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RESEARCH ARTICLE

EFFECT OF DIFFERENT *HIBISCUS SABDARIFFA* EXTRACTS ON GLUTATHIONE AND ITS RELATED ENZYMES IN MAMMALIAN LUNG TISSUE AND CULTURE CELLS UNDER INDUCED OXIDATIVE STRESS.

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Abstract

Oxidative stress refers to the excessive ROS production and the antioxidant system cannot be able to neutralize them. HPLC analysis of aqueous, 30% and 70% ethanol extracts of the polyphenol rich *H. sabdariffa* calyx (the highest phenolic, flavonoid, anthocyanin contents and antioxidant capacities) showed that cyanidin 3-*O*-glucoside chloride, chlorogenic acid and delphinidin derivatives were the major constituents. Production of H₂O₂ (prooxidant capacity) increased by increasing the ethanol extracts concentration and incubation time at pH 7.4. Gallic acid was the greatest in the production of H₂O₂. Pretreatment of rats with *H. sabdariffa* ethanol extract followed by tert-butyl hydroperoxide (t-BHP) injection partially blocked the effect of the t-BHP, as indicated by the oxidative stress markers. In rat lungs homogenates, glutathione, malondialdehyde levels and lactate dehydrogenase activity returned back almost to the normal level. The antioxidant enzymes catalase and glutathione S-transferase (GST) were increased, while glutathione peroxidase (GPx) and glutathione reductase (GR) were not affected. Histological studies indicated the presence of sever lung injury with t-BHP and *H. sabdariffa* extracts. In human lung fibroblast cells, *H. sabdariffa* extracts (150 µg and 300 µg) have almost no effect on GST activity while, resolved the inhibitory effect of H₂O₂ on GR and GPx activity.

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Abbreviations:-

CAT:	Catalase
CDNB:	1-chloro-2, 4-dinitrobenzene
DMSO:	Dimethylsulfoxide
DPPH:	1,1 Diphenyl -2-picryl-hydrazyl
GPx:	Glutathione peroxidase
GR:	Glutathione reductase
GSH:	Reduced glutathione
GST:	Glutathione S- transferase

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IC50:	Inhibitor concentration causing 50% inhibition
LDH:	Lactate dehydrogenase
MDA:	Malonyldialdehyde
MTT:	3, (4, 5-dimethylthiazol-2-yl) 2, 5 diphenyltetrazoliumbromide
ROS:	Reactive oxygen species
SE:	Standard error
TAC:	Total anthocyanin content
TBA:	2-thiobarbituric acid
t-BHP:	Tert-butyl hydroperoxide
TFC:	Total flavonoid content
TPC:	Total phenolic content

Introduction:-

In the biological system, reactive oxygen species (ROS) are highly reactive molecules resulted from normal cellular metabolism and several environmental factors. Several mechanisms counteract oxidative stress by producing antioxidants. A shift in the balance between oxidants and antioxidants in favor of oxidants is termed as “oxidative stress”. Oxidative stress refers to the excessive ROS production and the antioxidant system cannot be able to neutralize them. This imbalanced protective mechanism can lead to the damage of cellular molecules such as DNA, proteins, and lipids. Reactive oxygen species such as hydroxyl radicals, superoxide radicals and the non-radical hydrogen peroxide play important roles in the pathophysiology of a large number of diseases and oxidative damage (**Pham-Huy et al., 2008**).

In the process of cell respiration, mitochondrial oxidative metabolism produces ROS species and organic peroxides (Hussain et al., 2016). Lung is a site of major ROS production having a large surface that is constantly in contact with air oxygen and air pollutants that influenced the structure and function of the lung surfactant system (Juvin et al., 2002 and Anseth et al., 2005). Lung cells are recruited when air pollutants induce lung inflammation and released ROS, enhancing inflammation associated with tissue damage and other pathological effects (Macchionea and Garcia, 2011).

Reactive oxygen species has led to the evolution of an antioxidant defense system to protect lung tissue from substantial damage (**Tkaczyk & Vizek, 2007**). Antioxidants are naturally occurring substances that combat oxidative damage in biological entities. Antioxidants (reducing agent) are separated into two classes based on their mode of action as enzymatic and non-enzymatic antioxidants (**Abd El-Aal, 2012**).

Glutathione (GSH) is an important protective antioxidant against free radicals and other oxidants and has been implicated in inflammatory responses, modulation of redox-regulated signal transduction, regulation of cell proliferation, remodeling of the extracellular matrix (ECM), apoptosis and mitochondrial respiration. The GSH redox system is crucial in maintaining intracellular GSH/GSSG homeostasis, which is important to the normal cellular physiological processes, and represents one of the most important antioxidant defense systems in lung cells. This system uses GSH as a substrate in the detoxification of peroxides such as H₂O₂ and lipid peroxides, a reaction which involves glutathione peroxidase (GPx). This reaction generates GSSG which is then reduced to GSH by glutathione reductase (GR) in a reaction requiring NADPH (**Rahman and MacNee, 2000**). The most important antioxidant enzymes in lungs are superoxide dismutase (SOD), catalase (CAT) and GPx (**Patra et al., 2008**). Other antioxidants, such as glutathione-S-transferase (GST) and the redox proteins have crucial roles in the antioxidant defense system (**Birben et al., 2012**).

Phytochemicals such as polyphenols have been reported to be able to modulate ROS production, oxidative stress and inflammatory processes. Antioxidants such as polyphenols are natural compounds present in plants with numerous biological activities (**Hussain et al., 2016**). On the other side, antioxidants can exert a prooxidant activity in the presence of transition metal ions, generated phenoxyl radicals and ROS; induce cellular lipid peroxidation, DNA damage and apoptosis. Recently, the prooxidant property of the antioxidant compounds is not necessarily harmful for the biological systems and can be used therapeutically to treat oxidative stress condition (e.g. cancer treatment) (**Eghbaliferiz and Iranshahi, 2016**).

Species of the genus *Hibiscus* (family: Malvaceae) includes more than 300 species of annual herbs, shrubs or trees (Da-Costa-Rocha et al., 2014). Pharmacological investigations of the genus *Hibiscus* indicated their activities as anti-hypertensive, anti-inflammatory, hepatoprotective, anti-tumor, anti-diabetic, anti-convulsant, immunomodulators, antioxidant and anti-mutagenic agents (Rosa et al., 2007). The calyx of *Hibiscus sabdariffa* is a tropical plant, local name karkade (Arabic), roselle or red sorrel (English), rich in phenolic compounds with antioxidant activity and used in folk medicines against many complaints (Hirunpanich et al., 2006; Wang et al., 2011; Hassan et al., 2014).

This study was concerned with the investigation of the effect of different *H. sabdariffa* extracts on glutathione and its related antioxidant enzymes in rat lung tissue and human lung fibroblast (MRC-5) subjected to oxidative stress inducer. It was also concerned with the investigation of their possible roles in the antioxidant and prooxidant abilities involved in their biological potential in protection from oxidative stress condition.

Materials and Methods:-

Materials:-

Chemicals:-

Folin-Ciocalteu's (FC) reagent, Bovine serum albumin fraction IV (BSA), reduced glutathione (GSH), oxidized glutathione (GSSG), and 1-chloro-2, 4-dinitrobenzene (CDNB) were purchased from Merck Company. Delphinidin HCl, cyanidin HCl, α -tocopherol, ascorbic acid, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), 3, 4, 5-trihydroxy benzoic acid (gallic acid), nicotinamide adenine dinucleotide phosphate reduced form (NADPH) were products of Sigma-Aldrich Company. Doxorubicin, [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazoliumbromide] (MTT) and xylenol orange were purchased from Sigma Chemical Company. Dulbecco's Modified Eagle's/ F12 Medium (DMEM-F12), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serums (FBS) were purchased from Biowest. Butylated hydroxytoluene and ammonium ferrous sulphate were purchased from El-Gomhouria Company.

Plant material:-

The dried calyces of *H. sabdariffa* (family Malvaceae) were collected from different localities and markets at various periods from 2013 to 2016. The plants were botanically identified by the Botany Department, National Research Center, Egypt.

Methods:-

Phytochemical analyses:-

The dried calyces of *H. sabdariffa* were grounded, powdered and homogenized at a ratio 1:50 (w/v) with aqueous, 30% and 70% ethanol. The mixture was allowed to stand at 95°C for 15 min. The plant homogenates were centrifuged at 1000 g for 10 minutes, filtered through Whatman No. 1 filter paper and saved at -20°C for further analyses. Total phenolic content (TPC) was determined using the standard colorimetric method described by Singleton and Rossi, 1965. The total phenolic content of plant extract was represented as mg of gallic acid equivalent (GAE)/g calyx using the standard gallic acid calibration curve. The total flavonoid content (TFC) was measured according to the colorimetric method of Dewanto et al., 2002. Total flavonoid content was expressed as mg rutin equivalent (mg rutin/g calyx). Total anthocyanins (TAC) were measured according to the method described by Fuleki and Francis, 1968. The free radical scavenging activity of *H. sabdariffa* extract was determined using DPPH (1, 1-diphenyl-2-picryl-hydrazyl) according to the method described by Brand-Williams et al., 1995, with some modification (Leong and Shui, 2002). IC₅₀ concentrations were calculated after constructing the percent inhibition versus log extract concentrations curve.

Determination of *H. sabdariffa* phenolic and anthocyanin compounds using high performance liquid chromatography analysis:-

H. sabdariffa extracts and hydrolysable calyces were subjected to high performance liquid chromatography (HPLC) analysis in the National Research Center, central lab, Cairo, using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector (Lee et al., 2005 & Kim et al., 2006).

Determination of hydrogen peroxide production (prooxidant activity)

The ability to produce hydrogen peroxide (H₂O₂) by different *H. sabdariffa* extracts (30, 70% ethanol and aqueous) and some phenolic compounds were investigated in sodium phosphate buffer pH 7.4, deionized water and RPMI 1640 media using the ferrous ion oxidation – xylenol orange (FOX) assay (Long et al., 1999 and Banerjee et al.,

2003). The FOX assay was calibrated using a standard H₂O₂ solution to cover the range of 2-20 μM. A known volume of different *H. sabdariffa* extracts (500 μl) were added to 500 μl of deionized water and 100 mM sodium phosphate buffer, pH 7.4. The reaction mixtures were incubated at 37°C for 12, 36 and 48 h with shaking in the dark. Different *H. sabdariffa* extracts (50, 150, 300 μg/ml) diluted in RPMI 1640 media incubated at 37°C for the indicated period up to 48h with shaking in the dark. Different phenolic compounds (250 μM) such as gallic acid, protocatechuic acid, caffeic acid, catechin, quercetin, delphinidin, α-tocopherol and the powerful antioxidant standard compound (citric acid) were incubated in 50 mM sodium phosphate buffer, pH 7.4 at 37°C for 12, 36 and 48h with shaking in the dark.

Biological materials:-

Experimental animals:-

Fifty adult male albino rats weighing between 100-120 g were obtained from the animal house of the National Research Center, Giza, Egypt. The animals were kept under a 12 h light-dark cycle at room temperature. All animals were allowed to adapt to the environment for 2 weeks before the beginning of the experiment for normalization of laboratory condition.

Experimental Design:-

The rats were divided into 6 groups and each group includes 8 animals. **Group 1:** control group, healthy rats. **Group 2:** was given distilled water only for 5 consecutive days, on day 5, (0.2 mmol/kg body weight) of tert-butylhydroperoxide (t-BHP) was injected intra-peritoneal. **Group 3, 4:** were orally administered daily with aqueous extract of *H. sabdariffa* (250 mg/kg body weight) for 5 consecutive days, on day 5, (0.2 mmol/kg body weight) of t-BHP was injected intraperitoneal to group 4. **Group 5, 6:** were orally administered daily with 30 % ethanol extract of *H. sabdariffa* (250 mg/kg body weight) for 5 consecutive days, on day 5, (0.2 mmol/kg body weight) of t-BHP was injected intraperitoneal to group 6. The rats were sacrificed after 18 h of t-BHP intra-peritoneal injection. The animals were killed. The lungs were immediately removed and weighed and washed using ice-cold saline. Tissues of the lungs were homogenized with ratio 1:2 (w/v) in 25mM Tris- HCl buffer, pH 8, containing 1mM EDTA and 2mM β-mercaptoethanol. The lung homogenates were centrifuged at 10,000 xg for 20 min at 4°C and stored at -20°C for further analyses.

Cell culture:-

Human lung fibroblast cells (MRC-5) were purchased from Vacsera, Dokki, Egypt and cultured in DMEM-F12 medium which was supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100U/ml streptomycin. Growth curves for MRC-5 was determined under baseline conditions prior to investigation of cytotoxicity. The cytotoxicity effect of 30% ethanol extracts of *H. sabdariffa* against MRC-5 was investigated using MTT [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide] (MTT assay) assay, which yields a blue formazan product in living cells, but not in dead cells (**Kang et al., 2005**).

Experimental Design:-

For experiments, cells were cultured in 75 cm² flasks supplemented with 10% heat-inactivated FBS, 100U/ml penicillin and 100U/ml streptomycin and incubated at 37°C, 5% CO₂ until confluent (80-90%). The flasks were subjected to eight different treatments. 30% ethanolic extracts of *H. sabdariffa* were dissolved in DMSO (0.5%, v/v, final concentration) and added to culture medium to achieve the differently designed concentrations. The cells pre-treated with fresh media without serum containing the following different concentrations: 30% ethanol extracts of *H. sabdariffa* (150, 300 μg/ml), hydrogen peroxide (0.05, 1mM/ml), 30% ethanol extracts of *H. sabdariffa* (150 μg/ml) containing 1mM/ml of hydrogen peroxide, 30% ethanol extract of *H. sabdariffa* (300 μg/ml) containing 0.05mM/ml hydrogen peroxide, different possible controls as DMSO only and control (the media alone without serum). The cells were incubated for 24 h at 37°C, 5% CO₂. Media was removed and the cells were harvested (**Awad et al., 2014**). After incubation, the medium was removed. Cells were scraped. The cells washed twice with cold phosphate buffer saline. Cells were lysed in 0.1 M potassium phosphate buffer, pH 8 containing 5mM EDTA and 5 mM β-mercaptoethanol. The lysate was sonicated for 30 sec three times, centrifuged at 2000 rpm and preserved at -20°C for further analyses.

Biochemical analyses:-

Protein concentration was determined by the method of **Bradford, 1976** using bovine serum albumin as a standard. The total GSH was measured calorimetrically using the method of **Saville, 1958**. Level of lipid peroxidation was determined by measuring the formation malondialdehyde (MDA) using the method of **Ohkawa et al., 1979**.

Enzyme Assays:-

The Glutathione S- transferase (GST) activity was determined according to the method described by **Habig et al., 1974**. Glutathione peroxidase (GPx) was measured according to the method described by **Weinhold et al., 1990**. The Glutathione reductase (GR) activity was determined following the decrease in absorbance at 340 nm according to the method described by **Zanetti, 1979**.

Catalase (CAT) activity was assayed according to the method described by **Aebi, 1984**. Total Lactate dehydrogenase (LDH) activity was measured as described by **Fountain et al. (1970)**.

Histological analysis:-

The lung tissue of the experimental rats were immediately removed and fixed in 10% neutral-buffered formal saline for 72 h at least. Serial sections of 6 µm thick were cut and stained with Haematoxylin and eosin (**Carleton, 1980**).

Statistical analysis:-

Data were analyzed using means \pm standard deviation. Differences between two groups were determined by t-test. $P < 0.05$ significant, $P < 0.01$ highly significant and $P > 0.05$ insignificant.

Result:-**Phytochemical analyses:-**

The results in Table 1 showed that the 70% ethanol extract of *H. sabdariffa* at 95°C for 15 min have the highest phenolic content (39.19 ± 6.63 mg gallic acid equivalent /g dry calyx), flavonoid contents (1.33 ± 0.09 mg rutin equivalent/g dry calyx) and anthocyanin content (9 ± 3.8 mg anthocyanin/g dry calyx). The antioxidant capacity using DPPH scavenging activity showed that 30% ethanol and aqueous *H. sabdariffa* extracts at 95°C have a powerful antioxidant capacity with IC₅₀ values (5.68 ± 0.9 mg dry calyx /ml and 6.44 ± 1.163 mg dry calyx/ml, respectively) compared to 70% ethanol *H. sabdariffa* extract (IC₅₀= 11.04 ± 3.3 mg dry calyx /ml).

Analysis of *H. sabdariffa* phenolic and anthocyanin compounds using high performance liquid chromatography analysis:-

HPLC analysis of *H. sabdariffa* calyx phenolic compounds extracted with 30% ethanol, 70% ethanol, H₂O and hydrolyzed calyx were monitored at 280 nm, 320 nm and 360 nm. The results showed that chlorogenic acid was the highest concentration in the aqueous, 70% ethanol and 30% ethanol extracts representing 3.304, 1.543 and 1.105 mg/g calyx, respectively. Considerable amounts of caffeic acid were also observed in the three examined extracts. Alcoholic extraction of *H. sabdariffa* calyx indicated the presence of protocatechuic acid, gallic acid, quercetin and kaempferol in a concentration ranged from 0.0312 to 0.355 mg/g calyx. Protocatechuic acid and kaempferol were not found in aqueous extract. Cinnamic acid (0.0118 mg/g calyx) and sinapic acid (0.0113 mg/g calyx) were found only in aqueous extract. Hydrolysis of the calyx caused a decrease in the concentration of catechin to 0.2874 mg / g calyx. However, caffeic acid (2.088 mg/g calyx) and quercetin (0.696 mg/g calyx) increased in concentration by hydrolysis of *H. sabdariffa* calyx. Chlorogenic acid, rutin and kaempferol could not be detected after hydrolysis. *H. sabdariffa* pigment has been reported to be rich in delphinidin-3-sambubioside (70% of the anthocyanins) and cyanidin-3-sambubioside as the major pigments, with delphinidin-3-monoglucoside and cyanidin-3-monoglucoside. The content of cyanidin 3-*O*-glucoside chloride in the 30% ethanol, 70% ethanol and aqueous extracts of *H. sabdariffa* was found to be 13.8, 14.29, 17.7 mg/g calyx, respectively. The content of delphinidin derivative determined in 30% ethanol, 70% ethanol and aqueous extract of *H. sabdariffa* was found to be 2.08, 1.653 and 1.69 mg/g calyx, respectively (Table 2).

Hydrogen peroxide production (prooxidant activity):-

For the three *H. sabdariffa* examined extracts, no H₂O₂ was produced by incubation in deionized water at 37°C up to 48 h. 30% and 70% ethanol extracts produced H₂O₂ in 100 mM phosphate buffer, pH 7.4 after 24 h of incubation (0.58 ± 0.12 µg/ml and 5.9 ± 0.28 µg/ml, respectively) and its production increased by increasing the incubation time to 48 h (2.3 ± 0.33 µg/ml and 7.9 ± 0.05 µg/ml, respectively). Production of H₂O₂ of the three *H. sabdariffa* extracts was observed in RPMI 1640 media only at a concentration more than 50 µg/ml. After 48 h of incubation, H₂O₂ concentration was found at the concentration of 150 and 300 µg/ml of the aqueous extract (0.21 µg/ml, 0.45 µg/ml, respectively) (Fig.2 b). H₂O₂ concentration was 0.54 µg/ml and 0.45 µg/ml with 300 µg/ml of both *H. sabdariffa* alcoholic extracts after 24 h incubation (Fig.2 a). After 48h of incubation H₂O₂ generation was increased with 30% and 70% ethanol extracts at concentrations of 150 µg/ml (0.26 µg/ml and 0.53 µg/ml, respectively) and 300 µg/ml (0.93 µg/ml and 0.92 µg/ml, respectively) (Fig.2 b). The ability of some phenolic compounds (250µM)

which are abundant in *H. sabdariffa* extracts as gallic acid, protocatechuic acid, caffeic acid, catechin, quercetin, delphinidin, α -tocopherol and the standard antioxidant compound citric acid was incubated in pH 7.4 at 37°C. As shown in Fig.3, all polyphenols and the standard antioxidant compound citric acid produced amount of H₂O₂ in phosphate buffer after 24h. The production of H₂O₂ by gallic acid was the greatest. The yield of H₂O₂ was in the following decreasing order gallic acid > caffeic acid > quercetin > delphinidin > protocatechuic acid > catechin > citric acid > α -tocopherol. The production of H₂O₂ of all the examined phenolics was increased by increasing the period of incubation time to 48h, except quercetin and protocatechuic acid. They reached almost the same level at the equivalent concentration after 36h of incubation (Fig.4).

Animal study:-

Effect of *H. sabdariffa* extracts on some oxidative stress, lipid peroxidation and tissue damaged biomarkers:-

The lung non-enzymatic antioxidant defense was evaluated by total glutathione (GSH) level (Table 3). Rat group injected with t-BHP showed significant decrease ($0.05 < p < 0.1$) in the level of GSH in lung homogenate (85.35 ± 9.4 nmol /g tissue) compared with the control group (111.56 ± 9.06 nmol /g tissue). Oral administration with *H. sabdariffa* ethanol extract showed highly significant decrease ($p < 0.001$) in the concentration of GSH (62.55 ± 6.63 nmol /g tissue) compared to the control group. However oral administration with *H. sabdariffa* aqueous extract significantly ($p < 0.05$) increased the concentration of GSH (126.92 ± 20.74 nmol /g tissue) compared to the control group. Also the t-BHP treated rats after oral administration with aqueous and ethanol extracts showed significant increase in the concentration of GSH (135.9 ± 9.3 nmol/ g tissue and 112.45 ± 8.43 nmol /g tissue, respectively) compared to the t- BHP treated group.

The effect of *H. sabdariffa* extracts on t- BHP induced oxidative stress in rats using malondialdehyde (MDA) as a marker of lipid peroxidation are shown in Table 3. The MDA levels insignificantly decreased ($p > 0.1$) from 1647.8 ± 133 to 1329.6 ± 115.8 nmol/ g tissue in lung homogenate of rats treated with t-BHP. Pretreatment with aqueous extract of *H. sabdariffa* insignificantly decreased the MDA level ($p > 0.1$) in the lungs injected with either t-BHP or not (1451.7 ± 133.8 nmol/ g tissue, 1453.8 ± 144.7 nmol/ g tissue, respectively) compared to the control group. The reverse is true for the rats treated with *H. sabdariffa* ethanol extract where insignificant increase ($p > 0.1$) in the MDA level was detected (2124.9 ± 225 nmol/ g tissue) compared to control group. However, pretreatment with the ethanol extract of *H. sabdariffa* before injection of t-BHP significantly decreased ($0.05 < p < 0.1$) the MDA level (1227.6 ± 161.9 nmol/g tissue) compared to t-BHP treated rats.

Lactate dehydrogenase (LDH) activity in all the experimental groups was increased (Table 3). Significant increase in LDH activity was observed in t-BHP treated rats ($p < 0.01$) and rats administrated with aqueous ($p < 0.05$) *H. sabdariffa* extract (99.14 ± 11.99 unit/ g tissue, 80.14 ± 9.66 unit/ g tissue, respectively) compared to the control group (57.56 ± 3.79 unit/ g tissue). Treatment with ethanol *H. sabdariffa* extract showed insignificant increase in the LDH activity (63.58 ± 5.7 unit/ g tissue, $p > 0.1$) compared to the control group. In rats injected with t-BHP , pretreatment with aqueous and ethanol *H. sabdariffa* extracts, LDH activity insignificantly decreased (84.98 ± 7.56 unit/ g tissue, 75.89 ± 6.16 unit/ g tissue, respectively) compared to the t-BHP treated rats.

Significant increase in protein concentration ($p < 0.01$) was observed in the t-BHP treated rats and rats administrated with either aqueous or ethanol *H. sabdariffa* extract (55.5 ± 7.2 mg/ g tissue, 63.26 ± 8.2 mg/ g tissue, 58.6 ± 5.6 mg/ g tissue, respectively) compared to the control group (31.5 ± 3.4 mg/ g tissue). Pretreatment with ethanol or aqueous extracts of *H. sabdariffa* before injection of t-BHP insignificantly increased the protein concentration (66.6 ± 7.2 mg/g tissue, 64.7 ± 7.8 mg/g tissue, respectively) compared to the t-BHP treated rats (Table 3).

Effect of *H. sabdariffa* extracts on the activity of the antioxidant enzymes:-

In the lung tissue homogenates of in the t-BHP treated rats insignificant decrease in glutathione S-transferase (GST) activity was observed (1.199 ± 0.063 unit/ g tissue) compared to the control group (1.388 ± 0.21 unit/ g tissue, $p > 0.1$). Pretreatment with aqueous extract of *H. sabdariffa* before injection with t-BHP insignificantly increased the GST activity (1.229 ± 0.149 unit/ g tissue, $p > 0.1$) compared to the t-BHP treated rats (1.199 ± 0.063 unit/ g tissue). Treatment with *H. sabdariffa* aqueous extract have almost no effect on GST activity (1.308 ± 0.139 unit/ g tissue) when compared to control group. Oral administration of *H. sabdariffa* ethanol extract insignificantly increased the GST activity in lung tissue of rats either injected with t-BHP or not (1.313 ± 0.116 unit/ g tissue, 1.488 ± 0.143 unit/ g tissue, respectively, $p > 0.1$), (Table 4).

Decrease in the activities of glutathione peroxidase (GPx) was observed in lung tissue of all the examined groups (Table 4). GPx activity significantly decreased in the t-BHP treated rats (3.22 ± 0.324 unit/ g tissue, $p < 0.01$) and that administered aqueous extract of *H. sabdariffa* (3.11 ± 0.291 unit/ g tissue, $p < 0.05$) compared to the control group (5.616 ± 0.917 unit/ g tissue). Insignificant decrease in the GPx activity was observed in rats administered 30 % *H. sabdariffa* ethanol extract (3.68 ± 0.705 unit/ g tissue) compared to the control group. Insignificant increase was observed in the GPx activity of both aqueous and ethanol *H. sabdariffa* extracts before injection with t-BHP (3.84 ± 0.31 unit/ g tissue, 3.46 ± 0.433 unit/ g tissue, respectively) compared to the t-BHP treated rats group.

A highly significant decrease in glutathione reductase (GR) activity in lung tissue of t-BHP treated rats (1.97 ± 0.264 unit/ g tissue, $p < 0.01$) was observed compared to the control group (3.79 ± 0.459 unit/ g tissue). Oral administration with aqueous and ethanol extracts of *H. sabdariffa* caused insignificant decrease ($p > 0.1$) in the GR activity (2.77 ± 0.53 unit/ g tissue, 3.23 ± 0.399 unit/ g tissue, respectively) compared to the control group. The GR activity was significantly increased in rats orally administered *H. sabdariffa* aqueous extract before t-BHP injection (4.14 ± 0.389 unit/ g tissue, $p < 0.001$), but insignificantly increased in rats orally administered ethanol extract of *H. sabdariffa* before t-BHP injection (2.84 ± 0.509 unit/ g tissue, $p > 0.1$) when compared to the t-BHP treated rats. While when compared to the control group, GR activity increased in lung tissue of orally administered *H. sabdariffa* aqueous extract but decreased with ethanol extract administration (Table 4).

Data of catalase (CAT) activity in the lung of rats of all the experimental groups under investigation were increased (Table 4). The CAT activity was significantly increased in t-BHP treated rats (3.21 ± 0.376 unit/ g tissue, $p < 0.05$) and rats orally administered *H. sabdariffa* aqueous extract (3.47 ± 0.23 unit/ g tissue, $p < 0.01$) but insignificantly increased in rats orally administered *H. sabdariffa* ethanol extract, (3.5 ± 0.625 unit/ g tissue, $p > 0.1$) when compared to the control group (2.1 ± 0.374 unit/ g tissue). Aqueous *H. sabdariffa* extract administration have almost no effect on t-BHP injected rats, while ethanol *H. sabdariffa* extract administration significantly increased CAT activity after t-BHP injection (4.38 ± 0.477 unit/ g tissue, $0.05 < p < 0.1$) as compared to control and t-BHP treated rats (Table 4).

Histological examination:-

Injection of rats with t-BHP caused severe thickening of the alveolar walls and blood capillaries walls and infiltration of inflammatory cells in the alveolar wall. Treatment with aqueous extract of *H. sabdariffa* showed mild cellular infiltration close to the bronchiole and blood vessel walls thickening with severe dilatation and congestion. Treatment with ethanol extract of *H. sabdariffa* showed focal aggregation of inflammatory cells that invades the wall of the bronchiole under the epithelium and increase in alveolar septae thickening. Pretreatment of the rats with *H. sabdariffa* aqueous extract followed by t-BHP injection, showed focal aggregation of inflammatory cells and thickening of blood vessels walls but the alveolar septae look normal. Pretreatment of the rats with *H. sabdariffa* ethanol extract followed by t-BHP injection showed marked thickening with hypertrophy of muscle fibers in the bronchiole wall, the blood vessels show dilatation with congestion and fibrous tissue surrounded the bronchiole walls and the blood vessels (Fig. 4, 5).

Effect of *H. sabdariffa* ethanol extracts on human lung fibroblast (MRC-5) cells:-

Cytotoxicity of different concentrations of 30% ethanol *H. sabdariffa* extract (37.5 µg, 75.5 µg, 150 µg, 300 µg, and 600 µg/ml) against normal human lung fibroblast (MRC-5 cells) showed less than 10% reduction of cell viability, i.e. *H. sabdariffa* ethanol extract had almost no toxicity up to 600 µg. Glutathione level and the catalytic activities of GST, GPx, GR and protein concentration were also examined in human lung fibroblast cell (MRC-5) homogenates of six preparations 1) control 2) control DMSO 3) cells subjected to two concentrations of H₂O₂ (0.05 and 1mM) 4) cells treated with two concentrations of 30 % *H. sabdariffa* ethanol extract (150 and 300 µg dry weight) 5) cells treated with 300 µg *H. sabdariffa* extracts in the presence of 0.05mM H₂O₂ 6) cells treated with 150 µg *H. sabdariffa* extracts in the presence of 1mM H₂O₂ (Fig. 6). At low concentration of H₂O₂ (0.05 mM), the level of GST did not increase in the cells, however, increasing H₂O₂ concentration to 1mM increased the GST activity by 35%. Increasing *H. sabdariffa* extract concentrations from 150 µg to 300 µg increased the percent inhibition of GST activity from 41% to 65.2%. The two *H. sabdariffa* extracts (150 µg and 300 µg) have almost no effect on the activity of GST in the cells subjected to 0.05 and 1mM H₂O₂. H₂O₂ at concentration of 0.05mM or 1mM decreased GR and GPx activities. Treatment of MRC-5 cells with 300 µg of *H. sabdariffa* extracts increased the catalytic activity of GPx, while using 150 µg of *H. sabdariffa* extracts have no effect on GPx activity. Increasing *H. sabdariffa* extract concentrations from 150µg to 300 µg caused an increase in the GR and GPx activities. Both concentrations of *H. sabdariffa* extract resolved the inhibitory effect of H₂O₂ on GR and GPx activity. H₂O₂ did not affect the GSH level at its high concentration 1mM while the protein concentration increased. Treatment of cells

with 150µg of *H. sabdariffa* extracts decreased GSH level and increased the protein concentration. However treatment of cells with 300 µg of *H. sabdariffa* extracts decreased the protein concentration. Addition of 1mM H₂O₂ to 150 µg *H. sabdariffa* extracts increased GSH level. Addition of 1mM H₂O₂ to 150 µg *H. sabdariffa* extracts decreased the concentration of protein and addition of 0.05mM H₂O₂ to 300 µg *H. sabdariffa* extracts decreased the concentration of protein.

Discussion:-

Phytochemicals have been observed to be protective and defensive against many diseases. Most of phytochemicals possess antioxidant activity, scavenge reactive oxygen species (ROS), chelate metal ions, inhibit oxidases and activate antioxidant enzymes. They include phenols, phenolic acids, flavonoids and many sterols (Pacôme et al., 2014). *H. sabdariffa* is an important source of vitamins, minerals, and bioactive compounds, such as organic acids, phytosterols and polyphenols, some of them have antioxidant properties (Sayago-Ayerdi et al., 2007). Phenolic acids can occur in the free form (aglycones), but usually they are found in conjugated forms, as glycosides and esters. Also, they can be linked by ester bonds to the cell wall polysaccharides or they are associated with dietary fiber, namely lignin (Bravo, 1998 and Garcia-Salas et al., 2010).

In our study, chlorogenic acid had the highest concentration in the aqueous, 70% ethanol and 30% ethanol extracts representing 3.304, 1.543 and 1.105 mg/g calyx, respectively. Considerable amounts of caffeic acid were also observed in the three examined extracts. Owoade et al. (2016) reported the presence of chlorogenic acid, ferulic acid, naringenin, rutin and quercetin in *H. sabdariffa* extract. Pacôme et al. (2014) identified 18 compounds in *H. sabdariffa* petals: phenolic acids (chlorogenic acid and protocatechuic acid) and flavonoids (gossypetin, sabdaretin, gossypetin, luteolin, gossytrin, hibiscetin, rutin, hibiscetin, myricetin, eugenol, nicotiflorine, quercitrin, quercetin, kaempferol, astragalol and cyanoside). Phenolic acids also exist as insoluble bound complexes, which are either high molecular weight polyphenols or single phenolic compounds strongly bound to cell wall proteins or polysaccharides, and are not extractable by organic solvents (Arranz et al., 2010). Bound phenolic acids are typically liberated using base hydrolysis, acid hydrolysis or both (Mattila & Kumpulainen, 2002). Polyphenols are largely metabolized following ingestion in the stomach, small and large intestine, and liver with incomplete understanding of the absorption and metabolism of all polyphenols. Caffeic acid (2.088 mg/g calyx) and quercetin (0.696 mg/g calyx) increased in concentration by alkaline hydrolysis of *H. sabdariffa* calyx, while chlorogenic acid, rutin and kaempferol could not be detected after hydrolysis. Chlorogenic acid isomers can be hydrolyzed to quinic and caffeic acids (Sánchez Maldonado et al., 2014). This may be the reason for increasing caffeic acid from 0.862 to 2.088 mg/g calyx and complete degradation of chlorogenic acid after alkaline hydrolysis. The amount of caffeic acid released upon hydrolysis is lower than the amount expected from hydrolysis of chlorogenic acid. This may be due to hydrolysis with NaOH without protection using ascorbic acid and ethylenediaminetetraacetic acid (EDTA) that resulted in complete degradation of caffeic acid (Ross et al., 2009). HPLC showed the complete disappearance of rutin and the increase of quercetin from 0.1 to 0.69 mg/g calyx indicating the hydrolysis of rutin to quercetin (Table 2). This result is in accordance with the previous study of Wang et al. (2011) where acidic and enzymatic hydrolysis of rutin could produce isoquercitrin and quercetin. The observed decrease of catechin from 1.25 to 0.287 mg/g calyx, could be due to the degradation by alkaline hydrolysis or introducing of two double bonds in the C ring due to the presence of transition metals. One should note that *H. sabdariffa* contain iron, phosphorus, calcium, manganese (Sultan et al., 2014). The main constituents of *Hibiscus* anthocyanins were identified as delphinidin, delphinidin-3-glucoxyloside (Lin et al., 2007; Maganha et al., 2010 and Kuo et al., 2012). *H. sabdariffa* pigment has been reported to be rich in delphinidin-3-sambubioside and cyanidin-3-sambubioside as the major pigments, with delphinidin-3-monoglucoside and cyanidin-3-monoglucoside (Wong et al., 2002 and Frank et al., 2005). Our results indicated the presence of delphinidin derivatives and cyanidin 3-*O*-glucoside chloride in 30% ethanol, 70% ethanol and aqueous extracts. Cyanidin 3-*O*-glucoside chloride concentration represented 74.3%, 72.7% and 69%, respectively of total phenolic compounds. The content of delphinidin derivatives represented 9.1%, 8.4%, 6.6%, respectively of total phenolic compounds (Table 2).

The most important feature discussed for polyphenols is their antioxidant activity (Spanou et al., 2012 and Stagos et al., 2012). Actually, polyphenols can act as free radical scavengers and can interact with metal ions to form a chelate through their aromatic hydroxyl group. Emerging evidence indicates that these polyphenols may also behave as prooxidants initiating a reactive oxygen species (ROS) production, cellular DNA damage and cell death (Akagawa et al., 2003 and Mileo and Miccadei, 2016). Polyphenols such as caffeic acid, ferulic acid and apigenin, may have a prooxidant effect by activating ROS production and oxidative stress conditions (León-González et al., 2015).

The prooxidants activity of dietary flavonoids is linked to the total number of hydroxyl groups in the molecule, 2, 3-double bond in ring C and 4-oxo arrangement of flavons (**Procházková et al., 2011**). In *H. sabdariffa*, delphinidin (3 OH in the B ring) and quercetin (2, 3 double bond in the ring C beside 4-oxo arrangement) could be considered a strong prooxidant. In the present study, the prooxidant activity resulted from H₂O₂ production was monitored with the *H. sabdariffa* extracts. It showed that gallic acid was the greatest in the production of H₂O₂ due to the presence of three adjacent OH group. Production of H₂O₂ by the phenolic compounds tested indicated that, the production of H₂O₂ is related to their structures and only ortho and para phenolic compounds may undergo autoxidation. **Akagawa et al. (2003)** investigated the production of hydrogen peroxide by polyphenols, epigallocatechin, epigallocatechin gallate, epicatechin, epicatechin gallate and catechin which are abundant in green tea and black tea and chlorogenic acid and caffeic acid which are abundant in coffee. Flavonoids generally occur in foods as *O*-glycosides with sugars bound at the C3 position. Glycoside modification of the OH substitutions leads to inactivation of transition metal-initiated prooxidant activity of a flavonoid (**Rahal et al., 2014**). Transition metals could also mediate the prooxidant activity of polyphenols, through the reduction of metal ions involved in redox-cycling and promoting the generation of hydroxyl radicals through Fenton-reaction (**León-González et al., 2015**). In our results, ethanol extracts of *H. sabdariffa* increased the production of H₂O₂. It had to mentioned that most buffers and *H. sabdariffa* extracts contained trace metal ions. Iron, copper, zinc and lead were detected in *H. sabdariffa* calyx as reported by **Bakare-Odunola and Mustapha, 2014**. Also, high manganese levels (453.71 ± 14.07 mg/kg) followed by iron (219.8mg/kg) in *H. sabdariffa* flower was pointed out in the research of **Mihaljev et al. (2014)**.

Lungs are vulnerable target for oxidative stress (OS), because of their location, anatomy and function (**Bargagli et al., 2009**). The lung is a highly vascularized organ, vulnerable to pathogens, pollutants, oxidant, gases and toxicants, thus making it susceptible to OS (**Piao et al., 2008**). Lung under hypoxic condition increased inflammatory cell-derived prooxidants, lipid peroxidation products and change antioxidant enzymes activities (**Hoshikawa et al., 2001; Minko et al., 2002 and Araneda et al., 2005**). Hypoxia induced cell membrane structure and function alterations, caused acute lung injury and damage to components of the extracellular matrix (**Nagato et al., 2012**).

Tert-butyl hydroperoxide (t-BHP) is a short-chain analog of lipid hydroperoxide and used as a model substance to investigate the mechanism of cell injury resulting from acute oxidative stress in cells and tissues. There are two pathways by which t-BHP is metabolized to free radical intermediates, first, provided by cytochrome P450 or by hemoglobin in erythrocytes, leads to production of peroxy and alkoxy radicals (**Davies, 1989**). These radicals subsequently initiate lipoperoxidation of membrane phospholipids and membrane permeability alterations. The other pathway employs glutathione peroxidase; t-BHP is converted to *tert*-butanol and glutathione disulphide (GSSG) (**Crane et al., 1983 and Kucera et al., 2014**). **Kucera et al. (2014)** reported that t-BHP induced hepatocytes injury, changes of cell viability and oxidative stress. Hydrogen peroxide is present in exhaled air of humans and rats. Also, it is one of the major ROS that induce OS (**Halliwell et al., 2000**). It easily react with intracellular ions such as iron and copper to generate the highly reactive hydroxyl radicals and attack cellular components to cause various oxidative damages and inflammation leading to chronic diseases such as aging, atherosclerosis, rheumatoid arthritis and cancer (**Piao et al., 2008**).

Inflammation is an important protective response (is a natural defense mechanism) to cellular/ tissue injury (**Hussain et al., 2016**). The purpose of this process is to destroy and remove the injurious agent and injured tissues, thereby, promoting tissue repair. When this crucial and normally beneficial response occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results in chronic inflammation and destruction of normal tissue. Reactive oxygen species liberated by phagocytes recruited to sites of inflammation, are proposed to be a major cause of the cell and tissue damage, including apoptosis, associated with many chronic inflammatory diseases. Lung cells, alveolar epithelial type II cells, are susceptible to the injurious effects of oxidants (**Rahman and Macnee, 2000**).

Glutathione (GSH), ubiquitously present in all cell types, is the major non-enzymatic antioxidant and regulator of intracellular redox homeostasis (**Liu et al., 2002**). The antioxidant GSH has been shown to be critical to the lungs' antioxidant defenses, protecting airspace epithelium (membrane integrity) from oxidative/free radical-mediated injury and inflammation. Alterations in the levels of GSH in the lung lining fluid have been shown in various inflammatory conditions (**Rahman and Macnee, 2000**).

The present results showed that t-BHP reduced the level of GSH an index of OS, in rat lung. Pretreatment with *H. sabdariffa* aqueous extract and 30% ethanol extract before injection with t-BHP effectively blocked these phenomena, as indicated by slight increase in GSH level. The group treated with 30% ethanol *H. sabdariffa* extract showed a significant decrease in GSH level. **Liu et al. (2002)** reported a slight decrease in GSH level in rat liver treated with protocatechuic acid alone. *H. sabdariffa* 30% ethanol and aqueous extracts contain gallic acid, catechin, chlorogenic acid, caffeic acid, rutin and quercetin. However, protocatechuic acid and kaempferol could not be detected in aqueous extract. *H. sabdariffa* ethanol extract contain protocatechuic acid, higher amount of quercetin and caffeic acid beside the possible deglycosylation of rutin due to rutin hydrolysis in the rat stomach resulting in production of ROS. One should note that gallic acid, caffeic acid, quercetin and delphinidin are strong producers of H₂O₂ in buffer (pH 7.4). H₂O₂ is one of the factors that induce acute lung injury and it can easily cross membranes. GSH homeostasis may play a central role in the maintenance of the integrity of the lung airspace epithelial barrier (**Rahman et al., 2001 and Nagato et al., 2012**). In the present investigation, the increase in protein concentration and LDH activity were observed in rat lungs treated with ethanol extract, aqueous extract or t-BHP alone or with both. A slight decrease in LDH was observed in lungs of rats pre-treated with *H. sabdariffa* aqueous or ethanol extracts followed by t-BHP injection (84.98 unit/ g tissue, 75.89 unit/ g tissue, respectively) compared to the t-BHP treated rats (99.14 unit/ g tissue) . This may indicate slight protection by aqueous extract and ethanol extract to lung tissue. These results are in agreement with other studies that showed that the total protein concentration and LDH activity were increased in animals submitted to lung injury (**Dal-Pizzol et al., 2006 and da Cunha et al., 2011**). The increased protein concentration may indicate an increase in permeability of the alveolar capillary membrane (**da Cunha et al., 2013**). The increase in MDA, a marker for Lipid peroxidation was only observed in rat lungs treated with *H. sabdariffa* ethanol extract alone. This behaviour was also observed in GSH concentration. However, insignificant decrease in MDA level was observed in rat lungs treated with either aqueous extract or t-BHP alone.

A reduction in the activities of GST, GPx and GR in t-BHP treated rat lungs was observed in this study. This reduction may be due to an overwhelming oxidative modification of the enzymatic proteins by excessive generation of highly reactive free radicals, leading to loss of integrity and function of cell membranes (**Kalava & Mayilsamy, 2014**). Our result is in accordance with the previous study of **Yen et al. (2004)** that reported the reduction in the activity of GR and GPx in rat livers on t-BHP induction. Treatment with *H. sabdariffa* aqueous extract has almost no effect on GST activity compared to control. Oral administration of *H. sabdariffa* ethanol extract insignificantly increased the GST activity in lung tissue of rats either injected with t-BHP or not. In **Majid et al. (1991)** study, oral feeding of ellagic acid at varying dose levels for eight weeks resulted in a dose-dependent significant increase in the hepatic GST activity as well as in the hepatic and pulmonary GSH levels. However, the pulmonary GST activity was not affected, indicating that ellagic acid has a differential effect on GST activity in different organs. After ellagic acid administration, its elimination from the body through the bile and urine in the form of glutathione conjugates. **Majid et al. (1991)** deduced that the *in vitro* inhibition of GST by ellagic acid is due to the fact that GST has to conjugate ellagic acid with GSH in addition to conjugating CDNB with GSH. *In vivo*, ellagic acid enhances the hepatic GST activity. This increase may be due to the greater need of GSH and GST for the elimination of ellagic acid itself from the body. Since the lung is constantly exposed to the environment, there are significant differences in the kinetic, structural, and immunological properties among the tissue specific GSTs. Furthermore, different forms of GSTs differ among themselves in their ability to conjugate various xenobiotics to GSH.

GSTs function is to catalyze the conjugation of the sulfur atom of GSH to an electrophilic center of endogenous and exogenous toxic compounds, increasing their solubility and excretion (**Habig and Jakoby, 1981**). GSTP_i (GSTP₁) is responsible for more than 90% of the GST activity within the adult human lung epithelial cell population. Among the human GST classes, GSTP₁ was expressed more abundantly in the respiratory tissue (**Yan et al., 2010**). Most of GSTs composed of two identical subunits. A complete active site of each subunit composed of one site of binding for GSH (G site) and one site which binds a number of hydrophobic substrates (H site) adjacent to the G site (**Wilce and Parker, 1994**). Glutathionylation of proteins is considered as a primary line of defense. This reversible modification reaction can be performed non-enzymatically, but it has proposed that GSTP₁ may enhance this modification of thiol proteins during oxidative stress and/or nitrosative stress (NS). Because of their GSH high affinity and specificity, GSTs are structurally well equipped to accommodate diverse GSH-bound substrates such as glutathionylated proteins. GSTP₁ can potentially serve as a cellular NO donor and/or carrier (**Vasieva, 2011**), regulating the activities of other thiol redox-modifying enzymes (e.g. GR) (**Mazzetti et al., 2015**). Decrease in GPx and GR activity of lungs in rat treated with either ethanol extract or aqueous extract may be due to *H. sabdariffa* calyx had been analyzed to contain phytic acid, tannin and glycosides such as delphinidin-3-monoglucoside and delphinidin which are toxic to animal and human tissues at high doses (**Morton, 1987; Nnamonu et al., 2013**).

Catalase is not essential for some cell types under normal conditions; it plays an important role in cell adaptation to OS (Mates et al., 1999). In the present study, CAT activity was significantly higher in rat lung treated with aqueous extract ($p < 0.01$) or t-BHP alone ($p < 0.05$) or with rat lungs pretreated with ethanol extract followed by t-BHP injection ($0.05 < p < 0.1$). Insignificant increase in catalase activity of rat lungs treated with ethanol extract or pretreated with aqueous extract followed by t-BHP injection ($p > 0.1$) was observed. The activation of CAT activity could contribute to the detoxification of ROS (Ilieva et al., 2014). Pretreatment with ethanol extract followed by t-BHP injection partially blocked the effect of the t-BHP. The OS markers, GSH, MDA, LDH levels returned back almost to the normal level. However, OS enzyme markers including GR and GPx did not return back to the normal level. CAT, the enzyme detoxifying the effect of high concentration of H_2O_2 increased significantly ($0.05 < p < 0.1$). Pretreatment with aqueous extract followed by t-BHP injection increased the GSH level almost to the normal level (abolish the effect of t-BHP). MDA was not affected; however, LDH level and protein concentration (indicating the increase in permeability of the alveolar capillary membrane) could not block the effect of t-BHP. GR activity returned back to almost the normal level, however, GPx and CAT activity could not abolish the effect of t-BHP. The changes in the examined OS markers and antioxidant enzymes activities suggested lung injury in rat lungs injected with t-BHP or pretreated with both *H. sabdariffa* aqueous extract and ethanol extract. However, pretreatment with *H. sabdariffa* extracts followed by t-BHP injection could abolish the effect of t-BHP in some respects.

the histopathological analyses were done to verify the presence of inflammatory infiltrates and so validate the lung injury model. The injection of rats with t-BHP caused severe thickening of the alveolar walls and blood capillaries walls and infiltration of inflammatory cells in the alveolar wall. Loss of alveolar epithelial integrity results in the accumulation of protein-rich and highly cellular edema fluid in the alveoli (Proudfoot et al., 2011). This is confirmed with the increase in the total protein concentration and LDH activity in lung homogenate, decrease in the GSH level, decrease in GPx and GR activity and increase in CAT activity. Treatment with aqueous extract of *H. sabdariffa* showed mild cellular infiltration close to the bronchiole and blood vessel walls thickening with severe dilatation and congestion and treatment with ethanolic extract of *H. sabdariffa* showed focal aggregation of inflammatory cells that invades the wall of the bronchiole under the epithelium and increase in alveolar septae thickening. This may be due to the phenolics and flavonoids present in the extracts which are strong producers of H_2O_2 (a major ROS) resulting in production of ROS that induce acute lung injury. This behavior agreed with the increase in protein concentration and LDH activity in rat lungs treated with either ethanol extract or aqueous extract and the decrease in GSH level in rat treated with ethanol extract only. The antioxidant enzymes, GPx and GR activity decreased while an increase in CAT activity in rat lungs pretreated with either ethanol extract or aqueous extract was observed. Pretreatment of the rats with *H. sabdariffa* aqueous extract followed by t-BHP injection, showed focal aggregation of inflammatory cells and thickening of blood vessels walls but the alveolar septae look normal. The accumulation of excess fibrous connective tissue (the process called fibrosis) resulted in the end stage of organ failure. The pathogenesis of fibrosis in many diseases is thought to involve aberrant or over-exuberant wound-healing processes initiated to protect from injurious stimuli (Sakai & Tager, 2013). Fibroblasts play a key role during wound healing that produce large quantities of extracellular matrix components, cytokines and repair growth factors (Quesnel et al., 2010). One of the key steps in the tissue response is the deposition of collagen fibers at the sites of injury where lung fibroblasts release procollagen peptides into the extracellular space in order to create a scar. So collagen deposition must not be viewed as a late response to abnormal healing, but as an early phenomenon (Gonzalez-Lopez & Albaiceta, 2012). Initiation of fibrous tissue surrounded the bronchiole walls and the blood vessels was observed in histopathological examination of the rat lungs pretreated with ethanol extract followed by t-BHP injection which could indicate the start of lung tissue repair including fibroblast. Pathological accumulations of fibroblasts are the main source of the extracellular matrix proteins accumulation in the fibrotic area. Lung injury resulted in fibroblast proliferation, differentiation and migration. Therefore, fibroblast cells were chosen for the present cell line study.

Toxicity, cytotoxicity and genotoxicity were reported as a result of addition of phenolic compounds from plant sources to the cell culture media. This was attributed mainly to the H_2O_2 production by the oxidation of the phenolic compounds in some plants such as green tea, grape seed, apple extract (Halliwell, 2014). Polyphenols can be oxidized in cell culture media. Cell culture media are frequently deficient in antioxidants especially tocopherols and ascorbate and also deficient in selenium (Halliwell, 2008). Cells require transition metal ions, especially iron and copper, in order to grow. The components of the cell culture medium, including the various inorganic salts, vitamins, and amino acids, contribute to, but are not completely essential for the generation of H_2O_2 by a polyphenol (Babich et al., 2011). The widely used Dulbecco's modified Eagle's medium (DMEM) contains added iron (III) nitrate, $Fe(NO_3)_3$. Free iron is also present in Ham's F10 medium, often used to study the oxidation of low density

lipoprotein (LDL) *in vitro* (Firth & Gieseg, 2007). In other media, iron is mostly supplied in transferrin-bound form, usually in the widely used calf serum. Whereas transferrin-bound iron will not normally catalyze free radical reactions, contaminating (or added) free iron ions can be prooxidant, as can be copper and many other transition metal ions (Aruoma & Halliwell, 1987 and Halliwell, 2014).

In the present results, *H. sabdariffa* extracts (aqueous, 30% ethanol and 70% ethanol) in RPMI 1640 media produce substantial amounts of H₂O₂. H₂O₂ increased by increasing the concentration to 300 µg/ml of *H. sabdariffa* extract. Insignificant changes in H₂O₂ production was observed by increasing the extract concentration to 300µg either in 30 or 70% ethanol. Halliwell et al. (2000) reported that ascorbate-induced apoptosis in HL-60 cells was significantly greater in DMEM medium than in RPMI medium, correlating with the increased rates of H₂O₂ generation on addition of ascorbate to DMEM. This result is in accordance with the previously reported effects relative to epigallocatechin and epigallocatechin gallate, catechin and quercetin, showing that phenolic compounds are oxidized in cell culture media and lead to H₂O₂ generation (Long et al., 2000). Caffeic, chlorogenic and ferulic acids, were effective inducing DNA cleavage in human promyelocytic leukemia HL-60 cells in the presence of Cu (II) ions. Other phenolic compounds, such as piceatannol, a stilbene naturally present in grapes and rhubarbrhizome, among others, can interact with copper ions in their oxidized forms, Cu (II), promoting the Haber-Weiss and Fenton reactions that generate ROS, inducing DNA cleavage (León-González et al., 2015). One should note that the values of H₂O₂ produced was due to the phenolic compounds in the extract and any H₂O₂ produced in the control was decomposed to O₂ and water by the addition of CAT. H₂O₂ (strong ROS) at the level of 0.05mM or 1mM caused a decrease in GR (44%, 59.7%) and GPx (65.7%, 49%) activity, respectively, however it did not affect the GSH level at its high level (1mM) and increased the protein concentration. This may indicate that the antioxidant capacity of the cells could not compensate the prooxidant effect of the phenolic compounds. Treatment of MRC-5 cells with low concentration of H₂O₂ (0.05 mM) and high concentration of *H. sabdariffa* extract (300 µg) abolished the inhibitory effect of H₂O₂ to either GR or GPx activity. The activity of GR and GPx increased almost to the normal level. Increasing the concentration of H₂O₂ to 1mM and decreasing the *H. sabdariffa* extract to 150 µg resolved the inhibitory effect of H₂O₂ to both GR and GPx activity; however they did not go back to the normal level. Addition of *H. sabdariffa* extract (300µg/ml) to the cell culture is associated with a high decrease in protein concentration. This could be due to toxicity of polyphenol, anthocyanin, flavonoid and other compounds producing ROS in the media in presence of metal ions. However, decreasing the *H. sabdariffa* extract concentration to 150µg/ml and increasing the H₂O₂ concentration to 1mM increased the protein concentration. This may confirm that this effect is mainly due to *H. sabdariffa* and not to H₂O₂. GST activity decreased by 41%, 65.2% by increasing the *H. sabdariffa* extract concentration from 150 µg to 300 µg, respectively. One has to note that in the DMSO control, an increase in the GST activity was observed. This could be due to the detoxification by GST needed to cell survival. DMSO is a toxic material at high concentration but its use at low level did not affect the growth. At low concentration of H₂O₂, the level of GST did not increase, however at high concentration (1mM) it caused increase in GST required for detoxification and cell survival. Pretreatment of MRC-5 cells with high or low concentration of *H. sabdariffa* extract almost did not affect the GST activity. GSH level decreased in cells treated with 150µg *H. sabdariffa* extract by 60.8%. However, addition of 1mM H₂O₂ to 150 µg *H. sabdariffa* extracts increased GSH level and goes back to almost the normal level. This was also detected in the rat lungs pretreated with ethanol extract followed by t-BHP injection.

It must be realized that cells in culture are different from those *in vivo* in many ways. The normal cell matrix (which has important influences on cell morphology and function) is absent, as are other cell types that normally surround the cells in question and communicate with them. Cells that survive and grow in culture are often not representative of cells *in vivo* in terms of gene expression, enzyme levels and metabolism. Most cells *in vivo* are exposed to low O₂ concentrations except for the cells lining the respiratory tract, skin epidermis and cornea of the eye, however cell culture is commonly performed under 95% air 5% CO₂. Levels of O₂ to which cells in culture are exposed can affect many of their properties. The production of ROS rates by cellular enzyme systems and by leakage of electrons from electron transport chains are limited at normal cellular levels, elevated O₂ will thus increase ROS production in cells in culture. Increased ROS production might cause increased cell proliferation or lead to senescence, cell death, or adaptation. For human fibroblasts, a value of 40-50 doublings was originally suggested. Cell culture media are frequently deficient in the antioxidants that are normally obtained in the human diet and delivered to cells by the bloodstream (Halliwell, 2014).

Phytochemicals such as polyphenols have been reported to be able to modulate the inflammatory processes (Hussain et al., 2016). Much attention is currently focused on the role of natural polyphenols on modulating

intracellular ROS levels. Several polyphenols were demonstrated to interfere with enzymes driving the epigenetic alterations which modulate inflammation process, as such; it will be a challenge for future anti-inflammatory therapies (Mileo and Miccadei, 2016). Anti-inflammatory effects of polyphenols can be realized via binding to various receptors. Flavonoid (e.g.resveratrol, epigallocatechin gallate and curcumin) induced receptor stimulation can modulate active state of different kinases thus influence differentiation and apoptosis, cell survival (inhibition of apoptosis) and inflammatory response. In a study of Trebatická and Durackova (2015), conducted on human brain, polyphenols can be inhibiting the inflammation mediated by macrophages through the reduction of proinflammatory cytokines formation and inhibition of platelet aggregation.

Declarations:-

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Availability of data and materials:-

Data and materials related to this work are available upon request.

Competing interests:-

The authors declare that they have no competing interests.

Consent for publication:-

All authors approve the manuscript for publication.

Ethics approval and consent to participate:-

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee. All experiments were carried out in accordance with the Egyptian laws and National Research Centre guide lines, and the informed consent was obtained from all of the patients.

Table 1:- Phytochemical analyses of *H. sabdriffa* extracts

Extraction solvent	TPC mg GAE /g dry plant	TFC mg RE /g dry plant	TAC mg anthocyanin E/g dry plant	Antioxidant capacity using DPPH IC ₅₀ *
30% ethanol	20.96 ± 3.69 (14.8, 27.6)	0.39 ± 0.07 (0.28, 0.52)	4.24 ± 0.33 (3.67, 4.8)	5.68 ± 0.9 (4.7, 7.5)
70% ethanol	39.19 ± 6.63 (26, 47)	1.33 ± 0.09 (1.15, 1.58)	9 ± 3.8 (2.7,15.9)	1.04 ± 3.3 (6.4, 17.6)
H ₂ O	19.08 ± 2.67 (13.7, 21.9)	0.43 ± 0.03 (0.38, 0.495)	3.2 ± 0.23 (2.8, 3.6)	6.44 ± 1.16 (4.1, 7.8)

Values are represented as mean ± SE of 3 to 4 experiments

Values between parentheses are the highest and the lowest value

IC₅₀ (amount of extract which cause 50% inhibition of DPPH free radical)

Antioxidant capacity is represented by IC₅₀ (mg dry plant/ml)

Table 2:- Characterization of phenolic and anthocyanin compounds extracted from *H. sabdariffa* calyx using high performance liquid chromatography analysis.

Compound	Rt	Area	Extraction solvent			
			30% ethanol	70% ethanol	H ₂ O	Hydrolyzed Calyx
Gallic acid	5.40	3208.7	0.125	0.144	0.1469	0.1388
Protocatechuic acid	9.30	1618.7	0.212	0.355	0	0.1232
Catechin	17.50	608.1	0.821	0.4562	1.257	0.2874

Syringic acid	21.80	1529.4	0	0	0	0
Vanillic acid	23.50	2294.6	0	0	0	0
Coumarin	35.80	2779.3	0	0	0	0
Cinnamic acid	42.00	2192.4	0	0	0.0118	0
Chrysin	50.90	2761	0	0	0	0
Gentisic acid	16.50	1296.9	0	0	0	0
Chlorogenic acid	19.60	1054.7	1.105	1.543	3.304	0
Caffeic acid	20.20	2808.5	0.665	0.826	0.862	2.088
Ferulic acid	30.90	3798.6	0	0	0	0.0373
Sinapic acid	32.30	2897.5	0	0	0.0113	0.0186
Rosmarinic acid	39.60	2590.5	0	0.0926	0.0499	0.0434
Rutin	35.30	1668.9	0.051	0.1486	0.1898	0
Quercetin	42.70	3582.2	0.0762	0.1066	0.0689	0.696
Kaempferol	45.60	3848	0.0388	0.0312	0	0
Cyanidin 3-O-glucoside chloride	9.88	1307.8	13.8	14.29	17.7	-
Delphinidin derivatives	7.11	6193.7	1.69	1.653	2.08	-

Values are represented as mg/g calyx

Rt: Retention time

Table 3:- Effect of different extracts of *H. Sabdariffa* on GSH, protein, malondialdehyde (MDA) levels and lactate dehydrogenase activity (LDH) of lung tissue treated with tert-butyl hydroperoxide (t-BHP).

Treatment	GSH (nmol/g tissue)	Protein (mg/g tissue)	MDA (nmol/g tissue)	LDH (unit/g tissue)
Control	111.56 ± 9.06	31.5 ± 3.4	1647.8 ± 133.1	57.56 ± 3.79
t-BHP (0.2 mmol/kg)	85.35 ± 9.4	55.5 ± 7.2	1329.6 ± 115.8	99.14 ± 11.99
t-BHP vs. control	p < 0.1 < 0.05	p < 0.01	p > 0.1	p < 0.01
Aqueous <i>H. sabdariffa</i> (250 mg/Kg)	126.92 ± 20.74	63.3 ± 8.2	1453.8 ± 144.7	80.14 ± 9.66
Aqueous <i>H. sabdariffa</i> vs. control	p < 0.05	p < 0.01	p > 0.1	p < 0.05
Aqueous <i>H. sabdariffa</i> + t-BHP	135.86 ± 9.3	64.7 ± 7.8	1451.7 ± 133.8	84.98 ± 7.56
Aqueous <i>H. sabdariffa</i> + t-BHP vs. control	p < 0.1 < 0.05	p < 0.01	p > 0.1	p < 0.01
Aqueous <i>H. sabdariffa</i> + t-BHP vs. t-BHP	p < 0.01	p > 0.1	p > 0.1	p > 0.1
30% ethanol <i>H. sabdariffa</i> (250 mg/Kg)	62.55 ± 6.63	58.6 ± 5.6	2124.9 ± 225	0.58 ± 5.763
30% ethanol <i>H. sabdariffa</i> vs. control	p < 0.001	p < 0.01	p > 0.1	p > 0.1
30% ethanol <i>H. sabdariffa</i> + t-BHP	112.45 ± 8.43	66.6 ± 7.2	1227.6 ± 161.9	75.89 ± 6.16
30% ethanol <i>H. sabdariffa</i> + t-BHP vs. control	p > 0.1	p < 0.01	p < 0.1 < 0.05	p < 0.05
30% ethanol <i>H. sabdariffa</i> + t-BHP vs. t-BHP	p < 0.1 < 0.05	p > 0.1	p < 0.1 < 0.05	p > 0.1

Values are represented as mean ± SE of n=7

P < 0.01 highly significant, P < 0.05 significant and P > 0.1 insignificant

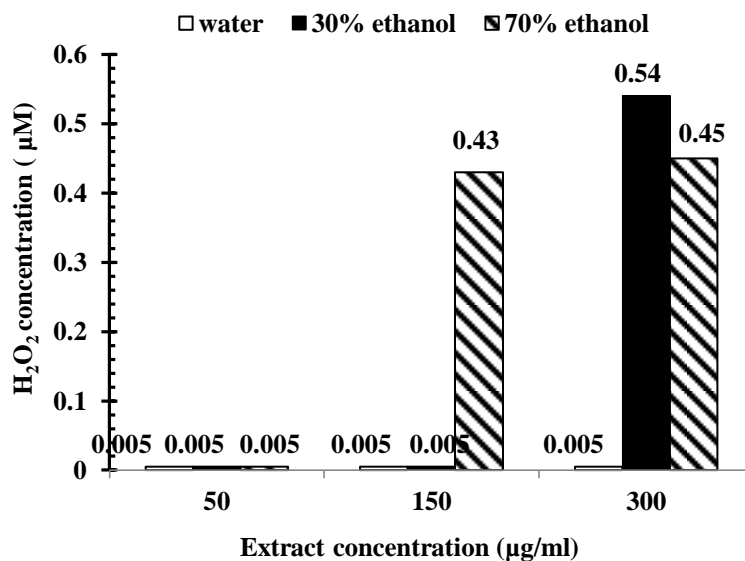
Table 4:- Effect of different *H. sabdariffa* extracts on the activity of some antioxidant enzymes in lung tissue of rats treated with tert-butylhydroperoxide (t-BHP)

Treatment	GST unit/g tissue)	GPx (unit/g tissue)	GR (unit/g tissue)	CAT (unit/g tissue)
Control	1.388 ± 0.21	5.616 ± 0.917	3.79 ± 0.459	2.1 ± 0.374
t-BHP (0.2 mmol/kg)	1.199 ± 0.063	3.22 ± 0.324	1.97 ± 0.264	3.21 ± 0.376
t-BHP vs. control	p > 0.1	p < 0.01	p < 0.01	p < 0.05
Aqueous <i>H. sabdariffa</i> (250 mg/Kg)	1.308 ± 0.1397	3.11 ± 0.291	2.77 ± 0.53	3.47 ± 0.23
Aqueous <i>H. sabdariffa</i> vs. control	p > 0.1	p < 0.05	p > 0.1	p < 0.01
Aqueous <i>H. sabdariffa</i> + t-BHP	1.229 ± 0.149	3.84 ± 0.307	4.14 ± 0.389	3.1 ± 0.377
Aqueous <i>H. sabdariffa</i> + t-BHP vs. control	p > 0.1	p > 0.1	p > 0.1	p < 0.1 < 0.05
Aqueous <i>H. sabdariffa</i> + t-BHP vs. t-BHP	p > 0.1	p > 0.1	p < 0.001	p > 0.1
30% ethanol <i>H. sabdariffa</i> (250 mg/Kg)	1.488 ± 0.143	3.68 ± 0.705	3.23 ± 0.399	3.5 ± 0.625
30% ethanol <i>H. sabdariffa</i> vs. control	p > 0.1	p > 0.1	p > 0.1	p > 0.1
30% ethanol <i>H. sabdariffa</i> + t-BHP	1.313 ± 0.116	3.46 ± 0.433	2.84 ± 0.509	4.38 ± 0.477
30% ethanol <i>H. sabdariffa</i> + t-BHP vs. control	p > 0.1	p > 0.1	p > 0.1	p < 0.01
30% ethanol <i>H. sabdariffa</i> + t-BHP vs. t-BHP	p > 0.1	p > 0.1	p > 0.1	p < 0.1 < 0.05

Values are represented as mean ± SE of n=7

$P < 0.01$ highly significant, $P < 0.05$ significant and $P > 0.1$ insignificant

(a)



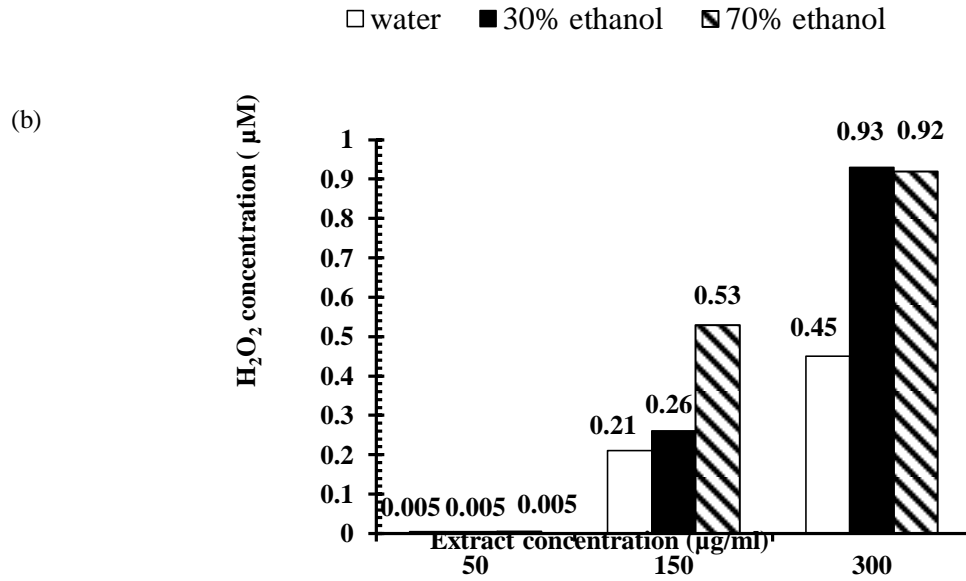


Fig. 1: H₂O₂ concentration in RPMI cell culture medium containing 50,150 and 300 µg/ml of *H. sabdariffa* different extracts: a) after 24h of incubation and b) after 48h of incubation.

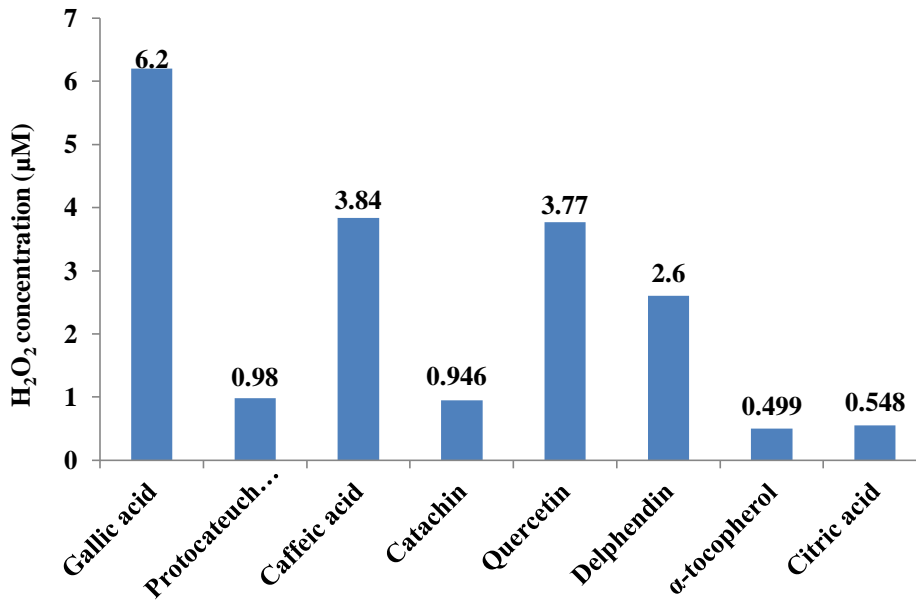


Fig 2:- Production of H₂O₂ by some phenolic compounds and the standard antioxidant compound citric acid (250 µM) after 24 h of incubation at pH 7.4.

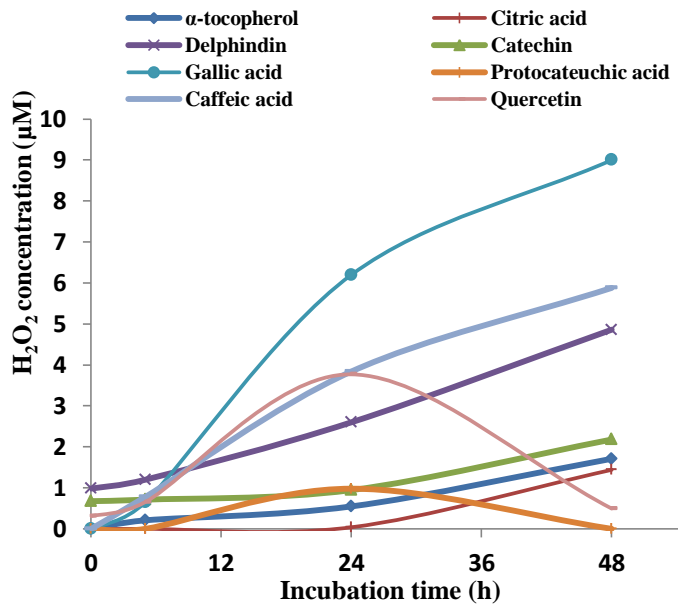
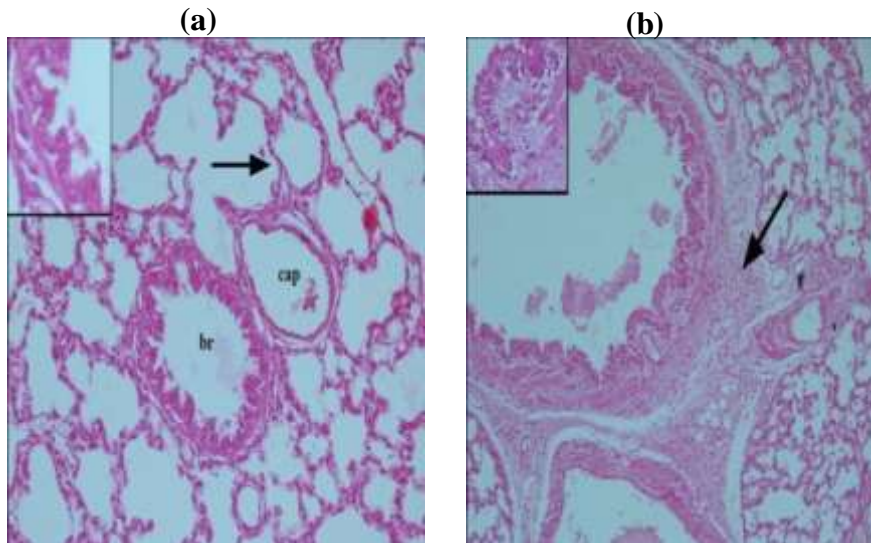


Fig. 3:- Effect of incubation time on H₂O₂ production by different phenolic compounds and the standard antioxidant compound citric acid.



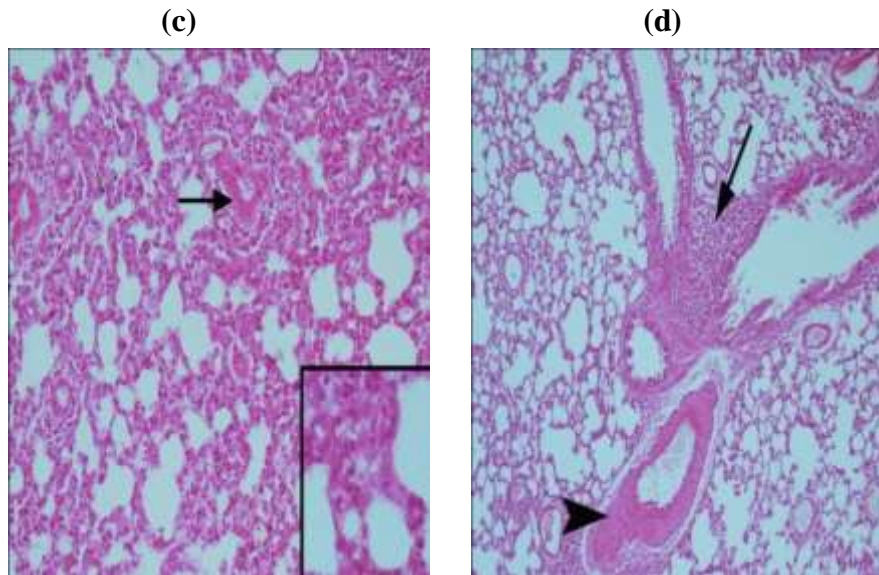
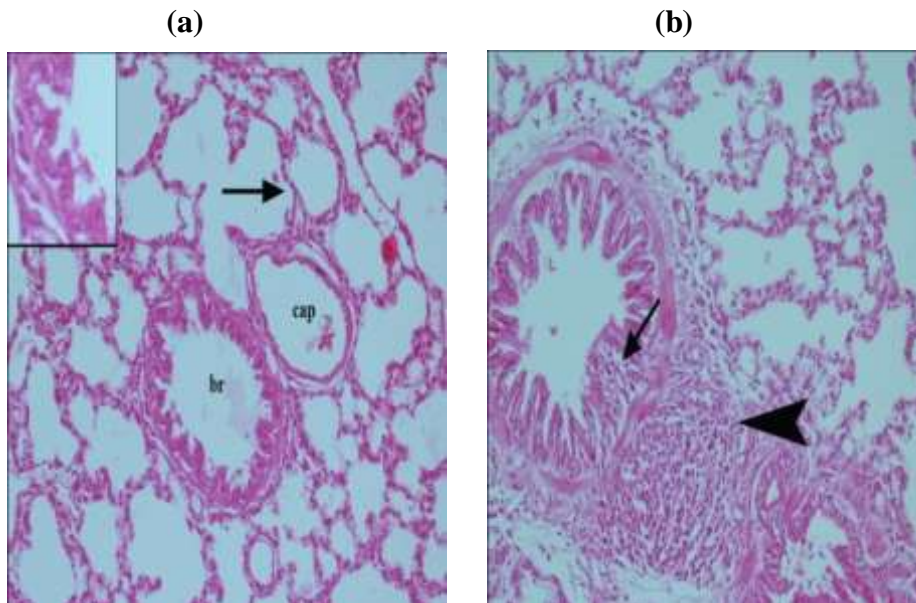


Fig. 4:- Histological findings for (A) control (thin walls of the alveoli (arrow), (B) rat lung treated with *H. sabdariffa* ethanol extract (focal aggregation of inflammatory cells (arrowhead) that invades the wall of the bronchiole under the epithelium (arrow), (C) rat lung injected with t-BHP (severe thickening of the alveolar walls and blood capillaries walls (arrow)) and (D) rat lung pretreated with ethanol extract followed by t-BHP (marked thickening with hypertrophy of muscle fibers in the bronchiole wall (arrowhead). The blood vessels show dilatation with congestion (arrow), (H&E section).



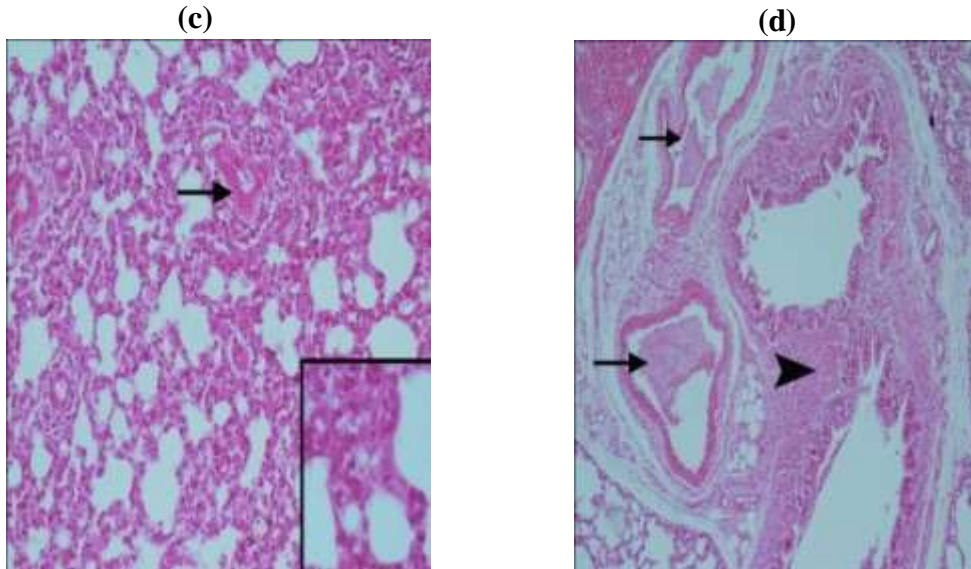
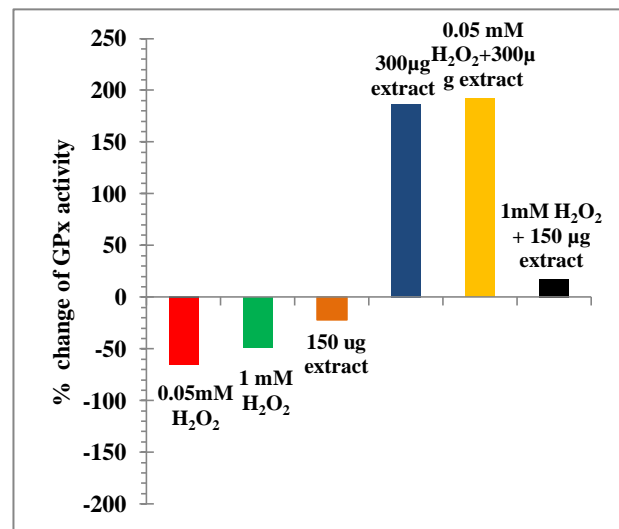
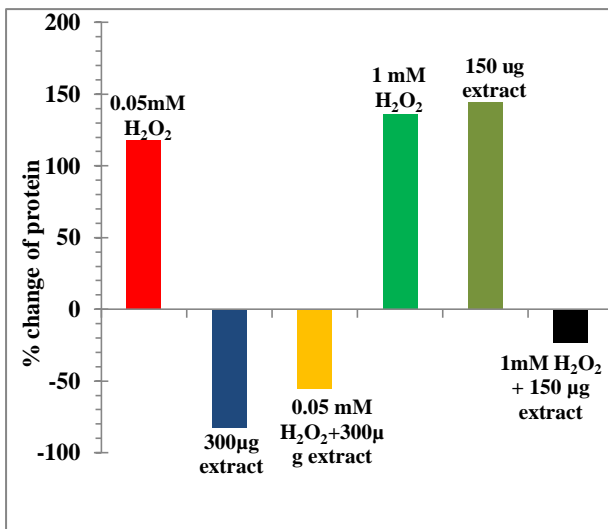
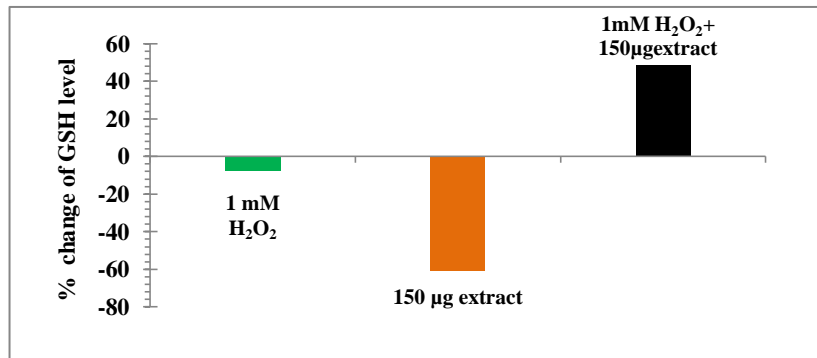


Fig. 5:- Histological findings for (A) control (thin walls of the alveoli (arrow), (B) rat lung treated with *H. sabdariffa* ethanol extract (focal aggregation of inflammatory cells (arrowhead) that invades the wall of the bronchiole under the epithelium (arrow), (C) rat lung injected with t-BHP (severe thickening of the alveolar walls and blood capillaries walls (arrow)) and (D) rat lung pretreated with ethanol extract followed by t-BHP (marked thickening with hypertrophy of muscle fibers in the bronchiole wall (arrowhead). The blood vessels show dilatation with congestion (arrow), (H&E section).



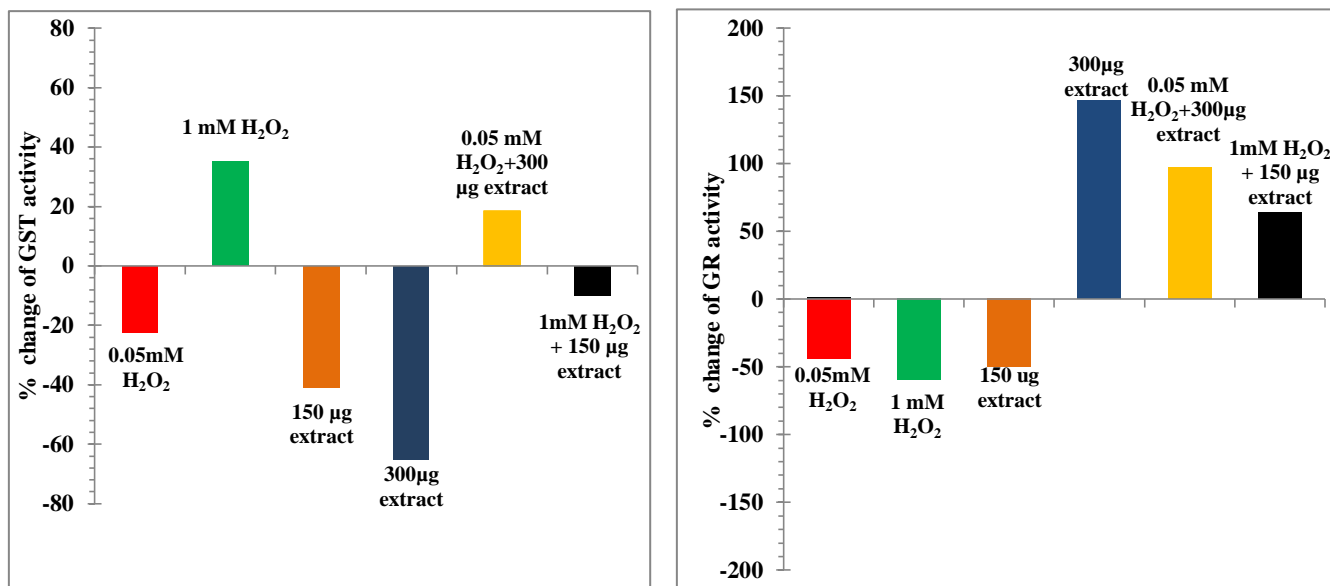
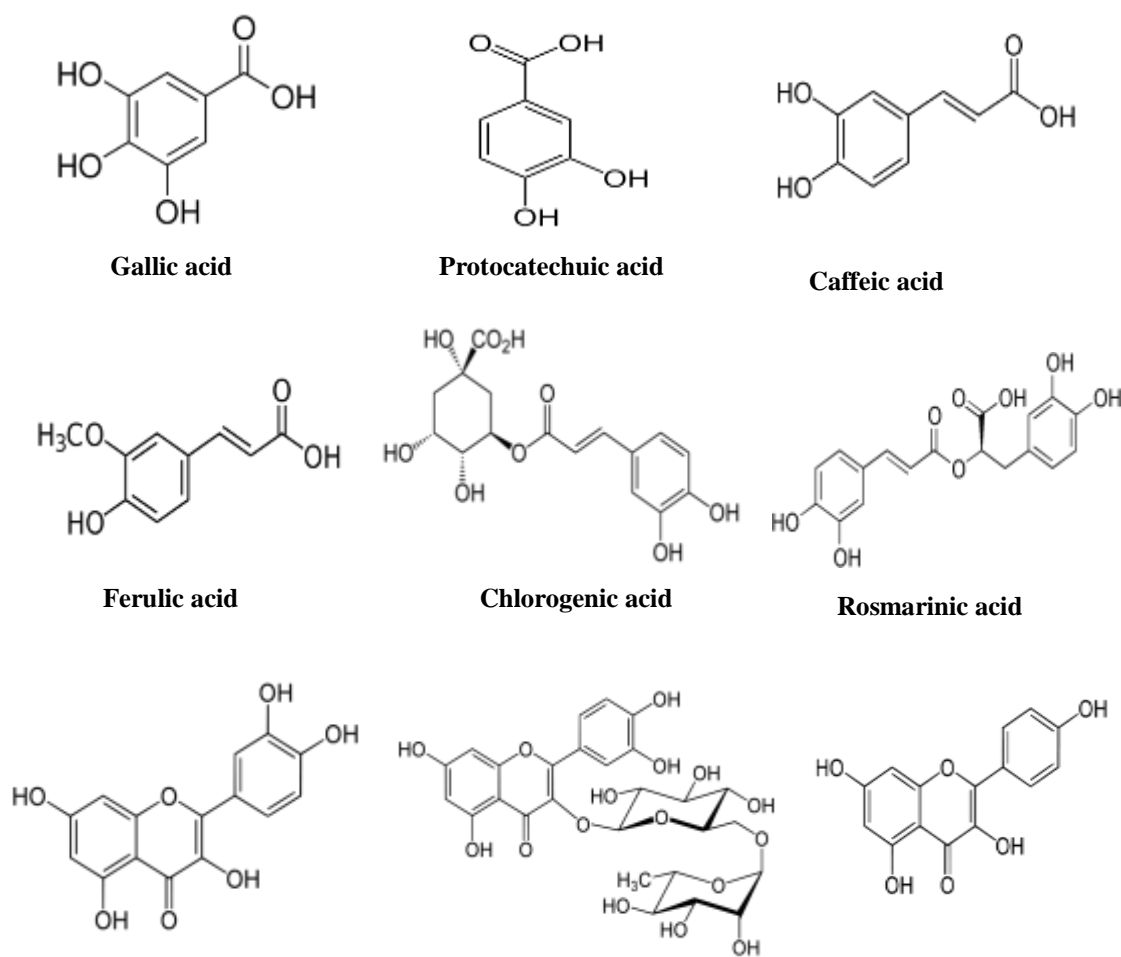


Fig. 6: Effect of *H. sabdariffa* extracts (150 and 300 µg) on glutathione, protein concentration and some antioxidant enzymes catalytic activities of MRC-5 cells subjected to H₂O₂ (0.05 and 1 mM).



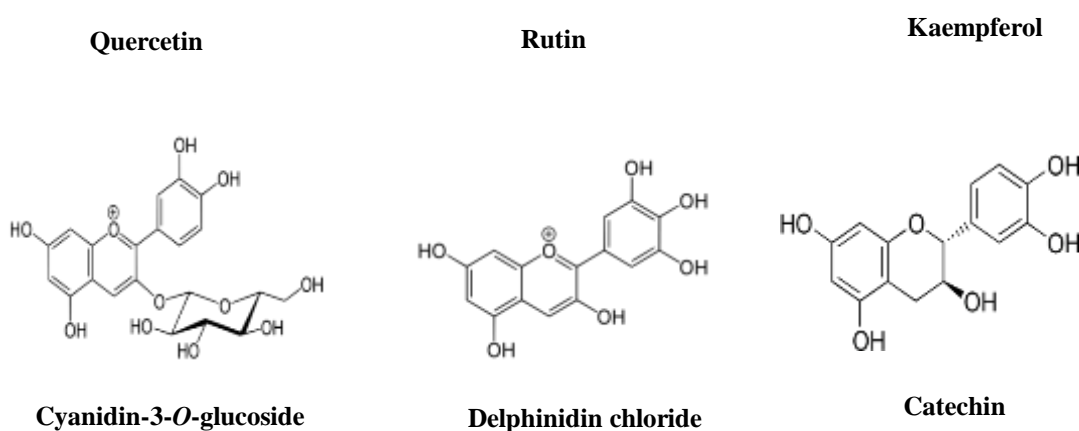


Fig. 7:- Structure of the major *H. sabdariffa* phenolic compounds as indicated from the present HPLC analysis.

Conclusion:-

Phenolic compounds can act as antioxidants or prooxidants depending on their chemical structure, concentration, environmental conditions such as the cell type and redox stress. Lungs are vulnerable target for OS, because of their location, anatomy and function. Evaluation of the OS condition is a hot topic in biology and medicine. Oxidative stress is viewed as an imbalance between the production of ROS and their elimination by protective mechanisms, which can lead to chronic inflammation. H_2O_2 production by all the examined *H. sabdariffa* extracts indicated the involvement of flavonoids and their related structures (results of HPLC analysis) in their prooxidant activities. The changes in the examined OS markers and antioxidant enzymes activities suggested lung injury in rat lungs injected with t-BHP or pretreated with both *H. sabdariffa* aqueous extract and ethanol extracts. However, pretreatment with *H. sabdariffa* extracts followed by t-BHP injection could abolish the effect of t-BHP regarding GSH, protein levels, antioxidant enzyme activities and some histopathological observations. Further research studies should not only focus on understanding the mechanism of phenolics to exert their effects, ROS production but also their effects on other organs.

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