The English Summary – Ph.D. Thesis

PHARMACOLOGICAL STUDY OF THE POSSIBLE PROTECTIVE EFFECTS OF SOME ANTIOXIDANTS AGAINST EXPERIMENTALLY-INDUCED HEPATOTOXICITY IN RATS

Thesis

Thesis Submitted for the Degree of

Doctor of Philosophy in Pharmaceutical Sciences

(Pharmacology and Toxicology)

By

Basim Anwar Shehata Messiha

B. Pharm. Sci. M. Sc.

Assistant Lecturer

Pharmacology and Toxicology Department

Faculty of Pharmacy, Beni-Sueif University

Summary & Conclusion

In the present investigation, the possible hepatoprotective effects of three natural extracts, namely aloe vera leaf pulp extract, grape seed extract and nutmeg extract were studied in comparison with N-acetylcysteine (NAC) as a standard hepatoprotective agent. To achieve this goal, two models of hepatotoxicity were performed; an *in vivo* model of rat hepatic ischemia/reperfusion (IR) injury and an *in vitro* model using freshly-isolated rat hepatocyte suspension incubated with carbon tetrachloride (CCl_4) as a chemical hepatotoxicant.

For setting of the *in vivo* model, hepatic IR was performed by clamping the portal triad (hepatic artery, portal vein and bile duct) of fasted adult male albino rats (one group was left as sham-operated control operation) for different time periods (15 or 30 minutes) followed by reperfusion (15, 30 or 60 minutes). According to the obtained results, hepatic IR injury model was set as 30 minutes of ischemia followed by 30 minutes of reperfusion.

Test agents (NAC: 150, 300 and 600 mg/kg/day; aloe extract: 5, 10 and 20 ml/kg/day; grape seed extract: 200, 400 and 800 mg/kg/day and nutmeg extract: 250, 500 and 1000 mg/kg/day) were administered orally on daily basis for seven days to normal animals to check the presence of any effect on normal liver functions and to choose suitable doses to conduct in the current experiments.

According to the published literature as well as pilot trials, all test agents (NAC 300 mg/kg/day, aloe 10 ml/kg/day, grape seed 400 mg/kg/day and nutmeg 500 mg/kg/day) were administered orally on daily basis for seven days followed by an additional dose 30 minutes before hepatic ischemia to study their hepatoprotective effects against hepatic ischemia (30 minutes)/reperfusion (30 minutes) injury.

The degree of hepatic injury was assessed by measuring serum alanine transaminase (ALT) and aspartate transaminase (AST) activities, serum total, direct and indirect bilirubin (tBil, dBil and iBil) levels, liver weight/body weight ratio, hepatic myeloperoxidase (MPO) activity, hepatic contents of thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) as well as histopathological changes.

In the *in vitro* experiment, freshly isolated rat hepatocyte suspensions were prepared according to the collagenase perfusion method and divided into 16 flasks. Doses of test agents were selected according to the published literature as well as pilot trials. All flasks, except one left as normal control, were incubated with 5 mM CCl_4. All other flasks, except the one left as CCl_4 control, were incubated with test agents and/or with nitric oxide synthase (NOS) inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME; a non-specific inhibitor) and aminoguanidine (AG; a specific inhibitor to inducible nitric oxide synthase or iNOS enzyme) 30 minutes before CCl_4. Concentrations of the test agents used were as follows: NAC (5 mM), aloe extract (100 µl/ml), grape seed extract (100 µg/ml), nutmeg extract (100 µg/ml), L-NAME (5 mM) and AG (1 mM). Combinations between test agents and NOS inhibitors were also done as follows: NAC + L-NAME, NAC + AG, aloe + L-NAME, aloe + AG, grape seed + L-NAME, grape seed + AG, nutmeg + L-NAME and nutmeg + AG.

Assessment of hepatocyte injury was performed by measuring percent lactate dehydrogenase (LDH) leakage, TBARS production, cellular GSH content, total nitrate/nitrite (NO_X) production, cellular calcium ($[Ca^{2+}]_i$) content and cellular

adenosine triphosphate (ATP) content. These parameters were measured in hepatocyte samples removed from the flasks at times -30, 0, 30, 60, 90 and 120 minutes of hepatocyte incubation with CCl_4.

The main findings of the present investigation can be summarized as follows:

I. *In vivo* Experiments:

1. Daily administration of oral NAC (150, 300 or 600 mg/kg/day), aloe extract (5, 10, 20 ml/kg/day), grape seed extract (200, 400 or 800 mg/kg/day) or nutmeg extract (250, 500 or 1000 mg/kg/day) did not significantly affect serum ALT or AST activities of normal animals.

2. Hepatic ischemia for 15 minutes followed by reperfusion for 15, 30 or 60 minutes did not result in significant elevation of serum transaminases ALT and AST activities compared to sham-operated animals.

3. Hepatic ischemia for 30 minutes followed by 30 minutes of reperfusion significantly elevated serum transaminases ALT and AST compared to sham-operated animals and the elevation was even significantly higher when the reperfusion period was increased to 60 minutes.

4. Hepatic ischemia for 30 minutes followed by 30 minutes of reperfusion significantly increased serum tBil and dBil levels, increased hepatic content of TBARS, decreased hepatic stores of GSH, increased hepatic MPO activity and resulted in significant histopathological changes like inflammatory infiltration, blood congestion and loss of normal hepatic architecture. On the other hand, no significant effect was noted regarding iBil or liver weight/body weight ratio.

5. Pre-treatment of rats with NAC (300 mg/kg/day; p.o.), aloe extract (10 ml/kg/day, p.o.), grape seed extract (400 mg/kg/day, p.o.), nutmeg extract (500 mg/kg/day, p.o.) or a combination of aloe, grape seed and nutmeg extracts for 7 days, plus an additional dose 30 minutes before ischemia, significantly reduced hepatic IR-induced injury as evidenced by suppression of serum transaminases ALT and AST as well as elevation of hepatic GSH content. The histopathological findings strongly inforced the obtained results of the biochemical evaluations.

6. Pre-treatment of rats with NAC (300 mg/kg/day; p.o.), aloe extract (10 ml/kg/day, p.o.), or a combination of aloe, grape seed and nutmeg extracts significantly reduced hepatic MPO activity compared to ischemic control.

7. Pre-treatment of rats with NAC (300 mg/kg/day; p.o.), aloe extract (10 ml/kg/day, p.o.), grape seed extract (400 mg/kg/day, p.o.) or a combination of aloe, grape seed and nutmeg extracts for 7 days, plus an additional dose 30 minutes before ischemia, significantly reduced hepatic TBARS content compared to ischemic control.

8. On the other hand, neither of the test agents or their combination resulted in significant effects regarding serum tBil, dBil and iBil values or liver weight/body weight ratio.

II. *In vitro* Experiment:

1. Incubation of freshly-isolated suspended rat hepatocytes with CCl_4 significantly increased LDH leakage, TBARS production and $[Ca^{2+}]_i$ content coupled with decreased cellular GSH content. These effects were observed as early as 30 minutes following CCl_4 addition and continued till the end of the experiment. In addition, CCl_4-incubation also significantly elevated NO_X production and decreased cellular ATP content after 60, 90 and 120 minutes of incubation as compared to normal flasks.

2. Pre-incubation of CCl_4-intoxicated hepatocytes with NAC significantly prevented CCl_4-induced LDH leakage and significantly increased cellular GSH content after 30 minutes of addition of CCl_4 and continued till the end of the experiment. It also restored TBARS production as well as $[Ca^{2+}]_i$ and ATP contents after 60 minutes of addition of CCl_4 and continued till the end of the experiment. NAC also significantly suppressed NO_X production after 90 and 120 minutes of incubation with the hepatotoxin.

3. Pre-incubation of CCl_4-intoxicated hepatocytes with aloe extract significantly suppressed CCl_4-induced LDH leakage and $[Ca^{2+}]_i$ elevation and significantly increased cellular GSH content after 60 minutes of addition of CCl_4 and continued till the end of the experiment. It also

suppressed TBARS and NO_X production and increased ATP contents after 120 minutes of addition of CCl_4.

4. Pre-incubation of CCl_4-intoxicated hepatocytes with grape seed extract significantly prevented CCl_4-induced LDH leakage and significantly increased cellular GSH content after 30 minutes of addition of CCl_4 and continued till the end of the experiment. The extract significantly decreased $[Ca^{2+}]_i$ content after 60 minutes of incubation with the hepatotoxin and continued till the end of the experiment. It also suppressed TBARS and NO_X production and increased ATP contents after 90 and 120 minutes of addition of CCl_4.

5. Pre-incubation of CCl_4-intoxicated hepatocytes with nutmeg extract significantly suppressed CCl_4-induced elevation of $[Ca^{2+}]_i$ content after 30 minutes of addition of CCl_4 till the end of the experiment. It also suppressed LDH leakage after 60 minutes of addition of CCl_4 and continued till the end of the experiment. The extract significantly suppressed TBARS and NO_X production and increased GSH and ATP contents after 90 and 120 minutes of incubation with the CCl_4.

6. Pre-incubation of CCl_4-intoxicated hepatocytes with L-NAME significantly increased LDH leakage, TBARS production and $[Ca^{2+}]_i$ content and decreased GSH content after 30 minutes of incubation with CCl_4. It significantly suppressed NO_X after 30 minutes of incubation with CCl_4 and the effect continued till the end of the experiment.

7. Pre-incubation of CCl_4-intoxicated hepatocytes with AG significantly reduced LDH leakage, TBARS production and $[Ca^{2+}]_i$ content and increased GSH and ATP contents after 120 minutes of incubation with the toxicant. It significantly suppressed NO_X production after 90 and 120 minutes of incubation with CCl_4.

8. Pre-incubation of CCl_4-intoxicated hepatocytes with L-NAME plus NAC, aloe extract, grape seed extract or nutmeg extract significantly elevated LDH after 30 minutes (regarding aloe and nutmeg), 60 minutes (regarding NAC and grape seed) and 90 minutes (regarding NAC, aloe and grape seed), elevated TBARS after 90 minutes (regarding aloe and grape seed)

and 120 minutes (regarding NAC and grape seed), suppressed NO_X after 30, 60, 90 and 120 minutes (with all agents) and elevated $[Ca^{2+}]_i$ after 60 minutes (regarding NAC and aloe) and 90 minutes (regarding grape seed) of incubation with CCl_4 as compared to the respective controls not receiving L-NAME.

9. Pre-incubation of CCl_4-intoxicated hepatocytes with AG plus aloe, grape seed or nutmeg (but not NAC) significantly suppressed NO_X after 120 minutes of incubation with CCl_4 as compared to the respective controls not receiving AG.

The following conclusions could be deduced from the present investigation:

1. Hepatic IR injury and freshly isolated hepatocytes intoxiacated with CCl_4 present good hepatotoxicity models *in vivo* and *in vitro*, respectively, for studying effects of different hepatoprotective agents. Free radical production, GSH and ATP depletion, inflammatory reactions, NO production and calcium deregulation are mainly involved.

2. N-acetylcysteine as well as aloe, grape seed and nutmeg extracts could be promising hepatoprotective agents by virtue of their anti-oxidant, anti-inflammatory, immunomodulatory, calcium channel blocking, liver microsomal enzyme inhibitory, cytosolic anti-oxidant enzyme stimulatory, anti-platelet, metal chelating and/or hemodynamic potentials.

3. Non-selective NOS inhibition by L-NAME potentiates CCl_4-induced hepatotoxicity on freshly isolated rat hepatocytes and decreases the hepatoprotective effects of NAC as well as aloe, grape seed and nutmeg extracts. Alternatively, selective iNOS inhibition by AG reduces this toxicity without affecting the protective effects of the test agents. Possible expectation is that induction of iNOS is responsible for toxic production of massive amounts of nitric oxide (NO) through oxidative and nitrosative stress. On the other hand, constitutive nitric oxide synthase (cNOS) is responsible for the production of cytoprotective amounts of NO leading to favorable liver microsomal enzyme suppression and anti-oxidant potentials.

According to the present study, natural extracts like aloe, grape seed and nutmeg could be promising hepatoprotective agents against different models of liver injury, and NO production might play a very important role in modulationg their protective effects.

Dr. Basim Anwar Shehata Messiha

Pharmacology and Toxicology Dept.