Egypt. J. Chem. **Vol. 64,** No. 2 pp. 631 - 647 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Evaluation of Hepato- and Neuroprotective Effect of Chemical Constituents in *Saussurea costus* Extract against the Toxicity Induced by Chloropyrifos Ethyl in Rats

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Abstract

The health hazardous occurred in both human and animals as a result of the continuous misuse of pesticides drives the researchers for looking about the solution. Natural plant extracts contain active phyto-constituents with antioxidant potency enables them to inhibit production of the free radicals that induce damage of the cells. The present study aimed to reveal efficiency of *Saussurea costus (S. costus* extract) against the hepato- and neurotoxicity induced by Chloropyrifos ethyl (CPF) in experimental animals (rats). The gas chromatography / mass spectrometer (GC/MS) that used for analyzing the active constituents in *S. costus* extract showed that the extract contains 11 potent active compounds and Dehydrocostuslactone represents about 77.37% and considered as the most dominant compound in the extract. Both of CPF and *S. costus* extract were studied on the rats that were divided into 6 groups as the following: Group 1 (control) received distilled water orally. Groups 2&3 (*S. costus* extract treated groups) received *S. costus* extract orally at a dose of 0.25 and 0.50 ml, respectively. Group 4 (CPF injected group) was injected with CPF at a dose of 3 mg/kg.bw. Groups 5&6 (CPF + *S. costus* extract group) injected with CPF then treated with *S. costus* extract at two tested doses respectively. The most hematological and biochemical measurements declined significantly ($P \leq 0.05$) in CPF injected group. *S. costus* extract restored all tested parameters towards the control values. Moreover, the electrophoretic isoenzyme showed that the physiological alterations occurred in the esterases (ESTs) as a result of CPF injection were represented by hiding normal EST types associated with existence of abnormal ones. Therefore, the similarity index (SI%) and genetic distance (GD%) values were altered with α-EST (SI=80.00%; GD=20.00%) and β-EST (SI=50.00%; GD=50.00%) patterns in CPF injected group. The *S. costus* extract at a dose of 0.5 ml restored integrity of these isoenzymes pattern by restoring the absent types with hiding the abnormal ones. Therefore, this group became physiologically similar to control group (SI=100.00%; GD=0.00%). These results were supported by histopathological examination for the target organs (brain, liver and kidney) that were affected by CPF and the *S. costus* extract improved architecture of these organs and restored their histopathological integrity to normal structure.

Keywords: Chloropyrifos Ethyl; Toxicity; *Saussurea costus;* GC/MS; Isoenzymes; Rats.

1. Introduction

In both developing and developed countries, several hundred pesticides have been used widely in agricultural practices for controlling unwanted insects and disease vectors [1]. Globally, average amounts of the used pesticides, herbicides and fungicides were about 342000, 566,000 and 353,000 tons during 1990, 2000 and 2010, respectively [2]. In 2017, Bernhardt *et al*. [3] reported that a total global use of pesticides is about 6 million metric tons and Europe is considered as the largest consumer of insecticides followed by China and the United States of America (USA). In developing countries, use of

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Received date: 30 December, 2020; revised date: 5 January 2021; accepted date: 6 January 2021
DOI: 10.21608/EJCHEM.2021.55840.3199

insecticides is around 25% with an exceptionally high application to vegetables [4]. Utilization of pesticides and the misuse of uncensored pesticides continue in the environment and leads to severe contamination affecting human health [5]. Pesticide residues are characterized by their affinity to accumulate in fruits, vegetables, grains and honey in addition to similar products such as wines and juices based on farmers' practices and treatments [6]. They include derivatives of pesticides such as metabolites, transformation products and reaction products in addition to the impurities that are considered to be of significant toxicity [7]. The deleterious effect induced as a result of the exposure to pesticides may be acute or chronic based on concentration of the toxic active ingredients, exposure time and status of the healthy individual [8]. Their adverse effects include headache, vomiting, neurotoxicity, itching, skin irritation, insomnia, dizziness, mental confusion, subconscious, allergic reaction and difficulties of breathing. Moreover, they may lead to long-term harmful effects such as kidney problem, birth defects, and cancer [9]. Organophosphorus compounds (OPs) are the most widely used group of pesticides to control pests in Agriculture and public health. OPs exert their toxic effects by inhibiting acetylcholinesterase (AChE) resulting in accumulation of acetylcholine (ACh) at synapases and neuromuscular junctions followed by hyper-activation of receptors resulting in neurological and imbalance disorders and finally death [10,11]. Therefore, AChE activity is considered as a biomarker of the toxicity induced by OP pesticides [12].

Chlorpyrifos ethyl (CPF) is the commonly used (OP) insecticide that utilized extensively to control Adenoma, Diptera, Homoptera and Lepidoptera in soil or on foliage in many crops as well workers in vector control programs throughout the world under different registered trademarks [13,14]. The exposure to CPF caused a variety of cellular modifications such as reducing synaptogenesis and differentiation of cells and problems in genetic transcription and DNA synthesis, alterations in expression factors of transcription, expansion in DNA grooves and destruction of the aqueous layer that consequently enhances the DNA degradation [15].

Traditional and folk medicine has been around for centuries. In developing countries, it was stated that about 80% of the population relied on utilization of plant extracts for health care despite development of the modern medicine [16]. Presence of the different groups in the secondary metabolites such as the phenolic compounds that have many positive biological activities are responsible for the health-promoting properties of herbs [17]. The plant extracts are rich in the bioactive compounds that have biological properties to exhibit efficiency against the oxidative stress induced as a result of the exposure to OPs through decreasing the peroxidation reaction and restoring the antioxidant system [18]. A number of studies have shown that plant polyphenols can be used as antioxidants against the diseases induced by oxidative stress [19,20]. The plant polyphenols belong to the antioxidant compounds that used for the prevention and/or treatment of many disease affecting millions of people worldwide [21].

A (*S. costus*) root contains wide range of the active phyto-constituents and high concentrations of phenolics and flavonoids and the maximum number of phytochemicals were detected in the methanolic extract. High antioxidant activity of methanolic extract can be related to the high amount of phenols and flavonoids [22]. It was reported that *S. costus* roots exhibited good biologically active properties including anticancer, antimicrobial, anti-ulcer, anti-hypertensive, anti-hepatoprotective, hypoglycemic, antispasmodic, anticonvulsant, anti-diarrheal and antiviral activities [23-25]. Therefore, the present study was designed to focus on studying effect of *S. costus* roots extract against the hepato- and neurotoxicity induced by CPF in rats.

2. Material and methods

Preparation of Aqueous Plant Extract

Two hundred grams of *S. costus* powder were extracted with excessive ethyl alcohol: distilled water at room temperature for 24 h. The extract was separately filtered then concentrated under vacuum using a rotary evaporator.

Chromatographic Analysis of the Active Constituents

Trimethylsilyl (TMS) derivatization was carried out based on the protocol described by **Moldoveanu and David [26]**. Briefly, the dried samples were re-suspended in 10 µL and 50 µL of *N*,*O*-Bis(trimethylsilyl) trifluoro-acetamide (BSTFA) then, incubated at 70 °C for 60 min in a dry block heater. The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analysis were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min at a split 50:1, injection volume of 0.5 µl and the following temperature program: 50 °C for 1 min; rising at 8 °C /min to 300 °C and held for 20 min. The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 30-700 and solvent delay 8 min. The mass temperature was 230°C and Quad 150 °C. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Animals and Treatments

Healthy thirty six (36) adult male *Sprague Dawley* rats with age 2 months old were obtained from the Animal House Colony, National Research Centre, Giza, Egypt. They were divided into 6 groups and housed in cage (filter-top) under controlled normal environmental and nutritional

conditions. Rats within different groups were daily treated for 17 days as the following:

Group 1 (Control group): rats were fed with normal diet and received tap water without any treatment. Group 2 (*S. costus* extract (0.25 ml) treated group): rats were fed with normal diet associated with administration of aqueous *S. costus* extract orally at a dose of 0.25 ml by stomach tube. Group 3 (*S. costus* extract (0.5 ml) treated group): rats were fed with normal diet associated with administration of extract orally at a dose of 0.5 ml by stomach tube. Group 4 (CPF injected group): rats were fed with normal diet associated with injection of CPF at a dose of 3 mg/Kg. bw. Group 5 (CPF + *S. costus* extract (0.25 ml) treated group): rats were fed with normal diet associated with injection of CPF and administration of extract at a dose of 0.25 ml. Group 6 (CPF + *S. costus* extract (0.5 ml) treated group): rats were fed with normal diet associated with injection of CPF and administration of extract at a dose of 0.5 ml.

Collection of Blood Samples and Tissues

Rats were anaesthetized at end of the experimental period. For the hematological measurements, the heparinized blood samples were collected from retro orbital plexus. The blood samples were centrifuged for 15 min at 4000 rpm and the clear serum were collected for biochemical measurements. Rats were sacrificed by decapitation then liver, kidneys and brain tissues were autopsied and fixed immediately in formal saline (10%) for histopathological examination.

Hematological Measurements

All the hematological measurements including hemoglobin (HB), red blood cells (RBCs), hematocrit (HCT), corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV) and platelet count (PLT) and white blood cells (WBCs) in addition to their differential blood cells (lymphocytes, monocytes and granulocytes) were quantified in heparinized blood samples using an automatic blood analyzer (ABX Micros 60 manufactured by HORIBA ABX SAS).

Biochemical Measurements

The biochemical parameters including liver enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)), renal functions (urea and creatinin), lipid profile (total cholesterol (TC) and triglycerides (T.Gs)) and protein profile (total protein and albumin)) were measured in sera samples by conventional colorimetric methods using spectrophotometer and following instruction of the commercial kits (Spectrum Diagnostics Egyptian Company for Biotechnology). Moreover, acetylcholinestrase (AChE) was assayed by Ellman Method **[27]**. The total antioxidant capacity (TAC) and total protein carbonyl content (TPC) that were used as markers of the oxidative stress, were quantified spectrophotometrically and expressed as mM/L **[28]** and nmol of reactive carbonyl compounds per mg protein of tissue **[29]**, respectively.

Statistical Analysis

Data were expressed Tables and illustrated in Figures as mean ± standard error (SE). All results were statistically analyzed by one-way analysis of variance (one-way ANOVA) test using the Statistical Package for Social Sciences (SPSS for windows, version 11.0) followed by Bonferoni test as Post-Hoc. At a "P" value of less than 0.05, the differences between the groups were considered statistically significant.

Electrophoretic Assays

The samples were prepared by pooling equal volumes of the individual serum samples for each group and used as one sample. Total protein was quantified in all pooled samples to ensure that the samples loaded in all wells with equal protein concentration **[30]**. After electrophoretic run, activity of esterases (ESTs) was detected by incubating the gel in conditioning buffer then staining it in reaction mixture consisting of specific substrates as α- and β-naphthyl acetate for α- and β-EST, respectively at the concentration 5.58 X 10-3 mM (pH 7.5) along with dye coupler Fast Blue RR. The EST pattern appeared as brown bands for α-EST and pink bands for β-EST **[31]**.

Data Analysis

The gel plates were photographed then analyzed using the Quantity One software (Version 4.6.2) to determine the relative mobility (Rf), band percent (B%) and relative band quantity (Qty%) of the bands separated electrophoretically in addition to the similarity index (SI%) and genetic distance (GD%) molecular weights (Mwts) that were calculated by the method suggested by **Nei and Li [32]**.

Histopathological Examination

The tissues specimens (liver, kidneys and brain) that were collected from all rats and fixed in formal saline (10%) washed with tap water, dehydrated and cleared then embedded in blocks of paraffin wax. The tissue was prepared as sections with thickness 4-5 micron then stained with Haematoxylin and Eosin according to the method suggested by **Suvarna *et al.* [33]** to be examined by light microscope (Olympus BX50, Tokyo, Japan). Histopathological damages were scored and ranged from 0-3. The damage score might be no damage (0), mild (1), moderate (2) or severe damage (3), while the grading was determined by the percentage that might be <30% for mild changes, <30% - 50% for moderate changes and >50% for the severe changes **[34]**.

3. Results and Discussion

The phytochemical constituents were analyzed in the *S. costus* extract and identified using GC/MS and the obtained results indicated that the extract contains 11 potent active compounds. As recorded in **Table 1** and illustrated in **Fig. 1**, it was noticed that Azuleno[4,5-b]furan-2(3H)-one, decahydro-3,6,9-tris(methylene), [3aS-(3a.alpha.,6a.alpha.,9a.alpha.,

9b.beta.)] represented the highest value (77.37%). Dehydrocostuslactone [DHE (3aS,6aR,9aR,9bS)-decahydro-3,6,9-tris(methylene) azuleno[4,5-b]furan-2(3H)-one)] is categorized as a medicinal plant-derived sesquiterpene lactone **[35]**. It is well known that the DHE was extracted from *S. lappa* and *Aucklandia lappa* and possess antifungal activity **[36]** and exhibits a cytotoxic efficiency against HeLa cell lines **[37,38]**. Also, *S. costus* extract contains other phytochemical compounds represented by eicosapentaenoic acid, TMS derivative, 9,12,15-Octadecatrien-1-ol, (Z,Z,Z) at the level of 4.5 and 4.4%. Costunolide $C_{15}H_{20}O_2$ from *S. lappa* (Asteraceae) root used successfully in gastrointestinal ailments. Presences of the costunolide a sesquiterpene lactone provides the plant extracts with narcotic and anticancer properties. The costunolide exhibited anticancer potential against gastric cancer and it was studied on AGS and BGC-823 cell lines and its mechanism of action has not been fully elucidated **[39]**. It was noticed that *S. lappa* exhibited anticancer activity against AGS cells and this might be attributed to its efficiency in stimulating apoptosis and cell cycle arrest at G2 phase in a time- and dose-dependent manner through induction of p^{53} and p^{21} Waf1 proteins and concomitant reduction of cyclin B1. Moreover, its anticancer activity might refer to suppression of antiapoptotic Bcl-2 in addition to activation of the proapoptotic molecules including Bax and caspase-3 **[40]**. In 2011, **Sandhyavali *et al*. [41]** supported that the 9,12,15-Octadecatrien-1-ol, (Z,Z,Z) was detected in *S. simpsoniana* (Asteraceae) and it exhibited antioxidant and antibacterial activities against *Staphylococcus aureus* ATCC 6538 **[42]**. In addition, it was found that this extract contains other constituents at low concentration represented by eicosapentaenoic acid TMS derivative, 10,12-Tricosadiynoic acid TMS derivative, 9,12-Octadecadiynoic acid, trimethylsilyl ester, 2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)], Tributyl acetylcitrate, Hi-oleic safflower oil, 2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)] and (E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1*H*)-ylidene)propan-1-ol, respectively.

It was necessary for the haematological parameters to be assessed for determining the adverse effect on blood constituents in rats and to reveal effect of the chemical compounds/plant extract that related to the blood constituents **[43]**. It is well known that CPF increased rate of chromosomal aberrations in spleen and bone marrow cells **[44]**. Also, it could induce DNA damage and hence accelerate cells apoptosis **[45]**. Therefore, it was necessary for undergoing the study at the hematological level. As shown in **Table 2**, the *S. costus* extract caused no significant alterations in all hematological measurements when it was administrated alone at both doses (0.25 and 0.5 ml). CPF caused a significant ($P \leq 0.05$) decrease in the most hematological measurements (RBCs, HB, HCT, MCV, RDW, MPV, PLT, WBCs and its differential (lymph, mono and gran.%) as compared to control group. Alterations of the haematological status of rats might be attributed mainly to the oxidative stress that stimulates abnormalities at haematological level **[46]**. As regard to MCH and MCHC, it caused no significant changes in their levels. Treatment with *S. costus* extract at a dose of 0.25 ml caused no significant ($P \leq 0.05$) changes in the altered measurements as compared to CPF treated group. When it was administrated at a dose of 0.5 ml, it increased these measurements significantly ($P \leq 0.05$) as compared to the group injected with CPF alone and that treated with *S. costus* extract at a dose of 0.25 ml. Administration of *S. costus* extract restored these measurements to normal levels due to presence of wide range of the polyphenolic compounds that well known by their ability to exhibit antioxidant activity against ROS generated as a result of CPF.

Liver is the most sensitive organ susceptible to be attacked by reactive oxygen species (ROS) **[47]**. It is well known that ALT is the major enzyme found principally in cytoplasm of hepatocytes and its activity is considered as an important index to determine the damage degree in cell membrane. Therefore, it is specific marker for hepatic damage **[48]**. AST is the most abundant enzyme in cytoplasm and mitochondria of hepatocytes. Moreover, it is present in heart and brain in addition to the skeletal muscles. It is considered as an indicator of mitochondrial damage that contains about 80% of this enzyme **[49]**.

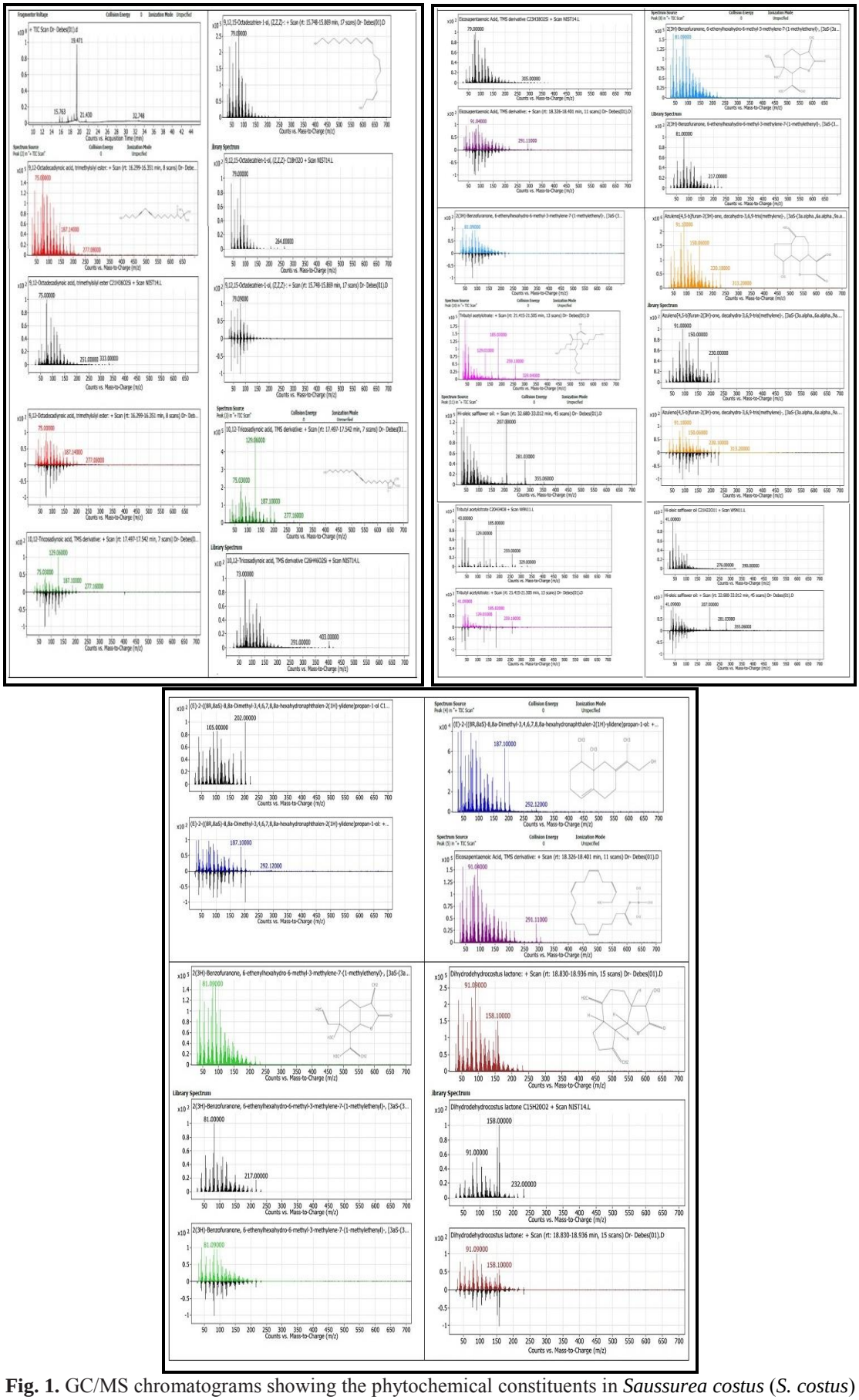


Fig. 1. GC/MS chromatograms showing the phytochemical constituents in *Saussurea costus* (*S. costus*) extract

TABLE 1. Natural phytochemical constituents of *Saussurea costus* (*S. costus*) extract using gas chromatography / mass spectrometer (GC/MS)

Peak	RT	Name	Formula	Area	Area Sum (%)
1	15.763	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	$C_{18}H_{32}O$	23704071	4.46
2	16.321	9,12-Octadecadiynoic acid, trimethylsilyl ester	$C_{21}H_{36}O_2Si$	11568471	2.18
3	17.512	10,12-Tricosadiynoic acid, TMS derivative	$C_{26}H_{46}O_2Si$	13191082	2.48
4	17.58	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propan-1-ol	$C_{15}H_{24}O$	5435294.9	1.02
5	18.348	Eicosapentaenoic Acid, TMS derivative	$C_{23}H_{38}O_2Si$	14193226	2.67
6	18.853	Dihydrodehydrocostus lactone	$C_{15}H_{20}O_2$	23966997	4.51
7	19.169	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-	$C_{15}H_{20}O_2$	5474521	1.03
8	19.305	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-	$C_{15}H_{20}O_2$	8423198.9	1.59
9	19.471	Azuleno[4,5-b]furan-2(3H)-one, decahydro-3,6,9-tris(methylene)-, [3aS-(3a.alpha.,6a.alpha.,9a.alpha.,9b.beta.)]	$C_{15}H_{18}O_2$	410989217	77.37
10	21.43	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	8076978.3	1.52
11	32.748	Hi-oleic safflower oil	$C_{21}H_{22}O_{11}$	6193295.1	1.17

RT: Retention Time

TABLE 2. Effect of *S. costus* extract against chlorpyrifos (CPF) induced toxicity on different hematological measurements in rats

		C	*S. costus* Extract		CPF	CPF + *S. costus* extract	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Formed Elements	**RBCs** (10^6/ul)	5.75 ± 0.02	5.71 ± 0.02	5.76 ± 0.02	3.78 ± 0.01[a]	3.79 ± 0.02[a]	5.73 ± 0.02[bc]
	HB (g/ dl)	12.97 ± 0.05	13.05 ± 0.07	13.09 ± 0.07	10.19 ± 0.02[a]	10.19 ± 0.02[a]	12.96 ± 0.03[bc]
	HCT (%)	33.80 ± 0.23	33.81 ± 0.14	33.85 ± 0.15	21.98 ± 0.10[a]	21.98 ± 0.13[a]	33.80 ± 0.09[bc]
	MCV (um^3)	56.86 ± 0.27	57.12 ± 0.28	57.12 ± 0.09	69.69 ± 0.17[a]	69.62 ± 0.16[a]	56.85 ± 0.05[bc]
	MCH (pg)	22.31 ± 0.34	22.95 ± 0.13	22.94 ± 0.08	22.92 ± 0.12	22.94 ± 0.08	22.30 ± 0.01
	MCHC (g/ dl)	39.19 ± 0.31	39.66 ± 0.35	39.65 ± 0.28	39.65 ± 0.34	39.77 ± 0.29	39.23 ± 0.18
	RDW (%)	14.40 ± 0.10	14.26 ± 0.03	14.28 ± 0.02	8.28 ± 0.02[a]	8.30 ± 0.02[a]	14.39 ± 0.04[bc]
	MPV (um^3)	8.72 ± 0.04	8.71 ± 0.02	8.71 ± 0.07	4.40 ± 0.02[a]	4.39 ± 0.03[a]	8.71 ± 0.02[bc]
	PLT (10^3/ul)	452.25 ± 1.65	454.40 ± 1.51	454.60 ± 1.49	251.40 ± 1.24[a]	251.40 ± 1.62[a]	453.60 ± 1.65[bc]
	WBCs (10^3/ul)	7.77 ± 0.04	7.75 ± 0.03	7.76 ± 0.04	4.69 ± 0.02[a]	4.71 ± 0.02[a]	7.78 ± 0.02[bc]
Differential Count	**Lymp.** (10^3/ul)	5.83 ± 0.03	5.81 ± 0.02	5.82 ± 0.03	3.52 ± 0.01[a]	3.53 ± 0.01[a]	5.83 ± 0.01[bc]
	Mono. (10^3/ul)	0.77 ± 0.00	0.77 ± 0.00	0.77 ± 0.00	0.46 ± 0.00[a]	0.47 ± 0.00[a]	0.77 ± 0.00[bc]
	Gran. (10^3/ul)	0.70 ± 0.00	0.70 ± 0.00	0.70 ± 0.00	0.42 ± 0.00[a]	0.42 ± 0.00[a]	0.70 ± 0.00[bc]

Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from CPF injected group, **c:** significant difference from CPF injected group and treated with *S. costus* extract (0.25 ml) at P≤0.05.

Data depicted in **Table 3** revealed that no significant changes were noticed as a result of administration of *S. costus* extract alone at both doses in all biochemical functions. CPF caused a significant (P≤0.05) elevation in levels the most biochemical measurements (ALT, AST, TC, T.Gs, urea and creatinine). This was in agreement with **Khanalizadeh and Najafian [50]** who postulated that levels of these enzymes were elevated due to necrosis of the parenchymal cells in the liver as supported by our histopathological examination. In addition, levels of these enzymes might be elevated due to altering in the antioxidant system that consequently leads to production of ROS in excessive rate and the homeostasis will be disturbed resulting in oxidative stress and hence leads to liver injury **[51]**. Levels of urea and creatinine in sera were used as indicators of kidney function and their elevated levels used as an index of kidney dysfunction **[52]**. In the present study, it was found that CPF increased their levels and this might be attributed to inflammation of the kidney tissues that consequently leads to degeneration of the tubules and tissue necrosis in addition to deposition of the protein cast within tubular lumina **[53]**.

Although the AChE used as an indicator of liver function and its activity depends on the catabolic and anabolic pathways and its activity altered in case of hepatocellular lesions, its activity is considered as a biomarker of the brain toxicity induced by OP pesticides **[12]**. During the current study, it was noticed that CPF caused a significant (P≤0.05) decrease in activity of AChE significantly (P≤0.05) as compared to control group. This in accordance with **O'Malley [54]** who reported that CPF affects the nervous system by inhibiting AChE and this inhibition results in over stimulation of the parts of nervous system that contain Ach muscarinic receptor in post-ganglionic fibers of central and peripheral nervous systems. Consequently, this activity leads to an excessive accumulation of Ach and overstimulation of cholinergic neurons **[55]**. CPF do not directly inhibit AChE, rather it is first metabolized to the corresponding oxygen analogue (CPF-oxon), a more potent inhibitor of AChE **[56]**. Activation of CPF into CPF-oxon is mediated by cytochrome P450 mixed function oxidases, primarily within the liver **[57]**; however, extrahepatic metabolism has been reported in other tissues including the brain **[58]**. As regard to total protein and albumin, it caused no significant changes in their levels. No ameliorative effect was noticed with administration of *S. costus* extract at a dose of 0.25 ml. It caused no significant (P≤0.05) changes in the altered measurements as compared to CPF treated group. The extract at a dose of 0.5 ml decreased levels ALT, AST, TC, T.Gs, urea and creatinine associated with increasing activity of AChE significantly (P≤0.05) as compared to the group injected with CPF alone and that treated with *S. costus* extract at a dose of 0.25 ml. The extract restored these measurements to normalcy. Administration of *S. costus* extract restored all the biochemical measurements to normal values and this might refer the polyphenolic compounds that are able to stimulate tools of the antioxidant system to maintain integrity of cell membranes against ROS that stimulate the oxidative stress **[59]**.

The oxidative stress is considered as one of the mechanisms associated with the exposure to CPF **[60]**. The TAC and TPC levels used biomarkers of the oxidative stress that were studied during the present study. As illustrated in **Fig. 2**, it was found that CPF caused elevation in levels of these measurements significantly (P≤0.05). This was in agreement with **Uchendu *et al.* [61]** who postulated that CPF has been reported to induce oxidative stress by increased levels of ROS, hydrogen peroxide (H_2O_2), nitrate (NO_3^-) and nitrite (NO_2^-). The *S. costus* extract at a dose of 0.25 ml caused no significant (P≤0.05) changes in these measurements as compared to CPF treated group. As regard to the treatment with the extract at a dose of 0.5 ml, levels of these measurements restored to normal values and decreased significantly (P≤0.05) as compared to the group injected with CPF alone and that treated with extract at a dose of 0.25 ml. This was in accordance to **Hsouna *et al.* [62]** and supported by **Sebai *et al.* [63]** who emphasized that presence of the active phenolic molecules that are considered as the first line of a complex defense system against the reactive stressors and provide the extract with the ability to scavenge against the $OH^{\bullet}$ which is the major cause of protein oxidation.

Esterase-like albumin activity is considered as marker for many chronic diseases **[64]**. It can be expressed as several molecular forms characterized by their hydrodynamic properties and identified by their molecular weights **[65]**. It can be visualized by staining the substrate in the presence of dye coupler (Fast Blue RR salt) using α- and β-naphthyl acetate **[31]**. Mutations may occur at qualitative level by hiding one or more of normal bands with existence of abnormal ones. Otherwise, the alterations may occur at quantitative level by altering quantities of the bands that identified at normal mobilities. The SI is only related to the qualitative alterations and it is inversely proportional to the GD. The low SI values are associated with high GD values indicating differences in number and arrangement of the bands **[66-68]**.

TABLE 3. Effect of *S. costus* extract against chlorpyrifos (CPF) induced toxicity on different biochemical measurements in rats

		C	*S. costus* Extract		Toxic	Toxic + Extract	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Liver Enzymes	ALT (U/L)	42.28 ± 0.01	42.28 ± 0.01	42.28 ± 0.01	58.95 ± 0.05[a]	58.95 ± 0.04[a]	42.28 ± 0.01[bc]
	AST (U/L)	98.18 ± 0.09	98.19 ± 0.09	98.18 ± 0.08	116.00 ± 0.07[a]	116.17 ± 0.07[a]	98.19 ± 0.08[bc]
	AChE (U/L)	35.88 ± 0.08	35.91 ± 0.08	35.89 ± 0.07	18.78 ± 0.03[a]	18.78 ± 0.04[a]	35.86 ± 0.05[bc]
Lipid Profile	TC (mg/dl)	73.55 ± 0.16	73.59 ± 0.15	73.59 ± 0.15	125.03 ± 0.06[a]	125.07 ± 0.06[a]	73.60 ± 0.16[bc]
	T.Gs (mg/dl)	67.07 ± 0.05	67.12 ± 0.05	67.12 ± 0.04	112.13 ± 0.07[a]	112.18 ± 0.05[a]	67.08 ± 0.03[bc]
kidney Functions	Urea (mg/dl)	48.70 ± 0.01	48.69 ± 0.01	48.69 ± 0.01	65.60 ± 0.04[a]	65.61 ± 0.02[a]	48.69 ± 0.01[bc]
	Creatinine (mg/dl)	0.91 ± 0.01	0.90 ± 0.00	0.90 ± 0.00	1.39 ± 0.01[a]	1.39 ± 0.01[a]	0.89 ± 0.01[bc]
	Total Protein (g/dl)	8.25 ± 0.01	8.27 ± 0.02	8.26 ± 0.01	8.27 ± 0.01	8.27 ± 0.01	8.26 ± 0.00
	Albumin (g/dl)	3.73 ± 0.01	3.73 ± 0.00	3.73 ± 0.00	3.73 ± 0.00	3.74 ± 0.00	3.73 ± 0.00

Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from CPF injected group, **c:** significant difference from CPF injected group and treated with *S. costus* extract (0.25 ml) at P≤0.05.

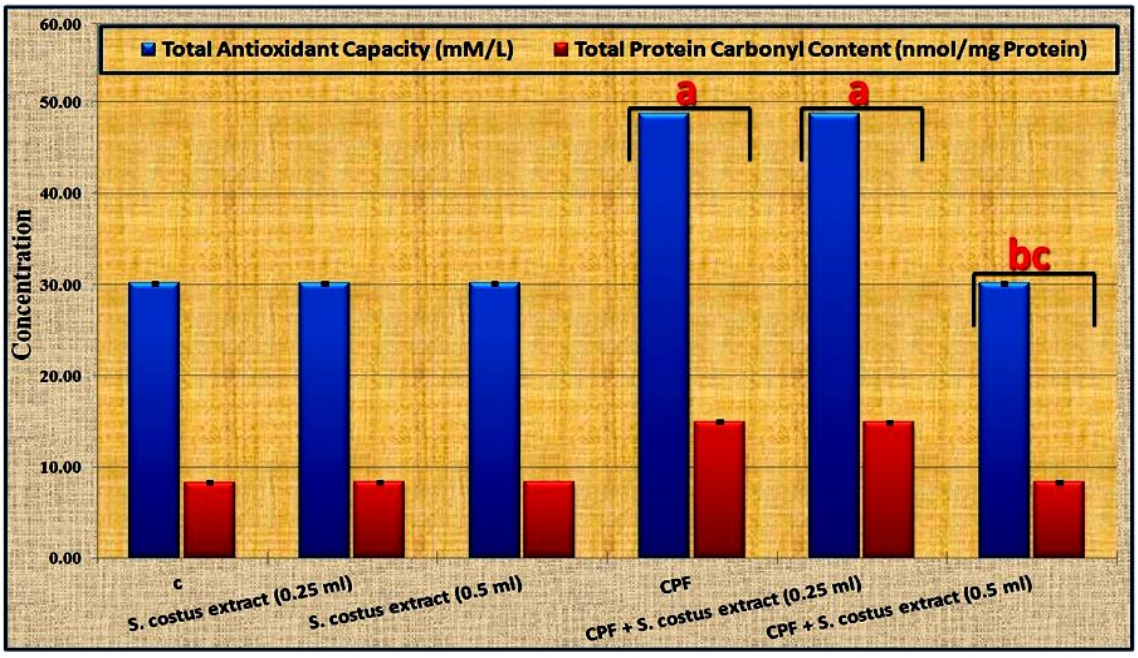


Fig. 2. Effect of *S. costus* extract against chlorpyrifos (CPF) induced toxicity on major markers of oxidative stress in rats. Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from CPF injected group, **c:** significant difference from CPF injected group and treated with *S. costus* extract (0.25 ml) at P≤0.05.

As shown in **Fig. 3**, it was found that electrophoretic α-EST isoenzyme was represented in serum of control rats by 4 types identified at Rfs 0.09, 0.26, 0.40 and 0.54 (B% 32.34, 30.38, 19.73 and 17.55 ; Qty% 7.54, 22.54, 4.60 and 9.67, respectively). All the α-EST types in control group are considered as common bands and no characteristic bands were identified. The *S. costus* extract alone at both doses (0.25 and 0.5 ml) caused no physiological variations from control group. CPF caused alterations in the α-EST pattern represented by existence of 2 abnormal bands identified at Rfs 0.34 and 0.69 (B% 17.73 and 11.90 ; Qty% 2.63 and 11.66, respectively) without hiding normal α-EST types. Therefore, the SI% value (SI=80.00%) decreased with increasing GD% value (GD=20.00%) in CPF injected group. The treatment with *S. costus* extract at a dose of 0.25 ml could not improve the isoenzyme pattern and also the physiological changes were represented by appearance of 2 abnormal bands identified at Rfs 0.34 and 0.69 (B% 18.54 and 11.11 ; Qty% 6.16 and 3.69, respectively). The SI% and GD% values were equal to those in CPF injected group (80.00 and 20.00%, respectively). The treatment with *S. costus* extract at a dose of 0.5 ml restored the physiological state of the α-EST isoenzymes pattern through hiding the abnormal types. Therefore, the α-EST isoenzyme pattern became completely similar to that in control group (SI=100.00% ; GD=0.00%).

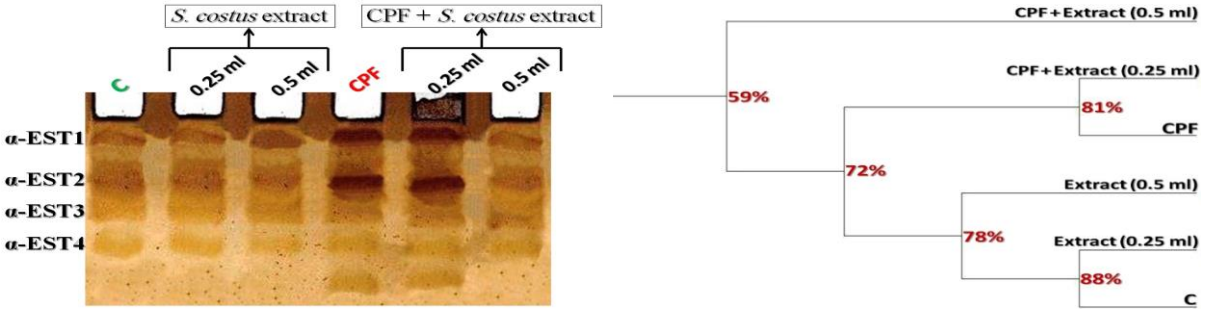
As shown in **Fig. 4**, it was found that electrophoretic β-EST isoenzyme was represented in serum of control rats by 2 types identified at Rfs 0.35 and 0.65 (B% 61.05 and 39.00 ; Qty% 10.51 and 11.08, respectively). The 1st β-EST type (β-EST1) in control group is considered as common band and no characteristic bands were identified. No physiological changes were identified in the groups that treated with *S. costus* extract alone at both doses (0.25 and 0.5 ml). The CPF caused physiological alterations in the β-EST pattern represented by hiding the 2nd EST type (β-EST2) with appearance of one abnormal band identified at Rf 0.75 (B% 38.03 ; Qty% 1.70). Therefore, the SI% value (SI=50.00%) decreased with increasing GD% value (GD=50.00%) in CPF injected group. The treatment with *S. costus* extract at a dose of 0.25 ml could not improve this isoenzyme pattern and also the physiological alterations were represented by hiding the 2nd EST type (β-EST2) with existence of one abnormal band identified at Rf 0.74 (B% 34.61 ; Qty% 4.90). Therefore, the SI% and GD% values were equal to those in CPF injected group (50.00 and 50.00%, respectively). The treatment with *S. costus* extract at a dose of 0.5 ml restored the physiological state of the β-EST isoenzymes pattern through hiding the abnormal type with re-appearance of the normal one identified at Rf 0.66 (B% 45.54 ; Qty% 4.69). Therefore, the β-EST isoenzyme pattern became completely similar to that in control group (SI=100.00% ; GD=0.00%). The alterations in electrophoretic EST pattern might occur as a result of overproduction of the ROS that induced by oxidative stress and consequently leads to altering structure and function of albumin molecules **[69]**. Also, this might occur as a result of variations in rates of protein expression secondary to DNA damage and hence affecting the isoenzymes. If there was no alteration in the protein expression, enzymatic activity of these two proteins was not altered. Furthermore, the alterations in the electrophoretic isoenzymes might refer to attack of the free radicals that are directly targeting on the nucleic acids responsible for the enzyme biosynthesis **[70,71]**. The *S. costus* extract exhibited antioxidative properties due to presence of the polyphenols that exhibit an effective role in protection and/or maintaining the macromolecule integrity against products of the oxidative reactions **[72]**. Also, these polyphenols enhance activity of the antioxidant enzymes to overcome the reactive species targeting these biomacromolecules **[59]**. Microscopic examination of liver in control rats revealed normal histological structure of hepatic parenchyma **(Fig. 5a)**. Meanwhile, liver of rats from groups 2 & 3 appeared histologically normal and showed no histopathological changes **(Fig. 5c & d)**. In contrast, the sections examined from group 4 showed histopathological alterations described as activation of Kupffer cells, vacuolar degeneration of hepatocytes, fibroplasia in the portal triad and newly formed bile ductuoles **(Table 4 & Fig. 5b)**. This was in agreement with **Ki *et al.* [73]** who postulated that CPF could generate a hepatic dysfunction in addition to the immunological abnormalities, embryotoxicity, genotoxicity, teratogenicity, neurochemical and neurobehavioral changes. Moreover, it was found that CPF even at lower dose induced alterations moderately in hepatic parenchyma, cytoplasm vacuolization and a lymphoid infiltration. Also, it induced formation of oedema and dislocation in wall of the central vein besides the hepatocellular necrosis randomly distributed throughout the parenchyma.

Our findings were supported by **Ezzi *et al.* [74]** who revealed that histological changes in the liver were proportional to the dose. Furthermore, **Solati [75]** showed that CPF caused mild alterations represented by mild mononuclear cells infiltration and periportal inflammation, hepatocytes necrosis, sinusoid hyperaemia and periportal lymphocytic infiltration. On the other hand, the liver sections from group 5 showed obvious improvement and showed no changes except slight hydropic degeneration of some hepatocytes **(Fig. 5e)**. Moreover, remarkable regressions of lesion in addition to activation of slight Kupffer cells were seen in liver sections from group 6 **(Fig. 5f)**.

Microscopically, kidneys of control rats showed normal histological structure of renal parenchyma (normal glomeruli and renal tubules) **(Fig. 6a)**. However, kidneys from groups 2 & 3 revealed no histopathological alterations **(Fig. 6c & d)**. On contrary, kidneys from group 4 revealed histopathological lesions manifested by vacuolar degeneration of renal tubular epithelium, necrobiotic changes of renal tubular epithelium and interstitial nephritis (interstitial mononuclear inflammatory cells infiltration) **(Table 4 & Fig. 6b)**. This was in accordance with **Rekha *et al.* [76]** who illustrated that CPF induced significant histomorphological changes that occurred in the kidneys as a result of the renal toxicity especially at high dose level. Meanwhile, mild changes were noticed in kidneys from group 5 and represented by slight vacuolar degeneration of epithelial lining some renal tubules **(Fig. 6e)**. Furthermore, apparent normal histological architecture was demonstrated in kidneys from group 6 **(Fig. 6.)**

TABLE 4. The histopathological score showing effect of S. costus extract against the lesions induced by chlorpyrifos (CPF) in different tissues

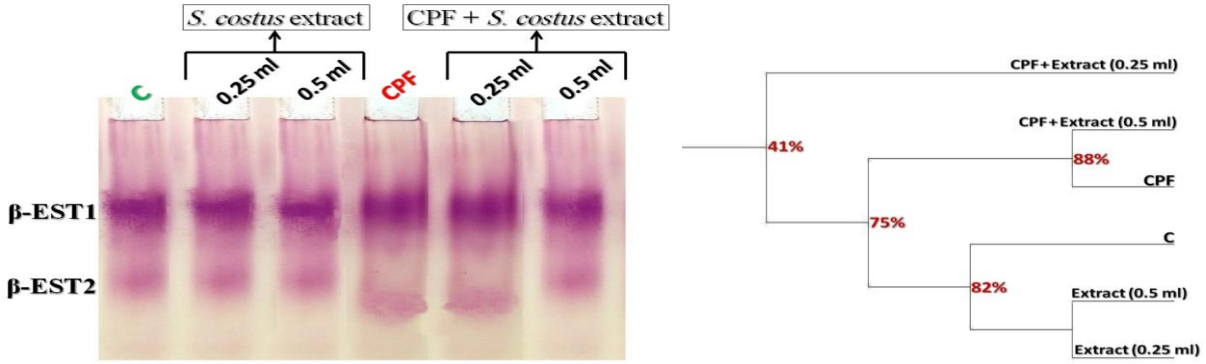
Organs	Lesions	C	*S. costus* Extract		CPF	CPF + *S. costus* Extract	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Liver	Kupffer cells activation	0	0	0	2	1	1
	Vacuolar degeneration of hepatocytes	0	0	0	3	1	0
	Fibroplasia in the portal triad	0	0	0	2	0	0
	Newly formed bile ductuoles	0	0	0	2	0	0
Kidney	Vacuolar degeneration of renal tubular epithelium	0	0	0	3	1	0
	necrobiosis of tubular epithelium	0	0	0	2	0	0
	inflammatory cells infiltration.	0	0	0	2	0	0
Brain	Pyknosis & necrosis of neurons	0	0	0	3	1	1
	Neuronophagia	0	0	0	2	0	0
	Cellular oedema	0	0	0	2	0	0
	Focal gliosis	0	0	0	1	0	0



Control			*S. costus* Extract						CPF			CPF + *S. costus* Extract					
			0.25 ml			0.5 ml						0.25 ml			0.5 ml		
Rf	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty %**
0.09	32.34	7.54	0.08	31.09	10.71	0.09	29.96	10.02	0.09	21.08	10.01	0.08	20.24	10.38	0.07	28.82	16.67
0.26	30.38	22.54	0.26	31.19	17.71	0.25	29.16	17.06	0.26	23.24	17.25	0.27	23.50	15.61	0.28	27.89	8.36
-	-	-	-	-	-	-	-	-	0.34	17.73	2.63	0.34	18.54	6.16	-	-	-
0.40	19.73	4.60	0.40	19.71	5.99	0.39	21.61	10.84	0.40	14.15	5.88	0.41	14.30	7.77	0.41	24.35	12.52
0.54	17.55	9.67	0.53	18.00	9.86	0.54	19.27	2.82	0.54	11.90	4.59	0.54	12.30	8.17	0.55	18.94	6.90
-	-	-	-	-	-	-	-	-	0.69	11.90	11.66	0.69	11.11	3.69	-	-	-

Rf: Relative Mobility, **B%:** Percent of Band Intensity, **Qty%:** Percent of Band Quantity.

Fig. 3. Native Electrophoretic α-esterase (α-EST) Isoenzymes Pattern Showing Physiological Effect of *S. costus* extract against chlorpyrifos (CPF) induced toxicity on Bands Number, Arrangement and Similarity Percents of this enzyme in sera of rats



Control			*S. costus* Extract						CPF			CPF + *S. costus* Extract					
			0.25 ml			0.5 ml						0.25 ml			0.5 ml		
Rf	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty %**
0.35	61.05	10.51	0.35	65.89	5.35	0.35	63.28	5.37	0.36	59.87	44.22	0.36	65.39	2.32	0.36	54.46	19.42
0.65	39.00	11.08	0.66	34.11	14.86	0.65	36.72	16.34	-	-	-	-	-	-	0.66	45.54	4.69
-	-	-	-	-	-	-	-	-	0.75	38.03	1.70	0.74	34.61	4.90	-	-	-

Rf: Relative Mobility, **B%:** Percent of Band Intensity, **Qty%:** Percent of Band Quantity.

Fig. 4. Native Electrophoretic β-esterase (β-EST) Isoenzymes Pattern Showing Physiological Effect of *S. costus* extract against chlorpyrifos (CPF) induced toxicity on Bands Number, Arrangement and Similarity Percents of this enzyme in sera of rats.

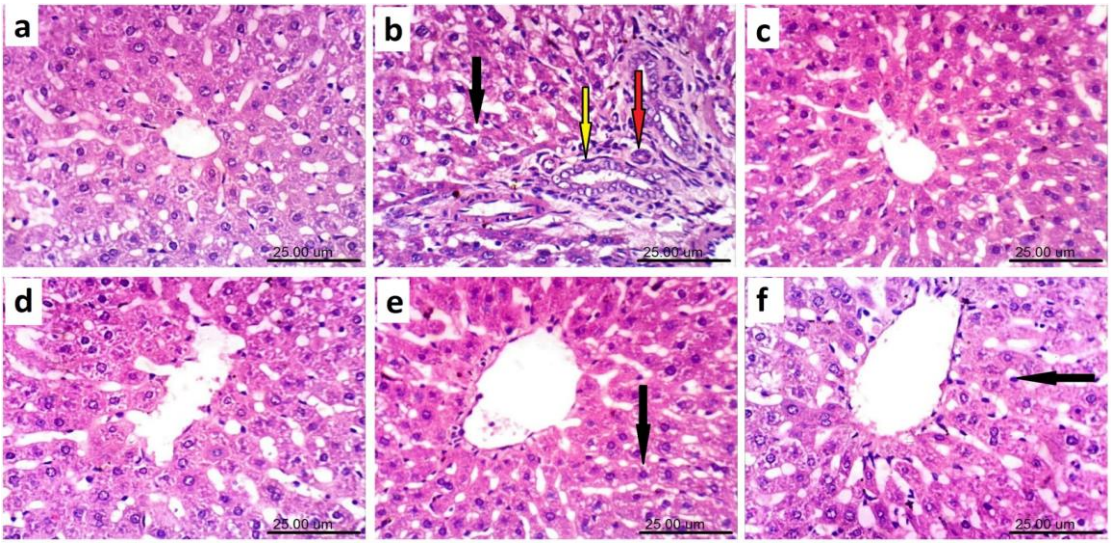


Fig. 5. Photomicrograph of histological H&E stained liver tissue of rats: a) group 1 (control) showing the normal histological structure of hepatic parenchyma, b) group 4 (CPF injected group) showing vacuolar degeneration of hepatocytes (black arrow), fibroplasia in the portal triad (yellow arrow) and newly formed bile ductuoles (red arrow), c) & d) groups 2 (*S. costus* extract (0.25 ml) treated group) & 3 (*S. costus* extract (0.5 ml) treated group), respectively showing no histopathological alterations, e) group 5 (CPF + *S. costus* extract (0.25 ml) treated group), showing slight hydropic degeneration of some hepatocytes (black arrow) and f) group 6 (CPF + *S. costus* extract (0.5 ml) treated group) showing slight Kupffer cells activation (black arrow) **(scale bar 25um, X400)**.

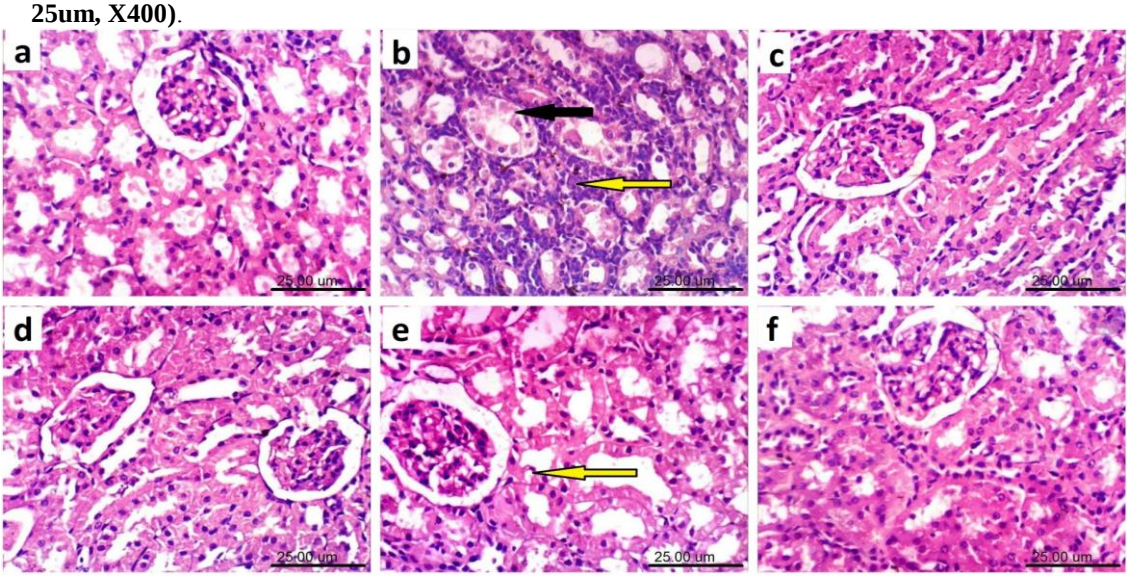


Fig. 6. Photomicrograph of histological H&E stained kidney sections of rats: a) group 1 (control) showing the normal histological structure of renal parenchyma, b) group 4 (CPF injected group) showing necrobiotic changes of renal tubular epithelium (black arrow) and interstitial nephritis (yellow arrow). c) & d) groups 2 (*S. costus* extract (0.25 ml) treated group) & 3 (*S. costus* extract (0.5 ml) treated group), respectively showing no histopathological alterations. e) group 5 (CPF + *S. costus* extract (0.25 ml) treated group) showing slight vacuolar degeneration of epithelial lining some renal tubules (yellow arrow) and f) group 6 (CPF + *S. costus* extract (0.5 ml) treated group) showing apparent normal histological architecture **(scale bar 25um, X400)**.

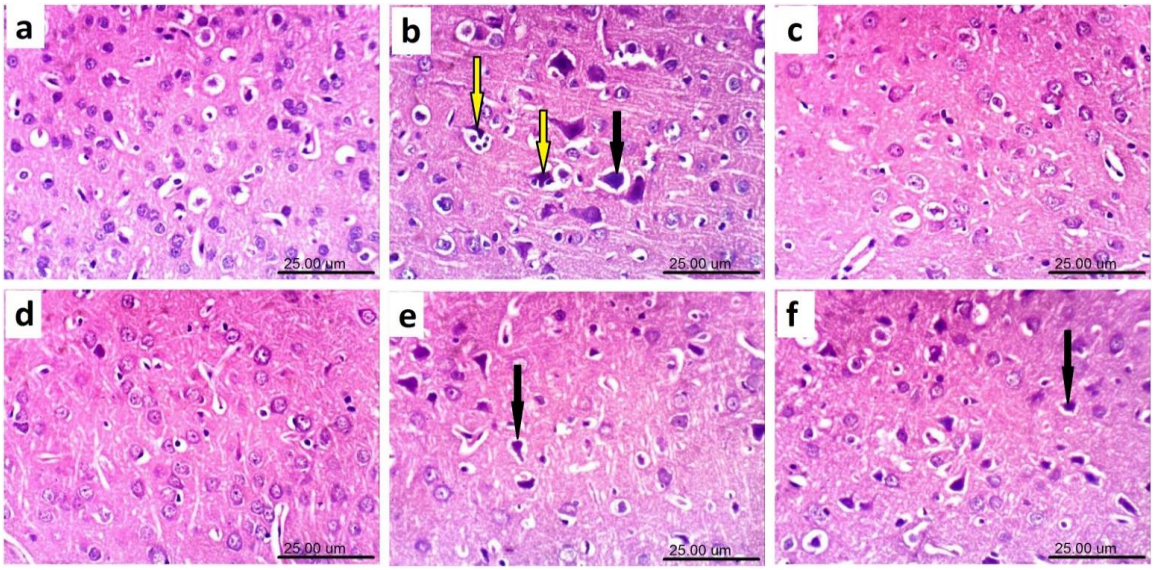


Fig. 7. Photomicrograph of histological H&E stained brain sections of rats: a) group 1 (control) showing no histopathological changes, b) group 4 (CPF injected group) showing pyknosis and necrosis of neurons (black arrow) as well as neuronophagia of necrotic neurons (yellow arrows). c) & d) groups 2 (*S. costus* extract (0.25 ml) treated group) & 3 (*S. costus* extract (0.5 ml) treated group), respectively showing no histopathological alterations, e) group 5 (CPF + *S. costus* extract (0.25 ml) treated group) showing necrosis of some neurons (black arrow) and f) group 6 (CPF + *S. costus* extract (0.5 ml) treated group) showing necrosis of some neurons (black arrow) **(scale bar 25um, X400)**.

Brain tissue of control rats revealed no histopathological changes **(Fig. 7a)**. No histopathological alterations were recorded in brain from groups 2 & 3 **(Fig. 7c & d)**. Meanwhile, the brain sections from group 4 manifested histopathological alterations described as pyknosis and necrosis of neurons, neuronophagia of necrotic neurons, cellular oedema and focal gliosis **(Table 4 & Fig. 7b)**. This was in accordance with **Solati [75]** who emphasized that the brain tissue had hippocampus, necrosis of neuron cells and edema, also, neurons with condensed chromatin are seen (arrows), the brain section showed hemorrhagic and necrotizing encephalitis (perivascular lymphoid cuffing). Meanwhile, hyperemia, hemorrhage and necrosis of neuron cells with focal gliosis were observed in brain section. On the other hand, the brain sections from groups 5 & 6 revealed obvious improvement and regression of lesions in addition to necrosis of some neurons **(Fig. 7e & f)**.

Administration of *S. costus* showed ameliorative effect against the deleterious effects induced by CPF in all studied organs (liver, kidney and brains). This was in agreement with **Lin *et al.* [77]**. This was attributable to the presence of costunolide in addition to the other polyphenolic compounds that exhibit antioxidant and anti-inflammatory activities **[78,79].** On the other hand, Essential oils, aqueous an organic solvent of plant extracts (contained polyphenolic compounds) and silver nanoparticles have been used as antifungal, antivirus and antibacterial to avoid a contamination in food **[80-83]**. It was postulated that the aqueous extract showed scavenging activities against attack of the ROS that stimulate the oxidative stress in addition to cytotoxic activities and its ability to regulate cell apoptosis by inhibiting proliferation of cancer cells and to down regulate the expression of both P^{53} and Bcl2 genes **[84]**.

4.Conclusion

The study concluded that *S. costus* root extract contains 11 potent active compounds and Dehydrocostuslactone is the most dominant compound (77.37%). *S. costus* extract restored all hematological and biochemical measurements that altered due to CPF injection towards the control values. The CPF caused physiological alterations in the ESTs represented by hiding normal EST types associated with existence of abnormal ones. Therefore, the SI% and GD% values were altered with α-EST (SI=80.00%; GD=20.00%) and β-EST (SI=50.00%; GD=50.00%) patterns in CPF injected group. The *S. costus* extract at a dose of 0.5 ml restored integrity of these isoenzymes pattern by restoring the absent types with hiding the abnormal ones. The histopathological examination in the target organs (brain, liver and kidney) showed that CPF

caused severe tissue lesions and the *S. costus* extract restored structure of these tissues to normal architecture. The *S. costus* have a potent active phytochemical compounds as antioxidant agents and could be used as anticancer to eliminate the unbalance in oxidative stress. Our current promised obtained results in moderate the health hazards induced by CPF opening the way to make *S. costus* as a drink in package form easy ready to use.

5.Conflict of Interests

The authors who are responsible theoretically and practically for the manuscript declare that there are no conflicts of financial and non-financial interests.

6- Acknowledgment

The author Acknowledge the Food Toxicology and Contaminants Dept., National Research Centre, Dokki, Egypt.

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