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Biochemical studies on the effect of prostaglandin inhibitors and willow bark extract on liver cirrhosis induced by acetylsalicylate in rats

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Abstract

Prostaglandins (PGs) are arachidonic acid metabolites produced by the action of the enzyme cyclooxygenase (COX). Although PGs are important mediators of inflammation in various diseases, The deciduous herb, *Salix mucronata* Thunb (commonly called cape silver willow or safsaf willow), is widely distributed along the Nile River in Egypt. Like other willow trees, extracts from safsaf have also been used in traditional medicine. The anti-inflammatory effects of *Salix mucronata* are the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin synthesis. **The study aims** to assess the induced cirrhotic effect of high doses of prostaglandin inhibitors as acetylsalicylate administration on the liver of rats to determine the protective impact of willow bark (*Salix mucronata*) extract on liver cirrhosis in rats with studying the role of cyclooxygenases (COX-1) and (COX-2) in diagnosis and prognosis of liver cirrhosis. **Material and methods:**

For this study, a total of 68 male albino rats weighing 90- 100 gm were obtained from the Holding company for Biological Products and Vaccines (Vaccera), Helwan- Giza, Egypt, and allocated to plastic cages covered with metal grids and allowed to acclimate for 10 days in the animal facility conditions before being divided into groups. For experimentation they were divided into 7 groups as follows: Group 1 (G1) (8 rats): Animals of this group received no treatment and served as a control group. Group 2 (G2) (10 rats): Each rat was orally administrated with a gavage tube extract of Salix mucronata daily for 8 weeks (150mg/kg). Group 3 (G3) (10 rats): Injected by Ibuprofen (IBP) only dissolved in sterile saline intraprotenial (I.P.) daily for 8 weeks (40mg/kg). Group 4 (G4) (10 rats): Administrated by Acetylsalicylate (ASA) only (300mg/kg) daily for 8 weeks to induce liver fibrosis. Group 5 (G5) (10 rats): Administrated first with Acetylsalicylate (ASA) and at the same time injected with Ibuprofen (IBP) to investigate the effect of Ibuprofen on the infected rats. Group 6 (G6) (10 rats): Administrated first with Acetylsalicylate and simultaneously each rat was given an oral extract of *Salix mucronata* to investigate the effect of the extract on the infected rats. Group 7 (G7) (10 rats) was Administrated first with Acetylsalicylate (ASA) and at the same time treated with a combination of both extract and Ibuprofen. **Results:** Changes in CAT, GSH, MDA, hydrogen peroxide, and nitric oxide Rats injected with ASA (G4), and rats injected with ibuprofen (G3) showed a significant decrease in GSH and CAT and a significant increase in MDA, hydrogen peroxide, and nitric oxide levels if compared with G1. Treatment with extract and ibuprofen (G7) showed close levels of CAT,

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GSH, MDA, hydrogen peroxide, and NO to G1. Changes in AFP as a tumor marker, IL-6, TNF-alpha, and 5 – Nucleotidase. Rats injected with ASA (G4) and rats injected with ibuprofen (G3) showed a significant increase in AFP, IL-6, TNF-a, and 5 -Nucleotidase levels if compared with G1 (control). While their levels exhibited a significant decrease in rats treated with extract and ibuprofen (G7). Changes in COX-1 and COX-2; In rats injected with only ibuprofen (G3) and rats injected with ASA (G4) exhibited a significant decrease in COX-1 and COX-2 concentration if compared with G1 (control). By treatment with extract and ibuprofen (G7), the concentration of COX-1 and COX-2 increased returning to the normal ranges of close to control.

Keywords: Prostaglandin inhibitors-willow bark extract- liver cirrhosis - acetylsalicylate - rats.

Introduction:

A total loss of liver function could lead to death within minutes, demonstrating the liver's great importance (Ozougwa & Eyo, 2014). Prostaglandins (PG) are involved in the regulation of many physiological processes in the liver and play a major role in the pathophysiology and treatment of liver diseases (Peltekian et al., 1996). The blood flows through liver sinusoids empties into the central vein and exits the liver from the hepatic vein (Wang, et al., 2017). Cirrhosis results from different mechanisms of liver injury that lead to necro-inflammation and fibro-genesis; histologically it is characterized by diffuse nodular regeneration surrounded by dense fibrotic septa with subsequent parenchymal extinction and collapse of liver structures, together causing pronounced distortion of hepatic vascular architecture (Dooley et al., 2018). Chronic injury to the liver results in inflammation, necrosis, and subsequently fibrosis (Kumar & Clark, 2009; Hessien et al., 2010). Stimulation of Kupffer cells, neutrophils, and T-cells cause the secretion of various cytokines and profibrotic mediator to convert quiescent to activated hepatic stellate cells (HSCs) (Sadek et al., 2016). Cirrhosis is an extremely heterogeneous condition, extending from an early asymptomatic stage to an advanced disease with various complications, rather than a terminal stage of different liver injuries. The evolution of cirrhosis is a multi-step series of events. To distinguish the heterogeneous phases of cirrhosis a five-stage system is proposed (Friedman, 2014).

Prostaglandins (PGs) are arachidonic acid metabolites produced by the action of the enzyme cyclooxygenase (COX). They act via high-affinity G-protein coupled receptors: four EP receptors for PGE2 termed EP1- EP4, IP receptor for prostacyclin, DP receptor for PGD2, FP receptor for PGF2a. These receptors are linked to the different signal transduction pathways (Ricciotti & FitzGerald, 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most used drugs in inflammatory diseases since they are effective in the management of pain, fever, redness, and edema arising as a consequence of inflammatory mediator release (Ferreira, 2002). Studies have shown that both the therapeutic and side effects of NSAIDs are dependent on cyclooxygenase (COX) inhibition (Helmy et al., 2015). The non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of minor pain and the management of edema and tissue damage resulting from inflammatory joint disease (arthritis) also in chronic joint disease, musculoskeletal pain, headache, menstrual pain, and dental pain. Choosing an NSAID for its analgesic and antipyretic effect in indications like fever, common cold, dental pain, minor soft tissue injuries, and non-specific body aches is not difficult as in most circumstances the drug is to be used for a short duration only (Buer, 2014; Jahnavi et al., 2019). Ibuprofen is one of the most widely used analgesic-antipyretic-anti-inflammatory drugs today (Abraham et al., 2005). It is a non-selective inhibitor of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Chavez & DeKorte, 2003). Ibuprofen is supplied as tablets with a potency of 200 to 800 mg. The usual dose is 400 to 800 mg three times a day (Jones et al., 2007). Dysmenorrhea, fever, and headache: Non-prescription ibuprofen is useful for

managing minor aches and pains, reducing fever, and relieving symptoms of dysmenorrhea (Grimes et al., 2006). In some studies, ibuprofen showed superior results compared to a placebo in the prophylaxis of Alzheimer's disease, when given in low doses over a long time (Melton et al., 2003). It induced apoptosis significantly in the early and late stages, suggesting that these anti-inflammatory agents might inhibit microbial proliferation (Chen et al., 2005). Ibuprofen exhibits few adverse effects, the major adverse reactions include the effects on the gastrointestinal tract (GIT), the kidney, and the coagulation system (Wilcox et al., 2005). Ibuprofen was a potential cause of GI bleeding (Rocca et al., 2005), apoptosis, heart failure, and hyperkalemia (Gambero et al., 2005). It is used for versatile purposes which include anti-inflammatory, and anti-platelets (Marciniak et al., 2007). Even though aspirin was not identified as a natural product, it is widely used by many plant scientists in their experiments. The reason is the similarity in their physiological effects (Al-Janabi et al., 2005). The pharmacological properties of aspirin are like those of salicylates, but also the biological actions attributed to salicylate itself, and it has other independent effects due to its reactive acetate group (Luigi et al., 2001). Both components, salicylate, and acetate groups, are biologically active and act independently of each other at different sites (Schror, 2016). Conventional NSAIDs inhibit COX-1 and COX-2 and this feature accounts for both therapeutic and side effects. Inhibition of COX-1 activity is considered a major contributor to NSAID GI toxicity. COX-2 is considered an inducible isoenzyme, although there is some constitutive expression in the kidney, brain, bone, female reproductive system, neoplasias, and GI tract. The COX-2 isoenzyme plays an important role in pain and inflammatory processes (Mbonye et al., 2008). The phenolic compounds isolated, salicylic glycosides, were the most abundant with reported analgesic, antipyretic, anti-inflammatory, and antirheumatic properties (Biegert et al., 2004). The Salix family is famous due to its endogenous salicylate compounds. The anti-inflammatory properties of extract of the Salix family may be related to its phytochemicals such as salicin, myricetin, kaempferol, quercetin, rutin, and luteolin. These compounds have immune-modulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors (Lopez-Parra et al., 2002). Herbal medicines usually contain total extracts from the constitutive plant(s) that are composed of many different compounds (Qin & Sun, 2005).

Material and methods:

The main objective of the present study is to determine the effect of high doses of prostaglandin inhibitors on liver cirrhosis in rats induced by acetylsalicylate where ibuprofen was used as a reference drug. Evaluate the effect of Willow bark (*Salix mucronata*) extract and assess the role of cyclooxygenases (COX-1 and COX-2) in the diagnosis of the progress of liver cirrhosis in rats.

Plant sample collection:

Specimens of *Salix mucronata* were collected by specialized farmers from June to August 2017 from Damanhour, El Beheira Governorate, Egypt. The collected specimens were brought to the laboratory after collection. In the laboratory, voucher specimens of the whole plant were identified by the department of Botany, Faculty of Science, Damanhour University, Egypt.

Preparation of willow extract.

The stem bark of the plant (*S. mucronata*) was dried in shade, grinded with an electric mill to a fine powder, and kept in dry conditions for the extraction process. 20 g of powder was extracted with 100 ml of methanol in the Soxhlet apparatus for a period of 72 h, then it was filtered with the Whitman filter paper then HPLC analysis of the extract was performed.

HPLC analysis of phenolic compounds:

The high-performance liquid chromatography (HPLC) analysis was carried out for the methanol extract of *S.*

mucronata using Agilent Technologies 1100 series liquid chromatography equipped with an autosampler and a diode-array detector. The separation and determination were performed on the XDB-C18 column (150 x 4.6 |im). The mobile phase consisted of acetonitrile (solvent A) and 2 % acetic acid in water (v/v) (solvent B). The total run time was 70 minutes at a flow rate of one ml/min with gradient programmed as follows: 100 to 85% (in 30 min), 85 to 50 % (in 20 min), 50 to 0 % (in 5 min) and 0-100 % (in 5 min) of solvent B. There was 10 min of post-run for reconditioning. The obtained peaks were monitored simultaneously at 280, 320, and 360 nm. All samples were filtered through a 0.45 |im Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with the standards. HPLC phenolic compounds standards were gallic, protocatechuic, p-hydroxybenzoic, gentisic, cateachin, chlorogenic, vanillic, syringic, caffeic, ferulic, sinapic, p- coumaric, rutin, apigenin-7- glucoside, rosmarinic, cinnamic, qurecetin, apigenin, kaempferol and Chrysin (El-Shazly et al., 2012).

Animal adaptation:

Male albino rats initially 90 -100 gm were used. Four or five animals were housed in a cage under controlled conditions (22± 2 ° C, 50-60 % relative humidity, and 12h light-dark cycles). Healthy male albino rats were obtained from the Holding company for Biological Products and Vaccines (Vaccera), Helwan- Giza, Egypt, and allocated to plastic cages covered with metal grids with dry husk padding and allowed to acclimate for 10 days in the animal facility conditions before being divided into groups for experimentation. This diet was composed of (v/v) a percentage of dried grass, soybean, wheat bran, corn, mixed vitamins, minerals (salts), bone powder, and bean straw. These contents were presented with different percentages as follows: Crude protein: 14%, Corn oil: 15%, and Crude fiber: 11.43%. Ingestion energy: not more than 488.76 K.Cal/100g. Animals were carefully observed every day and their body weights, food consumption, and water intakes were measured precisely every week to follow up on any signs of toxicity or abnormality during the experiment. During the experiment minimizing the risk of suffering rats including (pain) with providing good animal welfare was taken into consideration.

Experimental Groups:

A total of 68 male albino rats were divided into 7 groups as follows: Group 1 (G1) (8 rats): Animals of this group received no treatment and served as a control group. Group 2 (G2) (10 rats): Each rat was orally administrated with a gavage tube extract of *Salix mucronata* daily for 8 weeks (150mg/kg) (Abdel-Aty et al., 2018; Wahid et al., 2016). Group 3 (G3) (10 rats): Injected by Ibuprofen (IBP) only dissolved in sterile saline intra-peritoneal (I.P.) daily for 8 weeks (40mg/kg) (El-Sayed et al., 2015). Group 4 (G4) (10 rats): Administrated by acetylsalicylate (ASA) only (300mg/kg) daily for 8 weeks to induce liver fibrosis (Aziz et al., 2018). Group 5 (G5) (10 rats): Administrated firstly with acetylsalicylate (ASA) and at the same time injected with Ibuprofen (IBP) to investigate the effect of Ibuprofen on the rats. Group 6 (G6) (10 rats): Administrated firstly with acetylsalicylate and simultaneously each rat was given orally extract of *Salix mucronata* to investigate the effect of the extract on the infected rats. Group 7 (G7) (10 rats): Administrated firstly with acetylsalicylate (ASA) and at the same time treated with a combination of both extract and Ibuprofen. The whole experiment duration was 8 weeks.

Chemicals: Acetylsalicylate (ASA) was obtained from Oxford Laboratories reagent Neminath Industrial Estate, Navghar, Vasai East, Palghar-410210, Maharashtra, India. Ibuprofen (IBP) was obtained from Abbott Company and manufactured by Kahira Pharmaceutical & Chemical Industries Company, 4 Abd Elhamid Eldeeb St. Shoubra- Cairo, under license

from Abbott Laboratories limited USA and its subsidiary in Pakistan.

Clinical observations:

All animals were observed every day for systematic clinical status. The weight of the rats was measured weakly throughout the experimental period and all procedures were performed at a high institute of public health, Damanhour University, Egypt. Cirrhosis and mortalities were recorded precisely till the end of the experiment. Liver cirrhosis induction: Cirrhosis development was recorded after 8 weeks of taking acetylsalicylate (ASA). Estimation of serum aspartate aminotransferase levels (AST) (EC 2.6.1.1). Aspartate aminotransferase activity in serum was assayed according to the method of (Reitman &Frankel) (Livio et al., 1989).

Table 1: Weights of rats for different groups

Group	Number of rats in each group	Average of body wt (g) of each group First week	After 8 weeks
G1 (Control)	8	95	230
G2 (Extract)	10	96	200
G3 (ASA)	10	94	140
G4 (IBP)	10	97	170
G5 (ASA and IBP)	10	98	180
G6 (ASA and extract)	10	96	220
G7 (ASA, IBP, and extract)	10	95	250

Table 2: List of chemicals

Chemicals	Supplier
Bromophenol blue	Sigma Cat# D114391
Ethidium bromide dye	Sigma Cat# 54457 BioUltra
HEPES	Sigma Cat# I8896
Molecular grade water	Mediatech, Inc. Cat# 46-000-CM
Primers	Sigma Aldrich
Protease Inhibitor	Sigma SIGMAFAST™ Cat#S8829
Proteinase K	Sigma Chemical Co. - USA
List of Kits	
Kits	Source
TRIzol Total RNA Extraction kit	Life Technologies, Inc Cat# 15596-026
HiSenScript^{TM}RH[-] cDNA Synthesis Kit	iNtRON Biotechnology, Cat# 25014
cDNA synthesis kit	Thermo Scientific RevertAid first strand cDNA synthesis kit Cat# K1622
SYBR green qPCR master Mix, no ROX	Thermo Scientific, Cat# K0251

Estimation of serum alanine aminotransferase levels (ALT) (EC 2.6.1.2): ALT activity in serum was estimated according to the method of (Reitman &Frankel, 1957). Estimation of serum Alkaline Phosphatase levels (ALP) (EC 3.1.3.1): Alkaline phosphatase level was estimated using the method of (Belfield &Frankel, 1957). Estimation of serum total bilirubin: Total protein in serum was assayed by using the method of (Walter & Gerade, 1970). Albumin in serum was assayed by using the method of (Doumas et al., 1971). Estimation of serum urea levels: Urea in serum was assayed by using the method of (Fawcett and Scott, 1960). Estimation of serum creatinine: Creatinine in serum was assayed by using the method of (Schirmeister et al., 1964). Determination of GammaGlutamyltransferase (GGT) (EC 2.3.2.2): y-Glutamyltransferase (GGT) was assayed by using the kit of Sigma-Aldrich Company, Catalog Number MAK089.

Determination of 5 -Nucleotidase (5'-NT) (EC 3.1.3.5):

5 -Nucleotidase level was assayed using the kit of Cusabio Company, catalog number CSB- E07405r. Glutathione reduced level was determined by a commercial kit supplied by Biodignostic, Giza, Egypt according to the method of (Beutler et al., 1963). Determination of catalase (CAT) in liver tissues (EC 1.11.1.6): Catalase level was determined by a commercial kit supplied by Biodignostic, Co, Giza, Egypt according to the method of (Aebi, 1984). The determination of hepatic malondialdehyde (MDA) as a marker of lipid peroxidation level was assayed by using the method of (Ohkawa et al., 1979). Nitric oxide was assayed by using the method of (Montogomery and Dymock, 1961). Hydrogen peroxide was assayed by using the method of (Abei, 1984). Alpha-fetoprotein (AFP) level was assayed using a kit from Panomics Company, catalog number BC 1009. Principle: The AFP ELISA test is based on the principle of a solid-phase enzyme-linked immunosorbent assay. The assay system utilizes a goat anti-AFP antibody directed against intact AFP for solid Phase immobilization. A monoclonal anti-AFP antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. Tumor necrosis factor-alpha (TNF-alpha) level was assayed using a kit from BioSource Company, catalog number RK00029. Interleukin 6 level was assayed using a kit from BioSource Company, catalog number MBS726707. IL-6 ELISA kit applies the competitive enzyme immunoassay technique utilizing a polyclonal anti-IL-6 antibody and an IL-6-HRP conjugate. The assay sample and buffer are incubated together with IL-6-HRP conjugate in a pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for the HRP enzyme.

Primer design: The majority of SYBR Green-based assays used for this project were designed using the Primer3 program (Rozen and Skaletsky, 1999) and BLAST (Ye et. al., 2006). The mRNA sequences were taken from the NCBI database. In the case of genes known to have two or more splicing variants, the primers were designed in a common part, so that all mRNA variants are detected. The length of the PCR product was ideally between 75 and 150 bp (and for all primer pairs designed, it was 50-200 bp). The annealing temperature for all the designed primers was 60°C so that the standard cycling conditions could be used, and TaqMan assays could be run together with SYBR Green-based assays on one plate. To avoid detection of genomic DNA, primer pairs were routinely tested for PCR- efficiency on serially diluted cDNA.

Procedure: Isolation of total RNA:

Total RNA was extracted from frozen liver tissues according to Chomczynski and Sacchi procedure (Chomczynski and Sacchi, 1987) using TRIzol Total RNA Extraction kit (Phenol and Guanidine isothiocyanate Method). Briefly, 50 - 100 mg tissue was homogenized in 700 - 100 |l of TRIzol reagent

using glass Teflon and incubated in ice for 10 minutes (to permit complete dissociation of the nucleoprotein complex). Then, 150 - 200 ^l of chloroform was added to the homogenate and mixed by vigorous vortexing for 15 - 20 seconds and incubated in ice for 10 - 15 minutes then centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was transferred into a new tube and the same volume (about 0.5 ml) of 100% isopropanol was added, mixed, and incubated in ice for 10 - 20 minutes. The mixture was then centrifuged at 12,000 rpm for 10 min at 4°C and the aqueous/isopropanol supernatant was pipetted out and discarded, leaving the RNA pellet. The pellet was washed twice with 1ml of ice-cold 75% ethanol solution then the tube was centrifuged at 7500 rpm for 5 minutes at 4°C and the wash solution is discarded. The RNA pellet, then, was left to air-dry for 5 - 10 minutes. Finally, the air-dried RNA pellet was resuspended in 50 - 100 |il RNase-free H2O (bypassing the solution up and down several times through the pipette tip) and stored at -80°C for downstream applications.

Determination of RNA concentration and purity: RNA concentration was determined by measuring the absorbance at 260 nm (RNA solution was diluted 1/100 |il with RNase-free water). The RNA concentration, in ^g/ml, was calculated using the following formula: [A260 x D.F x 40]; depending on the fact that 1 absorbance unit at 260 nm corresponds to an approximate concentration of 40 |ig/ml of single-stranded RNA. RNA purity was estimated according to the ratio of absorbance readings at 260 nm and 280 nm [A260 / A280 ratio]. Pure preparations of RNA should have ratios of 1.8 - 2.0.

Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression:

Total RNA was extracted from ~500 mg of frozen brain tissue using BIOZOL reagent (Life Technologies, Inc.) as described previously. cDNA was reverse transcribed from total RNA samples using a cDNA Reverse Transcription kit (Thermo scientific RevertAid). The resulting cDNA was amplified by PCR using MicroRNA specific primers with universal primer (as shown in table) in CYBER Green PCR Master Mix and analyzed with a 7500 ABI PRISM Sequence Detector System according to the manufacturer's instructions (Applied Biosystems, Cheshire, UK). The relative levels of miRNAs, TH, TNF-alpha, IL-ip, IL-6, a-synuclein, iNOS, COX-2, APE1, Notch, p53, and BDNF mRNAs expression were calculated from the relevant signals by normalizing with the signal for U6 miRNA & P-Actin expression. The fold change of miRNA & mRNA expression was calculated based on the threshold cycle (Ct) value using the following formula: $2^{-\Delta\Delta CT}$ (Abei, 1984).

Complete blood counts (CBC): Complete blood count was counted by the automated method using Dirui BCC-3600, MA, USA automated hematology analyzer.

Statistical analysis: The obtained results were statistically analyzed using GraphPad instat, Version 3.06 (GraphPad Software Inc., San Diego, California USA), and the data were expressed as mean ± standard deviations (SD) and statistically analyzed by one-way ANOVA (Analysis of Variance) for multiple comparisons. P values less than 0.05 were considered significant. The charts were plotted using Microsoft excel software (2010 version).

Table 3: List of primer sequences:

Primer	Sequence
PTEN-F	AAT TCC CAG TCA GAG GCG CTA TGT
PTEN-R	GAT TGC AAG TTC CGC CAC TGA ACA
COX2-F	AGG CCT CCA TTG ACC AGA
COX2-R	TCA TGG TAG AGG GCT TTC AAC
COX-1 -F	CTG CAT GTG GCT GTG GAT GTC ATC
COX-1 -R	GGT CTT GGT GAG GCA GAC CAG

RESULTS

Determination of AST in the serum of rats:

G3 (rats injected with only ibuprofen) and G4 (rats injected with ASA) showed a highly significant increase in AST concentration compared with G1 (control) which ranged between 33-37, while G2 (rats administered with only extract) showed a high significant decrease in AST concentration when compared with G4 (rats injected with ASA). By treatment with either ibuprofen, extract, or a combination of ibuprofen and extract (G5, G6, and G7, respectively) the results showed a highly significant decrease in AST concentration if compared with G4 (rats injected with ASA) which was representing excellent regression.

Determination of Alkaline Phosphatase in the serum of rats:

Rats injected with ibuprofen, rats injected with ASA, rats injected with both ibuprofen and ASA, rats treated with extract, and rats treated with both extract and ibuprofen (G3, G4, G5, G6, and G7) respectively showed a significant increase in ALP concentration if compared with G1 control which ranged between 89 - 93. Rats administrated with extract only G2 showed a highly significant decrease in ALP concentration if compared with G4, by treatment with ibuprofen or extract or combination of both (G5, G6, G7) respectively the results showed a highly significant decrease in ALP concentration if compared with G4 (rats injected with ASA) which was representing excellent regression.

Determination of GGT in rats

Rats injected with only ibuprofen (G3) and rats injected with only ASA (G4) showed a highly significant increase in GGT concentration if compared with (G1) control which ranged between 36 - 38, while (G5) rats injected with both ASA and ibuprofen revealed a very significant increase in GGT concentration is also compared with (G1). (G2) rats administered with only extract showed a highly significant decrease in GGT concentration, while in treatment with either extract only (G6) or a combination between extract and ibuprofen (G7) the results showed a very significant decrease in GGT concentration if compared all with (G4) mice injected with ASA which was representing excellent regression.

Table (4): AST assessment in serum of rats of different groups

AST (U/ml)	Mean ± S.D
G1(Control)	34.00 ± 1.41
G2 (Extract)	38.00 ± 4.24 [b]
G3 (Ibuprofen)	86.50 ± 2.12 [a]
G4 (ASA)	133.50 ± 2.12 [a]
G5 (ASA+Ibuprofen)	85.00 ± 2.82 [b]
G6 (ASA + Extract)	68.50 ± 2.12 [b]
G7 (ASA+Extract+Ibp.)	42.50 ± 4.94 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Table (5): ALP assessment in the serum of rats of different groups

ALP (IU/L)	Mean ± S.D
G1 (Control)	91.00 ± 1.41
G2 (Extract)	101.51 ± 2.12 [b]
G3 (Ibuprofen)	122.50 ± 3.53 [a]
G4 (ASA)	204.50 ± 2.12 [a]
G5 (ASA+Ibuprofen)	174.50 ± 3.53 [a], [b]
G6 (ASA+Extract)	138.00 ± 2.82 [a], [b]
G7 (ASA+Extract+Ibp.)	100.50 ± 3.53 [a], [b]

a: significant if compared with G1(control). b: significant if compared with G4(rats injected with ASA)

Table 6: GGT assessment in tissues of rats of different groups

GGT (U/ml)	MEAN ±S.D
G1 (Control)	37.50 ± 0.0707
G2 (Extract)	39.00 ± 1.414 [b]
G3 (Ibuprofen)	53.50 ± 2.12 [a]
G4 (ASA)	55.00 ± 1.414 [a]
G5 (ASA+ Ibuprofen)	51.00 ± 2.82 [a]
G6 (ASA+Extract)	40.50 ± 4.94 [b]
G7 (ASA+Extract+Ibp.)	39.00 ± 1.414 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Determination of total bilirubin in the serum of rats

Rats injected with ASA (G4) showed a significant increase in total bilirubin concentration also rats injected with ibuprofen (G3) and rats injected with both ASA and ibuprofen (G5) showed a very significant increase in total bilirubin concentration if all these groups compared with (G1) control which ranged between 0.245 - 0.328. Rats administrated with only extract (G2) showed a very significant decrease in total bilirubin concentration if compared with (G4). By treatment with either extract or a combination of both extract and ibuprofen, the results showed a significant decrease in total bilirubin concentration if compared with (G4) rats injected with ASA only.

Determination of urea in the serum of rats

Rats injected with ibuprofen only (G3) showed a significant increase in urea concentration, while rats injected with ASA only (G4) and rats injected with both ASA and ibuprofen (G5) showed a highly significant increase in urea concentration if compared with G1 (control) which ranged between 28 - 34. Rats administrated with only extract (G2) showed a very significant decrease in urea concentration, while by treatment with extract or a combination of extract and ibuprofen (G6, G7 respectively) the result showed a very significant decrease in urea concentration if compared with G4 (rats injected with ASA) which was representing excellent regression.

Table (7): Total bilirubin assessment in serum of rats of different groups

T.BIL (Mg/dl)	Mean ± S.D
G1 (Control)	0.287 ± 0.0414
G2 (Extract)	0.27 ± 0.028 [b]
G3 (Ibuprofen)	0.53 ± 0.0141 [a]
G4 (ASA)	0.45 ± 0.0565 [a]
G5 (ASA+Ibpuprofen)	0.445 ± 0.0141 [a]
G6 (ASA+ Extract)	0.315 ± 0.035 [b]
G7 (ASA+ extract+ Ibp.)	0.289 ± 0.078 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Table (8): Urea assessment in serum of rats of different groups

Urea (Mg/dl)	Mean ± S.D
G1 (Control)	31.8 ± 3.11
G2 (Extract)	33.01 ± 4.24 [b]
G3 (Ibpuprofen)	44.10 ± 4.38 [a]
G4 (ASA)	51.65 ± 4.73 [a]
G5 (ASA+Ibpuprofen)	50.75 ± 1.76 [a]
G6 (ASA+Extract)	33.55 ± 2.05 [b]
G7 (ASA+Extract+Ibp.)	29.05 ± 1.48 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Determination of creatinine in the serum of rats

Rats injected with ASA (G4) and rats injected only with ibuprofen (G3) showed a significant increase in creatinine concentration if compared with G1 (control) which ranged between 0.237 - 0.392, while rats administered with only extract (G2) showed a significant decrease in creatinine concentration if compared with G4 (rats injected with ASA). In treatment with ibuprofen, extract or a combination of extract and ibuprofen (G5, G6, and G7 respectively) the results showed a significant decrease in creatinine concentration in comparison with G4 (rats injected with ASA) which was representing good regression.

Determination of GSH in tissues of rats

Rats injected with ASA (G4), rats injected with only ibuprofen (G3) rats injected with both ASA and ibuprofen (G5) showed a highly significant decrease in GSH concentration if compared with (G1) control which ranged between 63- 67. By treatment with extract or a combination between extract and ibuprofen (G6, G7) respectively the results showed a very significant increase in GSH concentration if compared with G4 while rats administrated with only extract (G2) showed a highly significant increase if also compared with G4.

Table (9): Creatinine assessment in the serum of rats of different groups

Creatinine (Mg/dl)	Mean ± S.D
G1 (Control)	0.315 ± 0.0777
G2 (Extract)	0.32 ± 0.0141 [b]
G3 (Ibuprofen)	0.51 ± 0.0565 [b]
G4 (ASA)	0.705 ± 0.0919 [a]
G5 (ASA+ Ibuprofen)	0.525 ± 0.0212 [b]
G6 (ASA + Extract)	0.425 ± 0.0919 [b]
G7 (ASA+ Extract +Ibp.)	0.45 ± 0.0565 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Table (10): GSH assessment in tissues of rats of different groups

GSH (Mg/g)	Mean ± S.D
G1 (Control)	65.60 ± 2.33
G2 (Extract)	73.52 ± 4.94 [b]
G3 (Ibuprofen)	42.40 ± 1.27 [a]
G4 (ASA)	34.71 ± 5.65 [a]
G5 (ASA+Ibuprofen)	44.00 ± 0.98 [a]
G6 (ASA+Extract)	61.00 ± 3.25 [b]
G7 (ASA+Extract+Ibp.)	55.91 ± 4.52 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Determination of MDA in tissues of rats

Rats injected with ASA (G4), rats injected with ibuprofen (G3), rats injected with both ASA and ibuprofen (G5), rats treated with the extract (G6), and rats treated with both extract and ibuprofen (G7) all showed a significant increase in MDA concentration if compared with G1 (control) which ranged between 4.32 - 5.88. Rats administrated with only extract showed a highly significant decrease in MDA concentration if compared with G4 (mice injected with ASA). By treatment with ibuprofen or extract or combination of both (G5, G6, G7) the results showed a very significant decrease in MDA concentration if compared with G4 (mice injected with ASA) which was representing excellent regression.

Determination of CAT in tissues of rats

Rats injected with ibuprofen and rats injected with ASA (G3, G4) respectively showed a highly significant decrease in CAT concentration compared with G1 (control) which ranged between 17- 21. In comparing other groups G2, G6, and G7 (rats administrated with only extract, rats treated with extract, and rats treated with a combination of extract and ibuprofen) respectively with G4 (rats injected with ASA) the results showed a very significant increase in CAT concentration.

Table (11): MDA assessment in tissues of rats of different groups

MDA (nM/mg protein)	Mean ± S.D
G1 (Control)	5.10 ± 0.782
G2 (Extract)	5.65 ± 0.777 [b]
G3 (Ibuprofen)	24.70 ± 1.55 [a]
G4 (ASA)	38.40 ± 0.919 [a]
G5 (ASA+Ibuprofen)	24.00 ± 3.25 [a], [b]
G6 (ASA+Extract)	10.45 ± 3.04 [a], [b]
G (ASA+Extract+Ibp.)	8.35 ± 1.2 [a], [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Table (12): CAT assessment in tissues of rats of different groups

CAT (U/mg protein)	Mean ± S.D
G1 (Control)	19.15 ± 1.48
G2 (Extract)	17.35 ± 1.48 [b]
G3 (Ibuprofen)	10.25 ± 0.777 [a]
G4 (ASA)	6.85 ± 1.06 [a]
G5 (ASA+Ibuprofen)	10.12 ± 1.83 [a]
G6 (ASA+Extract)	13.65 ± 2.19 [a b]
G7 (ASA+Extract+Ibp.)	18.85 ± 2.19 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Determination of Hydrogen peroxide in rats

Rats injected with ASA (G4) and rats injected with Ibuprofen (G3) showed a highly significant increase in hydrogen peroxide concentration if compared with G1 (control) which ranged from 108 - 111. Rats administrated with only extract (G2) showed a highly significant decrease in hydrogen peroxide concentration if compared with (G4) rats injected with ASA. By treatment with ibuprofen only or extract only or a combination of both (G5, G6, G7) respectively the results showed a significant decrease in hydrogen peroxide concentration if compared with (G4) rats injected with ASA.

Determination of nitric oxide (NO) in the serum of rats of different groups

Rats injected with ibuprofen only (G3) or with ASA only (G4) or both ASA and ibuprofen (G5) showed a very significant increase in nitric oxide concentration if compared with (G1) control which ranged between 3 - 7. Rats administrated with only extract (G2) showed a highly significant decrease in nitric oxide concentration if compared with (G4) rats injected with ASA. By treatment with either extract or a combination of both extract and ibuprofen (G6, G7) respectively the results showed a highly significant decrease in nitric oxide concentration and excellent regression if compared with (G4) rats injected with ASA. Rats injected with ASA (G4) and rats injected with ibuprofen (G3) showed a very significant increase in AFP concentration if compared with (G1) control which ranged between 4- 9. Rats administrated with extract G2 showed a highly significant decrease in AFP concentration if compared with G4 (mice injected with ASA), by treatment with ibuprofen or extract or combination of both (G5, G6, and G7) respectively the results showed a very significant decrease in AFP concentration if compared with G4 (rats injected with ASA) which was representing excellent regression.

Table (13): Hydrogen peroxide assessment in rats of different groups

Hydrogen peroxide (mM/g. tissue)	Mean ± S.D
G1 (Control)	109.60 ± 1.41
G2 (Extract)	116.50 ± 1.31 [b]
G3 (Ibuprofen)	126.10 ± 1.34 [a]
G4 (ASA)	151.31 ± 3.04 [a]
G5 (ASA+Ibuprofen)	138.72 ± 4.59 [b]
G6 (ASA+ Extract)	131.21 ± 3.04 [b]
G7 (ASA+Extract+Ibp.)	122.12 ± 4.24 [b]

a: significant if compared with G1(control). b: significant if compared with G4(rats injected with ASA)

Table (14): NO assessment in the serum of rats of different groups

NO (^mol/L)	Mean ± S.D
G1(Control)	4.70 ± 0.141
G2 (Extract)	6.52 ± 0.282 [b]
G3 (Ibuprofen)	11.45 ± 1.767 [a]
G4 (ASA)	23.85 ± 1.767 [a]
G5 (ASA+Ibuprofen)	19.55 ± 2.474 [a]
G6 (ASA+Extract)	12.45 ± 1.06 [b]
G7 (ASA+Extract+Ibp.)	9.75 ± 1.202 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Determination of TNF-alpha in the serum of rats of different groups

Rats injected with ASA (G4), rats injected with ibuprofen (G3) and rats injected with both (G5) showed a highly significant increase in TNF-alpha concentration if compared with G1 (control) which ranged between 90 - 94, while rats injected with only extract (G2) showed highly significant decrease if compared with (G4). By treatment with ibuprofen only, extract only or a combination of both (G5, G6, and G7 respectively) the results showed a very significant decrease in TNF-alpha concentration if compared with G4 (rats injected with ASA) which was representing excellent regression. Rats injected with ibuprofen (G3), rats injected with ASA (G4), and rats injected with both (G5) showed a highly significant increase in IL-6 concentration if compared with G1 (control) which ranged between 64 - 67. Treatment with either ibuprofen, extract, or a combination of both (G5, G6, and G7 respectively) highly significant decrease in IL-6 concentration if compared with G4 (rats injected with ASA) which was representing excellent regression. (G2) rats administrated with only extract showed also a highly significant decrease in IL-6 concentration if compared with G4. G1 Control G2 Extract G3 Ibuprofen G4 ASA G3 (rats injected with only ibuprofen) and G4 (rats injected with ASA) showed a significant increase in 5'-nucleotadase concentration if compared with G1 (control) which ranged between 190-195. Rats administrated with only extract (G2) showed a highly significant decrease in 5'-neucletodase concentration if compared with G4 (rats injected with ASA). By treatment with either ibuprofen, extract or a combination of ibuprofen and

extract (G5, G6, and G7 respectively) the results showed a highly significant decrease in 5'-nucleotadase concentration if compared with G4 (rats injected with ASA) which was representing excellent regression.

Determination of COX-1 of rats of different groups

G3 (rats injected with only ibuprofen) and G4 (rats injected with ASA) showed a very significant decrease in COX-1 concentration if compared with G1 (control) which ranged between 1.0 -1.33. By treatment with either ibuprofen, extract or a combination of ibuprofen and extract (G5, G6, and G7 respectively) the results showed a significant decrease in COX-1 concentration if compared also with G1 (control) which ranged between 1.0 - 1.33.

Determination of COX-2 of rats of different groups

G3 (rats injected with only ibuprofen), G4 (rats injected with ASA), and G5 (rats treated with ASA+ ibuprofen) showed a significant decrease in COX-2 concentration if compared with G1 (control) which ranged between 1.0 -1.12. Rats administered with extract only (G2) or treated with extract or a combination of ibuprofen and extract (G6 and G7 respectively) the results showed a significant increase in COX-2 concentration if compared also with G4 (rats injected with ASA).

Determination of complete blood count of rats of different groups

The results showed normal hemoglobin concentration (Hb), erythrocyte (RBC) count, packed cell volume (PCV), mean cell hemoglobin (MCH), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), leucocyte (WBC) count, and platelet count in rats administrated with extract (G2) and in rats treated with ibuprofen, extract, or combination of both (G5, G6, G7 respectively). Rats injected with ASA (G4) and rats injected with ibuprofen (G3) showed lower erythrocyte (RBC) count, hemoglobin (Hb), and packed cell volume (PCV) as compared with the control group (G1). The mean cell hemoglobin (MCH) and the mean cell volume (MCV) were significantly greater than controls, while the mean cell hemoglobin concentration (MCHC) was similar. The Total leucocyte (WBC) count, absolute neutrophil count, and lymphocyte count were higher if compared with G1 (control). To detect the effect of prostaglandin inhibitors induced hepatocellular damage and treatment by Salix extract on the hepatocytes. Light microscope preparation was used. It is firstly important to point out and illustrate the histological appearance of the normal liver of rats. This trend will suggestively give a typical model for comparison between normal and pathological conditions and offer a more accurate assessment and better evaluation of any pathological alternations that occurred in experimental animals in the present work.

Table (15): TNF-alpha assessment in the serum of rats of different groups.

TNF-alpha (Pg/ml)	Mean ± S.D
G1 (Control)	92.15 ± 3.04
G2 (Extract)	72.95 ± 4.87 [b]
G3 (Ibuprofen)	176.65 ± 4.73 [a]
G4 (ASA)	207.70 ± 1.69 [a]
G5 (ASA+Ibuprofen)	193.71 ± 3.81 [a b]
G6 (ASA+ Extract)	161.00 ± 2.82 [b]
G7 (ASA+Extract+Ibp.)	155.71 ± 1.83 [b]

a: significant if compared with G1 (control). b: significant if compared with G4 (rats injected with ASA)

Table (16): COX-1 assessment in rats of different groups

COX-1	Mean ± S.D
G1 (Control)	1.0 ± 0.33
G2 (Extract)	1.02 ± 0.34 [b]
G3 (Ibuprofen)	0.57 ± 0.14 [a]
G4 (ASA)	0.33 ± 0.12 [a]
G5 (ASA + Ibuprofen)	0.54 ± 0.17 [a]
G6 (ASA + Extract)	0.83 ± 0.09 [a, b]
G7 (ASA + Extract + Ibuprofen)	0.76 ± 0.2 [a, b]

a: Significant if compared with G1 (control) b: Significant if compared with G4 (rats injected with ASA)

Table (17): COX-2 assessment in rats of different groups

COX-2	Mean ± S.D.
G1 (Control)	1.0 ± 0.12
G2 (Extract)	0.98 ±0.22 [b]
G3 (Ibuprofen)	0.77 ± 0.13 [a]
G4 (ASA)	0.59 ± 0.14 [a]
G5 (ASA + Ibuprofen)	0.64 ± 0.13 [a]
G6 (ASA + Extract)	0.91 ± 0.11 [b]
G7 (ASA + Extract + Ibuprofen)	0.88 ± 0.08 [b]

a: Significant if compared with G1 (control) b: Significant if compared with G4 (rats injected with ASA)

Table (18): Complete blood picture of rats of different groups

	G1	G2	G3	G4	G5	G6	G7	UNIT
Hemoglobin concentration	13.2	14.3	12.8	9.2	11.9	13.7	14.1	g/dl
Red Cell Count	6.56	7.5	8.2	4.9	5.8	6.9	7.1	M/ul
Haematocrit (PCV)	40.3	40.5	42.3	34.2	36.5	39.5	40.1	%
M.C. V	61.5	54	51.6	71.5	67.1	57	60.7	fL
M.C.H	21.9	19.1	18.1	27.9	23.8	21.1	19.7	Pg
M.C.H.C	30.3	35.5	34.9	34.1	33.9	34.9	32.5	g/dl
R.D.W-CV	19.5	19.5	16.8	17.5	19.7	16.7	17.5	%
Platelet Count	490	652	610	519	520	515	502	x1000/ul
(WBC)	5.2	6.6	11.2	12.2	10.1	9.2	7.8	x1000/ul

Discussion:

Treatment strategies for liver problems and detection methods depend on the amount and progression of liver fibrosis and the rate of cirrhosis development (Beltagy et al., 2020; Gabr et al., 2020). This study aims to assess the induced cirrhotic effect of high doses of prostaglandin inhibitors such as acetylsalicylate (ASA) administration on the liver of rats. Ibuprofen was used as a reference drug to determine the protective impact of willow bark (*Salix mucronata*) extract on liver cirrhosis in rats with studying the role of cyclooxygenases (COX-1) and (COX-2) in the diagnosis and prognosis of liver cirrhosis. This study investigates the effect of a high chronic dose of ASA, IBP, and the protective, therapeutic effect of ibuprofen also after injection with ASA. The measurement of serum activities of ALT and AST is relevant for the diagnoses of liver dysfunction as these enzymes are present in the liver cytosol (ALT) and mitochondria (AST) thus a significant increase in the serum level of ALT suggests severe hepatic damage (Toosi, 2015). Results showed that ALT and AST concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by two-fold and three-fold, respectively. While in the extract group (G2), ALT and AST activities were very close to the normal values obtained for the control group (G1). By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7, respectively) the concentration of ALT and AST decreased returning to the normal ranges as the best regression was achieved in G7. From the obtained results ibuprofen increased the level of serum ALT and AST when it is used for a long duration as aspartate aminotransferase (AST) was increased dose- and time-dependently, ALT was affected only after 28 days of exposure to a high dose of ibuprofen (40 mg/kg) used in this study, the effect of ibuprofen on liver function strongly correlates positively with the dose and the duration of exposure that are in line with previous studies (Lees, 1994). High chronic doses of ASA play a role in the progression of liver fibrosis and can cause hepatic problems and these results agreed with previous studies (Aprioku et al., 2014).

The results showed that GGT concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by one-and-a-half-fold, respectively. While the extract group (G2) showed nearly normal values of GGT. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of GGT decreased returning to the normal ranges as the best regression was in G7. From the obtained results, ibuprofen and ASA increased the level of serum GGT when it is used for a long duration and high chronic dose, and these results are in agreement with previous studies (Chavez et al., 2012). Serum alkaline phosphatase level is generally used in toxicological studies to evaluate hepatic function. Elevation in serum alkaline phosphatase (ALP) by ibuprofen in this study is indicative of cellular injury to the liver. The results showed that ALP concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by 1.5-fold and 3 folds, respectively. While extract-administered rats (G2) showed very close to the normal values of ALP in control rats. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7, respectively) the concentration of ALP decreased returning to the normal ranges as the best regression was in G7. As noticed from the obtained results, ibuprofen and ASA cause a significant increase in serum ALP concentrations when it is used for a long duration and in high chronic doses. These results are in line with previous studies (Elkamshoushi et al., 2020).

ASA and IBP are capable of preventing lipid peroxidation and increasing the levels of glutathione, one of the most important oxidative defenses (Gabr et al., 2020; Beltagy et al., 2020) but this study showed that high chronic doses of ASA and IBP loss their ability to prevent lipid peroxidation while the level of lipid peroxidation indicators return to increase when ibuprofen used as a treatment for a short period after

injection with ASA so of ibuprofen has two effects on a long duration of administration and short duration. The extent of lipid peroxidation can be determined in liver homogenates by measuring the formation of malondialdehyde (MDA) (Jain et al., 2012). MDA is a major oxidation product of peroxidized polyunsaturated fatty acids. The monitoring of MDA levels in different biological systems can be used as important indicators of lipid peroxidation and oxidative stress both in-vitro and in-vivo for various (Aprioku & Uche 2013). which showed an increase in MDA indicating the effects of oxidative stress on lipids. Increased levels of oxidative stress have been associated with various disease patterns. In this study, results showed that MDA concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by fivefold and sevenfold respectively while in the extract group (G2) very close to the normal values of MDA were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of MDA decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of MDA in liver tissues of rats when it is used for a long duration also a high chronic dose of ASA plays a role in the increased production of MDA these results are in agreement with previous studies which reported that ASA increases MDA levels even at low (Sarihan et al., 2017) so along-term injection with ASA impaired the antioxidant enzyme levels and increased lipid peroxidation products. In addition, the liver and renal functions were slightly impaired.

GSH concentration decreased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by onefold and twofold respectively while in the extract group (G2) very close to the normal values of GSH were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of GSH increased returning to the value of the normal range in G7. From the obtained results ibuprofen decreased the level of GSH in liver tissues of rats when it is used for a long duration also a high chronic dose of ASA plays a role in the decreased production of GSH these results are in agreement with previous studies (Beltagy et al., 2015; Gabr et al., 2020) which reported that ASA decreases GSH levels. Aspirin induces the formation of Nitric oxide (NO) radicals in the body. This reduces leukocyte adhesion, which is an important step in the immune response to infection (Kesik et al., 2008). The results showed that nitric oxide concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by threefold and fivefold respectively while in the extract group (G2) very close to the normal values of nitric oxide were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of nitric oxide decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of nitric oxide when it is used for a long duration and a high chronic dose of ASA these results are in agreement with previous studies (Paul-Clark et al., 2004).

From the obtained results ibuprofen decreased the level of CAT in liver tissues of rats when it is used for a long duration also a high chronic dose of ASA plays a role in the decreased production of CAT these results are in agreement with previous studies (Cheng & Harris, 2005) which reported that ASA decreases CAT levels. The measurement of serum urea and creatinine is relevant for the diagnoses of renal dysfunction as results showed that urea and creatinine concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by one-fold and two-fold respectively while in extract group (G2) very close to the normal values of both urea and creatinine were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of urea and creatinine decreased

returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of serum urea and creatinine when it is used for a long duration. Also, a high chronic dose of ASA impaired renal functions and these results agree with the previous studies (Iida et al., 2010).

The results showed that albumin concentration decreased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by one-fold and three-fold respectively while in the extract group (G2) very close to the normal values of albumin were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of albumin increased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen decreased the level of serum albumin when it is used for a long duration also a high chronic dose of ASA impaired the liver and renal functions and these results are in agreement with previous studies (Gomaa et al., 1990). The results showed that total bilirubin concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by twofold and one-fold respectively while in the extract group (G2) very close to the normal values of albumin were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7); respectively. The concentration of total bilirubin decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of serum total bilirubin when it is used for a long duration also a high chronic dose of ASA impaired liver functions and these results agree with previous studies (Ghonemi & Eldahshan, 2012).

From the obtained results ibuprofen increased the level of 5 nucleotidase when it is used for a long duration also a high chronic dose of ASA increased the concentration of it and these results are in agreement with previous studies (Reynolds, 1982). The results showed that AFP concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by half fold respectively while in the extract group (G2) very close to the normal values of AFP were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of AFP decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of AFP when it is used for a long duration also a high chronic dose of ASA increased the concentration of it and these results are in agreement with previous studies. Tumor necrosis factor (TNF –alpha) and interleukin (IL-6) are considered major hepatotoxicity mediators in several experimental models of liver injuries (Wong et al., 2015). TNF-alpha was originally identified as a circulating factor. It has been implicated in some liver diseases and is an important mediator of many physiological conditions. TNF-alpha is expressed by both infiltrating inflammatory cells and hepatocytes in chronic liver injuries, and it has been proposed to play an important role during tissue damage (Kovalovich et al., 2000). Our data provide further evidence for the role of hepatic fibrosis. The results showed that TNF-alpha concentration decreased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by twofold and two and a half fold respectively while in the extract group (G2) very close to the normal values of TNF-alpha were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of TNF-alpha decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of TNF-alpha when it is used for a long duration and also a high chronic dose of ASA.

IL-6 might be vitally involved in fibrotic changes, partly by modulating the intrahepatic expression of other cytokines (Zhen et al., 2000). The results showed that IL-6 concentration increased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by half-fold and one-fold respectively while in the extract

group (G2) very close to the normal values of IL-6 were observed. By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7) respectively the concentration of IL-6 decreased returning to the normal ranges as the best regression was in G7. From the obtained results ibuprofen increased the level of IL-6 when it is used for a long duration and a high chronic dose of ASA and that was in agreement with previous studies (Zhang et al., 2004). Results showed that COX-1 concentration decreased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by two-fold and three-fold, respectively. While in the extract group (G2), COX-1 concentration was very close to the normal values obtained for the control group (G1). By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7, respectively) the concentration of COX-1 increased returning to the normal ranges as the best result was achieved in G7. From the obtained results ibuprofen inhibits COX-1 when it is used for a long duration after 28 days of exposure to a high dose of ibuprofen (40 mg/kg) used in this study, which is in line with previous studies. Also, high chronic doses of ASA play a role in the inhibition of COX-1 and these results agree with previous studies (Abd-Elaziz et al., 2019).

Results showed that COX-2 concentration decreased in G3 (rats injected with ibuprofen) and G4 (rats injected with ASA) by two-fold and three-fold, respectively. While in the extract group (G2), COX-2 concentration was very close to the normal values obtained for the control group (G1). By treatment with extract or ibuprofen or a combination of both in (G5, G6, and G7, respectively) the concentration of COX-2 increased returning to the normal ranges as the best result was achieved in G7. From the obtained results ibuprofen inhibits COX-2 when it is used for a long duration after 28 days of exposure to a high dose of ibuprofen (40 mg/kg) used in this study, which is in line with previous studies. Also, high chronic doses of ASA play a role in the inhibition of COX-2, and these results agree with previous studies (Catella-Lawson et al., 2001). This illustrated that non-selective NSAIDs, at therapeutic doses, inhibit both COX-1 and COX-2. The anti-inflammatory benefits of these drugs are primarily derived from COX-2 inhibition, while inhibition of COX-1 often elicits gastrointestinal (GI) toxicity. Aspirin irreversibly inhibits COX-1 by acetylation of a single serine residue on the enzyme. Ibuprofen inhibits COX by substrate competition with arachidonic acid and produces fewer side effects than aspirin and this was indicated in previous studies (Mitchell et al., 1993).

Conclusion: *Salix mucronata* (Safsaf willow) extract has immune-modulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors. This study suggests that *Salix mucronata* (Safsaf willow) extract is a good source of natural antioxidants as it has rutin, chlorogenic, caffeic, rosmarinic, cinnamic, apigenin, chrysin, apigenin-7-glucoside, ^-coumaric acid, sinapic acid, ferulic acid, vanillic acid, salicin, and cateachin so it has anti-inflammatory activities, hepatoprotective effect and administration of it can reduce or almost prevent toxicological effect induced by administration of a high dose of ASA which was clear in G6 and G7.

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