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NATURAL ANTIOXIDANTS OF HUMIC AND FULVIC ACIDS AGAINST OXIDATIVE STRESS INDUCED BY HYDROGEN PEROXIDE: *IN-VIVO* STUDIES

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ABSTRACT

Fulvic acid (FA) and humic acid (HA) are formed through the degradation of organic substances by chemical and biological process. Fulvic acids and humic acid has been found to possess the highest activity as antioxidant scavenging. Treatments of humic acid at different concentrations with 10,50 ,100 and 150 mg/ Kg showed a significant differences in kidney function and liver function. The results exhibited a significant concentration-dependent manner of free radical scavenging activity as well as significant reduction of MDA level. The lowest lipid peroxidation has been reported with 150 mg/kg B.W. Humic acid and fulvic acid showed a significant decrease in the enzymes activity of glutathione peroxidase and superoxide dismutase indicating their direct protective effects against induced hydrogen peroxide. The antioxidant properties of the FA and HA partially support the health beneficial to enhance certain aspects of plant growth or mixed with fertilizers/chemicals.

KEYWORDS:
Fulvic acid, Humic acids, *In vivo* antioxidant scavenging capacity, kidney function, liver function.

INTRODUCTION

Humic substances are structurally complex large to macromolecules which occur in soils and natural waters as a consequence of the breakdown of plant and animal residues by microbial activity [1]. Oxidative stress occurs when pro-oxidant forces such as hydrogen peroxide oxygen radicals are believed to be a primary factor in various degenerative diseases, [2]. Some important antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (GR) are involved in the reaction mechanism and work as antioxidants [3]. Humic acid is ubiquitous in the environment and has been found to influence physiological functions of aquatic organisms [4] as well as in soil [5]. The application fulvic acids as antioxidant substance has been described [6]. The main reason for the increasing attention devoted to humic acids can be explain by their antiviral, anti-inflammatory and estrogenic activities [7]. The potential of humic substances to form chelate complexes with heavy metals (such as cadmium) enable them to be used for the elimination of heavy metals from living organisms [8].

To prevent the damage caused by reactive oxygen specie, tissues had developed an antioxidant defense system that includes nonezymatic antioxidants (*e.g.*, glutathione, uric acid, bilirubin and vitamins C and E) and enzymatic activities such as superoxide dismutase, catalase and glutathione peroxidase [9-10].

Therefore, the objective of this work was to evaluate, *in vivo* superoxide ion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), scavenging capacity by humic acid and Fulvic acid against oxidative damage by administrated hydrogen peroxide to rats which affects prokaryotic growth and hydrolysis of specific components of the organic matter [12].

MATERIALS AND METHODS

Extraction and purification of humic and fulvic acids. (1) Extraction of humic substances. Extraction of humic acids was run according to the method described by [13] as follows: compost sample (25 gm) was taken and shaken with 500 ml of dispersion agent 0.5 N NaOH solution. The freshly prepared solution suspensions were left overnight and centrifuged and hence the supernatant contain humic and fulvic acids.

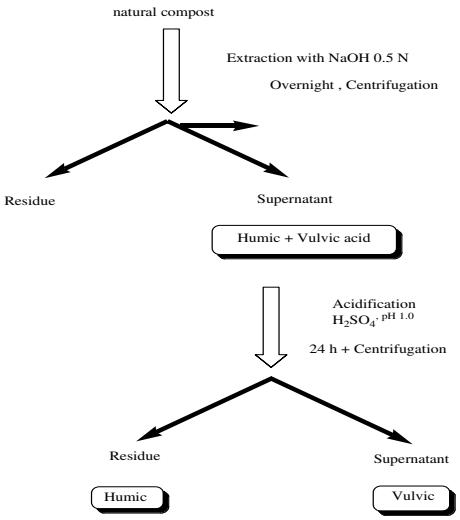
(2) Sepration of fulvic acid. The method described by [14] was followed for isolating and purifying humic acid. The extract of organic substances with alkali solution was adjusted first to pH 1.0 with H_2SO_4 conc. Then, it was allowed to stand 24 h to precipitate the humic acids. The supernatant contains the fulvic acids. The fulvic acid was then separated from the humic acid by centrifugation at 6000 rpm for 20 minutes. The supernatant contains the fulvic acid and the precipitate is identified as the humic

fraction of humus. The solution of fulvic acid passing through activated charcoal followed by elution of the charcoal. The solution was concentrated to small value by placing it inside an oven with a fan and left at room temperature. The concentrated solution was transferred to the membrane filter and electro dialyzed until the dialysate was free from chloride.

(3) Purification of Humic acid. The humic acid precipitate was washed several times with cold 0.05 N H_2SO_4 until the filtrate was colorless. The humic acid was re-dissolved in a small amount of 0.05 N NaOH solution, and then was transferred to a membrane filter. It was precipitated again by acidification to pH 1.0. The humic acid was transferred to cellophane bags and dialyzed against distilled water until the test for chloride in the distilled water outside the bags was negative [15] then humic acid was air-dried)

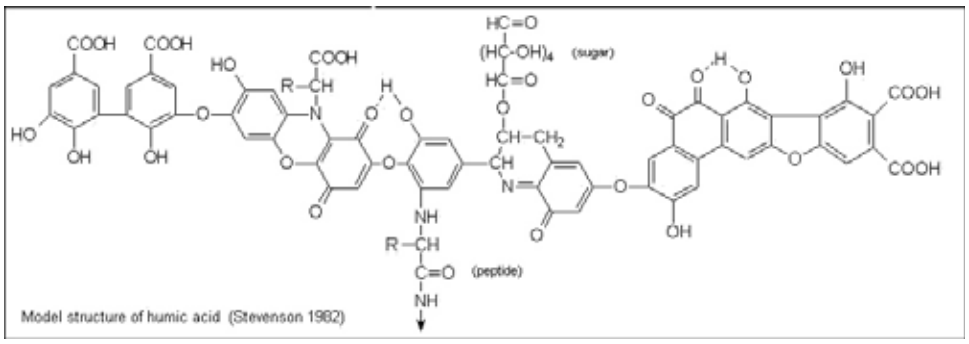
In order to reduce the ash content of the purified humic acid and to increase its purity, 10 g of the air dried humic acid were shaked with 100 ml of Khan's mixture (0.5 ml Conc. HCl + 0.5 ml HF 48% and 99 ml of H_2O) at room temperature for 42 hr after shaking, the acid mixture was centrifuged, the supernatant was removed, and the humic acid gel was dialyzed against distilled water until became Cl^- free. The precipitated humic acids gel was transferred to petri dishes and dried at room temperature (scrme 2,

The following scheme shows the separation of humic and fulvic were as flows (Scheme 1, 2, nd3):

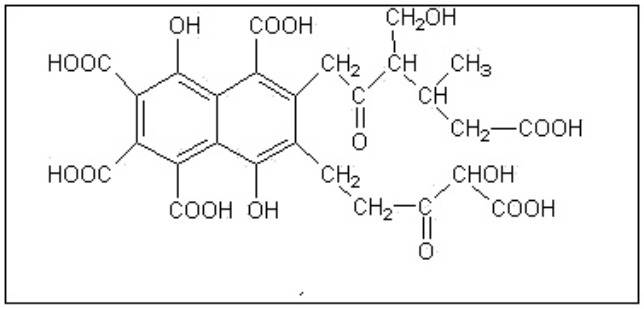


SCHEME 1
The separation of humic and fulvic

Structure of humic and vulvic acids are as follows:



SCHEME 2
Humic acid structure



SCHEME 3
Fulvic acid structure

Hydrogen peroxide and superoxide radical Scavenging Activity. (1) Assay of superoxide radical scavenging activity. Superoxide radical scavenging activity was carried out according to [16] based on the inhibition of formazan formation. Each 3ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, and 12mM EDTA, and 0.1 mg Nirobluetetrzolium (NBT) and 1ml sample solution. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract (10,50,100 amd 150 µg/ml) for 90 s. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the samples/standard.

(2) Determination of H_2O_2 scavenging capacity. The ability of humic and fulvic acids extracts to scavenge hydrogen peroxide was determined according to the method of [17].

***In vivo* Biological evaluation of humic acid and vulvic acid.** Rats Wistar used in this study were procured from the animal house olony at the National Research Centre (NRC), Egypt. All animals were housed under standard conditions of natural 12 h light and dark cycle with free access to food and water. All animal procedures were performed after approval from the Ethics Committee of The National Research Centre- Egypt and in accordance with the recommendations of the proper care and use of laboratory animals male rats (125-175 g) were divided into 9 groups (eight rats/ group). The first group represented the negative control, was administered 5% dimethylsulphoxide (DMSO). Groups two, three, four and five were treated with different concentrations of humic acid (10, 50 ,100 and 150 mg/Kg body weight). Groups six, seven, eight and nine were treated with analogous concentrations of fulvic acid. After four weeks rats were decapitated, samples were taken from various groups and prepared according to each method.

(1) Blood urea. It was estimated by the enzymatic method as described by [18].

(2) Serum creatinine. It was determined according to the method described by Faulkner and King (1976) [18].

(3) Determination of aspartate transaminase (AST) and alanine transaminase (ALT). Aspartate transaminase (AST) and alanine transaminase (ALT) were estimated according to the method described by [20].

***In-vivo* estimation of antioxidant enzymes activity.** Sprague - Dawley male rats (125-175 g) were divided into 11 groups (eight rats/ group). The first group represented the negative control, was administered 5% dimethylsulphoxide (DMSO). The second group, the positive control, was administered an oral dose of H_2O_2 + 5 % DMSO to induce oxidative damage. The single dose/week was 2 mL/Kg body weight. Groups three, four, five and six were treated with different concentrations of humic acid (10, 50, 100 and 150 mg/ Kg body weight). Groups seven, eight, nine and ten were treated with analogous concentrations of fulvic acid. Group eleven received an oral dose of Ascorbic acid 10 mg/ Kg body weight + 5% DMSO. After four weeks rats were decapitated, samples were taken from various groups and prepared according to each method.

TABLE 1
Superoxide Radical Scavenging Activity expressed as % inhibition.

Compound number	Superoxide scavenging activity 100 %			
	10 (µg/ml)	50 (µg/ml)	100 (µg/ml)	150 (µg/ml)
Ascorbic acid	64.82	90.73	99.74	100
Humic acid	28.05	36.77	60.01	67.12
Vulvic acid	32.51	42.91	67.89	79.00

TABLE 2
Hydrogen peroxide scavenging activity expressed as % inhibition.

Compound number	Hydrogen peroxide scavenging activity 100 %			
	10 (µg/ml)	50 (µg/ml)	100 (µg/ml)	150 (µg/ml)
Ascorbic acid	49.23	70.41	80.32	91.54
Humic acid	23.41	29.47	51.66	56.70
Vulvic acid	25.07	33.51	57.39	67.22

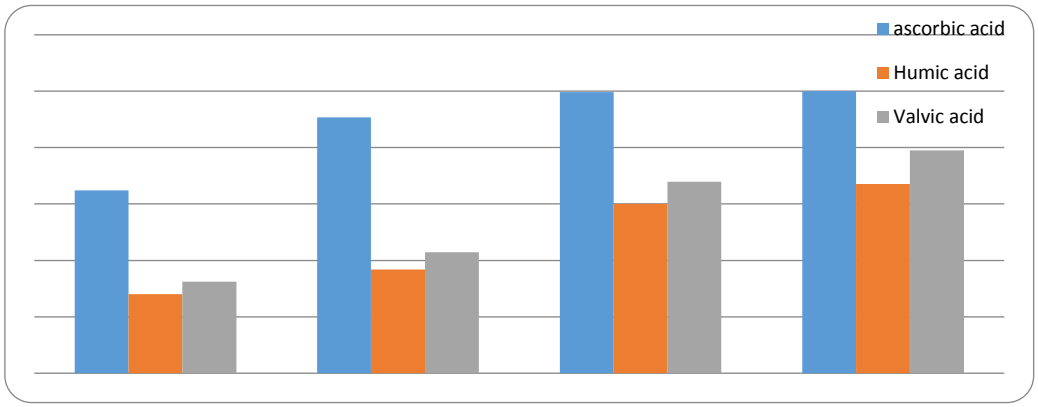


FIGURE 1
Humic and vulvic acid represent superoxide radical scavenging activity expressed as % inhibition

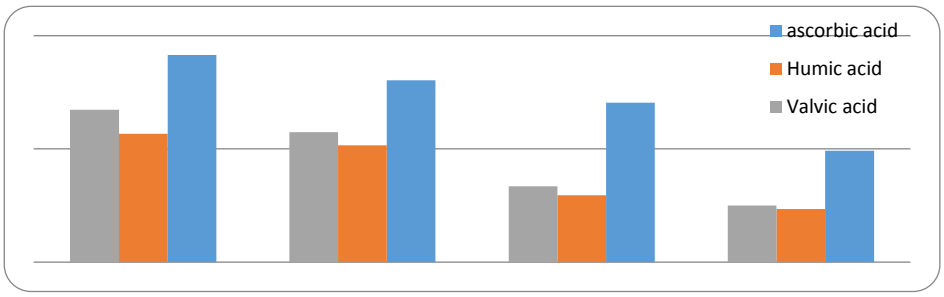


FIGURE 2
Humic and fulvic acid scavenge hydrogen peroxide expressed as % inhibition.

(1) Assay of Glutathione *peroxidase* (GPx) activity. Glutathione peroxidase was estimated by the method of [20].

(2) Assay of SOD activity. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of [21].

(3) Lipid peroxidation assay (MDA contents). Fresh liver samples (200 mg) were homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12,000×g for 20 min. The supernatant (1 ml) obtained was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA (or no TBA as the blank), and heated at 95°C for 30 min, then cooled in ice. The reaction product was centrifuged at 12,000×g for 15 min and the supernatant absorbance was measured at 400, 532 and 600 nm. The MDA equivalent was derived from the absorbance according to [22].

Statistical analysis. The results were analysed by an analysis of variance ($P < 0.05$) and the means were separated by Duncan's multiple range test. The results were processed by CoStat computer program (1986).
(Fig 2)

RESULTS

***In vivo* antioxidant activity of humic and vulvic acids.** The analysis of the fulvic acid and humic acids displays a scavenging activity compared to ascorbic acid reference compounds; although it was less efficient ($P<0.0001$). Scavenging activity of humic and vulvic acids were increased by increasing the concentration used in the experiments, by using 150 µg/ml for both compound. Superoxide scavenging activity represented by 79 and 76 for vulvic and humic compared to ascorbic acid (100 %) Table 2, Figure 1.

Hydrogen peroxide (H_2O_2) scavenging activity. The scavenging ability of various treatments with hydrogen peroxide is shown in Table 4 and illustrated in Figure 2 and used ascorbic acid as standards. It is noticed that all treatments are capable of scavenging hydrogen peroxide in an amount-dependent manner. The percentage of scavenging hydrogen peroxide is determined with 150 µg/ml of the humic acid and fulvic acid represented the highest scavenging activity expressed as % inhibition is as follows: humic acid (56.70%) and vulvic acid (67.22 %) against ascorbic acid standard at the same concentration (91.54%) (Table 1).

***In vivo* activity of antioxidant defense enzyme activities affected by administrated humic acid and fulvic acid under hydrogen peroxide oxidative stress.** Hydrogen peroxide-induced damage of liver cells results in oxidative damage through free-radical generation. In this work, the rats were treated with a single oral dose of hydrogen peroxide (2 ml/Kg body weight / week) to induce oxidative stress and liver damage. Humic acid and fulvic acid were evaluated for their protective effect against oxidative stress and induced liver damage in rats by estimating SOD, GPx activity as well as MDA levels. The results shown in Table 4 and illustrated in Figure 3 and Figure 4 revealed that, the administrated humic acid and fulvic acid showed a significant reduction in both enzymes indicating their protective effects against induced oxidative damage, *via* GPx and SOD defense enzymes modulation. Fulvic acid exhibited more potent protective activity at a dose of 150 mg/Kg.

The data also revealed a significant decrease in MDA level upon treatment with different concentrations of humic acid and fulvic acid. The best result was shown by Fulvic acid at a dose of 150 mg/Kg, which may be attributed to its higher antioxidant activity compared to humic acid. The data represented that, the humic acid and vulvic acid exhibited a significant concentration-dependent free radical scavenging activity as well as significant reduction of MDA level. Furthermore, both compounds, humic acid and fulvic acid, showed protective effect against lipid peroxidation in hepatic microsomes. This effect may be due to their inhibitory effect on inducible nitric oxide synthase and scavenging effect on peroxynitrite formation. The lowest MDA level was observed with compound fulvic acid at a dose of 150 mg/Kg b.w., Table 4 and Figure 4.

TABLE 3
Cytotoxicity effect of various concentrations of humic and fulvic acids on urea, creatinine and AST and ALT in rats

Compounds	Concentration mg/kg	Urea mg/dl	Creatinine mg/dl	AST U/ml	ALT U/ml
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Fulvic acid	10	$35.2^{d} \pm 0.88$	$1.08^{d} \pm 0.03$	$31.2^{d} \pm 1.3$	$21.2^{d} \pm 1.1$
	50	$39.2^{cd} \pm 1.25$	$1.16^{cd} \pm 0.08$	$32.1^{cd} \pm 1.4$	$23.8^{cd} \pm 1.6$
	100	$41.2^{bc} \pm 1.63$	$1.4^{bcd} \pm 0.08$	$37.4^{abc} \pm 2.3$	$22.9^{cd} \pm 2.0$
	150	$41.1^{bc} \pm 2.5$	$1.59^{bc} \pm 0.06$	$40.6^{ab} \pm 2.1$	$29.4^{ab} \pm 1$
Humic acid	10	$37.8^{cd} \pm 1.7$	$1.46^{bcd} \pm 0.18$	$35.3^{bcd} \pm 1.5$	$22.4^{cd} \pm 1$
	50	$42.5^{abc} \pm 2.8$	$1.57^{bc} \pm 0.11$	$37.1^{abc} \pm 1.2$	$25.6^{bcd} \pm 1$
	100	$44.8^{ab} \pm 1.68$	$1.71^{ab} \pm 0.14$	$40.4^{ab} \pm 1.8$	$27.2^{abc} \pm 1$
	150	$46.7^{a} \pm 2.04$	$2.1^{a} \pm 0.0.11$	$41.4^{a} \pm 1.63$	$31^{a} \pm 1.1$
Control negative		$40.1^{bcd} \pm 1.6$	$1.38^{bcd} \pm 0.28$	$36.2^{abcd} \pm 1.9$	$26.4^{abc} \pm 1$
$LSD_{0.05}$		4.870	0.4092	5.132	4.332

Each value represents the mean ± SE (Standard Error) and mean of three replicates; Values in the same column with the same letter are not significantly different at ($p \leq 0.05$)

TABLE 4
The antioxidant activity of compounds fulvic acids and humic substances against MDA formation, SOD and GPX in control and treated rats with H_2O_2 oxidative stress.

Compound	Concentration (mg/kg)	The measured parameters		
		SOD u/ml	GPX u/ml	MDA nmol/ mg protein
		Mean ± SE	Mean ± SE	Mean ± SE
Control (+)		$128.9^{a} \pm 31$	$5.69^{a} \pm 0.32$	$6.79^{a} \pm 0.3$
Control (-)		$35.77^{f} \pm 2.8$	$2.59^{d} \pm 0.26$	$3.5^{de} \pm 0.13$
Fulvic acid	10	$116.4^{ab} \pm 2$	$5.27^{ab} \pm 0.21$	$4.89^{b} \pm 0.24$
	50	$103.2^{abcd} \pm 2$	$4.72^{bc} \pm 0.35$	$4.02^{cd} \pm 0.23$
	100	$80.69^{cde} \pm 1$	$4.27^{c} \pm 0.2$	$3.88^{de} \pm 0.33$
	150	$70.97^{de} \pm 2$	$3.9^{c} \pm 0.18$	$3.27^{e} \pm 0.16$
Humic acid	10	$104.1^{abc} \pm 2$	$5.2^{ab} \pm 0.15$	$5.08^{b} \pm 0.07$
	50	$102.7^{abcd} \pm 3$	$4.7^{bc} \pm 0.25$	$4.65^{bc} \pm 0.23$
	100	$93.15^{bcd} \pm 2$	$4.2^{c} \pm 0.29$	$3.99^{cd} \pm 0.14$
	150	$86.71^{bcd} \pm 2$	$4.1^{c} \pm 0.12$	$3.82^{de} \pm 0.13$
Voltarine®	10	$63.31^{ef} \pm 3$	$3.01^{d} \pm 0.08$	$3.60^{de} \pm 0.28$
$LSD_{0.05}$		29.097	0.683	0.637

Each value represents the mean ± SE (Standard Error) and mean of three replicates; Values in the same column with the same letter are not significantly different at ($p \leq 0.05$)

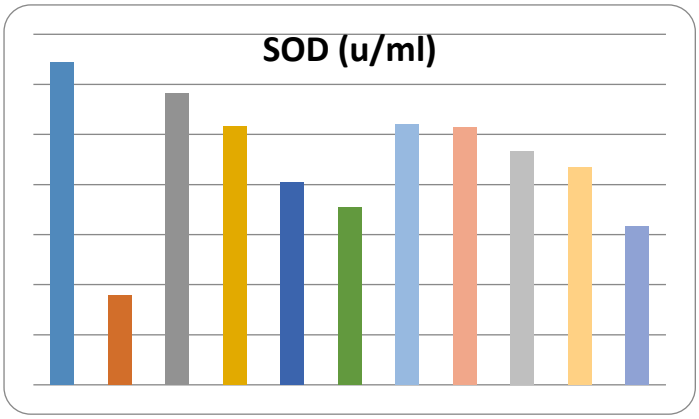


FIGURE 3
The modulating effect of administrated fulvic acids and humic substances on SOD activity under oxidative stress.

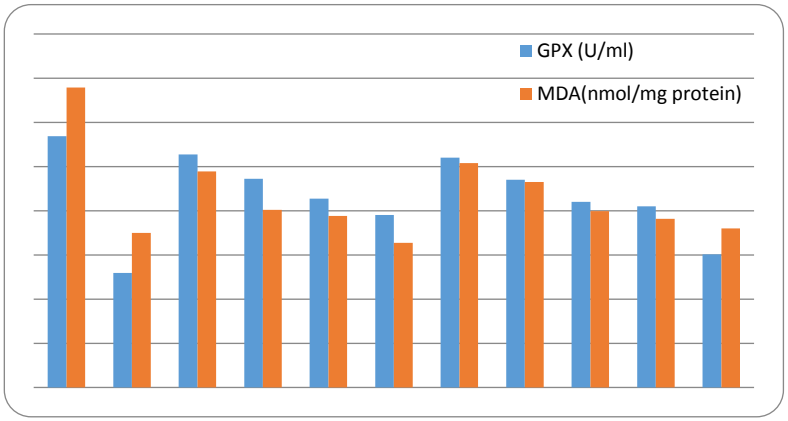


FIGURE 4
The modulating effect of administrated fulvic acids and humic substances on GPX activity and MDA formation under oxidative stress.

Effect of humic acid and vulvic acid on kidney and liver functions. The data obtained from Table 3 revealed that, humic acid and fulvic acid were tested for their possible effect on kidney and liver functions to assess their safety *in vivo* investigation. The results show that urea and creatinine levels as kidney function did not shows any significant toxicity when using fulvic acid with concentration of 10,50 ,100 and 150 mg/ Kg compared to negative control. On the other hand treatments of humic acid of different concentration with 10,50 and 100 mg/ Kg show significantly differences in kidney function Urea (mg/dl), Creatinine (mg/dl) and AST and ALT (U/ml) enzyme activities of the liver by using 150 mg/ Kg b.w. (46.7, 2.1, 41.4 and 31) respectively.

DISCUSSION

Vulvic and humic act as an antioxidant like other high molecular weight plant phenolics such as tannins [22-25]. The conversion of superoxide radical and H_2O_2 into more reactive species, e.g., hydroxyl radical, has been thought to be one of the unfavorable effects caused by superoxide radicals [9]. The results data was in accordance with [26] who found that, it has been known that phenolic hydroxyl group is the main active group which scavenges $OH^{\bullet}$ and favor's the encapsulation of the pro-oxidant iron species, which generates $OH^{\bullet}$ through the Fenton reaction [26]. This suggests that the phenolic hydroxyl group and metal-chelating ability by fulvic acid could explain the ROS scavenging activity observed.

Low concentrations of humic Acid, such as 25 and 50 µg/ml, reveal no increase induced lipid peroxidation compared to control conditions (28-29]. Also, the data reflected that at high doses of humic acid significantly affected the liver function and the kidney function that is may be due to the toxicity dose dependence of humic acid affecting the plasma membrane integrity and toxicity. Nevertheless, [30] reported that, the daily intake of humic acid by the average resident in these areas has been estimated to be as high as 400 mg. and has adversely affected the

health of inhabitants. Radioisotope tracing with iodinated humic acid in rats indicated that up to 60% of humic acid remained in the body 24 h after its administration. Humic acid disrupts intracellular calcium homeostasis and enhances the permeability of cell membranes to extracellular Ca^{+2}, resulting in sustained elevation of cytosolic Ca^{+2}/. These data was in accordance with [31], who found that, the antioxidant properties of fulvic acid explain some of the health beneficial effects of this compound since the excessive production of $O_2^{\bullet-}$, HOCl, H_2O_2, $OH^{\bullet}$, $ONOO^-$ and O_2 are involved in several pathologies. In addition, humic acid and fulvic compounds were able to prevent glutathione oxidation induced by H_2O_2 [28]. These results clearly indicated that SOD and GPx may play a role in the suppression of oxygen free radical formation in liver and kidney tissues and may be connected to the H_2O_2-induced increase in free radical generation or a decrease in amounts of protecting enzymes against lipid peroxidation. The decrease in SOD and GPx activity in the liver of H_2O_2 treated samples may indirectly cause oxidative DNA damage or mitochondrial damage in cells [32]. In accordance, the fulvic acids act as an antioxidant like other high molecular weight plant phenolics such as tannins. FA in vitro scavenged of $O_2^{\bullet-}$ and H_2O_2, in a concentration-dependent way. The presence of structural units O^- functionalized, including aromatic domains in fulvic acid, could explain their tendency to form molecular aggregates (hydrogen bridges, metal bridges and hydrophobic interactions) [33]. Treatments with humic acids in saline soils were associated with reduced soil electrical conductivity and reduced leakag as well as reducing potential as reported by double spraying with stimulator [34].

CONCLUSION

The antioxidant properties of humic and vularic acids explain some of the health beneficial effects which increase immune system function since the excessive production of free radicals are involved in several pathologies. Moreover, they could be a good candidate for use in pharmaceutical or food industries as an accessible source of natural antioxidants and for the improvement of food quality by retarding lipid oxidation. These substances may at times stimulate growth in both higher plants and soil microorganisms These compound could be applied directly to the soil or as a foliar spray, by itself or mixed with fertilizers/chemicals.

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