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Protective effect of some natural products against chemotherapy-induced toxicity in rats

Heba M.I. Abdallah [a], Rehab F. Abdel-Rahman [a,*], Sally A. El Awdan [a], Rasha M. Allam [a], Aliaa E.M.K. El-Mosallamy [a], Manal S. Selim [b], Sahar S. Mohamed [b], Mahmoud S. Arbid [a], Abdel Razik H. Farrag [c]

[a] Department of Pharmacology, Medical Division, National Research Centre, Giza, Egypt
[b] Department of Microbial Biotechnology, Genetic Engineering and Biotechnology Research Division, National Research Centre, Giza, Egypt
[c] Department of Pathology, Medical Division, National Research Centre, Giza, Egypt

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ABSTRACT

Aim: There is a great interest in combining anticancer drugs with natural products aiming at maximizing their efficacy while minimizing systemic toxicity. Hence, the present study was constructed aiming to investigate the protective potential of three natural products, 1,8-cineole an essential oil from *Artemisia herba alba*, exopolysaccharide (EPS) from locally identified marine streptomycete, and ellagic acid (EA), against chemotherapy-induced organ toxicity.

Methods: Isolation, production and characterization of EPS from marine streptomycete was done. Animals were allocated into five groups, GP1: normal control, GP2: cyclophosphamide (CYC), GP3: 1,8-cineole + CYC, GP4: EPS + CYC, GP4: EA + CYC. All drugs were administered orally 1 week before and concomitantly with CYC. Electrocardiography (ECG) analysis, liver enzymes (ALT and AST), cardiac serum markers (LDH and CK), oxidative stress biomarkers in hepatic and cardiac tissues (GSH and MDA), TGF-β1 and histopathological examination of hepatic and cardiac tissues were executed.

Results: The isolated stain produced EPS was identified as *Streptomyces xiamenensis*. EPS contains uronic, sulphate groups and different monosugars with *Mw* 4.65 × 10^4 g/mol and showed antioxidant activity against DPPH. Pretreatment of rats with 1,8-cineole, EPS and EA improved ECG abnormalities, decrease serum markers of hepato- and cardiotoxicity, prevent oxidative stress and decrease TGF-β1 in liver and heart tissues.

Conclusion: The present results demonstrate the hepatoprotective and cardioprotective effects of the above-mentioned natural products against CYC organ toxicity.

1. Introduction

Chemotherapy is one of the cancer treatment strategies which may be used alone or in combination with the other types, such as surgery and radiotherapy. It employs a wide group of drugs that have cytotoxic effects to one or more diseased tissues. Cyclophosphamide, an alkylating agent, is the most commonly used anticancer and immunosuppressant drug. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, and rheumatic arthritis and in preparation for bone marrow transplantation (Emadi et al., 2009; Nelius et al., 2010). However, patients receiving these antineoplastic agents experience severe side effects at the therapeutically effective doses. These side effects include general cell-damaging effects such as; decreased blood cell and myelosuppresion and immunosuppression. More specific side effects include nephrotoxicity, neurotoxicity, testicular dysfunction, cardiotoxicity and hepatotoxicity (Fraiser et al., 1991; Shanholtz, 2001; Prahalathan et al., 2004).

In general, regardless of the benefits of chemotherapy outweigh the disadvantages, it remains the commonest form of cancer therapy. Until now the development of antineoplastic agents that combine efficacy, safety and convenience for the patient remains a great challenge. Thus, there is a great interest in combining anticancer drugs with natural products aiming at maximizing their efficacy while minimizing systemic toxicity through the delivery of lower drug doses (Ismael et al., 2008).

Ellagic acid is a natural phenol that is found in various vegetables and fruits. It possesses significant health benefits due to its antioxidant and

* Corresponding author.
E-mail addresses: rehabs2001@yahoo.com, rf.abdelrahman@nrc.sci.eg (R.F. Abdel-Rahman).

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Table 1
Morphological, cultural, physiological and biochemical characterization of *Streptomyces xiamenensis*.

Spore chain morphology	Spore surface ornamentation-	Colour of spore mass	Pigmentation of substrate mycelium	Diffusible pigment	Melanin pigment production		Pectin decomposition	Nitrate reduction	H_2S production
					Iron agar	Tyrosine agar			
Straight > 40	Smooth	White	White	Light pink	–	–	–	+	–
Utilization of carbon sources									
+ve control (glucose)	I-inositol	Raffinose	D-xylose	D-mannitol	Rhamnose	Sucrose	D-fructose	L-arabinose	Galactose
+	–	–	–	+	+	+	+	+	+

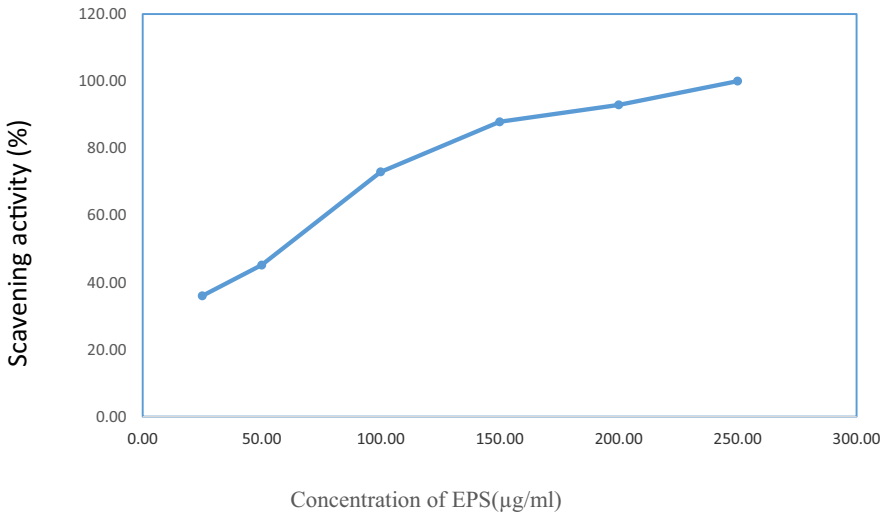

Fig. 1. The DPPH radical-scavenging activities of EPS.

antiproliferative activities. Ellagic acid showed protective effect on pulmonary, testicular, and nephrotoxicity induced by cyclophosphamide (Türk et al., 2010; Rehman et al., 2012; Saba et al., 2013). However, the prophylactic role of ellagic acid against cyclophosphamide-induced hepato- and cardiotoxicity has not been investigated yet.

Cineole (1,8-cineole), is a terpene present in many aromatic plants such as Artemisia herba alba, eucalyptus, sage, rosemary, and cardamom. Cardiovascular effects of cineole were described in normotensive rats. It was found that i.v. injection of cineole resulted in hypotension and this effect was suggested to be mediated by induced vascular relaxation (Lahlou et al., 2002). To the best of our knowledge, no reports are found regarding combining of cineole with cyclophosphamide aiming to decrease its side effects. However, recent report has pointed out to the promising anti-tumor effects of cineole that was explored against colon cancer (Murata et al., 2013). In addition, 1, 8-cineole was previously found to stimulate apoptosis in leukemia cell lines (Moteki et al., 2002). This natural product also showed other pharmacological effects such as anti-bacterial and anti-inflammatory activities (Juergens et al., 1998; Giamakis et al., 2001).

Medical benefits of extracellular polysaccharides isolated from marine microorganism by microbial fermentation have received a growing interest in the recent years (Vidhyalakshmi and Vallinachiyar, 2013). Recent advances in Biotechnology enabled high levels of polysaccharides to be produced in vitro from by controlling the growth conditions (Manivasagan and Kim, 2014). When comparing polysaccharides to the other bioactive constituents, it was found that they are highly effective, non-toxic natural substances having multiple pharmacological activities. There is a wide diversity in polysaccharides afforded by different microorganisms. Thus, it is warranted to use different species for obtaining polysaccharides with better properties (Elsakhawy et al., 2017). Exopolysaccharides were first isolated from marine *streptomycete* in Egypt by Selim et al. (2018) and their antitumor effect have been tested only in colon and breast cancer cell lines.

Taken together, the present study was constructed and aimed to investigate the protective effect of the aforementioned natural products; 1,8-cineole, ellagic acid (EA) and exopolysaccharide (EPS) produced from marine *streptomycete*.

2. Materials and methods

2.1. Chemicals

Cyclophosphamide, ellagic acid, 1,8-cineole, were purchased from Sigma Aldrich Co., Germany.

2.2. Isolation, production and characterization of marine exopolysaccharide (EPS)

2.2.1. Collection of samples and sampling site and isolation of streptomycetes

Sediment sample (5 cm depth) from Suez Canal (El-Ismailia governorate), Egypt was collected then the sample applied to serial dilution method (Hayakawa and Nonomura, 1987) using starch nitrate medium (Naguib et al., 1978) using 50% sea water. 0.1 ml inoculum of the appropriate dilution was plated on each plate. The plates were incubated at 28 °C for 7–14 days to allow the slow growing forms to develop. *Streptomycetes* were isolated based on their specific morphological characteristics and then subjected to purification.

2.2.2. Screening and production of EPS

Streptomycete strains were grown aerobically for four days in a fermentation medium (glucose 10 g, tryptone 5 g, yeast extract 5 g, NaCl

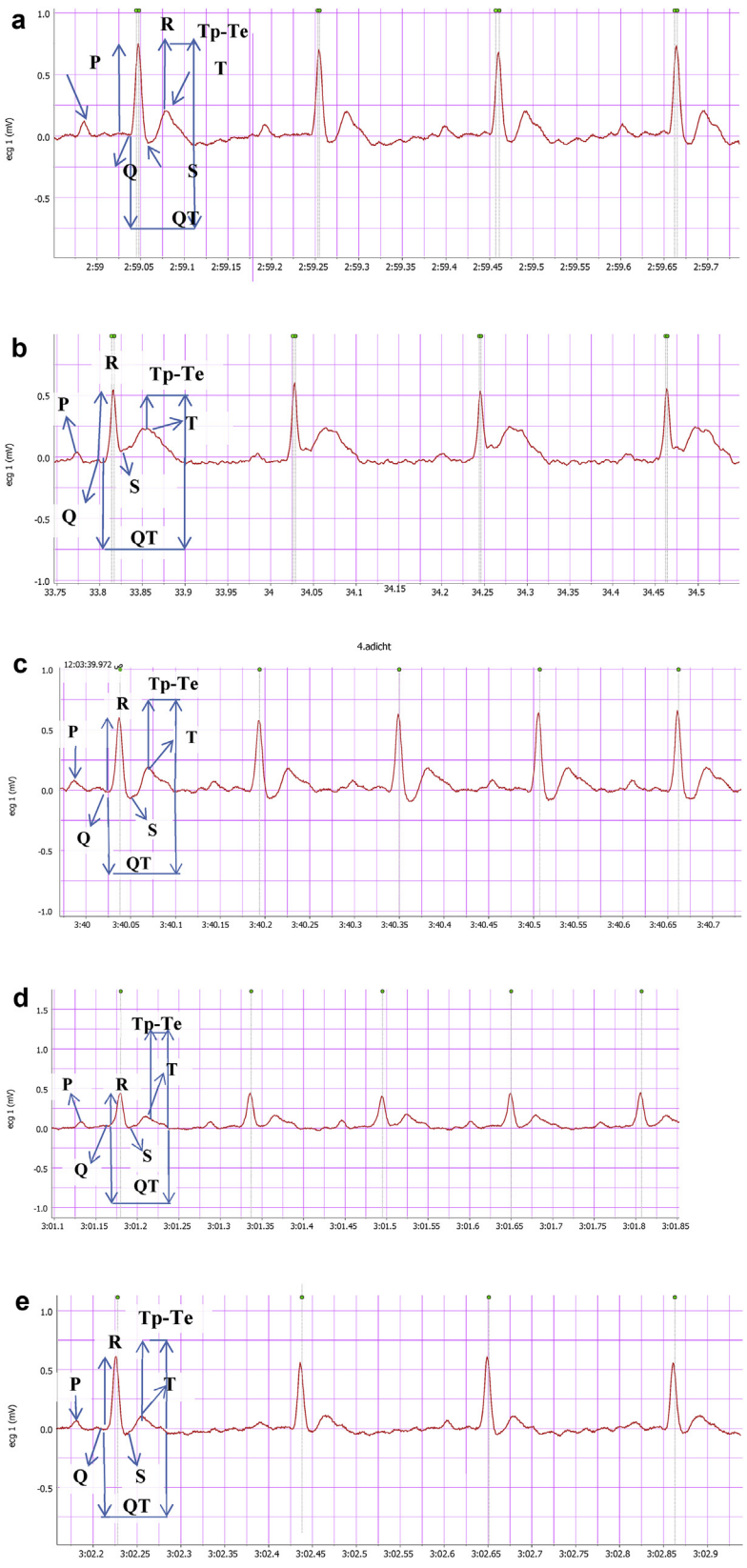

Fig. 2. (a). ECG chart of normal rat showing regular ECG normal pattern. (b). ECG chart of CYC-treated rat showing prolonged QT and Tp-Te intervals, depressed QRS complex, as well as ST-segment elevation. (c). ECG chart of CYC-induced cardiotoxicity pretreated with cineole (100 mg/kg) showing regaining of the QT and Tp-Te normal waves while the QRS complex is still depressed. (d). ECG chart of CYC-induced cardiotoxicity pretreated with EA (60 mg/kg) showing regaining of the QT and Tp-Te normal waves while the QRS complex is still depressed. (e). ECG chart of CYC-induced cardiotoxicity pretreated with EPS (100 mg/kg) showing regaining of the normal ECG pattern with a significant improvement in the prolonged QT and Tp-Te intervals and amelioration of the depressed QRS complex.

3 g, K2HPO4 3 g, KH2PO4 1 g, MgSO4·7H2O 0.5 g, CaCO30.5 g) in 75% Sea water at pH 7, 28 °C and 150 rpm on a rotary shaker (Manivasagan et al., 2013). Culture was centrifuged at 5000 rpm (Sigma-Laborzentrifugen, 2K15) for 30 min at 4 °C to remove streptomycete cells; the supernatant was subjected to deproteinization by Trichloroacetic acid TCA (5%). The pH of the supernatant was adjusted to 7.0 and dialyzed 3 times against flowing distilled water using dialysis tube (MWCO 3000). The supernatant was completed to two volumes with absolute ethanol and left at 4 °C to 24 h. The precipitated EPS was separated by centrifugation at 5000 rpm, for 25 min, re-dissolved in distilled water, dialyzed

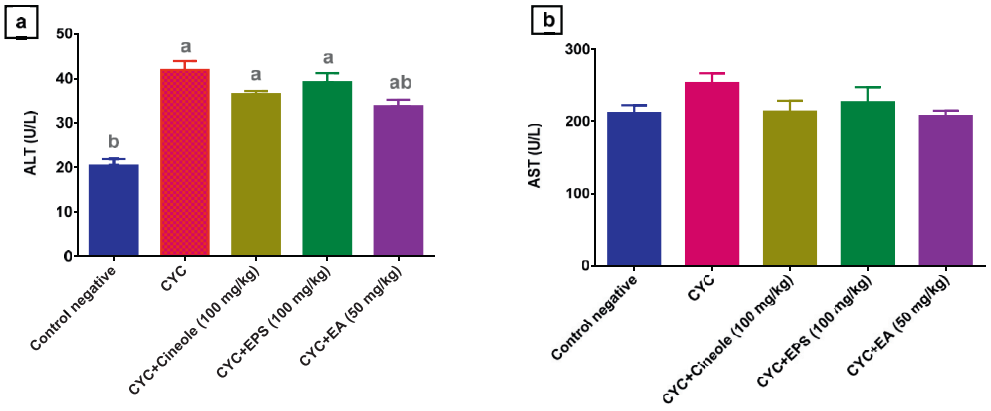

Fig. 3. Effect of 1,8-cineole, EPS and EA on ALT and AST levels in CYC-treated rats. Results are expressed as mean ± SEM and analyzed by one way ANOVA followed by Tukey's post-hoc test, $p < 0.05$. [a] significantly different from control negative group. [b] significantly different from CYC-treated group. CYC: cyclophosphamide, EPS: Exopolysaccharide, EA: Ellagic acid.

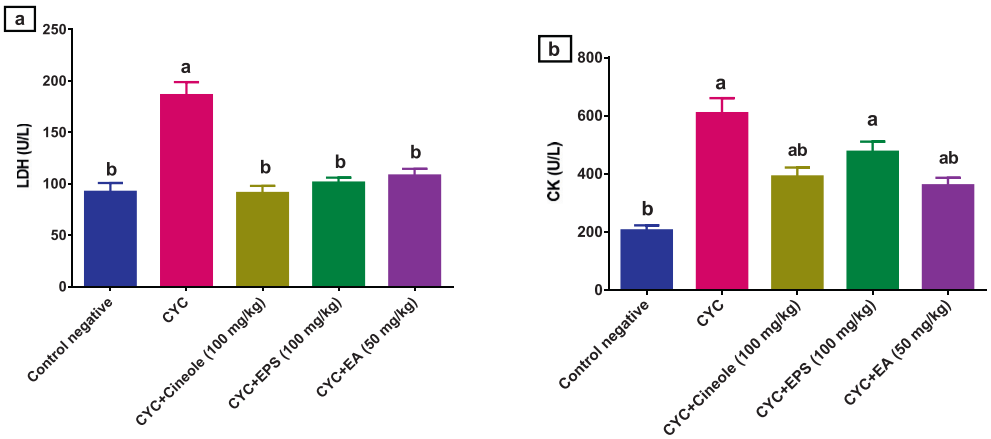

Fig. 4. Effect of 1,8-cineole, EPS and EA on LDH and CK levels in CYC-treated rats. Results are expressed as mean ± SEM and analyzed by one way ANOVA followed by Tukey's post-hoc test, $p < 0.05$. [a] significantly different from control negative group. [b] significantly different from CYC-treated group. CYC: cyclophosphamide, EPS: Exopolysaccharide, EA: Ellagic acid.

Table 2
Effect of 1,8-cineole, EPS and EA on hepatic and cardiac contents of GSH.

Groups	Hepatic GSH (ng/g tissue)	Cardiac GSH (ng/g tissue)
Control negative	8.30 ± 0.28^{b}	6.28 ± 0.39^{b}
CYC-treated	5.19 ± 0.31^{a}	3.21 ± 0.12^{a}
1,8-Cineole	8.55 ± 0.60^{b}	4.67 ± 0.29^{ab}
EPS	9.86 ± 0.39^{b}	3.27 ± 0.13^{a}
EA	11.29 ± 0.58^{ab}	3.86 ± 0.34^{a}

Results are expressed as mean ± SEM and analyzed by one way ANOVA followed by Tukey's post-hoc test, $p < 0.05$. [a] significantly different from control negative group. [b] significantly different from CYC-treated group. GSH: reduced glutathione, CYC: cyclophosphamide, EPS: Exopolysaccharide, EA: Ellagic acid.

Table 3
Effect of 1,8-cineole, EPS and EA on hepatic and cardiac contents of MDA.

Groups	Hepatic MDA (pg/g tissue)	Cardiac MDA (pg/g tissue)
Control negative	10.9 ± 0.27	10.0 ± 0.18^{b}
CYC-treated	11.1 ± 0.16	11.3 ± 0.06^{a}
1,8-Cineole	11.5 ± 0.06	10.6 ± 0.07^{b}
EPS	11.5 ± 0.05	10.1 ± 0.18^{b}
EA	11.6 ± 0.06	10.3 ± 0.14^{b}

Results are expressed as mean ± SEM and analyzed by one way ANOVA followed by Tukey's post-hoc test, $p < 0.05$. [a] significantly different from control negative group. [b] significantly different from CYC-treated group. MDA: malondialdehyde, CYC: cyclophosphamide, EPS: Exopolysaccharide, EA: Ellagic acid.

Table 4
Effect of 1,8-cineole, EPS and EA on hepatic and cardiac contents of TGF-β.

Groups	Hepatic TGF-β (pg/g tissue)	Cardiac TGF-β1 (pg/g tissue)
Control negative	148.4 ± 10.4^{b}	139.6 ± 1.0^{b}
CYC-treated	568.9 ± 20.1^{a}	240.1 ± 5.6^{a}
1,8-Cineole	98.8 ± 6.8^{b}	159.0 ± 5.3^{b}
EPS	232.8 ± 16.7^{ab}	177.5 ± 11.6^{ab}
EA	282.8 ± 16.3^{ab}	203.7 ± 8.3^{ab}

Results are expressed as mean ± SEM and analyzed by one way ANOVA followed by Tukey's post-hoc test, $p < 0.05$. [a] significantly different from control negative group. [b] significantly different from CYC-treated group. TGF-β1 transforming growth factor-beta, CYC: cyclophosphamide, EPS: Exopolysaccharide, EA: Ellagic acid.

with distilled water then washed by acetone and dehydrated by ether and dried at 50 °C. The yield of EPS was determined by Dubois method (Dubois et al., 1956).

2.2.3. Identification of the promising streptomycete isolate

2.2.3.1. Morphological characterization. The spore chain morphology and the number of spores per chain of the strains of 14 day old cultures grown on inorganic salts-starch agar were examined by light microscope (Shirling, and Gottlieb, 1966). The spore surface was examined using Em10 Carl-Zeiss transmission electron microscope (Tresner et al., 1961).

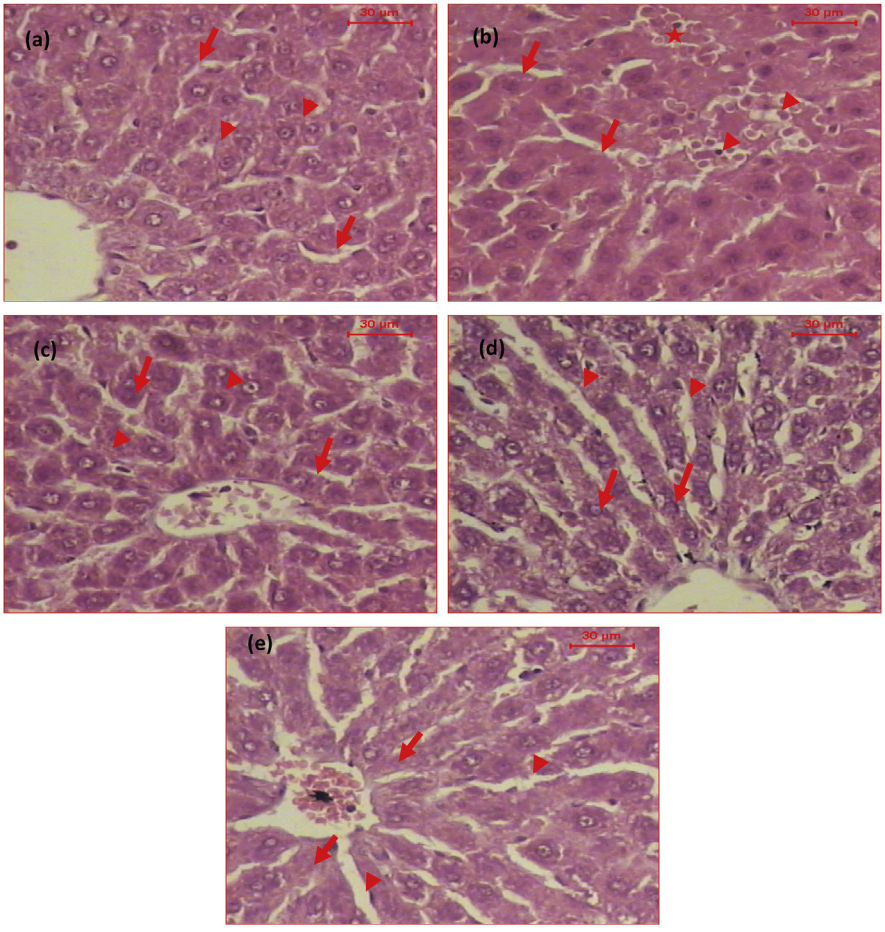

Fig. 5. (a) A section in the liver of normal group showing normal sinusoidal space (arrow) and normal hepatocytes (arrowhead) with polygonal shape, granulated cytoplasm and central nuclei; (b) A section of the liver of cyclophosphamide-treated group showing dilated and congested sinusoidal space (arrow), lymphocyte between hepatocytes (arrowhead) and necrotic small hepatocyte (asterisk); (c) A section in the liver of a cineole and cyclophosphamide group showing normal sinusoidal space (arrow) and normal hepatocyte with polygonal shape arrowhead); (d) A section in the liver of a polysaccharide and cyclophosphamide group showing normal hepatocellular architecture depicts hepatocytes (arrow) radiating from the central vein and congested sinusoidal space (arrowhead); (e) A section in the liver of ellagic acid and cyclophosphamide group showing normal hepatocellular architecture depicts hepatocytes (arrow) radiating from the central vein and congested sinusoidal space (arrowhead) (H&E; Scale Bar 30 µm).

2.2.3.2. Cultural characterization. The cultural characteristics of the strains were tested on the basis of the methods used in the International Streptomyces Project (ISP), using the media recommended by Shirling and Gottlieb (1966). The colours of mature sporulating aerial mycelium and substrate mycelium were monitored for 7, 14- and 21-day old cultures grown on starch nitrate medium. Diffusible pigments were detected on glycerol asparagine agar medium. Color determination was carried out using ISCC-NBS color charts (Kenneth, 1958).

2.2.3.3. Physiological and biochemical characterization. Physiological and biochemical characterization were determined according to the methods given by several authors as follows: (i) melanin pigment production and utilization of different carbon sources (Shirling and Gottlieb, 1966), (ii) nitrate reduction (Gordon, 1966), (iii) pectinase (Hankin et al., 1971) and (iv) hydrogen sulphide production (Cowan and Steel, 1974).

2.2.4. Fourier-transform infrared spectroscopy (FTIR)

The EPS was mixed with KBr powder, was ground and was pressed into a 1 mm pellets for FTIR measurements in the range of 400–4000 cm^{-1} on a Bucker scientific 500-IR Spectrophotometer (Ray, 2006).

2.2.5. Chemical analysis

Uronic acids were determined at 525 nm by the m-hydroxybiphenyl colorimetric method (Filisetti-Cozzi and Carpita, 1991). Sulfate was determined using the turbidly method (Dodgson and Price, 1962) and protein was determined using the Bradford method (Bradford, 1976). The monosaccharide composition was determined by HPLC on shim pack SCR-101N column (Shimadzu) with water deionized as the mobile phase at 0.5 mL/min (Liu et al., 2002).

2.2.6. Molecular weight determination

The molecular weight of BAEPS was determined to an Agilent 1100 HPLC with a refractive index detector (RID), Water Company Ireland according to Jun et al. (2006).

2.2.7. Antioxidant determination

The free radical scavenging activity of EPS was estimated by 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to Yang et al. (2008).

2.3. Animals

Male Wistar rats weighing between 130 ± 150 g were procured from the Animal House Colony at the National Research Centre (NRC), Egypt. The rats were adapted to the laboratory environment 2 weeks prior to the study. All animals were housed under standard conditions at (26 ± 2) °C, 12 h day and night cycle, and with food and water *ad libitum*. The investigation conforms to the guide for the care and use of laboratory animals according to the ethical guidelines of the institutional Animal Ethical Committee of the National Research Centre and according to the protocol approved by the NRC Ethics Committee (approval number: MREC-16-383).

2.4. Study design

Animals were randomly allocated into five groups:

Group 1: received normal saline (1 mL/kg) and considered control negative.

Group 2: received CYC (50 mg/kg, i.p.) for 3 consecutive days (Abdallah et al., 2015) and assigned as cyclophosphamide group.

Group 3: received CYC + 1, 8-cineole (100 mg/kg, p.o.) (Caldas et al.,

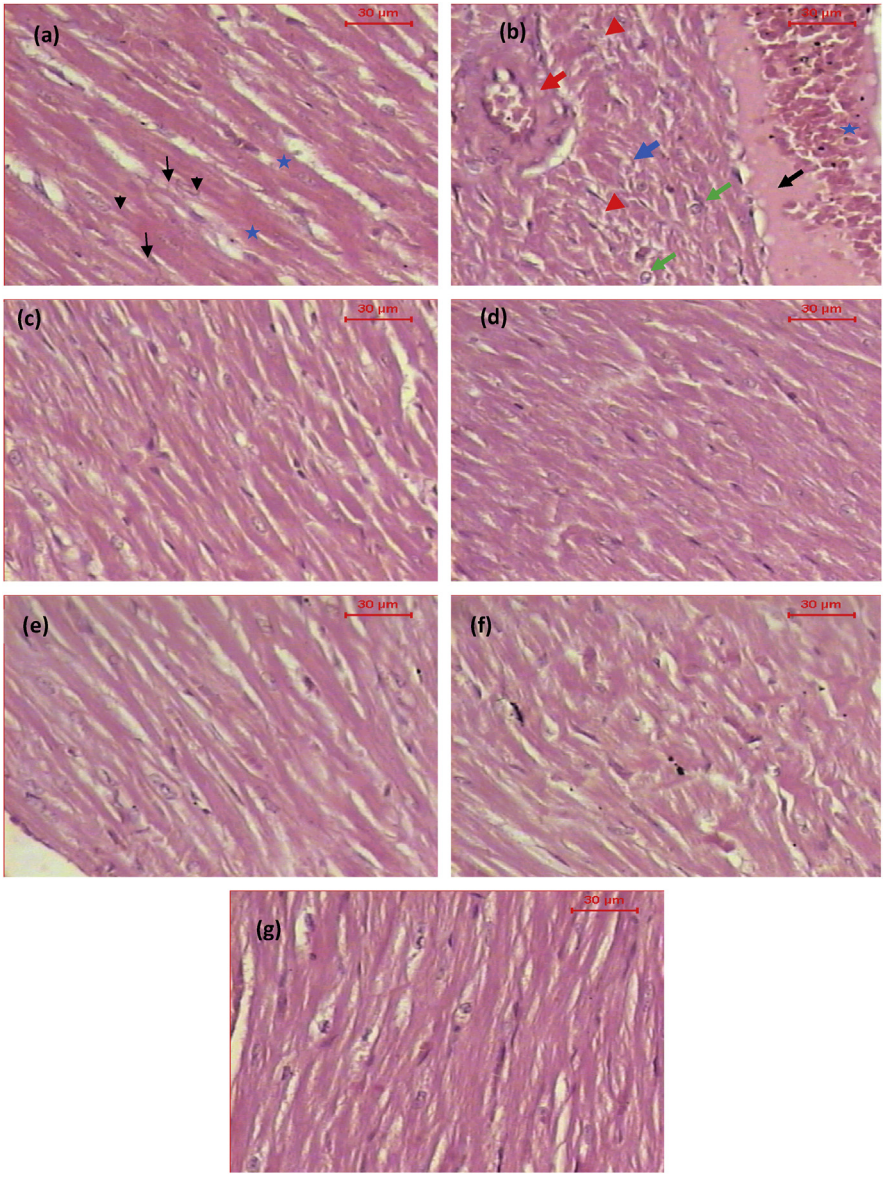

Fig. 6. (a) Longitudinal section of the cardiac muscle of a control animal showing normal branched striated cardiac myocytes with intact intercalated discs (arrows), faintly acidophilic sarcoplasm (asterisk) and centrally-located oval nuclei; (b) Longitudinal section of the cardiac muscles of a rat given cyclophosphamide showing disarrangement of cardiac myocytes, cytoplasmic vacuolation (arrow head), degeneration of muscle fibers (blue arrows), pyknotic nuclei (thin arrows), dilatation and congestion of blood vessels (arrow). Interstitial edema (black arrow) and extravasation of erythrocytes (asterisk) without infiltration of inflammatory cells nor lymphoma cells and edema; (c) Longitudinal section of the cardiac muscle of a rat given a cineole and cyclophosphamide showing relatively normal appearance of most cardiac fibers with small areas of vacuolated sarcoplasm (arrow); (d) Longitudinal section of the cardiac muscles of a rat given a cineole and cyclophosphamide showing disarrangement of cardiac myocytes; (e) Longitudinal section of the cardiac muscles of a rat given a polysaccharide and cyclophosphamide showing relatively normal appearance of most cardiac fibers; (f) Longitudinal section of the cardiac muscle of a rat given a polysaccharide and cyclophosphamide showing injured myocytes with scattered coagulative changes and thin bands of contraction necrosis; (g) Longitudinal section of the cardiac muscle of a rat given ellagic acid and cyclophosphamide showing normal histological appearance of cardiac myofibres and normal nuclei (H&E; Scale Bar 30 μm).

2015).

Group 4: received CYC + EPS (100 mg/kg, p.o.) (Mahmoud et al., 2016).

Group 5: received CYC + EA (60 mg/kg, p.o.) (Umesalma and Sudhandiran, 2011).

The drugs (1,8-cineole, EPS and EA) were administered for 7 days before beginning of CYC injection and lasted for other 3 days during CYC administration. At the end of the experiment, Serum samples were obtained as mentioned above and kept at −4 °C. Thereafter, animals were sacrificed; liver and heart tissues were dissected and homogenized. The obtained liver and heart homogenates were kept at −80 °C for further biochemical analysis. Aliquots of liver and heart tissues were fixed in formalin solution till histopathological examination.

2.4.1. Electrocardiogram (ECG) analysis

All the rats were anesthetized. A standard ECG was measured at the extremities, recorded (1 min), using a computer-based ECG device employing subcutaneous needle electrodes. The recordings were stored in a computer database using CardioSoft ECG software (Huston, Texas, USA).

2.4.2. Biochemical analysis

Colorimetrical determination of serum transaminases (aspartate aminotransferase, AST, and alanine aminotransferase, ALT), and the cadiotoxicity markers; lactate dehydrogenase (LDH) and creatine kinase (CK) were assessed using commercially available kits (Biodiagnostic, Egypt) for AST, ALT, LDH and (Salucea, Netherlands) for creatine kinase. Moreover, oxidative stress biomarkers; reduced glutathione (GSH) and malondialdehyde (MDA) were determined in liver and heart homogenates. Transforming growth factor-beta 1 (TGF-β1) was determined using ELISA kit (RayBio, USA) for rat in liver and heart homogenate.

2.4.3. Histopathological examination

At the end of the experiment, all rats were dissected. Liver and heart were excised out and instantaneously fixed in 10% formal saline for 24 hours (El-Alfy et al., 2012). The samples were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax. Sections of 6 μm thicknesses were prepared and stained with Haematoxylin and Eosin (H&E). The cytoplasm stained shades of pink and red and the nuclei gave blue color (Drury et al., 1976).

Semi-quantitative evaluation of H&E stained sections was done using light microscope (Leica QWin 500 Image Processing System; Cambridge,

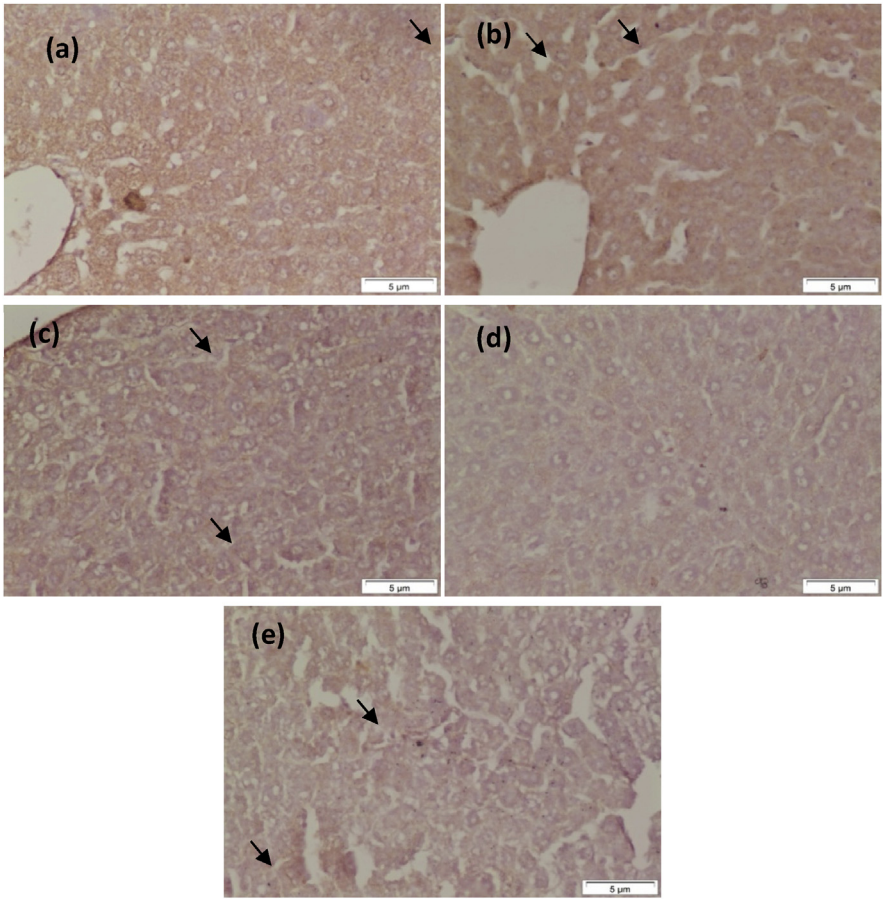

Fig. 7. Caspase-3 immunohistochemistry of a liver from (a) control rat showing caspase-3-positive cells are rarely present, (b) rat treated with cyclophosphamide (CYC) showing slightly number of positive hepatocytes suggesting a slight increase in apoptosis as compared to control animals (arrows), (c) rat treated with cinecole (Cin) + CYC showing slight decrease of caspase-3-positive cells (arrows), (d) rat treated with ellagic acid (EA) + CYC showing weak cytoplasmic and patchy weak nuclear (arrows) caspase 3 expression in the apoptotic hepatocytes as compared to control rats, (e) a rat treated with expolysaccharride (EPS) + CYC showing slight decrease of caspase-3-positive cells as compared to control rats (arrows) (Scale bar = 5 µm).

UK) to examine liver and heart damages. The scoring was used to evaluate the severity of hepatic injury according to the degree of sinusoidal dilatation, inflammatory cell infiltration, congestion, degeneration and cytoplasmic vacuolization. It was done as 0 (normal), 1 (mild), 2 (moderate), or 3 (severe) (Akbulut et al., 2014).

Concerning myocardial damage and its severity, the sections were given scores as follows: no changes = 0; mild = (myocytes damage or small multifocal degeneration with slight degree of inflammation); moderate = (extensive myofibrillar degeneration); marked = (necrosis with diffuse inflammatory process) (Zingarelli et al., 1998).

2.4.4. Immunohistochemical analysis

The immunohistochemical analysis of caspase-3 and p53 content of the hepatic and cardiac tissues were performed according to the methods described by Abdel-Rahman et al. (2017).

2.4.5. Statistical analysis

Results are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by Tukey's multiple comparisons post-hoc test.

3. Results

3.1. Production of EPS and identification of the promising isolate

Fifteen isolates were obtained from Suez Canal, (Ismailia governorate, Egypt). Four of them have the ability to produced EPS. The highest production (8.854 g/L) was chosen for further investigation strain no. 4. According to its characteristic as seen in Table 1 it was identified as *Streptomyces xiamenensis*.

3.2. Characterization of EPS

EPS produced by *Streptomyces xiamenensis* showed negative response to Bradford method indicating the absence of protein. The uronic acid was 69.2% indicating that the produced EPS was acidic while the sulphate content was 23.11%. The HPLC analysis of monosugars revealed that EPS was composed of glucuronic, galacturonic, glucose, xylose, mannose and galactose with molar ratio 31.6: 17.37: 1.51: 1.48: 1.47:1 individually. The weight average molecular weight (*Mw*), number average of molecular weights (*Mn*) and polydispersity (*Mw/Mn*) of the EPS were 4.65×10^4 g/mol, 3.82×10^4 g/mol, 1.217 respectively.

The FT-IR of EPS revealed characteristic functional groups. Stretching bands at 3500–3700 cm^{-1} are probably associated with the –OH. The absorption band at 1655.12 cm^{-1} indicating COO-, while band at 1710.31 cm^{-1} demonstrate C=O. The band at 1237.62 cm^{-1} showed S=O. Moreover, the band at 895 demonstrates β -glycosidic linkage.

3.3. Antioxidant determination

The DPPH radical-scavenging activity of EPS was illustrated in Fig. 1, from which it was obvious that the EPS exhibits scavenging efficiency with EC_{50} value of (68.54 µg/mL).

3.4. Effect on electrocardiography (ECG)

The normal rats were presented with a regular ECG pattern with defined P, QRS, and T waves (Fig. 2a).

Three days i.p. administration of CYC showed a marked fall in the QRS complex voltage (a measure of ventricular contraction), prolongation of the QT interval (a measure of ventricular repolarization), prolongation in the Tp-Te interval (a measure of transmural dispersion of

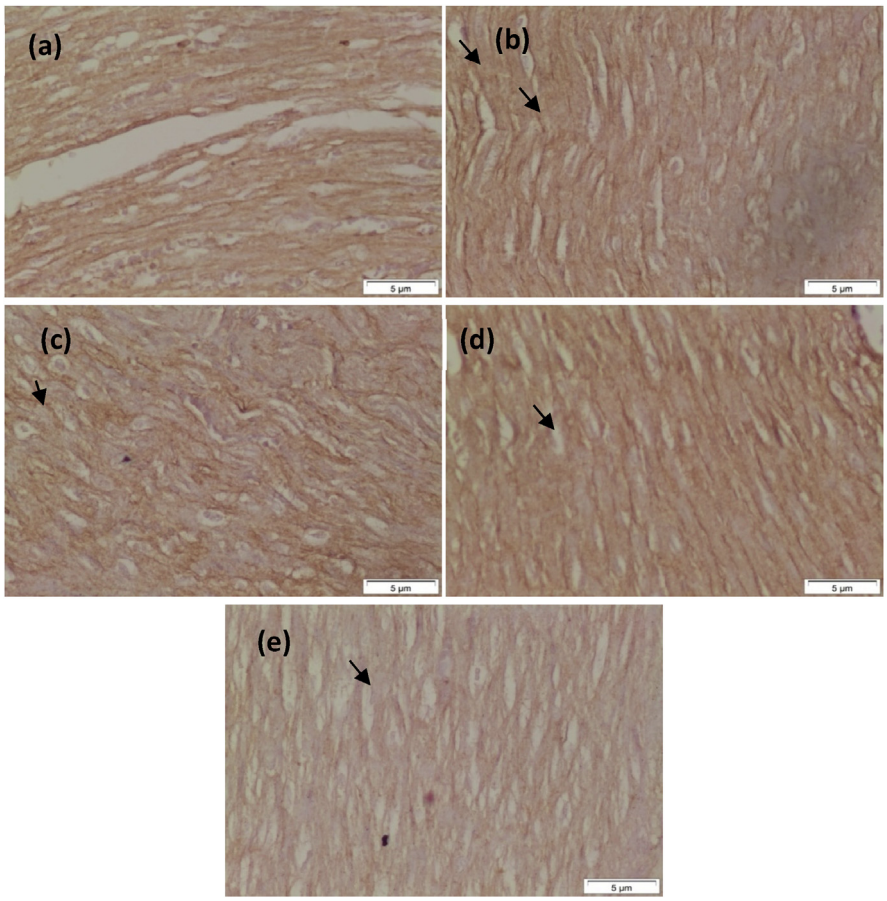

Fig. 8. Caspase-3 immunohistochemistry of cardiomyocytes from (a) control rat showing caspase-3-positive cells are rarely present, (b) rat treated with cyclophosphamide (CYC) showing strong expression of caspase-3 as compared to control, (c, d, e) the expression of cardiomyocytes of caspase-3 reveal reductions in rats treated with cinecole (Cin) + CYC, ellagic acid (EA) + CYC and expolysaccharride (EPS) + CYC, respectively (Scale bar = 5 µm).

repolarization) as well as ST- segment abnormalities compared with the normal control group (Fig. 2b).

However, no significant changes were observed on the intra-atrial conduction (P wave duration), the atrioventricular conduction (P-R interval), as well as on the heart rate by the administration of CYC.

Treatment with cineole (100 mg/kg) and EA (60 mg/kg) markedly succeeded in restoring the prolonged QT and Tp-Te in spite of that there was no significant changes were observed on the QRS complex (Fig. 2c, d).

However, treatment of rats with EPS was able to shorten the prolonged QT and Tp-Te intervals, and succeeded in restoring the depressed QRS complex (Fig. 2e).

3.5. Effect on liver enzymes (AST and ALT)

CYC (50 mg/kg) injection for three days significantly increased serum ALT activity (Fig. 3a) by about 2 folds; however, no significant change was noticed in serum AST activity (Fig. 3b). Treatment with EPS caused non-significant decrease in serum ALT activity. However, treatment with 1,8-cineole (100 mg/kg) and EA (60 mg/kg) significantly decreased ALT activity by 16 and 20%; respectively as compared with CYC-treated group.

3.6. Effect on cardiotoxicity markers (LDH and CK)

Injection of CYC for three days resulted in a significant increase (about 2-fold) in the serum activity of LDH as compared to the control negative group (Fig. 4a). Treatment with the three natural products significantly decreased LDH activity as compared to CYC-treated group by 51, 46 and 42% for 1,8-cineole, EPS and EA; respectively.

CYC also significantly increased serum activity of CK as compared to normal control group. However, treatment with 1,8-cineole and EA significantly decreased CK activity by 36% and 41% as compared to CYC-treated group (Fig. 4b).

3.7. Oxidative stress biomarkers (GSH and MDA) in liver and heart tissues

CYC injection caused prominent and significant depletion of hepatic and cardiac contents of GSH as compared to the normal control group. Treatment with 1,8-cineole and EPS re-normalized GSH contents in liver (Table 2). EA treatment increased liver GSH content by 36% above the normal level. In heart tissue, 1,8-cineole treatment could significantly increase GSH content as compared to CYC-treated group (Table 2).

Measurement of MDA as an indicator of lipid peroxidation revealed that CYC significantly increased lipid peroxidation in heart tissue as evidenced by increased MDA content. However, the investigated compounds could significantly reduce cardiac MDA as compared to CYC-treated group. Hepatic MDA content did show significant difference in all statistically analyzed groups (Table 3).

3.8. Effect on TGF-β1 in liver and heart homogenate

CYC induced a 4-fold and a 2-fold increase in TGF-β1 content in liver and heart tissues; respectively as compared to control negative group. Treatment with 1,8-cineole, EPS and EA significantly decreased hepatic TGF-β1 by 82, 59 and 50%; respectively and cardiac TGF-β1 by 34, 26 and 15% for the three agents respectively as compared to CYC-treated group (Table 4).

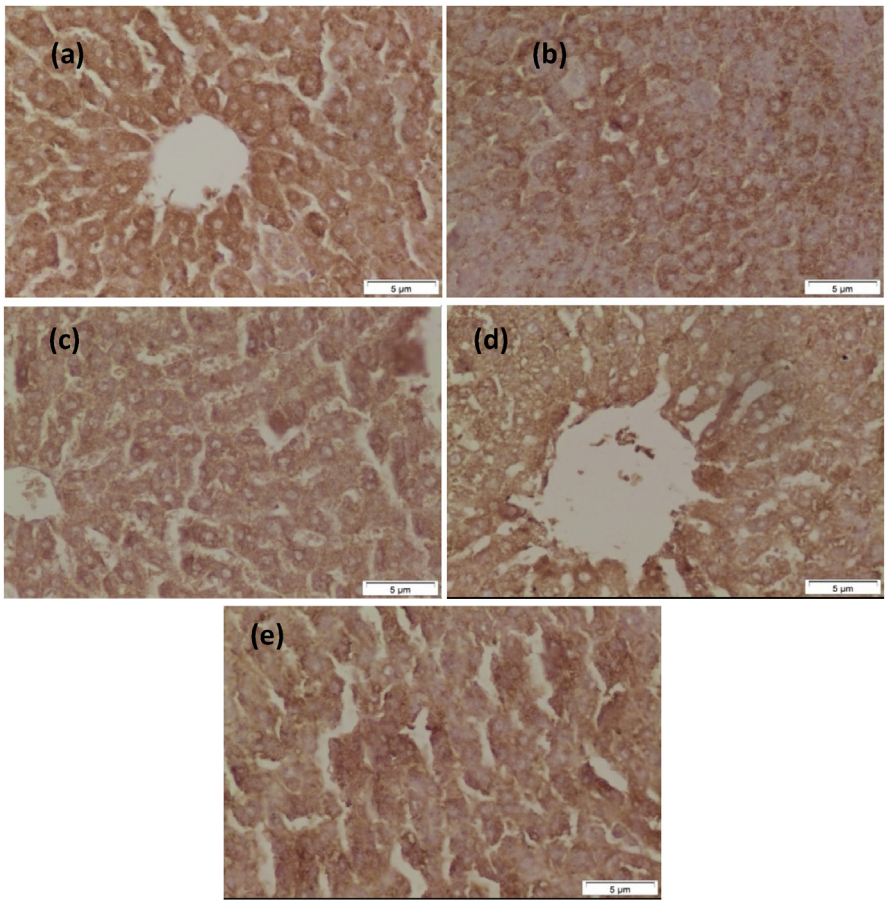

Fig. 9. p53 Immunohistochemistry in the liver sections from (a) rat of control showing few weakly stained nuclei of the hepatocytes, (b) rat of cyclophosphamide (CYC) group showing strong increase in p53-positive cells, (c, d, e) of cinecole (Cin) + CYC, ellagic acid (EA) + CYC and expolysaccharide (EPS) + CYC treated rats, respectively, showing reduction expression of both p53 as compared with cyclophosphamide (CYC) treated rat (Scale bar = 5 µm).

3.9. Histopathological findings

Microscopic examination of liver of control group shows the hepatic lobules formed of cords of single row of hepatocytes, which are polyhedral cells with one or rarely two large spherical nuclei and a granular eosinophilic cytoplasm and blood sinusoids in-between separated from hepatocytes by endothelial cells (Fig. 5a).

Histopathological investigation of liver of cyclophosphamide treated group revealed sever score, it showed dilated and congested portal tracts, with dilation and congestion of sinusoidal spaces, lymphocyte between hepatocytes and small necrotic area of hepatocytes (Fig. 5b).

Sections in the liver of a cineole and cyclophosphamide group revealed mild score, showing normal sinusoidal spaces and normal hepatocytes with polygonal shape (Fig. 5c). Sections in the liver of exopolysaccharide and cyclophosphamide group showed mild score, with normal hepatocellular architecture and congested sinusoidal spaces (Fig. 5d). Ellagic acid and cyclophosphamide treatment revealed mild score, with normal hepatocellular architecture depicts hepatocytes and congested sinusoidal spaces (Fig. 5e).

Likewise, histological examinations of control rats group indicate the normal structure of cardiac muscles. These muscles are striated, arranged in a linear array that branches and anatomizes in a specific pattern giving the appearance of a sheet. The cardiac muscle fibers joined by intercalated discs. They contain faintly acidophilic cytoplasm and centrally oval nuclei (Fig. 6a).

The rats of cyclophosphamide group showed disarrangement of cardiac myocyte, cytoplasmic vacuolation, and degeneration of muscle fibers, pyknotic nuclei, dilatation and congestion of blood vessels (sever score). The interstitial edema and extravasation of erythrocytes without infiltration inflammatory cells or lymphoma cells were noticed (Fig. 6b). Histopathological investigation of sections of the cardiac muscle of rats given cineole and cyclophosphamide showed mild score, with relatively normal appearance of most cardiac fibers with small areas of vacuolated sarcoplasm (Fig. 6c). In some rats, the cardiac muscles showed disarrangement of cardiac myocytes (Fig. 6d). Microscopic examination of sections of the cardiac muscle of rats given EPS and cyclophosphamide showed mild score, with relatively normal appearance of most cardiac fibers (Fig. 6e). In some cases, injured myocytes with scattered coagulative changes and thin bands of contraction necrosis were found (Fig. 6f). On the other hand, sections of the cardiac muscles of rats given ellagic acid and cyclophosphamide showed mild score, with normal histological appearance of cardiac myofibres and normal nuclei (Fig. 6g).

3.10. Immunohistochemistry of caspase-3

Liver of control negative group showed very occasional weak nuclear and cytoplasmic expression (Fig. 7a). In rats treated with cyclophosphamide (CYC), slightly numbers of positive hepatocytes suggesting a slight increase in apoptosis as compared to control animals (Fig. 7b). On the hand, slight decreases of caspase-3-positive cells of a liver from rats treated with ceinecole (Cin) + CYC (Fig. 7c). In rats treated with ellagic acid (EA), weak cytoplasmic and patchy weak nuclear caspase-3 expression in the apoptotic hepatocytes were found as compared to control rats (Fig. 7d). However, slight decrease of caspase-3-positive cells of rats treated with expolysaccharide (EPS) + CYC were present as compared to control rats (Fig. 7e).

Positive expression of caspase-3 is rarely detected in heart tissues from the control group with very week staining (Fig. 8a). However, the

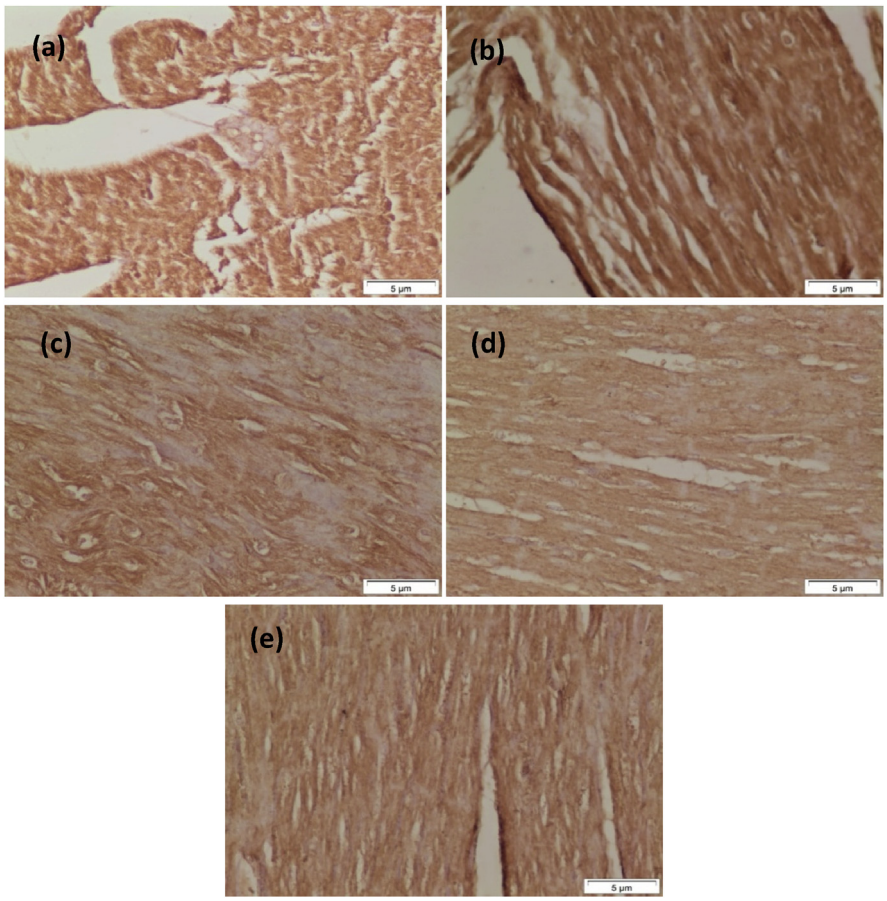

Fig. 10. p53 Immunohistochemistry in the heart sections from (a) rat of control showing weakly stained nuclei of the cardiomyocytes, (b) rat of cyclophosphamide (CYC) group showing very strong reaction in p53-positive cells, (c, d, e) of cinecole (Cin) + CYC, ellagic acid (EA) + CYC and expolysaccharride (EPS) + CYC treated rats, respectively, showing reduction expression of p53 as compared with cyclophosphamide (CYC) treated rat (Scale bar = 5 μm).

cyclophosphamide (CYC) group exhibited a strong expression of caspase-3 in the heart tissues as compared with controls group. Compared to the cyclophosphamide (CYC) group (Fig. 8b), the expression of cardiomyocytes caspase-3 revealed reductions in the group treated with cinecole (Cin) + CYC, ellagic acid (EA) + CYC and expolysaccharride (EPS) + CYC (Fig. 8c, d, e).

3.11. *Immunohistochemistry of p53*

The expression of the p53 was examined immunohistochemically in the rat liver of all the groups. The liver sections of negative control rats showed few positive cells (Fig. 9a). While, the expression of both p53 in cyclophosphamide (CYC) treatment led to strong increase in p53- positive cells (Fig. 9b); these expression levels were significantly reduced by cinecole (Cin), ellagic acid (EA) and expolysaccharride (EPS) treatment (Fig. 9c, d, e).

Immunohistochemistry of p53 in the heart sections from rat of control showing weakly stained nuclei of the (Fig. 10a). Rats of cyclophosphamide (CYC) group showing very strong reaction in p53- cardiomyocytes (Fig. 10b). Cinecole (Cin) + CYC, ellagic acid (EA) + CYC and expolysaccharride (EPS) + CYC treatment revealed a reduction in the expression of p53 as compared with cyclophosphamide (CYC) (Fig. 10c, d, e).

4. Discussion

Screening for natural substances with chemopreventive properties in order to reduce the negative effects of chemotherapeutic drugs is very urgent (Selim et al., 2018). So in this study three different natural product were used against chemotherapy-induced organ toxicity.

In the current study CYC injection caused significant elevation in serum ALT and decreased GSH content in liver tissue. In parallel, previous reports have described CYC-induced hepatotoxicity. CYC elevated circulating liver marker enzymes and produced a marked depression in antioxidant defense mechanism which was described as a major mechanism of CYC-induced hepatic damage. DNA damage is another proposed mechanism for CYC hepatotoxicity (Tuorkey, 2017). CYC could induce inflammation in liver tissue via up regulation of pro-inflammatory cytokines (Mahmoud et al., 2017). 1,8-cineole and EA could significantly decrease ALT activity. All the investigated drugs; 1,8-cineole, EA and EPS increased GSH content in the liver tissue indicating improvement in the endogenous antioxidant potential. Where EPS plays an important role as antioxidant, anti-inflammatory (Gu et al., 2017; Li et al., 2018). This role is because of the molecular weight and the structure of EPS where the presence of several units of glucuronic, galacturonic, glucose, xylose, mannose and galactose with uronic and sulphate groups increased its activity as antioxidant, anti-inflammatory and an antitumor agent. Li et al. (2018) stated that marine sulphated EPS is an important antitumor agent.

ECG pattern showed significant abnormalities upon injection of CYC such as marked fall in the QRS complex voltage (a measure of ventricular contraction), prolongation of the QT interval (a measure of ventricular repolarization), prolongation in the Tp-Te interval (a measure of transmural dispersion of repolarization) as well as ST- segment abnormalities. In agreement with current results, previous studies also have shown the abnormal changes in electrocardiogram (ECG) due to CYC injection such as increase in heart rate (HR) which was accompanied by transient decrease in QRS duration (Ogunsanwo et al., 2017). In addition, increase in QTc interval, increase in R–R interval, and decrease in the R wave

voltage were also described for CYC cardiotoxicity. These cardiotoxic signs were accompanied by depressed antioxidant defense mechanisms, increased inflammatory cytokines and apoptotic markers (El-Agamy et al., 2017). Administration of 1,8-cineole and EA improved ECG pattern, whereas, EPS treatment showed normalization of ECG compartments such as restoration of the depressed QRS complex. This indicates the cardioprotective effect of the investigated compounds.

Injection of CYC (50 mg/kg, i.p.) for three days also increased serum enzyme markers of cardiotoxicity as indicated by increased LDH and CK activities. It increased oxidative stress status in the heart tissue as reflected by decreased GSH content. CYC-induced cardiotoxicity has long been described and still represents a major clinical problem that faces its chemotherapeutic efficacy (Bhatt et al., 2017). The precise mechanism by which CYC induces cardiotoxicity is still under investigation. Suppression of antioxidants and alterations in lipid metabolism such as hypercholesterolemia, hypertriglyceridemia and decreased secretion of cardiac lipoprotein lipase are supposed mechanisms. This leads to lipotoxic cardiomyopathy and myocardial damage (Chakraborty et al., 2014). CYC is also claimed to be a direct cardiotoxic agent that causes endothelial damage and cardiomyocytes destruction resulting in release of CK-MB and LDH into the blood stream (Shanmugarajan et al., 2008).

1,8-Cineole and EA treatment attenuated the release of cardiotoxicity indices into the blood. EPS significantly decreased the activity of LDH enzyme. All these three natural products improved the abnormalities in ECG, increased the endogenous antioxidant; GSH and decreased lipid peroxidation that was induced by CYC as evidenced by decreased cardiac MDA contents. Collectively, this designates the ability of the investigated natural products to maintain the structural integrity of the heart, minimize cardiac damage and improve heart function demonstrating the cardioprotective effects of 1,8-cineole and EA against CYC -induced cardiotoxicity.

Transforming growth factor-beta (TGF-β1) is one of four types which belong to transforming growth factor superfamily of cytokines. The family of TGF-β is a pleiotropic and multifunctional cytokine which plays a major role in many diseases. TGF-β is a powerful cytokine that initiates and terminates tissue repair (Morikawa et al., 2016). TGF-β1 mRNA is expressed in hematopoietic, endothelial and connective tissue cells. Thus, it is the most implicated isoform in tissue fibrosis, including heart, kidney, liver and lung fibrosis (Border and Noble, 1994). It is produced by hepatic stellate cells in response to acute or chronic liver damage (Bissell et al., 2001).

The crosslink between TGF- β1 and hepatotoxicity has been demonstrated. It was found that TGF-beta β1 produced a loss of cell viability in hepatocytes, increased production of depletion of GSH and intracellular ROS, resulting in mitochondrial membrane damage and loss of membrane potential, which is followed by apoptosis (Zhuge and Cederbaum, 2006).

In addition, TGF-β1 stimulated collagen synthesis and participated in the remodeling process in heart failure. Moreover, its expression was increased in myocardium during cardiac hypertrophy. Together, it has been accepted that TGF-β1 is a regulator of extracellular matrix (ECM) composition in cardiac fibroblasts (Dobaczewski et al., 2011; Johnston and Gillis, 2017).

In parallel and in the shadow of mediating tissue injury, hepatic and cardiac TGF-β1 expression was increased in the present study due to CYC injection. Treatment with the 1,8-cineole, EPS and EA markedly decreased TGF-β1 contents in liver and heart tissues indicating their preventive effect on propagation of tissue injury. Previous studies have pointed to the protective effect of EA on ethanol-induced toxicity mediated by regulation of TGF-β1 production in liver cells (Sohn et al., 2013). Similarly, compounds that possessed cardioprotective effects could inhibit ROS generation and suppress cell apoptosis via blockade of TGF-β1 signaling pathway (Wang et al., 2017).

More interestingly, TGF-β1 level was found to be closely linked to tumor prognosis. In the early stages of carcinogenesis, TGF-β1 operates as a tumor suppressor factor. This is due to its anti-proliferative and pro-apoptotic effects (Padua and Massagué, 2009). Conversely, in advanced stages, TGF-β1 operates as tumor promoter, as cancer cells become refractory to its growth inhibitory effects. This renders TGF-β1 a useful biomarker for cancer prognosis and also a predictor of cancer recurrence (Principe et al., 2014). TGF-β1 is a hallmark of hepatocellular carcinoma (HCC) and represents one of the most important pathways to be targeted by anticancer drugs. TGF-β1 induces the epithelial-mesenchymal transition, which is crucial in the development of hepatocarcinogenesis and HCC metastasis (Ru et al., 2015).

Being effective in restoring TGF-β1 levels, the investigated natural products are suggested to be candidates for upcoming studies on treatment of liver cancer and reduction of chemotherapeutic agents' organ toxicity.

The immunostaining for apoptosis marker, caspase-3 was markedly elevated in CYC group compared to the control group in both hepatic and cardiac tissues. This finding is in line with previous findings which stated that CYC increased caspase-3 in cardiac, hepatic and renal tissues (El-Agamy et al., 2017; Caglayan et al., 2018). Previous reports declared that cardiomyopathy resulted from CYC is closely associated with its ability to induce apoptosis in cardiac tissue through DNA intercalation, activation of p53 protein and generation of ROS (Asiri, 2010).

Furthermore, p53 protein acts as a transcription factor and is a key role in regulating cell cycle progression and the activation of DNA repair. When an extensive damage occurs to the genetic material of cells; p53 is involved in the initiation of processes leading to programmed cell death, induction of pro-apoptotic genes and, finally, apoptosis (Chtourou et al., 2015).

P53 immunostaining significantly increased in CYC group as compared to control in both liver and heart. Administration of 1,8-cineole, EA and EPS decreased caspase-3 and p53 in liver and heart samples.

5. Conclusion

It could be concluded that cyclophosphamide injection produced marked hepatic and cardiac toxicity which was clearly evident through the ECG pattern, biochemical parameters and apoptosis markers. 1,8-cineole, exopolysaccharide and ellagic acid treatment showed beneficial hepatoprotective and cardioprotective effects against CYC-induced organ toxicity.

Declarations

Author contribution statement

Heba M.I. Abdallah: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Preparation and article reviewing.

Rehab F. Abdel-Rahman: Performed the experiments; Interpretation and drafting article and revising it critically; Wrote the paper.

Sally A. El-Awdan: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Rasha M. Allam: Performed the experiments; manuscript preparation.

Aliaa E.M.K. El-Mosallamy: Performed the experiments; Analyzed and interpreted the data.

Manal S. Selim: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sahar S. Mohamed: Contributed reagents, materials, analysis tools or data.

Mahmoud S. Arbid: Conceived and designed the experiments.

Abdel Razik H. Farrag: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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