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Antioxidant And Protective Effect Of Clove Extracts And Clove Essential Oil On Hydrogen Peroxide Treated Rats

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Abstract: The antioxidant, liver-protective and kidney-protective effects of clove extract and clove essential oil were carefully investigated. Total phenolic compound and total flavonoids for water, ethanol and acetone extracts of clove were determined, reducing power assay was used to evaluate these extracts compared with clove essential oil. Acetone extract of clove showed the highest total phenolic, flavonids contents compared with water and ethanol extracts. Both clove essential oil and clove acetone extract appeared the best activities in reducing power assay. The liver-protective, kidney-protective effects and antioxidant activity potential of acetone extract of clove and clove essential oil was also evaluated in male Wistar rats against hydrogen peroxide- (H2O2). Rats dived into four groups, First group was kept as control and feed on Basel diet and water, Second group was treated by 0.5% in drinking water without any other treatment, third group was given acetone extract of clove at 500mg/kg body weight prior to H2O2 administration (0.5% in drinking water), while fourth group was given clove essential oil at 200mg/kg body weight prior to H2O2 administration (0.5% in drinking water). Rats treated with acetone extract of clove and clove essential oil remarkably prevented the elevation of plasma AST, ALT, while increased both plasma total protein and albumin compared with H2O2 treated rats. Also both third and fourth group showed significant decreased in kidney markers (urea and creatinine) compared with H2O2 trated group. Plasma antioxidant state (MDA content and catalase activity) was affected by H2O2 treatment in second group, and both third and fourth group improved this markers. This study suggests that acetone extract of clove and clove essential oil has a liver-protective and kidney-protective effects against H2O2induced oxidative stress and bad effects on both liver and kidney and possess *in vitro* antioxidant activities.

Key words: Clove extract, clove essential oil, H2O2, antioxidant, liver, kidney.

Introduction And Experimental

In the past decades, oxidation mechanisms and free radical role in living systems have gained increased attention (**1**). Oxygen uptake inherent to cell metabolism produces reactive oxygen species (ROS). The reaction of this species with lipid molecules originates peroxyl radicals and their interaction with nucleic acids and proteins conduces to certain alterations and, therefore, functional modifications (**2**). This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity (**3**).

Exposure to H2O2 may cause elevation of superoxide anion and the dangerous hydroxyl (OH•) radical leading to glomerular dysfunction and renal damage (**4**)

The harmful action of the free radicals can, however, is blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (**5**). Antioxidants are compounds that can delay or inhibit the oxidation of lipid or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (**6**)

Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage (**7**). Antioxidants can protect the human body from free radicals and ROS effects.

At present, the most commonly used antioxidants are BHA, BHT, propyl gallate and tert butylhydroquinone. Besides this BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis (**8**). Therefore, there is a growing interest on natural and safer antioxidants (**9**).

Consequently, the need to identify alternative natural and safe sources of food antioxidants arose and the search for natural antioxidants, especially of plant origin, has notably increased in recent years (**10, 11**).

Clove water and ethanol extracts have powerful antioxidant activity against various antioxidant systems in vitro, moreover, clove buds can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical applications (**12)** .

Clove oil is obtained by distillation of the flowers, stems and leaves of the clove tree (*Eugenia aromatica* or Eugenia caryophyllata, Fam. *Myrtaceae*). Clove essential oils have been analyzed by GC-MS and 18 components found in essential oils. These components have been tested for antioxidant properties in an egg yolk-based thiobarbituric acid reactive substance (TBARS) assay and also undiluted in a -carotene agar diffusion assay. The essential oils and the components tested in the TBARS assay have demonstrated some degree of antioxidant activity (**13**).

Therefore, the present study was designed to explore the antioxidant effect of different clove extracts and the protective effect of clove acetone extract and clove essential oil in biological experiment on plasma liver functions, kidney functions and antioxidant status against H2O2 induced harmful effects.

Chemicals and procedures

1. Chemical reagents (Kits)

Kits for total protein, albumin, urea, creatinine and lipid peroxides (MDA) and ALT, AST, Catalase enzymes activity were obtained from Diamond Company, Cairo, Egypt.

2- Clove extracts preparation

Clove (*Eugenia caryophylata*) was collected and dried. The dried plant materials were powdered using a grinder. The extraction was done at room temperature. About 100 g of dried, ground plant materials were soaked in each solvent ethanol, water, and acetone (1 L) for 5-7 days separately. The soaked material was stirred every 18 h using a sterilized glass rod. The final extracts were passed through Whatman filter paper No.1. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40^oC and stored at 4^oC for further use.

3- Preparation of essential oil

Essential oil of clove was obtained by hydrodistillation method. The plant materials (about 100 g) were ground into small pieces and were placed in a flask (2 L) together with double distilled water (1.5 L). The mixture was boiled for 4 h. The extract was condensed in cooling vapour to collect the essential oil. The extracted oil was dried over anhydrous sodium sulphate. All essential oils were kept at freezing temperature until used.

4- Determination of total phenolic compounds:

The amounts of phenolic compounds in different extracts of clove were determined with Folin-Ciocalteu reagent using the method of (**14**). 2.5 ml of 10% Folin Ciocalteu reagent and 2 ml of Na_2CO_3 (2% w/v) was added to 0.5 ml of each sample of plant extract solution (1 mg/ml). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of Gallic acid dissolved in distilled water.

5- Estimation of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination according to (**15**). One millilitre (1 ml) of sample (1 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with UV visible spectrophotometer. The content was determined from extrapolation of calibration curve which was made by preparing quercetin solution in distilled water. The concentration of flavonoids was expressed in terms of mg/ml.

6- GC/MS analysis of clove essential oil

The chromatographic procedure was carried out using a Finnegan Mat SSQ 7000-GC-MS with autosampler. A methyl polysiloxane capillary column (DB-5, 50 m X 0.32 mm) was used. Helium was used as the carrier gas. The oven temperature used was maintained at 50ºC for 8 min. The temperature was then gradually raised at a rate of 3°C per min to 180°C per min and maintained at 180°C for 5 min. The temperature at the minjection port was 250°C. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature (**16**) and presented in the MS computer library (WILEY275.L).

7- In vitro antioxidant activity by reducing power assay

The reducing power of different extracts was determined according to the method of Yen and Chen (**17**). 2.5 ml of extract (25-800 µg/ml) in water were mixed with a phosphate buffer (2.5 ml, 0.2M, pH6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50oC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as a positive control.

8- invivo study for test the protective effect of best clove extract and essential oil against hydrogen peroxide administration:

8.1. Animal experiments

Rats were obtained from Research Institute of Ophthalmology, Giza, Egypt. And the work was carried out at its animal house. To study the protective effect of clove acetone extract and clove essential oil on hydrogen peroxide oral administration in albino rats; twenty four male albino rats (weighing between 90 and 110 g) were used for this investigation. The rats were fed *ad libitum* on a basal diet (BD) and water for 15 days as an adaptation period. There were housed individually in stainless steel cages and divided into four groups of six. All groups were fed the BD. Diet intake was monitored daily. The first group (C) was used as controls and received tape water as drinking water. The other three groups; received tape water containing hydrogen peroxide at a dose of (0.5%) in drinking water, daily for six weeks. The second group (H_2O_2 group) doesn't have any other treatment, while the third group (H_2O_2 + clove acetone extract group) was treated simultaneously by stomach tube with clove acetone extract (500 mg/Kg body weight), while the last group (H_2O_2 + clove essintial oil group) treated with cloove essintial oil (200 mg/Kg body weight). All rats fasted before blood sampling. The blood samples were drawn from eye plexuses, after 6 weeks , the rats were anesthetized using diethyl ether. The weight gain of the rats was recorded on a weekly basis.

8.2. Blood sampling and analysis

Blood samples were collected after six weeks in tubes contain heparin as an anticoagulant from the eye plexuses under diethyl ether anesthesia and then centrifuged at 3000 rpm for 20 min. to obtain plasma, which was kept frozen until analysis. The both of alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) activities were measured according to the method described by (**18**). Total protein was determined according to (**19**). And albumin was determined according to (**20**). The content of malondialdehyde (MDA) was determined spectrophotometrically at wave length 532 nm according to the method of Draper and Hadley (**21**). Catalase (CAT) activity was determined at wave length 510 nm according to the method described by Beers and Sizer (**22**). Urea was determined according to (**23**) and creatinine was determined according to (**24**).

9- Statistical analysis

The results of the animal experiments were expressed as the Mean ± SD and they were analyzed statistically using the one-way analysis of variance ANOV A followed by compare means with Duncan's multiple range test. In all cases p 0.01 was used as the criterion of statistical significance.

Results And Discussion

1- Total phenolic compounds and total flavonids in clove extracts:

Data in Table (1) showed that total phenolic content of acetone, ethanol and water clove extracts were (31.88, 10.06, 28.7 mg gallic acid equivalent/100g respectively). The total flavonoid of acetone, ethanol and water clove extracts were (10, 3.44, 8.3 mg gallic acid equivalent/100g respectively). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources and they have been shown to possess significant antioxidant activities (**25**). Studies have shown that increasing levels of flavonoids in the diet could decrease the occurrence of certain human diseases (**26**).

Acetone extract showed high total phenol and flavonoid contents. This result is consistent with the findings of **(27)** who studied the total phenolic content and antioxidant activity of three lentil seed extracts acetone, methanol, and hexane, and found the highest extraction rate of phenolic compounds for lentil seeds was obtained by acetone.

Table (1): total phenolic compounds and total flavonids in different ginger extracts.

	Phenolic content (mg/100g)	Flavonids content(mg/100g)
Acetone extract	31.88	10
Ethanol extract	10.06	3.44
Water extract	28.7	8.3

2- Essential oil chemical composition:

Table (1) showed the identified compounds in clove essential oil, Eugenol and eugenol acetate were the main components in clove essential oil, our data are agree with **(28, 29).**

Table (2): chemical composition of clove essential oil

Component	**%**	**Component**	**%**
Eugenol	83.661	5-Heptene-2-one 6 methyl	0.014
Eugenol acetate	12.118	P- Cymene	0.01
Caryophyllene oxide	1.572	D- Limonene	0.013
2,3,4 trimethoxy acetophenone	1.12	2- Hexanone 6-acetyloxy	0.035
Benzyl benzoate	0.185	Methyl salicylate	0.012
Ledol	0.496	Retinol acetate	0.017
Eucalyptol	0.068	(-) – Spathulenol	0.029
Linalool	0.078	3- Methyl cinnamic acid	0.052
Camphor	0.044	Coniferyl alcohol	0.022
2-Nonanone	0.028	Farnesyl acetate	0.008
- Pinene	0.036	Benzyl salicylate	0.026
Total identified	99.644	Number of compounds identified	22

2- In vitro evolution of antioxidant activity of different clove extracts and clove essential oil:

Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism in phenolic antioxidant action (**30**). In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe+3 to Fe+2 by donating an electron. The amount of Fe+2 complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose– response curves for the reducing powers of the

clove extracts and clove essential oil. It was found that the reducing powers of extracts also increased with an increase in their concentrations. At the highest concentration (200 ug/ml) of all tested materials clove essential oil showed highest activity (0.504) followed by acetone extract (0.411) , then water extract (0.401) and finally ethanol extract (0.344).

However, the inhibitory action of herb extracts could be enhanced by more recovery of phenolic compounds using suitable solvents because the connection of phenolics complex is not the same for all types of solvents used (**31**). It can be concluded that the clove essential oil were considerably more effective as antioxidant in reducing power assay followed by acetone extracts.

Clove essential oil showed the highest antioxidant activity compared with 16 essential oils tested by ferric reducing power assay (**32**)

Clove essential oil has been reported in previous studies as one of the strongest antioxidants, even higher than some synthetic antioxidants like BHT or butylated hydroxyanisole (**33 – 35**). The strong activity of clove essential oil can be due to the presence of eugenol, the main constituent of this essential oil, which is known to have antioxidant activity (**35, 36**).

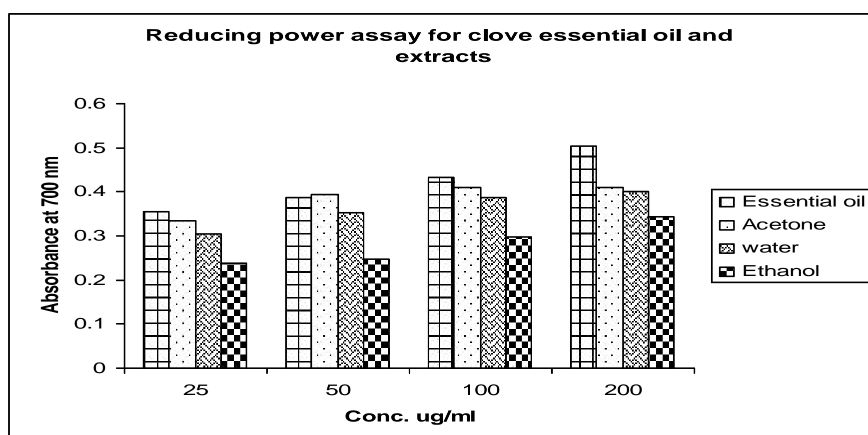

Fig. 1: Reducing power of clove extracts and clove essential oil.

8- In vivo study for test the protective effect of best clove extract and essential oil against hydrogen peroxide administration:

8.1. Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on liver functions:

Table (1) illustrates the effect of H2O2 and/or supplemented clove extract and clove essential oil on plasma liver functions parameters. In comparison with control group in group treated with 0.5% H2O2 revealed significantly increased AST and ALT activities, and decreased total protein and albumin. In rats subjected to H2O2 and supplemented with clove extract and clove essential oil, the enzyme liver marker indicate a decrease of AST (53.24±2.99 and 44.39±1.33) ALT(50.41±1.23 and 41.54±1.89) and increases the level of total protein (3.72±0.04 and 3.83±0.057) albumin (2.073±0.519 and 2.22±0.0935) as compared with group treated with H2O2 only.

Table (3): Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on liver functions

	Total protein (g/dl)	Albumin (g/dl)	AST (U/L)	ALT (U/L)
Control	3.94 ± 0.16 a	2.225 ± 0.179 a	30.96 ± 1.92 d	21.28 ± 1.43 d
H2O2 group	3.25 ± 0.17 b	1.902 ± 0.0879 b	92.32 ± 4.3 a	73.4 ± 3.05 a
H2O2 + acetone clove extract	3.72 ± 0.04 a	2.073 ± 0.519 ab	53.24 ± 2.99 b	50.41 ± 1.23 b
H2O2 + clove essential oil	3.83 ± 0.057 a	2.22 ± 0.0935 a	44.39 ± 1.33 c	41.54 ± 1.89 c
LSD $_{0.01}$	0.227	0.209	5.296	3.747

Each value is the mean ± SD. Means have different superscript letters indicate significant variation at (P 0.01), while the same letters indicate non significant variation.

H2O2 can be a potential source of damage to cells if it is decomposed through reduction to the highly reactive hydroxyl radical. As shown in the well-characterized Fenton reaction (Equation 1), this reduction requires the presence of unchelated ferrous iron:

$Fe^{+2} + H^{+} + H_2O_2$ ---------------- $Fe^{+3} + .OH + H_2O$

Lipid peroxidation is an auto-catalytic, free-radical mediated, destructive process, whereby polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (**37, 38**).

This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity (**3**). This is evidenced by an elevation in the serum marker enzymes namely AST, ALT, ALP, total bilirubin, GGTP and decrease in albumin and total protein.

Free radical mediated process has been implicated in pathogenesis of most of the diseases. The protective effects of clove extract and clove essential oil on H2O2 induced hepatotoxicity in rats appears to be related to inhibition of lipid peroxidation in addition to free radicals scavenging action. Preliminary phytochemical studies reveal the presence of Polyphenolic compound and flavonids present in clove extract (**12**) and clove essential oil (**13**). Polyphenolic compounds and flavanoids are hepatoprotectives (**39**). The observed antioxidant and hepatoprotective activity of clove may be due to the presence of polyphenolic compounds and flavanoids.

8.2. Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on kidney functions:

Data in Table (4) appeared a significant elevation ($P<0.01$) in serum urea and creatinine concentration in H2O2 treated group comparing to control group with mean value of (41.506 ± 1.92, 1.001 ± 0.042), (21.48 ± 0.7, 0.536 ± 0.046) respectively. However, acetone extract of clove and clove essential oil groups caused significant decrease ($P<0.01$) in mean value of previous parameters compared to H2O2 group.

Table (4): Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on kidney functions

	Urea (mg/dl)	Creatinine (mg/dl)
Control	21.48 ± 0.7 d	0.536 ± 0.046 d
H2O2 group	41.506 ± 1.92 a	1.001 ± 0.042 a
H2O2 + acetone clove extract	33.097 ± 1.13 b	0.854 ± 0.4 b
H2O2 + clove essential oil	28.27 ± 1.04 c	0.756 ± 0.0365 c
$LSD_{0.01}$	2.366	0.0768

Each value is the mean ± SD. Means have different superscript letters indicate significant variation at (P 0.01), while the same letters indicate non significant variation.

The role of oxidative stress as important contributing cofactors to cellular dysfunction including kidney, has substantially increased over the last years (**40**, **41**). We can hypothesized that exposure to H2O2 may cause elevation of superoxide anion and the dangerous hydroxyl (OH•) radical leading to glomerular dysfunction (**4**) with subsequent elevation in serum creatinine, blood urea, and serum uric acid concentrations (**42**). H2O2 exposure may lead to activation of a wide variety of inflammatory response like cytokines (**43**), thus diverse deleterious renal damage may occur with subsequent decrease in glomerular function which may result in elevation of kidney biomarkers.

Polyphenolic compound present in clove extract (**12**) and clove essential oil(**13**) may be responsible for the antioxidant capability of the plant and has protective effect against oxidative damage induced by H2O2.

8.3. Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on plasma antioxidants:

Hydrogen peroxide (H2O2) has been shown to induce oxidative stress in both human and animal models, leading to the generation of potent reactive oxygen species (ROS), such as hydroxyl radical (OH^{-}). Oxidative stress results when generation of reactive oxygen and/or nitrogen species and activity of the antioxidant defenses are unbalanced.

Lipid peroxidation is an auto-catalytic, free-radical mediated, destructive process, whereby polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (**38,39**). These latter compounds then decompose to form a wide variety of products, including low molecular mass hydrocarbons, hydroxy aldehydes, fatty acids, ketones, alkenals and alkanals, in particular malonaldehyde (MDA) (**44**), Thus, reduction of MDA production would indicate inhibition of lipid peroxidation.

Plasma MDA were significantly increased in rats treated with H2O2 as compares with control group (Table 5). Clove extract and clove essential oil resulted significant reduction of lipid peroxidation product induced by H2O2. Clove essential oil supplementation kept value of MDA in plasma with in the normal limit.

Table (5): Effect of acetone extract of clove and clove essential oil against hydrogen peroxide on plasma antioxidants

	MDA (nmol/dl)	Catalase (IU/ml)
Control	16.66 ± 1.097 c	34.76 ± 0.289 c
H2O2 group	36.56 ± 2.17 a	51.36 ± 1.97 a
H2O2 + acetone clove extract	24.016 ± 2.76 b	42.62 ± 2.267 b
H2O2 + clove essential oil	19.72 ± 1.48 c	35.32 ± 3.56 c
$LSD_{0.01}$	3.663	4.311

Each value is the mean ± SD. Means have different superscript letters indicate significant variation at (P 0.01), while the same letters indicate non significant variation.

Clove oil reduced tissue oxidative stress, shown by the significantly ($p < 0.05$) reduced MDA levels in the hearts of diabetic rats and the kidneys of normal rats (**45**).

Catalase, an enzyme predominantly located in peroxisomes and\ enriched in hepatocytes and erythrocytes, catalyzes the dismutation of hydrogen peroxide, forming O2 and H2O. (**46**)

CAT activity changes in the blood plasma implied that H2O2 treatment increased CAT levels, and that clove extract and clove essential oil tended to decrease CAT levels. These results could be deduced from the assumption that anti-oxidative clove extract and clove essential oil contributed to removing H2O2, the CAT substrate, as by ascorbic acid. Nevertheless, this reversion of the CAT level increase by the clove extract and clove essential oil suggests that the anti-oxidative of clove should protect the rats from cellular damage caused by ROS, similar to ascorbic acid.

Our findings are similar with (**47**) who suggested that the plant extracts decreased CAT levels in rats through the same mechanism as that of the antioxidant ascorbic acid and that they have potential as antioxidants.

Conclusion

Our present study indicates that clove essential oil and clove extracts is devoid of genotoxicity and pro-oxidant property. The enhanced liver functions, kidney functions, and antioxidant status observed in clove treated rats and its protective role against H2O2 induced cell damages might be due to the effect of active compounds which found in essential oil and plant extract.

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