Biochemical Evaluation of Some Natural Products against Toxicity Induced By Anti-tubercular Drugs in Rats

Zeinab Yousef Ali

Biochemistry department, National Organization for Drug Control and Research (NODCAR), Giza 12553, Egypt.
Zeinabyousef65@ymail.com

Abstract: Tuberculosis (TB) is a communicative disease caused by Mycobacterium tuberculosis bacteria that may cause death if it is left untreated. WHO (2010) recommended standard drugs as first line anti-tuberculosis (anti-TB) therapy involved (HRZE): isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) received as a fixed dose combination suspension according to the body weight. However, hepatic and renal toxicity are the most serious adverse effects of these drugs. Therefore, *In vitro* antioxidant studies were carried out include: antiradical activity by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) and metal chelating activity. Furthermore, this study aimed to evaluate the protective effects of the hydroethanolic extract of *Mentha peprita, Origanum vulgare* and *Pimpinella anisum* against toxicity induced during treatment with combinations of anti-tuberculosis drugs compared with silymarin in rats. *In vitro* study revealed that all the tested extracts considered as a good source of natural antioxidants due to their high content of phenolic and flavonoid compounds and thus exhibited good antioxidant potential that decreased in the order of *M. peprita* > *O. vulgare* > *P. anisum*. *In vivo* study using a total of 56 female Sprague-Dawely rats divided into seven groups (8 rats each) as follows: **Group 1** served as a normal control for 30 days; **Group 2** received a combined suspension of anti-TB drugs (HRZE) in a fixed dose of 6.75, 13.5, 36.0 and 24.8 mg/Kg b.w/day, p.o., respectively for 30 days. **Group 3-7** received a sole dose of *M. peprita, O. vulgare* or *P. anisum* extract or a combined polyherbal extract or silymarin (100 mg/kg b.w/day, p.o) 30 min prior to anti-TB drugs for the same period. The results demonstrated that administration of a combined anti-TB drugs induced hepatotoxicity as evidenced from a significant elevation in the serum enzyme activities [alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP)], total bilirubin (T. Bil) and decrease in total protein (T.P), associated with renal disorder as confirmed from a market elevation in serum urea, creatinine and uric acid as well as oxidative stress confirmed from a significant decrease in total antioxidant capacity (TAC) and reduced glutathione (GSH) along with marked elevation in both markers of lipid peroxidation [malondialdehyde (MDA), Conjugated dienes (CD) and total hydroperoxides (ROOHs)], protein oxidation (protein carbonyl, PC) and DNA fragmentation. However, co-adminstration of the tested extracts with anti-TB drugs showed good hepato- renal-protection as evidence from maintenance of the aforementioned biochemical changes near normal. This improvement was decreased in the order of silymarin $\simeq$ polyherbal preparation > *M. peprita* > *O. vulgare* > *P. anisum* may be due to their high content of phenolic and flavonoids which might offer hepato-renal protection. This study revealed the synergistic effect of the hydroethanolic extract of *Mentha peprita, Origanum vulgare* and *Pimpinella anisum* to protect the liver and kidney tissues against toxicity induced during treatment with combined anti-TB drugs through increasing antioxidant defence capacity. **In conclusion**: These tested extracts may be used as a dietary supplement in polyherbal preparation by patients taking anti-tuberculosis medications. Further studies will be needed in different animal model with different doses to delineate the precise mechanisms underlying the effects of this polyherbal preparation.

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1. Introduction

Tuberculosis (TB) is one of the major causes of death from a curable infectious disease. It mainly affects the poorest countries of Africa and Southeast Asia. TB is a commonly occurring respiratory infection and its incidence has increased globally in the past few years. It is displayed by infection with Mycobacterium tuberculosis bacteria (WHO, 2010; Villemagne et al., 2012). The World Health Organization (WHO, 2010) estimated the global burden of tuberculosis (TB) in 2010 as around 14 million prevalence and 2.38 million deaths from this curable infectious disease. The single use of anti-TB drug may result in the rapid development of resistance or failure of treatment (WHO, 2003). Therefore, WHO (2010) recommended a several

regimens available for the treatment of tuberculosis. In most developing countries, the preferred regimen for successful treatment that prevents acquired resistance and enhances efficacy involves two phases: an initial (or intensive) phase followed by continuation phase. The initial phase is a daily treatment with four drugs (HRZE): isoniazed (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) received as a fixed dose combination according to the body weight for 2 months, followed by a continuation phase (HR) of 4-6 months of isoniazed (H) and rifampicin (R). However, this regimen often causes serious adverse drug reactions, which may result in discontinuing the scheduled treatment.

Liver and kidney dysfunction posses a major problem for effective completion of the course of anti-tubercular chemotherapy and hence could influence compliance especially in the initial intensive phase therapy. Poor compliance may result in stoppage of therapy which not only precipitates recurrence of disease but also the development of drug resistance tuberculosis (WHO, 2010; Kim et al., 2012).

Adverse effects of anti-TB therapy are potentiated by multiple drug regimens such as HRZE. All these four drugs are potentially hepatotoxic independently, when given in combination their toxic effects are enhanced in a synergistic manner. The conversion of monoacetyl hydrazine, a metabolite of isoniazid, to a toxic metabolite via cytochrome P450 leads to hepatotoxicity. Rifampicin induces cytochrome P450 enzyme causing an increased production of toxic metabolites from acetyl hydrazine. Rifampicin can also increase the metabolism of isoniazid to isonicotinic acid and hydrazine, both of which are hepatotoxic (Eminzade et al., 2008). The plasma half life of acetyl hydrazine (metabolite of isoniazid) is shortened by rifampicin and acetyl hydrazine is quickly converted to its active metabolites by increasing the oxidative elimination rate of acetyl hydrazine, which is related to the higher incidence of liver necrosis caused by isoniazid and rifampicinin combination (Hussain et al., 2012). Pyrazinamide in combination with isoniazid and rifampicin is also, associated with an increased incidence of hepatotoxicity. Pyrazinamide decreases blood levels of rifampicin by decreasing its bioavailability and increasing its clearance (Sweetman, 2009; Ravishah et al., 2012).

Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for liver toxicity. In this concern, Bioactive phytochemical like phenolic acids, tannins, poly-phenols and flavonoids can scavenge free radicals, thus inhibit the oxidative mechanisms and reduce risk of the degenerative diseases (Ünver et al., 2009). Mono- and polyherbal preparations with potent antioxidant and antihepatotoxic activities made from traditionally have been described.

Mentha piperita L. (family: *Labiatae*) commonly known as peppermint with Arabic name nana is native to the Mediterranean region and nutrient rich. It is an aromatic and has stimulant and carminative properties. It is currently being used for alleviating nausea, flatulence and vomiting. It was revealed that M. piperita has antioxidant and antiperoxidant properties (Ünver et al., 2009; Rita and Animesh, 2011).

Origanum vulgare L. (family: *Lamiaceae*) commonly known as oregano is a rich source of natural phenolic antioxidants and has potential to be a source of nutritional ingredients for functional foods (Jałoszyński et al., 2008). It has been used in food preservation and in traditional medicine in the treatment of common ailments and has potential for positive modulation of oxidation-linked diseases (Kocić-Tanackov et al., 2012).

Pimpinella anisum (family: *Apiaceae*) commonly known as anise is an annual herb indigenous to the near east and widely cultivated in the Mediterranean regions. It has been used as an aromatic herb and spice since Egyptian times (Yazdani et al., 2009). The principal constituents of anise are volatile oil, coumarins, fatty acids, flavonoid glycosides, proteins and carbohydrates (Al Mofl et al., 2007). In addition, the antimicrobial properties of these species have been reviewed by several researchers (Sharma et al., 2007; Eteghad et al., 2009).

Therefore, the present investigation was designed to evaluate the antioxidant potential of hydroethanolic extracts of *M. peprinta, O. vulgare* and *P. anisum* in vitro as well as their possible protective activity on the hepatorenal injure induced by anti-tubercular drugs in rat, compared to silymarin as a reference drug.

2. Material and Methods

Drugs:

Rimactazide: (Novartis Pharma S.A.E. Co, Cairo) each capsule contains 150mg Isoniazid and 300mg Rifampicin (R). Pyrazinamide: (P.T.B Tablets, Amoun), each tablet contains 500mg Pyrazinamide. Ethambutol: (Etibi Tablets, Zoga/Memphis), each tablet contains 500mg ethambutol Hcl.

Chemicals

All chemicals of analytical grade were purchased from Sigma Aldrich (St. Louis, MO, USA.).

Plant extracts

The leaves of *Mentha peprita* and *Origanum vulgare* were collected, dried in shadow at room temperature and powdered, while the seeds of *Pimpinella* anisum were crushed. Each powdered plants were extracted with 90% ethanol for 48 h. The extracts were filtered, concentrated under reduced pressure by using rotary evaporator and stored at 0 – 4°C. Polyherbal extract were freshly prepared by mixing three tested extracts were mixed in equal ratio.

Total phenolics contents

The total contents of phenolic compounds were measured using the Folin–Ciocalteu method (Rakitzis, 1975). Briefly, 1ml of appropriately diluted samples or a standard solution of gallic acid was added to a 25mL volumetric flask containing 9 mL of distilled water. A reagent blank was prepared using distilled water. One milliliter of Folin–Ciocalteu phenol reagent was added to the mixture and mixed by shaking. After 5 min, 10 ml of 7% Na_2CO_3 solution were added with mixing. The solution was then immediately diluted to a volume of 25 ml with distilled H_2O and mixed thoroughly. After incubation for 90 min at 23°C, the absorbance relative to that of a prepared blank was read at 750 nm using a spectrophotometer (Model, USA). The total phenolic contents are expressed here in milligrams of gallic acid equivalents (GAE) per 100 g of dry extract.

Total flavonoids contents

The total flavonoid content was measured using a colorimetric assay developed previously (Zhishen et al., 1999). One milliliter of the extracts or standard solutions of catechin was added to a 10 mL volumetric flask. Distilled water was added to make a volume of 5 mL. At zero time, 0.3 mL of 5% (w/v) sodium nitrite was added to the flask. After 5 min, 0.6 ml of 10% (w/v) $AlCl_3$ was added and, then 6 min, 2 mL of 1 M NaOH were also added to the mixture, followed by the addition of 2.1 mL distilled water. Absorbance was read at 510 nm against the blank (water) and flavonoid content was expressed as mg catechin equivalents per 100 g of dry extract. Samples were analyzed in triplicate.

Free radical scavenging activity (DPPH$^•$ assay)

These models of assay involve investigation of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^•$) radical scavenging activity (Blois, 1958). Antiradical activity expressed as IC_{50} and compared with that of ascorbic acid as standards. The IC_{50} value was defined as the concentration of the sample in µg/ml required to scavenge 50% of free radicals. Therefore, the lower the IC_{50} value, the higher is the antioxidant activity of the tested sample.

Ferric reducing antioxidant power of serum (FRAP)

The antioxidant power of blood serum was determined using FRAP assay (Benzie and Strain, 1999). Briefly, 50 µl of the blood serum (normal as well as experimental cells) suspension was added to 1.5 ml of freshly prepared and pre-warmed (37°C) FRAP reagent (300 mM acetate buffer, pH = 3.6, 10 mM TPTZ (tripyridyl-s-triazine) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1) and incubated at 37°C for 10 min. The absorbance of the sample was read against reagent blank (1.5 ml FRAP reagent and 50 µl distilled water) at 593 nm. Standard solutions of Fe^{2+} in the range of 100 to 1000 mM were prepared from ferrous sulphate ($FeSO_4.7H_2O$) in distilled water. The data was expressed as mM ferric ions reduced to ferrous form per ml (FRAP value).

Metal chelating activity

The ability of the extract to chelate ferrous ions was estimated by modified method of Haro-Vicente et al. (2006). Briefly, different concentrations of extract were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixtures was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0-A_1)/A_0]$ x100, where A_0 the absorbance of the control, and A_1 of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

Experimental animals

Sprague-Dawely rats weighing 150-170 g were used. Animals were obtained from the animal house of National Organization of Drug Control and Research, (NODCAR), Cairo, Egypt. Animals were kept under standard conditions with temperature at 23 ± 2°C and a 12/12 hours light/dark cycle throughout the experiment. The animals were allowed free access to food and water. The standard guidelines of NODCAR were used in handling the experimental animals.

Oral acute toxicity

Oral acute toxicity was conducted according to the method of Organization for Economic Co-operation and Development guidelines No. 423 (OECD 2000). Five groups of six male albino rats each, weighing 150 ± 20 g b.wt was used. Animals were kept fasting providing only water, after which each plant extract was administered orally by gastric tube in different gradual doses. Animals were observed periodically for the symptoms of toxicity and death within 24 h and then daily for 14 days.

Induction of experimental hepatorenal toxicity

Rats were given daily fixed-dose combinations of essential anti-TB drugs of (HRZE): Isoniazid (H), Rifampicin (R), Pyrazinamide (Z) and Ethambutol (E) (6.75, 13.5, 36.0 and 24.8 mg/Kg b.w/day, p.o., respectively for 30 days) in suspension for 30 days that is equivalent to the WHO (2010) recommended doses for an adult human (Paget &Barnes,1964).

Experimental design

The rats were acclimatized for a week to the environment before the start of the experiment. A total of 56 albino rats were divided into equal seven groups with 8 rats in each as follows:

The treatment groups received the ethanolic plant extract alone or in combination along with antitubercular drugs for the same period. The activity of the extracts will be comparable to silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*), at a fixed dose of 100 mg/kg, p.o. A total of 70 rats will be used and will be divided randomly into equal seven groups (10 rats each) as follows:

Group 1 served as a normal control for 30 days;

Group 2 received a combined suspension of anti-TB drugs (HRZE) for 30 days;

Groups 3-7 co-administrated a sole dose (100 mg/kg b.w/day, p.o for 30 days) of *M. peprita, O. vulgare* or *P. anisum* extract or a combined polyherbal extracts or silymarin 30 min prior to anti-TB drugs (HRZE).

At the end of the experiment period, all animals were fasted for 12 hrs; blood samples were collected by puncturing retro-orbital plexus and sera were separated by centrifugation at 640 g at 4°C for 10 minutes. The sera were carefully collected and preserved at -20°C for different biochemical analysis. About 0.1 ml of heparinised blood was lysed directly in 0.9 ml of ice-cold distilled water and used for determination of the blood glutathione. The livers were quickly excised, rinsed in cold saline, blotted and weighed and used for determination of the DNA fragmentation.

Biochemical analysis

Assessment of liver function

Serum alanine aminotransferase (AST) and aspartate amino transferase (ALT) activities were determined using the method of Reitman and Frankel (1957), Total Bilirubin (T.Bil) was estimated by the method of Jendrassik and Grof (1938) and alkaline phosphatase (ALP) estimated using the method of Belfield and Goldberg (1971). While, the activity of serum lactate dehydrogenase was assayed according to the kinetic method of Bergmayer and Brent (1974). Total protein (T.P) was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Assessment of kidney function

Blood urea was assayed according to the colorimetric method described by Tabacco et al., (1979). Serum creatinine was determined by the method of Henery et al., (1974) and uric acid

Total antioxidant capacity (TAC) assay

The determination of the antioxidative capacity in liver and kidney homogenates is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual H_2O_2 is determined colorimetrically by an enzymatic reaction which evolves the conversion of 3, 5 dichloro-2-hydroxy benzensulphonate to a colored product Koracevic and Koracevic (2001).

Assessment of oxidative stress markers

Lipid peroxidation (LPO) was estimated by measuring thiobarbituric acid reactive substances (TBARS) and expressed in terms of malondialdehyde (MDA) content, according to the method of Buege and Aust (1978). Conjugated dienes (CD) and total hydroperoxides (ROOHs) are generated at intermediate stages of lipid peroxidation was determined by the method of Recknagel and Glende (1984) and Pryor and Castle (1984), respectively. Protein oxidation was monitored by measuring protein carbonyl (PC) contents following the method of Reznick and Packer (1994). Reduced glutathione (GSH) content was estimated by the method of Beutler et al. (1963).

DNA fragmentation assay

Liver tissues were homogenized with 5 volumes of an ice-cold lysis buffer (5 mM Tris–HCl) pH 8.0 containing 20 mM EDTA and 0.5% Triton X-100. DNA extraction was performed by shaking the tissues homogenates in lysis buffer for 1 h at 4°C (Homma-Takeda et al., 1997) and centrifuged to separate the intact chromatin and the fragmented DNA. The supernatant and the pellet containing intact chromatin were used for the colorimetric reaction by Diphenylamine (DPA) (Perandones *et al.*, 1993). Absorbance of the developed blue colour was measured at 578nm with spectrophotometer (Kyoto, Japan). Percentage of DNA fragmentation in each sample was expressed by the formula:

% DNA fragmentation = (O.D Supernatant / O.D Supernatant + O.D Pellet) × 100.

Statistical analysis

All results are expressed as means ± SE. All in vitro measurements were replicated three times. IC_{50} (the concentration required to scavenge 50% of free radicals) value was calculated from the dose-response curves. One-way analysis of variance

ANOVA using the SPSS17.0 software, followed by Duncan's multiple range test (DMRT) to calculate the statistical significance between various groups and Pearson's correlation coefficients were calculated to determine relationships among parameters at different parameters. A value of $P<0.05$ was considered as statistically significant.

3. Results and Discussion

The first line anti-tuberculosis drugs (anti-TB) that recommended by WHO (2010), continues to be the effective drugs in the treatment of TB. However, the transient abnormalities in liver and kidney functions are common during the early stages of anti-TB therapy, but sometimes hepatotoxicity may be more serious and require a change of treatment. Therefore, this study was aimed to evaluate and compare the hepatorenal protection of the hydroethanolic extracts of Mentha peprita, Origanum vulgare and Pimpinella anisum as sole or in combined polyherbal extracts during treatment with combined antitubercular drugs for 30 days in rat. In addition, their contents of phenolics and flavonoids were measured and correlated to their protective potential.

Phenolic and flavonoid compounds are the major active nutraceutical ingredients in plants. Both of these classes exhibited good antioxidant potential and metal chelators (Lobo et al., 2010; Ghasemzadeh and Neda Ghasemzadeh, 2011). The data in Table 1 indicated that the three tested plant extracts contain significant amounts of these compounds. Total phenols were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: y = 0.0008x, R2 = 0.9986). While, the flavonoids content was determined in terms of quercetin equivalent (the standard curve equation: y = 0.0236x, R2 = 0.9995). The obtained results in Table 1 revealed that *Mentha peprita* contains the highest concentration of phenolic and flavonoid compounds followed by Origanum vulgare and Pimpinella anisum, respectively.

Phenolic compounds considered as one of the main classes of secondary metabolites. They act as antioxidants in a number of ways. Their hydroxyl groups are good hydrogen donors. Hydrogen-donating antioxidants can react with different reactive species in the termination reaction to break the cycle of generation of new radicals. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals. Moreover, phenolic structures often have the potential to strongly interact with proteins, due to their hydrophobic benzenoid rings and hydrogen-bonding potential of the phenolic hydroxyl groups. This gives phenolics the ability to act as antioxidants also by virtue of their capacity to inhibit some enzymes involved in radical generation, such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase. Additionally, synergistic effects of phenolics with other antioxidants and regulation of the intracellular GSH levels have also been described (Pereira et al., 2009; Ghasemzadeh and Neda Ghasemzadeh, 2011).

Also, flavonoids have gained tremendous interest as potential therapeutic agents against a wide variety of diseases, most of which involve oxidant damage. The antioxidant potential of flavonoids is due to their ability to scavenge free radicals. Besides this mechanism, flavonoids regulate the signal transduction, suppression of tumor formation, stimulation of the immune system and DNA repair, and modulation of enzyme activity related to detoxification, oxidation and reduction (Aron and Kennedy, 2008; Jeremy and Crozier, 2012).

The results depicted in Table 1 confirmed that all the tested extracts exhibited antioxidant activities as confirmed by three different models of assay such as antiradical activity (DPPH assay), ferric reducing antioxidant power (FRAP) and metal chelating capacity. These antioxidant potential are related to their content of flavonoids and phenolics.

Table 1. Total phenolics, flavonoids and antioxidant potential of the tested plant

Plant	Total phenolics (mgGAE/100 g)	Flavonoids (mg CE/100 g)	DPPH IC_{50} (µg/ml)	FRAP (µmol/ml)	Metal chelating capacity
Mentha peprita	514.3 ± 3.93	230.7 ± 3.31	69.6 ± 0.94	287.4 ± 3.49	362.5 ± 9.67
Origanum vulgare	410.3 ± 5.76	221.4 ± 4.81	85.9 ± 1.02	405.3 ± 6.17	389.6 ± 10.4
Pimpinella anisum	220.7 ± 8.36	136.3 ± 5.24	103.5 ± 3.14	538.1 ± 8.69	407.3 ± 8.39

Total phenolic content expressed in milligrams of gallic acid equivalent (GAE)/100 g of dry extract.
Total flavonoid content expressed in milligrams of catechin equivalent (CE)/100 g of dry extract.
FRAP expressed in $FeSO_4.7H_2O$ equivalent (µmole/ml)

1,1-diphenyl-2-picrylhydrazyl ($DPPH^•$)

$DPPH^•$ assay is an easy, rapid and sensitive method for the antioxidant screening of plant extracts. $DPPH^•$ is a stable free radical at room temperature. $DPPH^•$ radical is scavenged by antioxidants through the donation of a proton, forming the reduced $DPPH_2$. The colour changes from purple to yellow after reduction, which can be

quantified by its decrease of absorbance at wavelength 517 nm (Blois, 1958). In the present study free radical scavenging potential was performed with different concentrations of the tested extracts. Among the three tested extracts, *Mentha peprita* exhibited the highest antiradical activity followed by *Origanum vulgare* and *Pimpinella anisum* with IC_{50} of 69.6, 85.9 and 103.5 µg/ml respectively.

Ferric reducing antioxidant power (FRAP)

The antioxidant activity was also assayed by measuring the ferric reducing antioxidant power (FRAP) that based on the ability of the antioxidants to reduce Fe^{3+} to Fe^{2}. Subsequently, the Fe^{2+} formed interact with colorless TPTZ at low pH providing a strong absorbance at 593 nm owing to the formation of a blue colored Fe^{2+}-TPTZ compound. Fe_2SO_4. $7H_2O$ was used as standard Fe^{2+} ($Y = 0.0007x$, $R^2 = 0.9979$).

This colorimetric method was initially developed to assay plasma antioxidant capacity, but it can be used to measure the antioxidant capacity from a wide range of biological samples and pure compounds (Benzie and Strain, 1999). The results depicted in Table 1 confirmed that *Mentha peprita* exhibited the highest FRAP value followed by *Origanum vulgare* then *Pimpinella anisum*.

Metal chelating activity

Metal-catalyzed oxidation of proteins occurs when a metal ion often Fe^{3+} or Cu^{2+} is released from its normal protected environment and binds to an unprotected site. There, it interacts with H_2O_2 in the Fenton reaction to produce hydroxyl radicals, which oxidize adjacent amino acid side chains. Carbonyl groups (PC) are formed on several amino acids and this modification can be tagged, e.g. by reaction with hydrazide derivatives (Møller et al., 2011).

In addition, Iron can stimulate and accelerate LPO by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes LPO (Ghasemzadeh and Neda Ghasemzadeh, 2011). Consequently, the tested extracts were screened for their potential iron Fe^{2+} chelating effects.

In the current investigation, Fe^{+2} ions can form complexes with ferrozine that prevented in the presence of chelating agents, resulting in a decrease in the red colour of the complex. Measurement of color reduction allows the determination of metal chelating activity. According to the obtained results in Table 1,the ferrous metal chelating activity of the three tested hydroethanolic extracts followed the order of *Mentha peprita* > *Origanum vulgare* > *Pimpinella anisum*.

On the basis of the obtained results, it can be concluded that a hydroethanolic extract of *Mentha peprita*, which contains the largest amount of phenolic and flavonoids compounds, exhibits the highest antioxidant that confirmed by antiradical, ferric reducing antioxidant power (FRAP) and metal chelating activities. These in vitro assays indicate that hydroethanolic extract of *Mentha peprita* is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses related disorders.

Oral acute toxicity test

the oral acute toxicity test revealed that three tested hydroethanolic extracts supplemented as sole or in combined therapy, had no adverse effect on rats up to the dose of 2000 mg/kg indicated their relatively safe and corroborates its use in traditional medicine. Therefore, one-twentieth of the maximum dose (100 mg/kg b.wt) was used in the current study.

Effect of Different Treatments on Liver Function

Liver is a central metabolizing organ, so it is more susceptible to metabolism-dependant injury (Vaghasiya et al., 2009). Sweetman (2009) reported that anti-TB drug-induced hepatitis usually occurs within the first few weeks of treatment. Isoniazid and pyrazinamide are thought to have the greater potential for hepatotoxicity than rifampicin.

As shown in table 2, daily administration of the (HRZE) for 30 days result in hepatic damage as confirmed by the elevated activities of the marker serum enzymes (ALT, AST and ALP) and T. bilirubin along with significant depletion in the protein level as compared with control group. These results are in agreement with previous studies of Adhvaryu et al. (2008), Jeyakumar et al. (2009) and Hussanin et al. (2012).

The elevated levels of serum enzyme activities may be attributed to damage of the structural integrity of liver result in increased release of these diagnostic marker enzymes into the systemic circulation (Bello and Wudil, 2012). Total bilirubin is a good indicator of liver function. Its high levels are indicative of hepatic cell damage or bile duct damage (Vaghasiya et al., 2009). A marked depletion in the serum protein level indicated the synthetic function of the liver has been markedly diminished (Jeyakumar et al. 2009).

However, co-administration of the tested extracts and silymarin at a daily dose of 100mg/kg with anti-TB drugs was significantly prevented these biochemical changes and confirmed the protection of the membrane integrity against anti-TB induced leakage of marker enzymes into the circulation, improvement in the secretary mechanism of the hepatic cell and stabilization of the hepatocellular membranes. In addition, the increase in the total

protein levels may be accelerated the regeneration of parenchyma cells and indicating their improvement the functional efficiency of the liver. The current result suggested that all three tested extracts were found to be effective in preventing hepatotoxicity partially with near similar efficacy. This protective effect was more pronounced in the polyherbal extract and silymarin and decrease in the order of Silymarin ~ Polyherbal > M. peprita > O. vulgare > P. anisum. Silymarin, a standard plant extract with strong antioxidant activity obtained from S. marianum, is known to be an effective agent for liver protection and liver regeneration. Eminzade et al. (2008) confirmed the protective actions of silymarin against hepatotoxicity induced by different combinations of anti-tuberculosis drugs. Jeremy and Crozier et al. (2012) provide evidences that phenolic and flavonoids exhibited antioxidant and protective activities against free radicals linked diseases.

Table 2. Effect of different treatments on liver function parameters

Groups	ALT (U/L)	AST (U/L)	ALP (IU/L)	T.Bil (mg/dl)	T.P (mg/dl)
Control	22.5 ± 0.76 [a]	50.5 ± 1.15 [a]	302.3 ± 3.61 [a]	0.77 ± 0.03 [a]	6.04 ± 0.21 [d]
(HRZE)	55.7 ± 1.39 [f]	102.6 ± 2.60 [e]	612.1 ± 5.17 [e]	1.49 ± 0.04 [d]	4.16 ± 0.16 [a]
M. peprita + (HRZE)	29.5 ± 1.21 [c]	69.0 ± 1.13 [c]	480.5 ± 5.64 [c]	1.10 ± 0.05 [bc]	5.05 ± 0.09 [b]
O. vulgare + (HRZE)	33.1 ± 0.83 [d]	79.5 ± 1.15 [d]	502.9 ± 6.12 [d]	1.11 ± 0.06 [c]	4.65 ± 0.11 [b]
P. anisum + (HRZE)	36.8 ± 1.66 [e]	83.5 ± 1.51 [d]	511.6 ± 6.92 [d]	1.02 ± 0.03 [bc]	4.73 ± 0.14 [b]
Polyherbal + (HRZE)	24.9 ± 0.92 [ab]	64.5 ± 1.76 [bc]	415.3 ± 11.3 [b]	0.94 ± 0.03 [b]	5.54 ± 0.17 [c]
Silymarin + (HRZE)	27.1 ± 0.48 [bc]	61.7 ± 1.70 [b]	427.2 ± 3.34 [b]	0.90 ± 0.03 [b]	5.75 ± 0.18 [c]

Each value represents the mean of 8 rats ± S.E. Values within a column followed by different letters are significant different (P<0.05) by DMRT. Alanine aminotransferase (AST); aspartate amino transferase (ALT); alkaline phosphatase (ALP); Total Bilirubin (T.Bil); Total protein (T.P)

In this context, WHO (2003) recommended that rifampicin (R) and isoniazid (H) are the most effective drugs available for the treatment of tuberculosis. However, the use of these drugs is associated with hepatotoxicity in some individual. Recent studies (Kim et al. 2012) showed that the first-line anti-TB drugs includes HRZE are ef¬fective, but this regimen often causes serious adverse drug re¬actions, which result in discontinuing the scheduled treatment. Hussain et al. (2012) provided evidence that these antitubercular drugs cause cellular damage through the induction of oxidative stress, a consequence of dysfunction of hepatic antioxidant defence system.

Effect on kidney function

Nephropathy is one of the important microvascular complications of anti-TB therapy (Sweetman, 2009). The results in Table 3 demonstrated that anti-TB treated group for 30 days exhibited a significant increase in the serum concentrations of creatinine, urea and uric acid compared with the control animals, indicated a significant degree of glomerular dysfunction mediated by anti-TB drugs. Meanwhile, thirty days treatment of the other groups with hydroethanolic extract of tested extracts or silymarin (100 mg/kg/day, p.o) led to a significantly less increase in serum creatinine, urea, and uric acid levels that associated with anti-TB treatment. This results is consistent with the previous study of Rekha et al. (2005) who demonstrated that rifampicin-induced acute renal injure is sometimes encountered in the treatment of tuberculosis. This renal injure is usually reversible if detected early and treated appropriately. Idilman et al. (2006) reported that the side effect is hepatotoxicity, ranging from asymptomatic transaminitis to fulminant hepatic failure.

Table 3. Effect of different treatments on the kidney function parameters

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Uric Acid (mg/dl)
Control	19.9 ± 0.72 [a]	0.66 ± 0.019 [a]	0.47 ± 0.018 [a]
(HRZE)	42.0 ± 0.73 [f]	1.33 ± 0.019 [d]	1.37 ± 0.028 [f]
M. peprita + (HRZE)	27.6 ± 0.69 [cd]	0.75 ± 0.029 [a]	0.63 ± 0.017 [c]
O. vulgare + (HRZE)	29.3 ± 1.32 [d]	0.85 ± 0.038 [b]	0.81 ± 0.024 [d]
P. anisum + (HRZE)	32.4 ± 1.16 [e]	0.96 ± 0.042 [c]	0.90 ± 0.030 [e]
Polyherbal + (HRZE)	23.9 ± 1.10 [b]	0.73 ± 0.023 [a]	0.55 ± 0.017 [b]
Silymarin + (HRZE)	25.8 ± 0.89 [bc]	0.75 ± 0.029 [a]	0.53 ± 0.012 [b]

Each value represents the mean of 8 rats ± S.E. Values within a column followed by different letters are significant different (*P*<0.05) by DMRT.

Several mechanisms may contribute to the onset of the progression of renal injure that occur in patients or animals during treatments with anti-TB drugs includes LPO and oxidative stress. This suggestion is supported by significant increase in MDA and conjugated diene concentrations, a marker of LPO of cellular lipids and protein, after administration of anti-TB drugs (Table 3). Also, Choi et al. (2012) reported that oxidative stress can promote the formation of a variety of vasoactive mediators that can affect renal functions directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and thus reduce glomerular filtration rate (GFR). Consequently, the mechanism of the protective effect of the tested extracts on anti-TB induced renal injury can be explained by their antioxidant activity.

Effect on oxidative stress markers

Lipid peroxidation (LPO) is a multistep reaction that can result in destruction of cellular membranes. The reaction starts when a free radical reacts with the double allylic bond of a polyunsaturated fatty acid (PUFA) to form a conjugate diene. This reacts with O_2 forming peroxyl radicals that react with $OH^{\bullet}$ atoms from other lipids, producing lipid hydroperoxides or forming cyclic peroxides, and several products are formed, including MDA. In addition, iron can promote the breaking of this compound forming lipid peroxyl radicals that can cause further reactions with other PUFA and thus propagate the chain reactions as long as substrates are available (Jomova and Valko, 2011).

As shown in Table 4, administration of anti-TB drugs alone for 30 days were significantly increased serum markers of LPO [conjugated diene (CD), lipid hydroperoxides (ROOHs) and malondialdehyde (MDA)], and protein oxidation (protein carbonyl, PC) levels that associated with significantly depletion in TAC and GSH as compared with control group indicate generation of oxidative stress.

The elevated level of serum MDA is a clear manifestation of excessive formation of free radicals and activation of LPO system result in enhancement of the oxidative modification of proteins (PC) may leading to the structural alteration and functional inactivation of many enzyme proteins (Jomova and Valko, 2011). Meanwhile, The depletion of intracellular antioxidant defenses (TAC) along with increased LPO followed by an imbalance in the redox status indicated enhanced oxidative stress (Jeyakumar et al., 2009, Ravishah et al., 2012). In addition, the decreased level of GSH during anti-TB regime could be a result of decreased synthesis or increased utilization due to increased oxidative stress in the untreated anti-TB group. These results were in agreement with that of Mohit et al. (2011) and Saraswathy and Devi (2012) and confirmed that oxidative stress is one of the mechanisms involved in the pathogenesis of anti-tubercular drugs-induced toxicity.

Table 4. Effect of different treatments on the antioxidant defense and oxidative stress markers

Groups	Antioxidant defense		oxidative stress markers			
	TAC	GSH	CD	ROOHs	MDA	PC
Control	4.34 ± 0.10 [c]	49.6 ± 1.61 [de]	4.09 ± 0.09 [a]	1.28 ± 0.03 [a]	1.59 ± 0.06 [a]	1.32 ± 0.02 [a]
(HRZE)	3.04 ± 0.12 [a]	33.7 ± 1.05 [a]	8.12 ± 0.14 [e]	2.66 ± 0.11 [e]	3.12 ± 0.13 [f]	2.45 ± 0.07 [f]
M. peprita + (HRZE)	3.73 ± 0.141 [b]	46.9 ± 0.83 [d]	5.01 ± 0.06 [c]	1.58 ± 0.04 [bc]	2.35 ± 0.02 [c]	1.69 ± 0.02 [cd]
O. vulgare + (HRZE)	3.64 ± 0.110 [b]	42.0 ± 0.78 [c]	5.38 ± 0.08 [d]	1.70 ± 0.02 [c]	2.64 ± 0.03 [bd]	1.81 ± 0.02 [e]
P. anisum + (HRZE)	3.49 ± 0.103 [b]	38.2 ± 0.66 [b]	5.05 ± 0.17 [c]	1.84 ± 0.02 [d]	2.86 ± 0.03 [e]	1.75 ± 0.02 [de]
Polyherbal + (HRZE)	4.83 ± 0.181 [d]	49.5 ± 0.92 [de]	4.84 ± 0.06 [c]	1.46 ± 0.04 [b]	2.12 ± 0.03 [b]	1.59 ± 0.02 [b]
Silymarin + (HRZE)	4.44 ± 0.141 [c]	51.6 ± 1.09 [e]	4.55 ± 0.06 [b]	1.52 ± 0.03 [b]	2.26 ± 0.04 [bc]	1.62 ± 0.01 [bc]

Each value represents the mean of 8 rats ± S.E. Values within a column followed by different letters are significant different ($P<0.05$) by DMRT. Total antioxidant capacity (TAC, mmol/L); Reducing glutathione (GSH); Conjugated Diene (CD, μmol/L); Lipid hydroperoxides (ROOHs, μmol/L); Malondialdehyde (MDA, μmol/L); Protein Carbonyl (PC, nmol/mg protein)

However, co-administration of the tested extracts as sole or in combined polyherbal extract or silymarin at a dose of 100 mg/Kg/day for the same period significantly prevented this biochemical changes confirming the protective action of the tested extracts that may be attributed to antioxidant potential against oxidative stress induced during combined TB drug therapy (HRZE). Decreased LPO levels in the treated groups were attributed to antioxidant and free radical scavenging activities of their phytochemicals present in the tested extracts. Protein carbonyl (PC) content is the most widely used marker of oxidative modification of proteins and suggested to be a reliable marker of oxidative stress. A significant decrease in the protein oxidation along with significant decrease in LPO confirms control over oxidative stress (Jomova and Valko, 2011). Therefore, our study suggested that the oxidative stress induced by the anti-TB drugs was eliminated by tested extracts and silymarin leading to marked improvement in the hepatorenal function tests. This protection effect was pronounced in the group treated with silymarin and polyherbal extract and decreased in order of silymarin ≃ polyherbal > *M. peprita* > *O. vulgare* > *P. anisum*.

Effect on the DNA fragmentation

ROS are able to enhanced lipid and protein oxidation, and consequently increasing DNA damage. Thus natural antioxidants can inhibit or to reduce spontaneous DNA alteration by direct react with mutagens or on the process of their activation. Phenolics and flavonoids have been reported to scavenge free radicals thus prevent their interaction with cellular DNA (Naowaratwattana et al., 2010, Bellver et al., 2011).

Figure 1 showed the mean percentage of the genetic damage induced by a combination of therapeutic exposure to anti-tuberculosis drugs (HRZE). On the contrary, the tested extracts were able to inhibit DNA fragmentation induced during administration of first line anti-tubercuor drugs (HRZE) in the order of silymarin ≥ *E.jambolana* > *C.sempervirens* > *C.scolymus* by percentage values of 56.91%, 54.68%, 46.44% and 39.89, respectively. This protection attributed to the inhibition of reactive metabolite and ROS formed during the processes of microsomal enzymes activation which are capable of breaking DNA strands (Machana et al., 2011; Bakhtyar and Hajizadeh, 2012).

Compared to the normal group, the data in Table 5 demonstrated that there was strong correlation between DNA fragmentation and serum levels of LPO and protein oxidation. In addition, significant negative correlation was observed between serum TAC and levels of lipids and proteins oxidation biomarkers. Also, marked negative correlation was observed between TAC and serum enzyme activities (ALT, AST and ALP), urea, creatinin and uric acid levels.

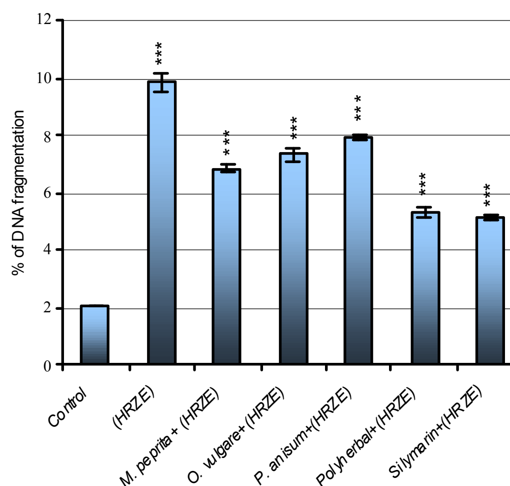

Figure 1. DNA fragmentation of rat liver cells by diphenylamine assay

Furthermore, in vitro study revealed the positive correlation between antioxidant activity of the tested extracts and their content of phenolics and flavonoids. Finally, the present study confirmed that oxidative stress has been found to be an important mechanism in toxicity of antitubercular drugs in rats, and revealed that polyherbal preparation containing the three tested extracts in equal ratio to exhibit maximum protective efficacy and may be used as a dietary supplement by patients taking anti-tuberculosis medications. In conclusion: the present study may help the industry to produce polyherbal preparation with fewer side effects, which are affordable and more effective in the maintenance of normal hepato-renal function during treatment with anti-TB drugs.

Corresponding author:
Dr. Zeinab Yousef Ali
Biochemistry department, National Organization for Drug Control and Research (NODCAR), 6 Abou Hazem St., Pyramids Ave., Giza 12553, Egypt.
Fax: 002-02-35855582
E. mail: Zeinabyousef65@ymail.com

Table 5. Pearson's correlation coefficient matrix for different parameters

Parameters	Liver Function					Kidney Function			Antioxidant States						DNA frag.
	ALT	AST	ALP	T.Bil	T.P	Urea	Creat-inine	Uric acid	TAC	GSH	CD	ROOH	MDA	PC	
ALT	1	--	--	--	--	--	--	--	--	--	--	--	--	--	--
AST	0.893**	1	--	--	--	--	--	--	--	--	--	--	--	--	--
ALP	0.852**	0.922**	1	--	--	--	--	--	--	--	--	--	--	--	--
T.Bil	0.836**	0.820**	0.820**	1	--	--	--	--	--	--	--	--	--	--	--
T.P	-0.712**	-0.771**	-0.769**	-0.644**	1	--	--	--	--	--	--	--	--	--	--
Urea	0.880**	0.859**	0.881**	0.852**	-0.652**	1	--	--	--	--	--	--	--	--	--
Creatinine	0.887**	0.862**	0.792**	0.817**	-0.635**	0.878**	1	--	--	--	--	--	--	--	--
Uric acid	0.929**	0.921**	0.862**	0.836**	-0.735**	0.887**	0.907**	1	--	--	--	--	--	--	--
TAC	-0.703**	-0.719**	-0.703**	-0.628**	0.699**	-0.660**	-0.682**	-0.728**	1	--	--	--	--	--	--
GSH	-0.813**	-0.866**	-0.768**	-0.666**	0.682**	-0.775**	-0.754**	-0.852**	0.690**	1	--	--	--	--	--
CD	0.918**	0.854**	0.824**	0.847**	-0.702**	0.842**	0.875**	0.909**	-0.610**	-0.734**	1	--	--	--	--
ROOH	0.910**	0.856**	0.845**	0.832**	-0.714**	0.858**	0.874**	0.923**	-0.697**	-0.774**	0.897**	1	--	--	--
MDA	0.780**	0.880**	0.901**	0.778**	-0.762**	0.827**	0.776**	0.827**	-0.662**	-0.779**	0.731**	0.800**	1	--	--
PC	0.889**	0.872**	0.885**	0.860**	-0.744**	0.886**	0.873**	0.921**	-0.663**	-0.737**	0.929**	0.934**	0.828**	1	--
DNA frag.	0.707**	0.804**	0.844**	0.710**	-0.740**	0.762**	0.670**	0.729**	-0.567**	-0.708**	0.715**	0.711**	0.883**	0.777**	1
N: 56, **. Correlation is significant at the 0.01 level (2-tailed).															

Pearson's correlation coefficient (r) is a measure of the strength of the association between the two variables.

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