Screening of Lipopolysaccharide - Induced Endotoxin Shock: Potential of Phytotherapy for Ameliorating Endotoxin Disorders

Abd El-Hamid Mohamed Elwy[1]and Ghada Tabl [2]

[1] Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Tanta University
[2] Zoology Department –Faculty of Science, Tanta University
ghada_tabl@yahoo.com

Abstract: The phytotherapy properties of bee propolis and garlic extracts were evaluated in mice intoxicated with lipopolysaccharide-induced endotoxin shock. The endotoxin is a multisystem disorder manifested by several pathogens changes associated with organ damage. The effects of endotoxin solely or in combination with eitherpropolis or garlic extract or both on mortality rate, serum TNF-α, and liver reduced glutathione (GSH), glutathione peroxidase (GPX), glutathione reductase (GR), oxidized glutathione (GSSG), superoxide dismutase (SOD) and lipid peroxidation product malondialdehyde (MDA) were investigated. The results revealed that endotoxicity exerted toxic effects manifested by elevated in serum level of tumor necrosis factor (TNF-α) and mortality rate. Moreover, endotoxicity caused markedly perturbation in the antioxidant system of liver as reflected by reduced GSH and GSSG, a decrease in GR as well as, GPX activity was reduced. MDA was elevated whereas; SOD activity was suppressed in liver tissue. The toxicity induced bylipopolysacchride (LPS) was ameliorated to great extent by the administration of bee propolis or garlic extract. In summary, the perturbation observed in most of the tested parameters was amended, but they did not return back to the normal values. This indicates that bee propolis and garlic extracts represent good sources of natural antioxidants with high potentials against oxidative damage. Furthermore, the phytotherapy significantly suppressed LPS-induced TNF-α production and reduced mortality rate.
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1-Introduction

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxin is invariably associated with gram negative bacteria whether the organisms are pathogenic or not. The term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin.Gram-negative bacteria and their endotoxin may be acasual or complicating factor in many serious diseases. The syndromes most commonly connected with bacterial endotoxin are sepsis and septic shock,which are systemiccomplication of many diseases *(Raetz and Whitfield, 2002)*.

Tumor necrosis factor (TNF-α) is implicated as a major mediator of endotoxin shock *(Beutler et al., 1985; Ogata et al., 1999)* and causes adult respiratory distress syndrome (*Ferrari-Balivieraet al., 1989),* hemodynamic and cardiovascular dysfunction (*Natason et al., 1989),* intravascular coagulation and multiple- organ damage (*Carrico et al., 1986; Blackwell et al., 2000).*

Ogata et al. (1991 & 1999) reported that when mice were pretreated intraperitoneally with carrageenan, even at low dose of endotoxin caused an enormous production of TNF-α in sera and even death. It was assumed that inflammation and suppression of the reticuloendothelial system (RES) played an important role in the enhancement of LPS- induced TNF-α production and lethality by pretreatment with carrageenan, because carrageenan is used as an inflammatory agent and as a RES blocker, even in the case of human patients, inflammation and depression of the RES (*Carrico et al., 1986& Ogata et al., 1999)*,are assumed to be important factors in developing endotoxin-induced mortality.

There is increasing evidence that inflammatory mediators such as platelet activating factor (PAF), leukotriene (LTs) *(Cohn et al., 1991)* and cyclooxygenase products are involved in the pathophysiology of endotoxemia. Platelet activating factor (PAF) is recognized and a potent mediator of inflammation, because it is produced by and activates a variety of cells involved in inflammatory reactions including platelets, neutrophils, eosinophils, endothelial cells, and macrophages *(Bonavida et al., 1990).* Previous studies suggested that many PAF antagonists prevent endotoxin-induced hypotension and lethality *(Rabinovici et al., 1990).* In response toedotoxemia induced by administration of lipopolysaccharide, a complex series of reactions occurs in mammalian tissues. During this inflammation response, cell produce

different mediators, such as reactive oxygen species (*Mirochnitchenko et al., 2000)*.

Over the last few decades, worldwide increase in the use of natural products for pharmacological purposes has been observed (*Wagh and Borkar, 2012).* Herbal remedies are considered both safe and effective and their use is on the rise (*Samuels, 2005).*

Recently, there has been a surge in interest in herbal medicine possibly, because they have fewer side effects than current therapy (*Hocaoglu et al., 2012).*Although non-steroidal medications can be effective, herbs and dietary supplements may offer a safer, and often an effective, alternative treatment for pain relief, especially for long-term use(*Maroon et al., 2010).*

Body's energy and heal diseases (*Bensky and Barole, 1990).* One of the major obstacles to understanding herb- drug interaction is the inconsistencies in the quantity and quality of the various preparations of the herbs. Herbal preparations and formulas may contain either large or, conversely insignificant amounts. The herbal formula is believed to harmonize the of active components *(Samuels, 2005).* It is therefore difficult to predict the effects of this formula in the commercial herbs on prevention or therapies uses and to avoid the increasing risk forcomplications .Today many standard formulas can be purchased over the counter in pharmacies with quantity and quality of the contents receiving minimal side effects *(Samuels, 2005).* In the recent years, extensive research work has been focused on the use of natural materials as antioxidants against thetoxic oxidative materials to ameliorate their toxic and cell damage effects. As far as Egyptian propolis, the natural materials were used in this study.

Propolis (bee glue) is a natural sticky resinous mixture produced by honey bees *Apismellifera* collected from various part of plant sources (*Banskota et al., 2001; Waghand Borkar, 2012).* Propolishas more than 300 phytocompounds. These compounds are collected from different leguminous plants and collectively termed as propoliswhich are valuable sources of new and biologically active molecules possessing antimicrobial property and these phytochemicals have inhibitory effects on all types of microorganisms (*Wagh and Borkar, 2012).* Propolis has been used in folk medicines and complementary therapies since 3.000 B.C in Egypt and in different nations(*Hegazi,1998)* and has become one of the most popular functional foods all around the world (*Wanget al., 2013)*. Propolis show a broad spectrum of bioactivities, such as antioxidant (*Sulaiman et al., 2011),* antibacterial *(Mascheroni et al., 2010),* immunomodulatory *(Sforcin, 2007),* antiviral (*Nolkemper et al., 2010),* cardioprotective*(Zhu et al., 2011),* and anti-inflammatory effects (*Paulinoet al. 2006).*

Egyptian propolis becomes a subject of research by biologists and chemists (*Kujumgiev et al., 1999; Hegazi et al.,2000a, 2000b;Hegazi and Abd El-Hady, 2002). Wang et al. (2013)* found that Chinese propolis exerted excellent anti-inflammatory effects *invivo* and evaluated its potential anti-inflammatory *in vitro*.

Garlic (*Allium sativa)* has been of interest to physician since antiquity (*Richard and Rivlin, 2006).* Bulbs of garlic have been found in excavations of ancient tombs in Egypt, Greece, China and Japan (*Rivlin, 2001).* Scientists from around the world have identified a number of bioactivesubstances in garlic. The validity of ancient medicine is now being evaluated critically in cell-free systems, animal models, and human populations(*Richard and Rivlin, 2006).* Prevention and therapeutic trials of garlic are still in early stages. There are many promising lines of research suggesting the potential effects of garlic.

The medicinal benefits of garlic may contribute to their antioxidant activities (*Chung, 2006).* Garlic fluid extract exerts antioxidant action by scavenging reactive oxygen species (ROS) and enhancing of the cellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase and increasing glutathione in the cells*(Borek, 2001;Banerjee et al., 2003).*Moreover, garlic has been long known to have antibacterial effect (*Shobanaet al.,2009).* Theour goal of this study is to investigate the potentials and the efficacy of beepropolisand garlic extract either solely or in combination with each other as phytotherapy against endotoxicity and correlate these effects with the ability of these natural products to maintain the anti-oxidative defense system .

2-Materials:

2.1. Chemicals and kits

- Carrageenan (CAR) was purchased from Sigma (St. Louis Mo.).
- Lipopolysaccharide (LPS) from *Escherichia coli*waspurchased from Sigma (St. Louis Mo).
- Propolis was purchased from Imtenancompany, Cairo, Egypt.
- Garlic powder from Tomaxtablets, produced by Atos for production of medicinal herbs (Atos Pharma).
- Kits of antioxidants were purchased from BioAssay Systems. 3191 Corporate Place, Hayward, CA 94545, USA.
- Kit of TNF-α was purchased from (Biosource, USA.)

All chemicals used were analytically grade and all solutions were prepared in double distilled and deionized water. All the experiments were carried out

at fixed time of the day (10-12 O'clock) to minimize daily fluctuation in the study parameters.

2.2. Preparation of propolis and garlic extracts:

- Preparation of water extract of propolis;
  Water extract of propolis was obtained as described by *Nagai et al. (2003)* as follows: 20.0 g of propolis were suspended and extracted with 5volumes of distilled water with shaking using shaker at laboratory temperature (25°C) for 24 hrs. The extracts were centrifuged at 3000 g for 20 min., and the supernatants were taken. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were taken. The obtained supernatants were combined and dialyzed against distilled water, and then the dialysate was lyophilized. solution (200 mg/ml water) was used as the sample solution for the test.

- Garlic extract was prepared by dissolving the garlic powder from Tomax tablets in distilled water. The dose used was based on the therapeutic level for the drug. In the present investigation, the adopted experimental dose level has been calculated as equivalent of human therapeutic dose .

2.3Animals:

The experimental models used in this study were male Balb/C micewith initial weight ranging from 30-35g. They were obtained from Breeding Unit of the Egyptian Organization for Vaccine and Biological preparations. All mice were kept under the same environmental conditions. The animals were fed *ad Libitum* with a standard diet and allowed free access of water and maintained in pathogen free animal laboratory. Using mice as animal model gives a good opportunity to know the real effect of the given drugs.

2.4. Experimental design for antioxidant assay:

After two weeks of acclimatization to the laboratory environment, selected mice of nearly a similar weight were divided into the following groups;

Control groups (n=15): In view of high sensitivity of the measured parameters, control groups have been always kept in parallel with the experimental groups and subjected to simultaneous investigation. Mice were divided into three sub groups:

- Subgroup one(n=5): negative control. This was carried out by injecting mice intraperitoneal (i.p.) with saline in a dose corresponding to LPS challenge group.
- Subgroup two (n=5): Positive control for garlic extract in water .Mice received an oral daily dose of garlic extract (1mg/kg) for 15days.
- Subgroup three (n=5): positive control for propolisexperiments. Each mouse was given an oral daily dose of propolis extract(200 mg/kg)for 15 days. , according to *Bhadauria et al. (2007)*
- LPS-challenged group: mice pretreated with carrageenan 5mg in 0.5 ml of saline i.p. as a priming agent 24h prior to challenge intravenously with8µg of LPS (1/10 LD_{50}).
- LPS-challenged and treated with propolis (200 mg/kg): Mice received a single dose of LPS (1/10 LD_{50}), followed by a daily oral dose of propolis (200 mg/kg) for 15 days.
- LPS-challenged and treated with garlic (1mg/kg): Mice received a single dose of LPS (1/10 LD_{50}), followed by a daily oral dose of garlic extract (1mg/kg) for 15 days.
- LPS-challenged and treated with both proplois and garlic: Mice received a single dose of LPS (1/10 LD_{50}), and treated with a daily combineddoses of propolis,200 mg/kg and garlic extract 1mg/kgfor 15 days.

3-Methods:

3.1. Induction of endotoxin shock

Endotoxin shock was induced in mice according to the method described by *Ogata et al. (1991)*with some modifications: briefly, the carrageenan 5mg in 0.5ml of physiological saline was injected intraperitoneally (i.p.) as a priming agent 24h prior to challenge with intravenous of 60 µg LPS in 0.5ml of physiological saline .

3.2. Median lethal dose (LD_{50}) of LPS

To determine the median 50% lethal dose (LD_{50}), various doses of LPS (0,20,40, 60,80,100,120,140,160 µg)were injected intravenously (i.v.) into the tail vein as an inducing agent and the 50% lethal dose LD_{50} was determined by counting deaths during a day (24hrs). A total number of 90 mice were used for the determination of median lethal dose.

3.3. Cumulative percentage of mortality

The mortality rate was determined by using 10 mice for each group and by counting the number of dead mice at 6, 18, 24, 36, 48 and 72 hrs after injection of various doses of LPS (0,10,20, 40,60,80,100,120,140). The cumulative percentages of mortality in mice receiving each dose of LPS were determined by counting the cumulative numbers of dead mice at 72hrsafter injection of LPS according to *Ogata et al. (1993).* Treated mice received once oral dose of 200 mg/kg propolis and 1 mg/kg garlic at the same time of LPS challenge. Total number of 180 mice were used for the determination of mortality rate.

3.4. TNF-α determination

Blood samples were usually collected after 2h post LPS challenge according to *Ogata et al. (1993).* The TNF-α was evaluated in serum with an ELISA kit (Biosource, USA) according to the manufacturer's

protocol. Treated mice received once anoral dose ofpropolis (200 mg/kg) or garlic (1mg/kg) and both of them at the same time of LPS challenge.

3.5. Determination of antioxidant activities

The mice were sacrificed and the liver samples from (right lobe) were homogenized in 5-10ml cold buffer, 50mM potassium phosphate, PH 7.5. (mMEDTA) per gram tissue, centrifuge at 300g for 20minutes at 4°C, the supernatants were removed and used for the antioxidant assays of superoxide dismutase (SOD) *(Nishikimi et al., 1972);* Lipid peroxide Malonialdehyde (MDA) *(Satoh, 1978);* reduced glutathione(GSH) (*Beutler et al., 1963),* glutathione reductase (GR) (*Goldberg and Spooner,1992);* oxidize glutathione (GSSG*) (Bartoli et al., 1978),* and glutathione peroxidase (GPX) (*Paglia and Valentine,1967).*

3.6. Statistical analysis:

Data of biochemical analysis were analyzed using the computer program of SPSS. All statements of significance were based on probability of $p \leq 0.05$.

4.Results

4.1. Median lethal dose of LPS.

As regarding the LD_{50} of LPS dose table (1) and figure (1). The data recorded showed that the LD_{50} was 80 µg of LPS/mouse at 24hrs.

4.2. LPS-induced mortality.

As illustrated in table (2) and figure (2). The cumulative percent mortality after various doses 50% lethal dose was 60 µg of LPS/mouse post 72 hrs.

4.3. LPS- induced TNF-α production.

As shown in table(3) and figure(3). The LPS challenge induced an increase in TNF-α production $p \leq 0.05$.

4.4. Antioxidant defense system.

The (1/10 LD_{50}) of LPS showed highly disturbances in antioxidant defense systemp≤0.05 (Table 4).

4.5. Phytotherapy treatment with propolis and garlic on LPS-induced mortality and TNF-α production.

Data in tables (2&3) and (Figures 2 & 3) revealed the oral administration of propolis in combination with garlic caused a significant amelioration in the mortality rate. Moreover, the oral treatment with propolis or garlic and both of them caused significant decrease in TNF-α production$p<0.001$ (Table 3and Figure 3).

4.6. The role of phytochemical substances propolisor garlic and their combination on antioxidant defense system

A significant increase in the mean level of all studied parameters except malondialdehyde (MDA) which showed significant $p \leq 0.05$ decrease (Table 4), but the effect of both propolis and garlic together in combination was more pronoun.

Table (1):Determination of median lethal dose(LD_{50})

LPS (µg)	Deaths' percentage
0	0%
20	10%
40	20%
60	30%
80	50%
100	70%
120	100%
140	100%
160	100%

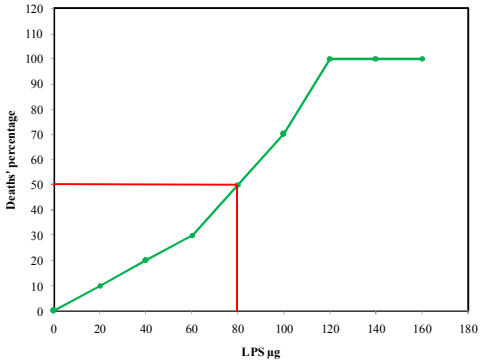

Figure (1): Median lethal dose of LPS (LD_{50})

Table (2): Cumulative percentage of mortality

LPS (µg)	Cumulative percent untreated	mortality treated + GP
0	0%	0%
10	10%	0%
20	20%	0%
40	30%	10%
60	50%	20%
80	70%	30%
100	100%	50%
120	100%	60%
140	100%	60%

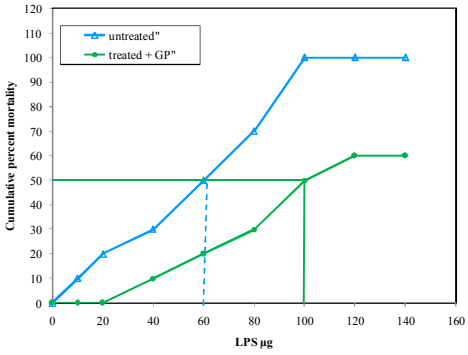

Figure (2): Cumulative percent mortality after injection of LPS into untreated mice. Cumulative percent mortality after injection of LPS into treated mice with garlic and propolis (GP).

Table (3): Effect of propolis or garlic and their combination on serum TNF-α production

groups	TNF-α U/ml	*p*
Untreated	109000 ± 3660	
Treated with propolis	57400± 1360[a]	<0.001*
Treated garlic	34600 ± 4740[a]	<0.001*
Treated propolis & garlic	19800± 1240[a]	<0.001*
F (p)	155436* (<0.001)	

Data was expressed in mean ± SE
pair wise between untreated group and each other group was assessed using Post Hoc test (Scheffe)
a: significant with untreated group

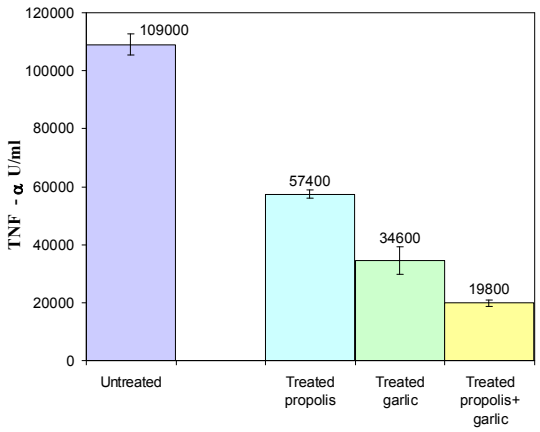

Figure (3): Effect of propolis or garlic and their combination on serum TNF-α production

Table (4):Effect of propolis (P) or garlic (G) and their combination (G+P) on LPS – induced disorders in antioxidant activity (SOD, MDA, GSH, GR, GSSG and GPX)

	Negative Control	Control G	Control P	LPS-Challenged	LPS-Challenged + P	LPS-Challenged+ G	LPS-ChallengedG+P
SOD U/g tissue	62.17 ± 0.77	61.80 ± 0.51	62.22 ± 0.55	25.31± 1.29[a]	50.47± 0.24[a]	57.08± 1.09[a]	59.93 ± 0.18
% change		↓0.60	↑0.08	↓59.29	↓18.82	↓8.19	↓3.61
p		1.000	1.000	<0.001*	<0.001*	0.008*	0.639
MDAμmol/gtissue	0.63 ± 0.01	0.63 ± 0.04	0.68 ± 0.07	0.94± 0.02[a]	0.7 ± 0.02	0.64 ± 0.01	0.61 ± 0.01
% change		↓0.32	↑7.30	↑49.84	↑17.46	↑2.22	↓3.17
p		1.000	0.972	<0.001*	0.351	1.000	1.000
GSH nmol/gtissue	4.45 ± 0.06	4.45 ± 0.09	4.39 ± 0.13	1.64± 0.04[a]	3.72± 0.04[a]	3.91[a] ± 0.06	4.19 ± 0.10
% change		↓0.09	↓1.30	↓63.12	↓16.40	↓12.26	↓5.93
p		1.000	1.000	<0.001*	<0.001*	0.007*	0.515
GR U/g tissue	32.14 ± 0.72	31.78 ± 0.15	32.43 ± 0.68	18.30± 0.65[a]	24.98± 1.17[a]	26.58± 1.28[a]	31.72 ± 0.44
% change		↓1.13	↑0.90	↓43.07	↓22.28	↓17.31	↓1.31
p		1.000	1.000	<0.001*	<0.001*	0.006*	1.000
GSSG nmol/g **tissue**	0.46 ± 0.03	0.46 ± 0.02	0.44 ± 0.02	0.17± 0.02[a]	0.29± 0.02[a]	0.40 ± 0.03	0.43 ± 0.01
% change		↑0.44	↓3.93	↓62.01	↓37.12	↓13.10	↓6.11
p		1.000	1.000	<0.001*	0.004*	0.791	0.994
GPX U/g tissue	7.64 ± 0.10	7.64 ± 0.18	7.56 ± 0.16	2.66± 0.08[a]	4.72± 0.22[a]	5.97± 0.04[a]	6.91 ± 0.05
% change		↑0.05	↓0.94	↓65.22	↓38.24	↓21.82	↓9.48
p		1.000	1.000	<0.001*	<0.001*	<0.001*	0.058

Values are expressed as means ± S.Ea: significant with control group *: Statistically significant at $p \leq 0.05$

5. Discussion

Mice pretreated with carrageenan (CAR) are convenient for the study of pathogenesis mechanism of endotoxin shock and multiple-organ failure (MOF), because carrageenan makes mice more sensitive to LPS endotoxin.

In the present study, LPS induced high mortality rate and significant increase in the level of TNF-α.The present data coincides with the finding of *Ogata et al. (1991) who* reported that mice pretreated with CAR, even in a small dose of LPS can enhance both the level of TNF-α production and the rate of mortality.

In addition, the present findings demonstrated that, the action of the two natural drugs under investigation; propolisandgarlic extracts at the tested doses and oral administration, showed discernible effect on the rate of mortality and significantly decreased the TNF-α production. This decrease reported herein may indicate the role played by PAF antagonist. Moreover, *Ogata et al. (1993)* demonstrated that PAF plays the important role of causing septic shock in endotoxin sensitized mice.

Garlic has many medicinal effects such as anti-platelets aggregation (*Steiner et al., 1996)* and antibacterial (*Shobana et al., 2009).* Garlic and propolis were found to protect mice against LPS mortality and to abolish clinical signs of LPS–induced inflammatory lesion. Nevertheless, the results presented here suggest that, garlic and propolis are potent drugs against LPS induced mortality and that is mechanisms of action involves suppressing PAF and cytokine production, TNF-α. Moreover, the present results, where propolis or garlic separately or in combination suppressed the high rate of mortality caused by endotoxin coincides with the findings of *Rabinovici et al. (1990)* and *Ogata et al. (1993)* who demonstrated that PAF antagonist suppressed TNF-α activity and mortality rate; these suggest that PAF

was produced in response to LPS endotoxin shock which is in a line with *Steiner et al. (1996)* who reported that garlic has anti-platelet aggregation.

The pathogenic sequence of reactions mediated by endotoxin leading to the production of sepsis involves the oxygen radicals or reactive oxygen species which produce tissue damage often observed during septic injury *(Mette etal.,2007).* Inactivation of these damaging radicals by antioxidants may be helpful for protecting sepsis. Concerning the intravenous injection of bacterial endotoxin Lipopolysaccharide induced shock and TNF-α production, the present study showed that LPS-injected mice developed changes in tissue antioxidant enzyme activities and produces TNF-α which caused mortality. The groups of mice treated with propolis or garlic separately or in combination revealed significant changes in the activity of antioxidant enzymes as compared with LPS challenged group. This means that propolis and garlic provided reasonable protection for the cell of liver from LPS-induced endotoxin shock and mortality.

Reduced glutathione (GSH) is an important natural antioxidant; the levels of GSH showed a significant depletion after LPS injection, this might have led to damage of liver cells due to enhancing lipid peroxidation. These results are in accordance with *Yoshikawa et al. (1994)* who demonstrated changes in tissue antioxidant enzyme activities and lipid peroxides in endotoxin- induced multiple organ failure.

Liver glutathione peroxidase (GPX), together with superoxide dismutase (SOD), form part of the cellular antioxidant defense system against reactive molecules and free radicals, which cause lipid peroxidation (*Machlin and Bedich, 1987).* Concerning, the phytotherapies, the level of malondialdehyde (MAD) in the present study, showed significant reduction alongside with significant increase in the activity of glutathione peroxidase (GPX) compared with mice group injected with LPS. These results are in parallel with that of *Machlin and Bedich (1987)*. Furthermore,the result is in agreement with *El-Ghazaly and Khayyal (1995)* who reported that treatment with aqueous propolis extract prior to irradiation which causes liver damage, reduced malondialdehyde (MAD).

With regard to the effect of LPS on liver superoxide dismutase(SOD), the results revealed that LPS significantly reduced the activity of this enzyme. The SOD is the first line of defense against superoxide anion through catalyzing dismutationof O_2^- to hydrogen peroxide and molecular oxygen. The treatment of LPS challenged mice group with propolis and garlic separately or combined, enhanced the activity of SOD and prevented damaging processes which means thatpropolis seems to stimulate and induce superoxide dismutase enzyme and consequently could possibly be a therapeutic value in protecting against toxic materials as reported by *El-Ghazaly and Khayyal(1995).* The obtained data revealed that, the treatment with propolis and garlic together was more pronounced and more effectual than when propolis or garlic was given separately.

So, in the last we can summarized that such a detail study about the effect of these natural products can be of potential therapeutic value against endotoxicity, because they have very good anticoagulant effects represented in decreasing TNF-α production and provide protection against free radical damages in the body tissues. Garlic and propolis, significantly reduced LPS-.induced mortality not only by suppressing LPS-induced TNF-α in serum but also by ameliorating the antioxidant enzymes defense system.

6. References

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