hepatic tissue. There is previous study showed effect of NOB in elevation of IL-10 (Yarim et al., 2022), but there is no previous study regarding the effect of NOB administered prior to 5-FU-induced hepatotoxicity, on IL-10 level in serum of rats; thus, we cannot have the chance to compare the results of the current study with those of others.

Furthermore, the current study showed that there was a significant increase ($P < 0.05$) in the heme oxygenase (HMOX-1) gene expression fold in Group III rats IP injected with a single toxic dose (150 mg/kg) 5-FU compared to previously-mentioned gene expression in control group I as showed in Fig. 5. The HMOX1 is the most important enzyme that degrades the pro-oxidant heme to -carbon monoxide (CO)/a vasodilatory gas that possess anti-inflammatory properties, -bilirubin/an anti-oxidant derived from biliverdin, and -ferrous ions (Adnan Atshan and Zalzala, 2024). Thus such enzyme has important role as anti-inflammatory, antioxidant, cytoprotective, antiapoptotic and immune regulating function (Khalaf et al., 2022). Although HMOX1 is a redox-regulated enzyme that is induced in many diseases and act against OS; but it can also promote cell death (Li et al., 2021). Moreover, as 5-FU induction generation of ROS's, lipid peroxidation and induction for apoptosis such drug also can induce the expression of the HMOX-1 gene indicative for OS (Sommer et al., 2017). In addition, the present study showed that in NOB only treated rats group II, there is a highly-significantly ($P < 0.01$) increase in the fold of HMOX-1 gene expression compared to that of the control group I rats; and may indicate that such polymethoxyflavone increase in expression of HMOX-1 gene.

Researchers mentioned that, there are several HO-1 inducers/modulators for therapy or potential functions, such as herbal medicines such as resveratrol, curcumin, propolis and others (Wu and Hsieh, 2022). Where, herbals (for example resveratrol) act as through the upregulation of Nrf2/Ho-1 and PI3K/Akt signalling pathways and anti-inflammation

(Yang et al., 2018).

Additionally, curcumin and propolis possessed protective effects that are mediated through improving the Nrf2/HO-1 pathway (antioxidant response) and by inhibiting the NF-κB, TLR4/RAGE, and MAPKs (ERK, p38, and JNK) signaling pathways (anti-inflammatory response) (Teter et al., 2019; Kunugi and Ali, 2020).

Dependent on structure of Nobiletin as flavonoid and has polymethoxy group give it structural activate relationship like very strong effect as antioxidant, act as scavenger for ROS's, chelating agent for transitional elements like metals and inhibits for many types of enzymes like: cyclooxygenase, monooxygenase, lipoxygenase, phospholipase A2 and xanthine oxidase (XO) (Cos et al., 2000).

Concerning the expression of the NAD(P)H quinone-1(NOQ-1) dehydrogenase, in rats orally-administered NOB group II. The results of the current study showed that, there is a significant ($P < 0.05$) increase in the expression of NQO-1 protein in NOB with 5-FU in group IV comparing to such protein expression in control group I (Fig. 6). Moreover, there is also significant increase ($P < 0.05$) in NOQ-1 dehydrogenase protein level in 5-FU/Group III rats compared to such protein level in group I (Fig. 6). As (Jaiswal, 2000) reported that the expression of NQO1 gene is coordinately-induced with other detoxifying enzyme genes in response to xenobiotics, antioxidants, oxidants, heavy metals, and radiations (Jaiswal, 2000). Furthermore, there was also a highly-significant increase ($P < 0.05$) in the expression of NQO-1 protein in NOB with 5-FU group IV compared to that in Fig. 6 and this may indicate the ability of NOB to activate the redox-sensitive transcription factors for NQO1 such as Nrf2 and c-Jun or Keap1. Which may then bind to the antioxidant response element (ARE) and activate NQO1 gene expression (Jaiswal, 2000). Moreover, in this study, in group IV which given orally NOB before a single toxic dose 150 mg/kg 5-FU, there is also a highly-significant increase ($P < 0.05$) in the fold of HMOX-1 gene expression compared to that in group III induction which only given IP injection of 5-FU and this may indicate the protective effect of NOB from the depletion of antioxidant, and preserved hepatic cells from induction of apoptosis. With the regard of histopathology, the present study showed that, the liver section of Group III rats/IP injected with a single dose of 150 mg/kg 5-FU (Fig. 7B), there is a pronounced dilation in central veins of hepatic tissue (approximately 95% of it). The sever dilation in central veins are indicative for inflammatory induction and starting for inflammatory cells infiltration also inflammatory markers indicated for inflammation start; moreover, the endothelial cells of central veins detached and perivascular fatty changes start with some loss of architecture of hepatocytes around central veins this indicated for starting of hepatocytes suffer from death. There is some area suffer from apoptosis and collagenous tissue formation with dilatation in sinusoids compare with control group (Fig. 7A). In case of NOB with 5-FU group less dilation of central veins compare with 5-FU group, no endothelial cells detached, less infiltration of inflammatory cells and less collagenous tissue formation compare with 5-FU group only because of protective effect of Nobiletin on hepatic tissue and antioxidant activate can protect hepatocyte from apoptosis. Nobiletin has ability to reduction inflammatory markers and reduce inflammatory cells infiltration.

This study will investigate the effects of orally-administered 10 mg/kg nobiletin daily for 14 days prior to 5-FU on parameters (1- The oxidative stress potential [MDA and GSH] levels, 2- The apoptosis marker caspase-3/(Casp-3) level, 3- Selected levels of inflammatory and anti-inflammatory markers; 4- The level of HMOX-1 enzyme level and 5- The liver architecture (histological examination). NOB where to be administered prior to 5-FU there are significant different in these parameters so depend on these findings can defined there is significant protective effect of NOB against 5-FU.

5. Conclusion

In conclusion, this study is first in this line. The finding of present study indicated that treatment with NOB can alleviate hepatic damage induced by a single IP toxic dose (150 mg/kg) of 5-FU in rats. NOB also enhances the normal level of biochemical tests, it has anti-inflammatory with prevent inflammation, antioxidant, and antiapoptotic property thus, in the present study concluded that NOB was protective against 5-FU-induced hepatotoxicity.

Credit authorship contribution

Safa Ahmed Yahya: who does animal experiment, Analysis and interpretation of data, and compose the article. Nada N. Al-Shawi: visualize and delineation of the study, the rectify manuscript, and presiding the all process.

Funding

"This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

Declaration of competing interest

None.

Data availability

The data that has been used is confidential.

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