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Doxorubicin-Induced Cardiotoxicity in Adult Male Albino Rats and the Ameliorative Effects of Achillea Fragrantissima Crude Extract, Its Combined Ethyl Acetate/n. Butanol Fraction, and Vitamin E

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- oxidative stress

Abstract

Doxorubicin (DOX) is a potent chemotherapeutic agent. Unfortunately, due to the incidence of dose-dependent cardiotoxicity, its clinical usage is hampered. Natural products are gaining popularity as a means of preventing DOX-induced cardiotoxicity. In the present study, we investigated the cardiotoxic effects of DOX and assessed the potential ameliorative effects of Achillea fragrantissima extract, its combined ethyl acetate/ *n*. butanol (EtOAc-Bu) fraction, and vitamin E in adult male albino rats. Rats were randomly divided into five groups: Control, DOX, DOX + A. fragrantissima crude extract, DOX + its (EtOAc-Bu) fraction, DOX + Vitamin E. A single dose of DOX (15 mg/kg) was given intraperitoneally (**ip**) in each group except the control. Rats were assessed before scarification for arterial blood pressure and ECG recordings. Serum and heart tissue were collected for biochemical, and histopathological assessment. Pre-treatment with vitamin E, *0*A. fragrantissima crude extract, and its (EtOAc-Bu) fraction ameliorated DOX-induced cardiotoxicity, in varying degrees and with varying significance, as evidenced by reversing the DOX-induced ECG changes, reducing serum cardiac enzymes, and improving DOX-induced histopathological changes. All the suggested protective measures, in varying degrees and with varying significance, reduced the oxidative stress, lipid peroxidation, and apoptosis markers (reduced NO, and MDA, down-regulated mRNA expression of both iNOS and caspase-3), and elevated the antioxidants markers (GR, SOD, and TAC). We concluded that such suggested natural products might offer a promising chemo-preventive approach against cardiotoxicity induced by DOX.

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INTRODUCTION

Anthracyclines are a class of cancer-fighting drugs. The survival rate of cancer patients has increased significantly as a result of this class of medications. Doxorubicin (DOX) is an anthracycline that is often recommended due to its potent anti-cancer properties. The anti-tumor activity of anthracycline depends on DNA damage. In this mechanism, doxorubicin interferes with DNA repair mediated by topoisomerase II-α, and cause accumulation of protein-linked DNA breaks, induction of apoptosis which ultimately result in DNA damage followed by cell death (1). Accordingly, it is frequently used as a first-line treatment for various malignancies, such as including sarcoma, lymphoma, multiple myeloma, leukemia, lung cancer, and breast cancer (1). Unfortunately, DOX clinical use is hindered by the occurrence of dose-dependent toxicity (2). The most common toxicity induced by DOX is cardiotoxicity (3). Such DOX-induced cardiotoxicity can be divided into three types: acute, early-onset chronic, and chronic progressive cardiotoxicity (1).

Many theories have been proposed to explain the mechanism of DOX-induced cardiotoxicity, including free radical generation, lipid peroxidation, inflammation, mitochondrial damage, and apoptosis (3). According to one of these concepts, DOX-induced cardiotoxicity is mostly caused by oxidative stress (4). The enzymes NADH dehydrogenase and cytochrome P-450 reductase convert doxorubicin to the semiquinone radical, which then forms a combination with iron to generate a DOX-iron (Fe^{+2}) free radical complex. This complex reduces oxygen to form superoxide and regenerate DOX; continued redox cycling culminates in the generation of hydrogen peroxide and the hydroxyl radical, which is one of the most dangerous chemical species. Eventually, the presence of this active radical causes lipid peroxidation, which damages the membrane structure and function irreversibly. Cellular enzymes are released, and oxygen delivery to the cardiac cell is decreased, resulting in hypoxic conditions (1). DOX-induced oxidative stress also promotes apoptosis either directly through localization in the mitochondria (extrinsic route) or via oxidative stress and excessive calcium buildup in the cell (intrinsic pathway). Activation of caspase-induced programmed cell death is the final stage in both pathways, and failure can occur as a result of either one (4–7). DOX can also increase nitric oxide (NO) production by stimulating the inducible nitric oxide synthase (iNOS) enzyme (8). As a result of low quantities of antioxidant enzymes in the heart, the oxidative stress generated by DOX is greater than in other organs (9). Detecting acute cardiotoxicity might be difficult due to the lack of a clear clinical manifestation. Despite this, there is evidence that early after DOX delivery, cardiac damage, in the form of ventricular dysfunction, begins (10). Acute cardiotoxicity is reversible, yet its beginning predicts the likelihood of subsequent heart failure. Subacute and chronic cardiotoxicity, on the other hand, is permanent and irreversible (8). Therefore, in case of acute DOX-induced cardiotoxicity, proposing a protective measure may be quite beneficial, figure (1). In the fight against DOX-induced cardiotoxicity, people are turning to natural products as a defense approach (11).

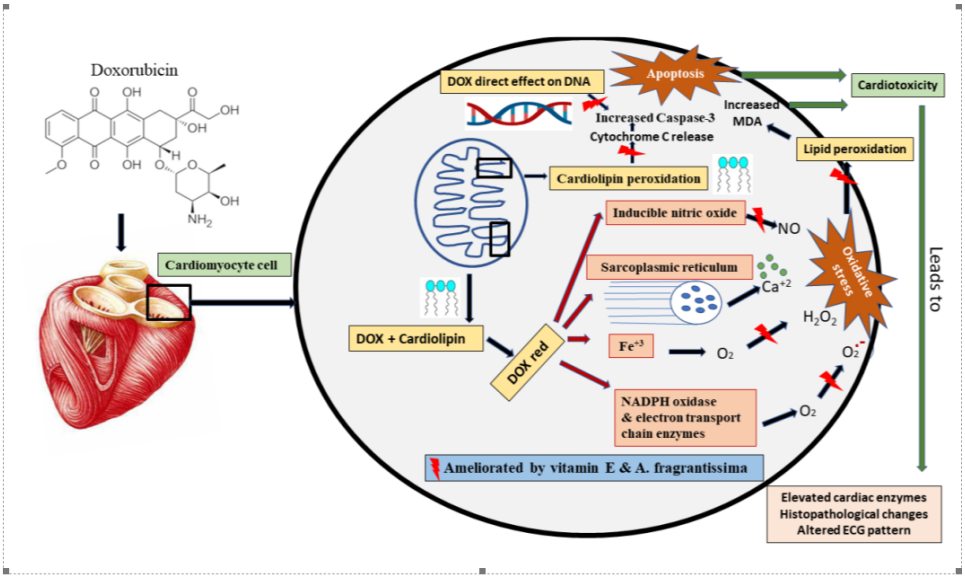

Figure (1) showing the mechanism **of Doxorubicin induced cardiotoxicity.**

Antioxidant compounds may help protect cells and tissues from the detrimental effects of free radicals caused by DOX (12). Incorporating vitamin E, which has antioxidant properties, may reduce DOX's damaging effect on cardiomyocytes without interfering with its ability to target cancer cells (13,14). Free radical scavenger activity in vitamin E protects cell membranes from free radical attack, which is an important first line of defense against lipid peroxidation. Polyunsaturated fats in cell membranes, which are critical for membrane structure and function, can also be protected by vitamin E (15). Additional benefits of vitamin E include maintaining healthy red blood cells, promoting better circulation and supporting healthy muscle and nervous system function (16).

Herbal medication is another option to guard against DOX-induced cardiotoxicity, as it may be more effective, less harmful, and less expensive in the long run than pharmaceuticals (17). Achillea (Asteraceae) is an important plant genus with immense ethnomedicinal uses. It comprises nearly 130 species that are vastly spread in Middle East and Eastern Europe regions(18,19). In Egypt, Achillea is represented by two species: Achillea fragrantissima (A. fragrantissima) (Forssk.) Sch. Bip. (Recognized as Qaysoom) and Achillea santolina (Recognized locally as Beatheran) (20). A. fragrantissima is a fragrant medicinal plant wildly grown in the Egyptian deserts especially in Sinai Peninsula (20,21). This plant is consumed by the Bedouins in the Arabian region for treatment of gastrointestinal disorders, arthritis, and fever(22,23). Plenty of studies have demonstrated that A. fragrantissima extracts had promising pharmacological activities such as strong antioxidant potential, anti-inflammatory, analgesic, anti-ulcerogenic activities, α glucosidase inhibitory effect, in addition to antidiabetic and hypolipidemic effects (18,24). Moreover, the anti-inflammatory and antioxidant functions of A. fragrantissima extract imply potential anticancer activity. A. fragrantissima also inhibits lipopolysaccharide (LPS) induced synthesis of nitric oxide (NO) and various proinflammatory mediators, like iNOS. A. fragrantissima extract can also induce differentiation, cell cycle arrest and apoptosis (25). Such diverse activities of the plant are correlated to its unique array of secondary

metabolites; particularly volatile oil, sesquiterpenoids and flavonoids (26,27).

Flavonoids are polyphenolic compounds known for their potent antioxidant activities arising from the hydroxy groups in their structure. These functional groups scavenge free radicals and/or chelate metal ions. Hence, Flavonoids as a dietary component have beneficial health-promoting properties. Several studies have shown that flavonoids help protect against cancer, cardiovascular disease, and other age-related diseases (28).

Based on the above-mentioned reasons, the aim of this study is to assess the protective abilities of *A.* fragrantissima crude extract, its (EtOAc-Bu) fraction, and vitamin E to ameliorate DOX-induced cardiotoxicity in adult male albino rats as manifested by electrocardiographic, biochemical, and histopathological changes.

2. Materials and Methods

2.1. Chemicals

Adricin (Doxorubicin HCL, 50 mg) vials were procured from EIMC United Pharmaceuticals, Badr City, Cairo, A.R.E. (Item number; 24995). Vitamin E capsules of yellow oily material (*α*-tocopherol, 1000 mg/ capsule) were purchased from Pharco Pharmaceuticals, Egypt. Spectrophotometric kits were purchased from diagnostic kits Biodiagnostic company, Giza, Egypt; and PCR kits were purchased from QIAGEN GmbH, Germany and willow fort, Birmingham, UK. Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich®, St. Louis, MO, USA.

2.2. Experimental animals

All experimental procedures were conducted in compliance with US National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and the Suez Canal University, Faculty of Medicine's Instituted Animal Care and Use Committee's guidelines.

Eighteen adult male albino rats were purchased from Ophthalmology Institute, Giza. The choice of albino rats was based on the similarity of their bodily reactions to human. Every 6 rats were placed in each cage. For all the rats, the environment was standardized. They were kept in plastic cages in a low-stress special room in a well-ventilated, clean and safe experimental animal house at Suez Canal University's Faculty of Medicine. Rats were fed rat pellets and had free access to water in a typical laboratory setting. Before being used in the experiment, rats were acclimatized to their surroundings for one week (room temperature; 12-hour light-dark cycle; moderate humidity [60 ± 5%]). Each rat was given a unique identification number (e.g., using a pen marker). Each cage had a card with the following information on it (Group name, number of the rats, name of the researcher).

2.3. A. fragrantissima plant Processing

2.3.1 Collection of A. fragrantissima plant

Aerial parts of A. fragrantissima were collected in June from the Sinai Peninsula in Egypt's El-Arbane valley. The plant was authenticated by a Botany professor at Suez Canal University's Faculty of Science. The plant was preserved in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Egypt (with the registration number Af-2006-1). The plant was gathered, left to dry at room temperature, and grinded.

2.3.2. Extraction and fractionation of A. fragrantissima

One and half kilogram of powdered A. fragrantissima was exhaustively extracted with 95% ethanol (EtOH) (2.5L, 48hr x 3). The combined extracts were vacuum concentrated at 40°C to produce a dark green residue (100gm).

Dried A. fragrantissima crude extract (80gm) was suspended in water before being extracted with n-hexane, chloroform, ethyl acetate, and n-butanol. All solvent extracts as well as the water-soluble fraction were evaporated to dryness under vacuum to afford 0.8 gm, 20.8 gm 5.28 gm and 7.36 gm of n-hexane (Hex.), chloroform ($CHCl_3$), ethyl acetate (EtoAc) and n-butanol (n- BuOH) fractions respectively and aqueous fraction was 25.84 gm. The ethyl acetate and n-butanol fractions exhibited similar TLC pattern therefore they were combined and called combined ethyl acetate/n. butanol fraction (EtOAc-Bu fraction).

2.4. Phytochemical investigations of A. fragrantissima crude extract and its fractions

The phytochemical analysis was done to choose the fraction with the highest total Phenolic Content (TPC), total flavonoid content (TFC), and in-vitro antioxidant activity to be used in the experimental study.

2.4.1. Phytochemical screening for the presence flavonoids in A. fragrantissima crude extract and its fractions

The crude A. fragrantissima extract and its fractions were screened for the presence of flavonoids according to the method described by ***Cock and Kukkonen 2011*** (29). In this method, 100 µL of aqueous sodium hydroxide was added to 1 ml of each extract. The presence of flavonoids was confirmed by the development of an intense yellow color which reversed to the original color of the extract by the addition of 100 µL of conc. HCl was added to the test solutions.

2.4.2. Estimation of TPC and TFC in A. fragrantissima crude extract and its fractions

These were quantified spectrophotometrically applying Folin-Ciocalteu's assay and AlCl3 method respectively (30,31).

2.4.3. Evaluation of invitro Antioxidant Activity of A. fragrantissima crude extract and its fractions

The invitro antioxidant activity of A. fragrantissima crude extract and its fractions was evaluated by three different methods: DPPH free radical scavenging activity, Ferric reducing antioxidant power (FRAP) and Total antioxidant capacity (TAC) according to the procedures mentioned in (30).

2.5. Experimental design

As shown in figure (2), adult male albino rats were assigned into five groups at random (n= 6) to help minimize bias: Group I (Control): was given distilled water (0.5 ml/kg) by gavage once/day for 10 days with a single dose of saline (0.5 ml/kg) on the 6th day intraperitoneally. Group II (DOX Group): was given distilled water (0.5 ml/kg) by gavage once/day for 10 days and a single dose of DOX (15 mg/kg) intraperitoneally (ip) on the 6th day of the experiment. Group III (DOX + A. fragrantissima crude extract): was given A. fragrantissima crude extract (300 mg/kg) by gavage once/day for 10 days and a single dose of DOX (15 mg/kg) intraperitoneally (ip) on the 6th day of the experiment. Group IV (DOX + A. fragrantissima EtOAc-Bu fraction): was given A. fragrantissima EtOAc-Bu fraction (300 mg/kg) by gavage once/day for 10 days and a single dose of DOX (15 mg/kg) intraperitoneally (ip) on the 6th day of the experiment. Group V (DOX + Vitamin E): was given Vitamin E (500 mg/kg) prepared in corn oil by gavage once/day for 10 days and a

single dose of DOX (15 mg/kg) intraperitoneally (ip) on the 6th day of the experiment.

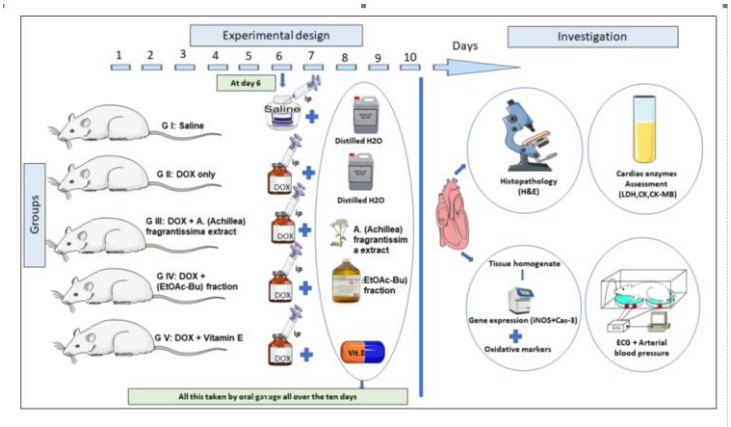

Figure (2) showing Schematic representation of the study protocol.

The DOX dose used in our study was chosen based on its induction of cardiotoxicity in rats as implicated by previous studies (32–39)

Vitamin E dose utilized in this study was based on earlier research showing that this dose is efficient in lowering lipid peroxidation and raising levels of antioxidant enzymes in experimental animal models (40–43).

According to previous studies, A. fragrantissima is a well-tolerated substance with a high margin of safety (44,45). Accordingly, the doses that were used in our study were considered benign.

A. fragrantissima EtOAc-Bu fraction was used based on our results of the phytochemical analysis as it showed the highest TPC, TFC, and in-vitro antioxidant activity as compared to other fractions.

2.6. Physiological assessment of the cardiovascular system

2.6.1 Electro-cardiography (ECG) assessment

Before sacrificing the rats, Electro-cardiography (ECG) recording was done using ECG 100C module, Biopac Acqknowledge software 4.0 (MP150 data acquisition system), allowing assessment of heart rate, cardiac cycle abnormalities, arrhythmias, myocardial ischemic signs, and ventricular hypertrophy. We corrected the QRS, QT and PR durations for their known dependency on heart rate change (46). Rate corrected ECG durations were assessed by calculating their percentage from the total cardiac cycle duration, as following:

Rate-corrected-QRS "cQRS" = (QRS duration in ms/RR interval in ms) x 100

Rate-corrected-QT "cQT" = (QT duration in ms/RR interval in ms) x 100

Rate-corrected-PR "cPR" = (PR duration in ms/RR interval in ms) x 100

Rats were weighed and anesthetized with a single intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). EL 405 needle electrodes were subcutaneously inserted to right arm, left arm, and right leg as V-, V+ and ground electrodes respectively, to record lead II ECG trace.

2.6.2. Assessment of Mean arterial blood pressure (MAP)

Experimental rats were assessed before scarification for Mean arterial blood pressure (MAP) using Biopac Acqknowledge software 4.0 (MP150 data acquisition system) using NIBP200A, and UIM100C units.

According to manufacturer instructions (BIOPAC Systems Inc., 42 Aero Camino, Goleta, CA 93117, USA, 2011), non-invasive assessment

of the rat blood pressure, using IRSENSOR cuff through which rat tail passed. Simultaneous recording of rat pulse, and pressure applied enabled measurement of systolic, diastolic, and mean ABP of the study rat.

2.7. Sample collection

At the end of the experiment, blood samples were drawn retro-orbitally from rats before they were slaughtered under anesthesia. The samples were kept at 4 °C for 6 hours before being centrifuged at 3000g for 10 minutes to obtain serum, which was then frozen at -80 °C for cardiac enzyme testing. Animals were then decapitated as a kind of sacrifice. In a matter of seconds, each animal's heart was extracted. A portion was stored at -20°C for the evaluation of oxidative stress biomarkers, another at -80°C for the step one real-time PCR and relative quantification technique of polymerase chain reaction (PCR), and the final portion was fixed in neutral-buffered formalin (10%) for histopathological investigation.

2.8. Biochemical analysis

2.8.1 Cardiac enzymes evaluation:

Serum was analysed for cardiac enzymes assessment. According to the manufacturer's instructions, serum creatine kinase (CK), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH) were evaluated using an automatic chemical analyser. The activity for serum heart enzymes is expressed as U/L.

2.8.2 Oxidant-Antioxidant biomarkers analysis:

Heart tissues were homogenized using 0.9% NaCl solution and centrifuged for 5 minutes by 3000g. Supernatants were used to evaluate the levels of Nitric oxide (NO), Lipid Peroxidation Marker, Malondialdehyde (MDA), Glutathione Reductase (GR) Superoxide Dismutase (SOD), and Total Antioxidant Capacity (TAC). The results of the Spectrometric TAC Analysis are given as micromole per litre, and U/mg for both GR, and SOD, Nmol/g tissue for MDA, μmol/mg for nitric oxide.

2.8.3 PCR analysis of Inducible Nitric Oxide Synthase (iNOS) and caspase 3

Total RNA was extracted from rat heart specimens (50 mg tissue: n = 6/group) according to the protocol of the miRNeasy Mini Kit (Cat. no. 217004 QIAGEN GmbH, Germany). The NanoDrop 1000 Spectrophotometer V3.8 (Thermo Fisher Scientific, UK) was used to analyse the amounts and integrity of total RNA at OD260/280 nm. QuantiTect Rev. Transcription Kit (Cat. no. 205311 QIAGEN GmbH, Germany) was used to reverse transcriptase total RNA (50 g) into complementary DNA (cDNA). Until used in a PCR experiment, synthesized cDNA was kept at (-80°C). Prime 3 software was used to create specific primers for the caspase-3 and iNOS genes, as shown in table 1. Quantitative real-time PCR was completed in a 20μL final volume using HERA SYBR® Green qPCR master mix (Willowfort, Birmingham, UK) and 10pmol of each primer and 50 ng of cDNA the final reaction was performed using StepOne™ Real-Time PCR System (Cat. no. 4376357, Thermo Scientific, UK).

The following PCR cycles were used: an initial denaturant at 95 °C for 5 minutes, followed by 40 cycles of denaturant at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds. The relative expression of messenger RNA (mRNA) was estimated using the 2-ΔΔCt technique for each gene, and expression was standardized to the mean expression of β-actin gene (47).

Table 1. Primers sequence for iNOS, Caspase -3 and β-actin genes.

iNOS	Upper: 5'TCTGTGCTAATGCGGAAGGTCATG3'
	Lower: 5'TTGTCACCACCAGCAGTAGTTGTTC3'
Caspase -3	Upper: 5'GCAGCAGCCTCAAATTGTTGACTA3'
	Lower: 5'TGCTCCGGCTCAAACCATC3'
β-actin	Upper: 5'TCCTCCTGAGCGCAAGTACTCT3'
	Lower:5'GCTCAGTAACAGTCCGCCTAGAA3'

2.9. Histopathological assessment

The formaldehyde-fixed hearts were embedded in paraffin, cut into sections, and stained with Haematoxylin-Eosin (H & E). After that, the slides were examined under a microscope. All images were taken with a calibrated standard digital microscope camera (Tucsen® ISH1000 digital microscope camera) and an Olympus® CX21 microscope, with a maximum resolution of 10 MP (megapixels) (3656 x 2740 pixels per image) and using "IS Capture" software for capture and image enhancements. All slides were photographed with a 200x magnification, a 20x objective lens, and an Olympus®, Japan UIS optical system (Universal Infinity System).

2.10. Statistical analysis

The Statistical Package for the Social Sciences version 22 was used for the statistical analysis (SPSS v.22 software). GraphPad Prism version.7 was used to create the figures (GraphPad Software, San Diego, CA, USA). The data were presented as Mean ± Standard Deviation. A one-way analysis of variance (ANOVA) was used to determine the mean difference between groups, followed by a least significant difference (LSD) post-hoc test, with a significance level of 0.05.

3. Results

3.1. Effects of pre-treatment with Achillea fragrantissima crude extract, its (EtOAc-Bu) fraction and Vitamin E on Doxorubicin-Induced changes in ECG pattern

ECG analysis was done by MP150 data acquisition system (biopac acqknowledge software 4.0). Figure 3 shows samples of ECG traces of different groups from which the following parameters of analysis were obtained.

Injection with DOX was associated with a significant increase in heart rate and in some cases atrial fibrillation. Pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction and vitamin E showed no cases of AF. Pre-treatment with A. fragrantissima crude extract and vitamin E did not have any significant effect on this increased heart rate. Pre-treatment with A. fragrantissima (EtOAc-Bu) fraction showed significant decrease of heart rate ($p \leq 0.05$) as shown in figure 4.A. The uncorrected ECG durations were not affected in all DOX-injected groups (figures 4.B, 4.D). However, as Dox injection results in significant increase in heart rate, we corrected the ECG durations for their known dependency on heart rate (46). Rate-corrected-QRS showed significant widening on DOX injection ($p \leq 0.001$) as shown in figure 4.C. Pre-treatment with A. fragrantissima (EtOAc-Bu) fraction and vitamin E improved the rate-corrected-QRS widening toward the normal group ($P > 0.05$). However, pre-treatment with A. fragrantissima crude extract did not show such improvement in the rate- corrected-QRS

parameter (P ≤0.05). Rate-corrected-QT interval showed significant increase on DOX injection (p≤0.05). Pre-treatment with (EtOAc-Bu) fraction improved the rate-corrected-QT interval toward the normal group (P > 0.05). However, pre-treatment with the crude extract, and vitamin E did not improve the rate-corrected-QT parameter (figure 4.E).

Other parameters as rate-corrected PR interval, ST segment, R wave amplitude or T wave inversion were not significantly affected in the DOX-injected group in comparison to the normal group.

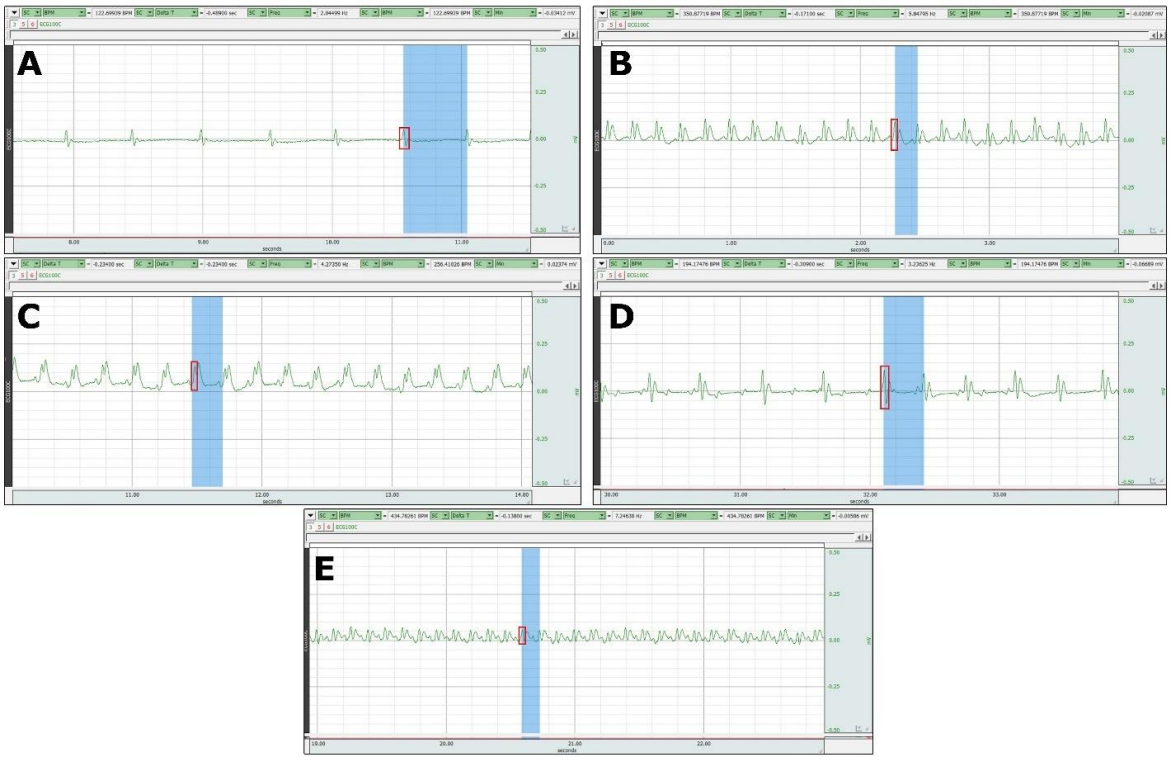

Figure 3. ECG tracing samples in different groups: (A) Group I (Control), (B) Group II (DOX Group), (C) Group III (DOX + *A. fragrantissima* crude extract), (D) Group IV (DOX + A. fragrantissima EtOAc-Bu fraction), (E) Group V (DOX + Vitamin E).

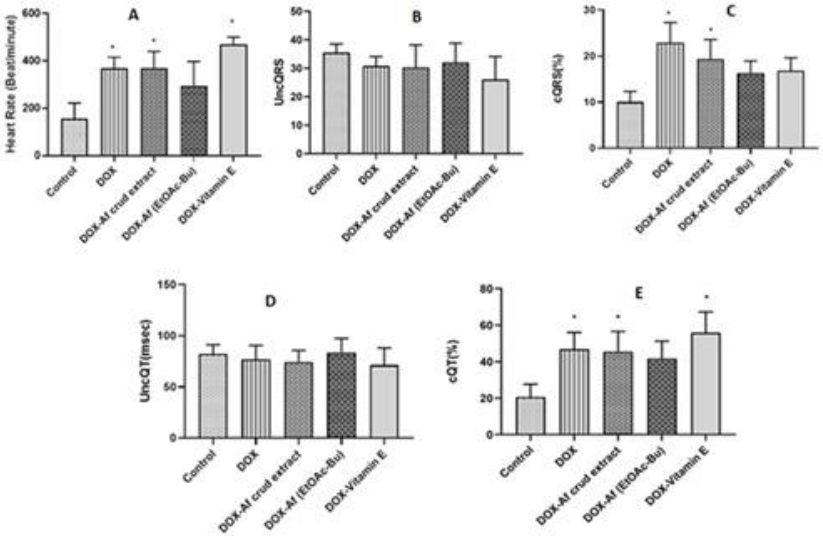

Figure 4. Bar Graph showing effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on (A) Heart rate (HR), (B) Uncorrected QRS duration, (C) rate corrected QRS duration, (D) Uncorrected QT interval, (E) rate corrected QT interval. Doxorubicin (DOX), Af (A. fragrantissima), Af (EtOAc-Bu) (A. fragrantissima combined ethyl acetate/ n. butanol fraction). * Significant versus control group. Significance at p ≤ 0.05.

3.2. Effects of pre-treatment with Achillea fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in Mean Arterial blood pressure

DOX injection as well as pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and vitamin E did not have any significant effects on either systolic, diastolic, nor mean blood pressure as evidenced by Biopac MP150 recording apparatus.

3.3. Effects of pre-treatment with Achillea fragrantissima crude extract, its (EtOAc-Bu) fraction, and vitamin E on Doxorubicin-Induced changes in serum cardiac enzymes

As shown in table (2), injection with DOX significantly ($p \leq 0.05$) elevated serum creatine kinase (CK), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) compared to the control group. Pre-treatment with A. fragrantissima (EtOAc-Bu) fraction, and Vitamin E significantly ($p \leq 0.05$) reduced serum CK, CK-MB and LDH levels compared to the DOX group. Pre-treatment with A. fragrantissima crude extract significantly ($p \leq 0.05$) reduced only the serum CK-MB level compared to the DOX group.

Table 2. Effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in serum creatine kinase (CK), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH).

Groups/(mean±SD)	CK (U/L)	CK-MB(U/L)	LDH(U/L)
Control	940.3±294.1	1145±305.7	533.7±217.9
DOX	1811.8±571.2[a]	2235.5±449[a]	1436.3±336.6[a]
DOX+Af crude extract	1206.7±264.9	1026.6±187.1[b]	1243.2±434.5
DOX+Af (EtOAc-Bu)	1024.6±164.5[b]	1222.3±435.5 [b]	818.3±209 [b]
DOX+Vit. E	878.5±246.3[b]	590.1±47.1[b]	865±225.9 [b]

Doxorubicin (DOX), Af (A. fragrantissima), Af (EtOAc-Bu) (A. fragrantissima combined ethyl acetate/ n. butanol fraction), CK (Creatine kinase), CK-MB (Creatine kinase-MB), LDH (Lactate dehydrogenase). [a] Significant versus control group, [b] Significant versus DOX-group. Significance at $p \leq 0.05$.

3.5. Effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in heart tissue concentration of Oxidant-Antioxidant parameters; nitric oxide (NO), lipid peroxidation marker malondialdehyde (MDA), Glutathione Reductase (GR), Superoxide Dismutase (SOD), and total antioxidant capacity (TAC).

As shown in table 3, injection with DOX significantly elevated at ($p \leq 0.05$) the oxidative stress marker NO, and the lipid peroxidation marker MDA, while significantly ($p \leq 0.05$) reduced GR, SOD and TAC as compared to the control group. Pre-treatments of rats with A. fragrantissima crude extract, (EtOAc-Bu) fraction, and Vitamin E significantly reduced MDA, while significantly elevated GR, SOD and TAC as compared to DOX-group. A. fragrantissima crude extract and Vitamin E significantly reduced NO.

Table 3. Effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in nitric oxide (NO), lipid peroxidation marker malondialdehyde (MDA), Glutathione Reductase (GR), Superoxide Dismutase (SOD), and total antioxidant capacity (TAC).

Groups/ (mean±SD)	NO (µmol/mg)	MDA Nmol/g tissue	GR (U/mg)	SOD(U/mg)	TAC µmol/L
Control	15.1± 6.8	0.4±0.2	748.5±32.8	9.5±0.8	1.7±0.1
DOX	38.8±15.9 [a]	1.6±0.1 [a]	254.4±101.4 [a]	7.6±0.3 [a]	0.8±0.1 [a]
DOX+ Af crude extract	23.7±1.9 [b]	0.4±0.2 [b]	621.3±157.7 [b]	9.3±0.8 [b]	1.8±0.03 [b]
DOX+ Af (EtOAc-Bu)	25.5±2.2	0.5±0.1 [b]	761±148.8 [b]	9.5±0.6 [b]	1.7±0.2 [b]
DOX+ Vit. E	22.2±3.7 [b]	0.7±0.2 [b]	573.8±140.3 [b]	10.8±1.0 [b]	1.2±0.3 [b]

Doxorubicin (DOX), Af (A. fragrantissima), Af (EtOAc-Bu) (A. fragrantissima combined ethyl acetate+n butanol fraction), [a] Significant versus control group, [b] Significant versus DOX-group. Significance at $p \leq 0.05$.

3.6. Effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in mRNA expression of Inducible Nitric Oxide Synthase (iNOS), and caspase-3

Figures 5 shows the mRNA expression of iNOS, and caspase-3 in all studied groups respectively. As regards to both iNOS, and caspase-3, they were increased significantly ($p \leq 0.05$) in DOX-group compared with control group, while pre-treatment of rats with (EtOAc-Bu) fraction, and Vitamin E significantly ($p \leq 0.05$) down-regulated the iNOS, and caspase-3 mRNA expression compared with DOX-group A. fragrantissima crude extract significantly ($p \leq 0.05$) down-regulated the iNOS mRNA expression only.

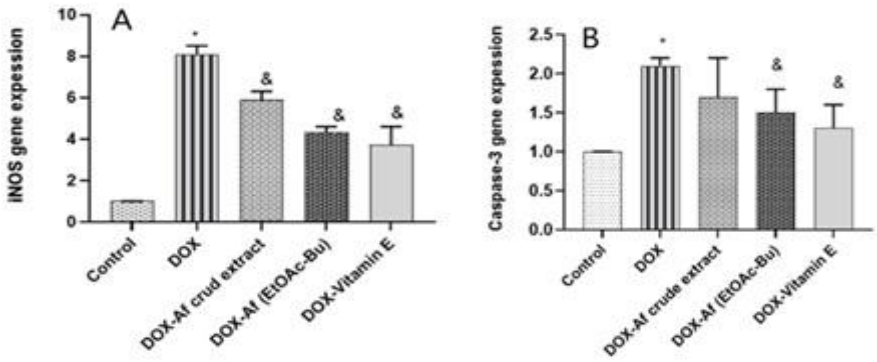

Figure 5. Bar Graph showing effects of pre-treatment with A. fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced changes in (A) Inducible Nitric Oxide Synthase (iNOS), (B) caspase-3. Doxorubicin (DOX), Af (A. fragrantissima crude extract), Af (EtOAc-Bu) (A. fragrantissima combined ethyl acetate/ n. butanol fraction), Inducible Nitric Oxide Synthase (iNOS). * Significant versus control group, & Significant versus DOX-group. Significance at $p \leq 0.05$.

3.7. Effects of Achillea fragrantissima crude extract, its (EtOAc-Bu) fraction, and Vitamin E on Doxorubicin-Induced histopathological changes in the heart tissue of rats

Figure 6 demonstrated the histopathological assessment of heart tissues. Figure 6-A showed control group of normal heart histology. The myocytes arranged into fascicles of muscle fibres; each cell had abundant eosinophilic cytoplasm

rich in myofibrils showing cytoplasmic striations with central oval vesicular nucleus. Fascicles separated by thin interstitial tissue showed scanty loose connective tissue with thin-walled vessels. On the contrary, DOX group shown in figure 6-B exhibited notable and prominent cardiac injury, where loss of fascicular pattern, marked degeneration of muscle cells with marked cytoplasmic vacuolation and loss of myofibrils with cytoplasm fragmentation and shrinkage, myofibril necrosis and infiltration of inflammatory were observed interstitial and perivascular oedema. Administration of A. fragrantissima crude extract before DOX injection as a prophylaxis has moderately improved the histopathological changes compared to DOX. group (Figure 6-C), where restored fascicular arrangement of muscle fibres myocytes, eosinophilic abundant cytoplasm with myofibrils, focal cytoplasmic striations and oval central vesicular nuclei were noted in addition to very few foci showing residual moderate congestion with mild perivascular oedema were also observed. Meanwhile, pre-treatment with A. fragrantissima (EtOAc-Bu) fraction resulted in mild alleviation of cardiac injury triggered by DOX where focally mildly restored fascicular arrangement with residual changes in the form of slight vacuolation, congestion and interfascicular and perivascular oedema were noted together with few degenerated myocytes (Figure 6-D). Pre-treatment with Vitamin E before DOX. administration caused the best amelioration of histopathological picture, with restored fascicular arrangement of muscle fibres, myocytes showed eosinophilic abundant cytoplasm with myofibrils and focal cytoplasmic striations with oval central vesicular nuclei. In addition, very few foci of residual mild congestion and interfascicular and perivascular oedema were observed (Figure 6-E).

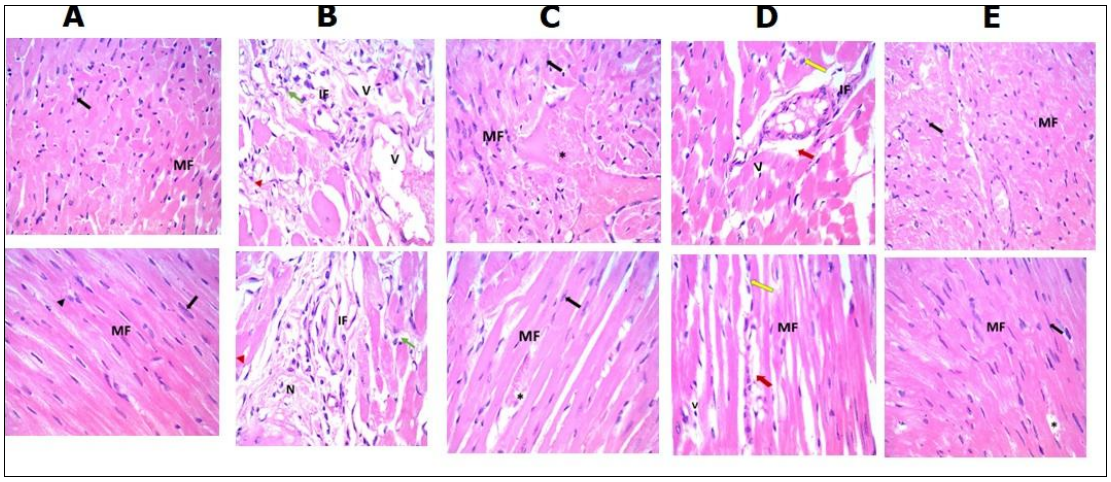

Figure 6. Photomicrography of the heart tissues from the experimental groups (stained with H& E; magnification power = 400, upper panel: transverse section, lower panel: longitudinal section). Control group (A) showed normal muscle fibres myocytes (MF) with oval vesicular nucleus myocytes (black arrow) and fascicles separated by thin interstitial tissue showed scanty loose connective tissue with thin-walled vessels (black arrowhead). Doxorubicin (DOX) group (B) displayed loss of fascicular pattern, marked degenerated muscle cells with cytoplasmic vacuolation (red arrowhead), infiltration of inflammatory cells (IF), necrotic myofibrils (N), cytoplasm fragmentation and shrinkage with increased nuclear number (green arrow) and marked vacuolation (V) and interstitial and perivascular oedema. A. fragrantissima crude extract pre-treated group (C) exhibited moderate improvement of the heart tissue with restoration of fascicular arrangement of muscle fibres myocytes (MF) with oval vesicular nucleus myocytes (black arrow) and few foci of moderate congestion (*). A. fragrantissima (EtOAc-Bu) fraction pre-treated group (D) showed mild improvement of heart tissue with focally mildly restored fascicular arrangement of myocytes (MF), moderate interfascicular and perivascular oedema (red arrow) moderate vacuolation (V) and congestion (*) and few degenerated myocytes (yellow arrow). Vitamin E pretreated group (E) demonstrated almost normal heart tissues with restoration of fascicular arrangement of muscle fibres myocytes (MF) with oval vesicular nucleus myocytes (black arrow) and mild congestion (*).

3.8. Phytochemical Analysis of A. fragrantissima crude extract, and its fractions based on which we have chosen to study the A. fragrantissima EtOAc-Bu fraction

3.8.1. Phytochemical screening for the presence of flavonoid:

The A. fragrantissima crude extract and its fractions were screened for the presence of flavonoid. Table 4 revealed that the EtOAc-Bu fraction demonstrated the highest colour intensity corresponding to the presence of flavonoid, so this fraction was described as The Flavonoid Rich Fraction.

Table 4. Phytochemical screening of A. fragrantissima crude extract and its fractions for the presence flavonoid.

Extract/ fraction	Presence of flavonoid
A fragrantissima crude extract	+
Hexane (Hex.) fraction	+
Chloroform (CHCl3) fraction	+ + +
Combined ethyl acetate/ n. butanol (EtOAc-Bu) fraction	+ + + +
Aqueous (AQ) fraction	+ + +

3.8.2. Total phenolics and flavonoids content:

TPC as well as TFC were quantified spectrophotometrically using Folin- Ciocalteu and AlCl3 methods. The obtained results were expressed as gallic acid equivalent GAE/g for TPC and rutin equivalent RE/g for TFC. Both of TPC and TFC of A. fragrantissima crude extract and its different fractions were solvent dependent, and figure (7) demonstrated that the amount of phenolic and flavonoid compounds in the crude extract and its fractions were vastly varied, ranging from 24.68± 1.45 and 136.58±10 mg GAE/g for TPC and 60.88± 4.117 and 162.4 ± 8.8 mg RE/g for TFC. The EtOAc-Bu fraction possessed the highest TPC and TFC in comparison with the crude extract and other fractions.

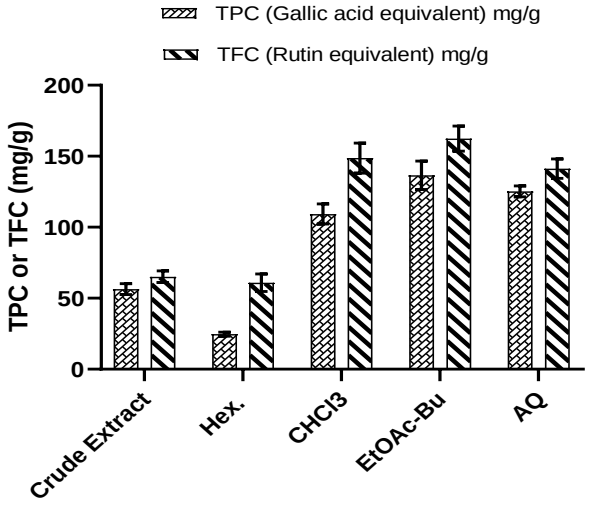

Figure 7. Bar Graph showing Total phenolics contents (TPC) expressed in Gallic acid equivalent (GAE) mg/g and total flavonoids contents (TFC) expressed in Rutin equivalent (RE) mg/g of A. fragrantissima crude extract and its fractions; (Hexane (Hex.) fraction, Chloroform (CHCl3) fraction, Combined ethyl acetate/n. butanol (EtOAc-Bu) fraction, Aqueous (AQ) fraction.

3.8.3. Evaluation of invitro Antioxidant Activity

Plant antioxidants constitute a wide variety of phytochemicals such as polyphenolics (phenolic acids and flavonoids), tocopherols and terpenoids. These compounds exert their antioxidant effect by different mechanisms such as quenching of singlet oxygen, hydrogen transfer, electron transfer and metal reduction and chelation (30,48). Therefore, and because of the diversity of natural products and their different scavenging modes of ROS (reactive oxygen species), the antioxidant potential of A. fragrantissima crude extract as well as its fractions was inspected simultaneously by three indicative assays (DPPH, FRAP, TAC).

3.8.3.1. DPPH free radical scavenging activity:

The DPPH radical has been frequently employed in model systems to explore the neutralizing actions of various natural substances such as phenolic compounds, anthocyanins, and crude plant extracts. Antioxidants can grab the free radical chain of oxidation and generate stable free radicals, stopping further oxidation propagation. Antioxidants can scavenge the DPPH radical by donating hydrogen, which results in the decolorization of the DPPH radical when higher concentrations of extracts are added, resulting in the reduced DPPH-H (49).

As shown in figure 8.A, among A. fragrantissima crude extract and its fractions, the EtOAc-Bu fraction exhibited a distinctive radical scavenging ability with IC50 = 27.7 ± 1.4 µg/ml compared to ascorbic acid as positive control (IC50 = 14.3 ± 1.1 µg/ml). In addition, all the crude extract and its solvent fractions exhibited DPPH scavenging rate in concentration-dependent manner (Table 5).

Table 5. Antioxidant activity of A. fragrantissima crude extract and its fractions via DPPH

Conc (µg/mL)	Crude extract	Hex. Fraction	CHCl3	EtOAc-Bu fraction	AQ fraction
1280	89.55±1.43	69.71±3.87	92.58±1.46	93.88±1.64	89.12±2.46
640	85.07±1.91	40.43±2.79	90.14±0.92	91.59±0.83	87.25±1.97
320	80.87±1.75	15.94±1.42	89.71±1.43	91.16±0.72	86.38±0.86
160	60.14±2.92	8.99±0.53	88.55±0.93	90.58±0.94	84.36±1.72
80	40.43±3.61	4.64±0.28	84.39±2.17	88.26±1.38	81.07±2.93
40	18.99±1.87	2.17±0.39	58.12±3.46	67.68±2.98	52.46±3.68
20	13.62±0.84	1.30±0.46	29.42±3.98	38.91±3.73	26.23±4.91
10	5.36±0.75	0.29±0.07	20.72±1.43	25.08±1.45	14.20±1.46
Hexane (Hex.), Chloroform (CHCl3), Combined ethyl acetate/n. butanol (EtOAc-Bu), Aqueous (AQ).					

3.8.3.2. Ferric reducing antioxidant power (FRAP) assay:

Since there is a direct correlation between the antioxidant potential of a substance and its reducing capacity (50), the FRAP assay is considered a reliable method to estimate the reducing capacity of a substance, which is an important parameter for a compound to be a good antioxidant (49,51). The antioxidant capacity of the investigated samples is linked to their ability to reduce ferric (III) iron to ferrous (II) iron in the FRAP reagent, which is an indicator of electron-donating ability and linked to a lower generation of oxidant species; this is thought to be an important mechanism of phenolic compounds as antioxidants (49,52). Figure (8.B) demonstrates the ferric ion reducing power of A. fragrantissma crude extract and its fractions expressed in terms of mMol Fe^{+2}/g. The EtOAc-Bu fraction

possessed the highest reducing power 4.25±0.73 mMol Fe+2 /g compared with A. fragrantissima crude extract and the other fractions. Ascorbic acid (2.75 ± 0.91 mMol Fe+2 /g) and BHT (7.26±0.68 mMol Fe+2 /g) were served as positive controls. Interestingly, the FRAP of EtOAc-Bu fraction was greater than that of ascorbic acid revealing the powerful antioxidant effect of this fraction which could be attributed to its phenolic content (49,52).

3.8.3.3. Invitro Total antioxidant capacity (TAC)

The invitro TAC of A. fragrantissima crude extract besides its fractions was estimated applying phosphomolybdate assay where BHT was employed as a positive control. This test depends on the formation of a green phosphate/MoV complex because of the reduction of phosphomolybdate ion by an antioxidant (30). Results were expressed as mg gallic acid equivalent per gram sample (mg GAE/g).

Figure (8.C) indicated that, the EtOAc-Bu fraction exhibited the highest activity (69.18±6.53 mg GAE/g) in comparison with the crude extract and the other fractions. The antioxidant capacity of EtOAc-Bu fraction was approaching that of BHT (the positive control ;74.86±3.92 mg GAE/g) determining its powerful ability to eliminate free radicals by electron transfer mechanism.

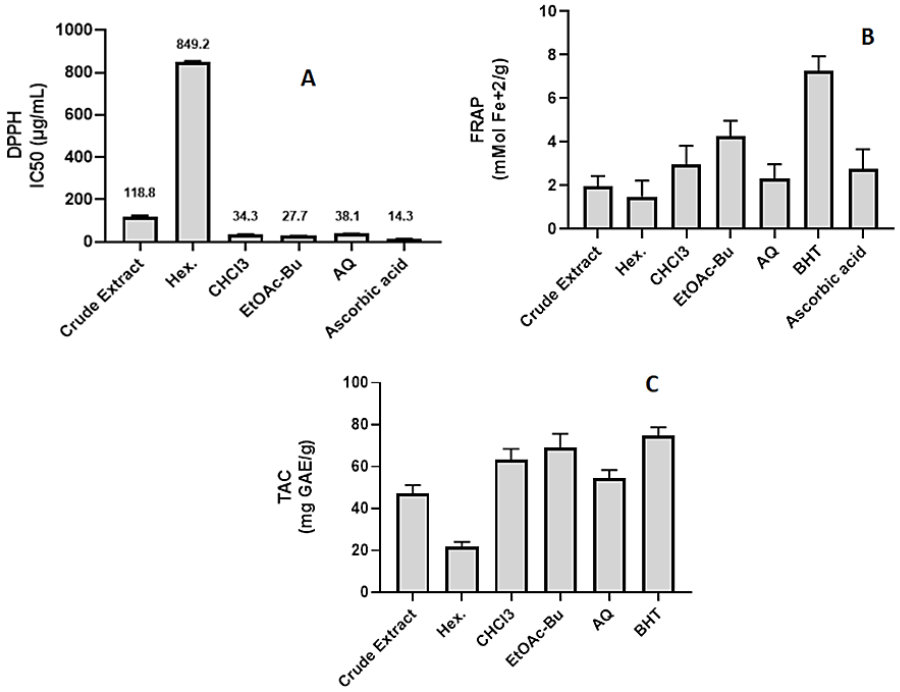

Figure 8. Bar graph showing Invitro Antioxidant Activity A. fragrantissma crude extract and its fractions, (A) The IC50 value of DPPH free radical scavenging, (B) Ferric ion reducing antioxidant power (FRAP) assay, (C) total antioxidant capacity (TAC) assay. (Hexane (Hex.) fraction, Chloroform (CHCl3) fraction, Combined ethyl acetate/n. butanol (EtOAc-Bu) fraction, Aqueous (AQ) fraction.

4. Discussion

Despite the ongoing development of new cancer treatment methods, systemic chemotherapy remains the gold standard. However, problems linked with cancer's toxic effects began to emerge within the context of effective cancer treatment. Antitumor medications that include anthracycline antibiotics, such as DOX, are among the most significant. However, its therapeutic efficacy is restricted by its hazardous toxic effects such as cardiotoxicity, which might jeopardize the patient's health (53,54).

DOX's cardiotoxic impact has a multifactorial pathophysiological basis that has yet to be fully elucidated (1). The most well-studied mechanism for DOX-induced cardiotoxicity is oxidative stress. In this study, oxidative stress was evident as follows: DOX significantly elevated NO, and MDA, significantly increased mRNA expression of iNOS, and significantly reduced TAC, and mRNA expression of GR, and SOD in DOX-group compared to the control group.

When the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), is unbalanced with low level of cardiac endogenous antioxidant systems, oxidative stress develops (55). A key contributor to DOX-induced oxidative stress is Nitric oxide (NO). As shown in current study, DOX-induced cardiotoxicity results in a rise in NO levels owing to NOS (NO-synthase) isoforms, namely inducible NOS. As a result, increased NO production leads to the build-up of nitro tyrosine in cardiac tissue (1,37,54,56).

Additionally, increased lipid peroxidation occurs because of oxidative stress, increasing MDA production, which causes immediate membrane disruption and the release of cellular enzymes such myocardial LDH and CK, indicators for heart injuries (3,57–59), as evidenced in current study.

Apoptosis is another studied mechanism for DOX-induced cardiotoxicity, evidenced by increased caspase 3 mRNA expression in the DOX group. Caspase-3 protein could be considered diagnostic for myocardial infarction and cardiotoxicity.

The results of current study regarding oxidative stress and apoptosis pathways were in accordance with similar studies that used the same studied dose of DOX (34,35,37,39). Moreover, our findings were also in line with previous studies of different models of DOX-induced cardiotoxicity (1,6,36,60–63).

The above results explain the underlying mechanism of the resultant DOX-induced cardiotoxicity which was manifested in the present study by significant elevation of serum CK, CK-MB and LDH, altered ECG pattern in the form of increased heart rate, widened rate-corrected-QRS complex, which is the best landmark of cardiotoxicity as proved by Zhao et al 2011 who used the same duration of usage of DOX (64), significant increase in rate-corrected-QT interval and in some cases atrial fibrillation, and histopathological changes in the heart tissue.

LDH and CK-MB are among the key indicators for cardiac injury. Such increase can be attributed to the cardiac damage in cellular membranes induced by DOX (37). Altered ECG patterns were additional signs of DOX-induced cardiotoxicity which impedes mitochondrial metabolism inducing cardiovascular contractile dysfunction (56,58,65). Gül and Aygün 2019 attributed the DOX-induced abnormal ECG alterations to DOX's degrading impact on cell membrane (3). DOX-induced cardiotoxicity was also manifested in the present study by the resultant histopathological changes in the heart tissue as moderate loss of fascicular pattern, marked degeneration of muscle cells with marked cytoplasmic vacuolation and loss of myofibrils with fragmentation of cytoplasm, shrinkage, and increased number of nuclei, marked interstitial oedema and perivascular oedema and marked vacuolation and congestion.

These histopathological parameters of DOX-induced cardiotoxicity were in accordance with similar experimental studies that used the same

DOX dose used in the present study. Al-Thubiani, Abuzinadah, and El-Aziz 2021 study results showed DOX-induced changes as substantial expansion in QRS complex, increase in serum LDH and CK-MB, and harmful histopathological changes as "degenerative changes in the cardiac muscle fiber, some vacuolated fibers, inflammatory cell infiltrates between the cardiac muscle fibers, congestion of blood vessels with perivascular Red blood cell extravasation" (39). Bin Jardan et al 2020 denoted a substantial rise in LDH and CK-MB levels, as well as a considerable loss of myofibrils and wavy fibers with DOX (37). Tamizhselvi et al 2020 study results showed increased CK-MB, LDH and evident histopathological changes in cardiac muscle as necrosis with aggregations, severely inflamed cells and injured vascular spaces (38). Kelleni, Amin, and Abdelrahman 2015 study declared that DOX causes an increase in serum LDH and CK-MB levels, as well as significant regions of degeneration, perinuclear vacuolization, inflammation, and interstitial haemorrhage (35). Dogan 2014 study showed that DOX-induced significant cytoplasmic vacuolization, myofibrillar disorganization and myofibrillar loss (34).

These findings were also in agreement to a great extent with previous studies of different models of DOX-induced cardiotoxicity. A single DOX dose of 10 mg/kg induced histopathological findings in the cardiac tissue and increased serum level of CPK, and LDH (59). Cumulative dose 15 mg/kg in the form of 2.5 mg/kg of DOX thrice weekly for two weeks showed significant histopathological lesions (6). On the other hand, other studies showed significant changes in ECG pattern and blood pressure. Hu et al 2020 study demonstrated that after 7 days of exposure to DOX (15 mg/kg, i.p.), ejection fraction decreased significantly (EF). DOX caused a drop-in heart rate, blood pressure, cardiac output, stroke effort, and a lengthening of relaxation time constants (Tau) (63). Younis 2019 displayed that with DOX (3 mg/kg/ every other day for 2 weeks), there were significant prolongation of QT, QRS intervals, elevated ST, shortening of the p wave, P-R, and R-R intervals indicating a variety of cardiac arrhythmias and conduction problems, substantial drop in all BP indices (61). The heart rate (HR) decreased after a total overall dosage of DOX (10 mg/kg) (66). These changes could be attributed to the subacute cardiotoxicity induced by cumulative doses of DOX compared to the single DOX dose used in current study.

In cell and animal models, a variety of pharmacological and non-pharmacological treatments targeted at mitigating cardiotoxicity have proved to be effective. However, there is currently no gold standard in clinical practice for the treatment of anthracycline-induced cardiotoxicity (56,63).

Antioxidants can help reduce the burden of free reactive radicals in cells when combined with chemotherapy. The current study aimed at examining the possible protective impact of *Achillea fragrantissima* crude extract, its (EtOAc-Bu) fraction, and vitamin E against DOX-induced cardiotoxicity in adult male albino rats.

The present study results demonstrated that pre-treatment with *A. fragrantissima* crude extract, its (EtOAc-Bu) fraction, and vitamin E, in varying degrees and with varying significance, ameliorated DOX-induced cardiotoxicity evidenced by reversing of some ECG changes as improving the rate-corrected-QRS widening induced by DOX, reducing serum cardiac

enzymes levels, and improving the DOX-induced histopathological changes as compared to the DOX group. Only pre-treatment with *A. fragrantissima* (EtOAc-Bu) fraction showed a significant decrease in heart rate that was increased by DOX and improved the rate-corrected-QT interval. Additionally, the suggested protective measures in this study reduced the oxidative stress, lipid peroxidation, and apoptosis markers (NO, MDA, and down-regulated mRNA expression of both iNOS, and caspase-3), and elevated the antioxidants markers (TAC, and mRNA expression of both GR and SOD) in varying degrees and varying significance.

Based on the present study results, Vitamin E showed cardioprotective potential as evidenced by significantly ameliorating different parameters induced by DOX as the oxidative stress markers (NO, iNOS, and caspase-3 mRNA expression), lipid peroxidation marker (MDA), antioxidant markers (GR, SOD, and TAC), and serum CK, CK-MB, and LDH levels. Moreover, vitamin E improved the rate-corrected-QRS widening and showed the best amelioration of the histopathological changes induced by DOX(32). Because of its oxygen-scavenging characteristics, vitamin E reduces lipid peroxidation and lowers oxygen generation, thereby protecting against oxidative damage (67). Therefore, vitamin E in conjunction with cancer chemotherapeutic drugs could be demonstrated as an appropriate cancer adjuvant therapy (68).

The results regarding vitamin E are in line with other previous studies as Abdel-Samia, Bushra, and Gomaa 2019 study. For two weeks, rats were given 100mg Vitamin E /kg/day orally, followed by one week of 4mg Dox /kg/day and 100mg Vitamin E /kg/day orally. When compared to the Dox-treated group, rats treated with Dox and Vitamin E showed a significant reduction in the histopathological changes, as well as a reversal of the elevated cardiac enzymatic activity induced by DOX (69). In Donia, Eldaly, and Ali 2019 study, they came to the conclusion that vitamin E (100 mg/kg orally) decreased oxidative stress, inflammation, and apoptosis in DOX-induced cardiomyopathy. They stated that vitamin E reduced LDH and CK levels, MDA levels, and caspase 3 activity, boosted GSH levels, dramatically improved architecture, and reduced myofibril loss (16). Another study showed that vitamin E (100 mg/kg) orally for one week, followed by a single dose of DOX (20 mg/kg), resulted in a substantial decrease in MDA, CK-MB, and LDH elevated by DOX (32). Pre-treatment with vitamin E (100 mg/kg) given orally for two weeks before DOX reversed the DOX-induced PR, QT, and ST segment alterations and reduced CPK-MB and LDH levels. As a result, they assumed that vitamin E protects the rat heart against DOX-induced damage (70).

On the other hand, these results of the present study aren’t in agreement with Reeja et al 2020 study. They discovered that Vitamin E (100 mg/kg orally) did not protect the heart against antitumor agent-induced cardiotoxicity (71). Bjelogrlic et al 2005 study demonstrated that vitamin E (100 IU/kg, orally) given 24 hours before DOX single dose (10 mg/kg) was not able to prevent DOX's immediate cardiotoxic effect. However, it did stop further advancement of heart muscle damage over time and dramatically decreased CK. On the other hand, hearts from animals given vitamin E were nonetheless considerably poorer than hearts from normal animals (72). This could be attributed to the short

duration of pre-treatment with Vitamin E in such studies as compared to our study.

As regards, *A. fragrantissima* crude extract, and its fractions (Hexane, Chloroform, (EtOAc-Bu) fraction, and Aqueous fractions) were compared as regards to the total phenolic and total flavonoid content, DPPH free radical scavenging activity, FRAP assay, and TAC. The (EtOAc-Bu) fraction demonstrated the highest TPC and TFC, FRAP assay, TAC by phosphomolybdenum method and a distinctive radical scavenging ability. That's why we focused in our study on both the *A. fragrantissima* crude extract, and its (EtOAc-Bu) fraction.

Based on the present study results, both *A. fragrantissima* crude extract, and its (EtOAc-Bu) fraction showed amelioration of the parameters evidencing DOX-induced cardiotoxicity. Pre-treatment with *A. fragrantissima (EtOAc-Bu)* fraction significantly reduced CK, CK-MB, LDH, MDA, and iNOS, and caspase-3 mRNA expression, significantly elevated the GR, SOD, and TAC, and mild improvement of the histopathological changes induced by DOX. Furthermore, administration of (EtOAc-Bu) fraction improved the rate-corrected-QRS widening, significantly decreased the heart rate, and improved the rate-corrected-QT interval. This is consistent to some extent with the *in vitro* analysis done which significantly proved the better antioxidant effect accomplished by the (EtOAc-Bu) fraction of *A. fragrantissima*. On the other hand, *A. fragrantissima* crude extract only significantly reduced CK-MB, NO, MDA, and iNOS mRNA expression, significantly elevated the GR, SOD, TAC, improved the rate-corrected-QRS widening, and moderately improved the histopathological changes induced by DOX. Furthermore, administration of *A. fragrantissima* crude extract before DOX injection failed to improve the heart rate, and the rate-corrected-QT interval.

A. fragrantissima is a prominent and significant medicinal plant in Arabic herbal medicine for preventing and treating a variety of health issues. This plant has antioxidant and anti-inflammatory effects. Genus *Achillea* is well known for its highly bioactive metabolites including terpenoids; amid which sesquiterpenes and sesquiterpene lactones predominate, flavonoids, phenolic acids, tannins, polyacetylenes and alkamides (19,27).

The antioxidant activity appears to be heavily influenced by radical scavengers as flavonoids (73). Additionally, Phenolics act as antioxidants at different levels as lowering oxygen concentrations, intercepting singlet oxygen, scavenging initial radicals, breaking down primary products of oxidation to nonradical species, and halting the extraction of hydrogen from compounds (74).

As shown in current study, *A. fragrantissima* (EtOAc-Bu) fraction protects against oxidative damage by detoxifying ROS owing to the existence of the flavones and flavanols classes (phenolic chemicals). Moreover, the tannin content of *A. fragrantissima* exceeds 8%. Such a high proportion of tannins serves as antioxidants and aids in the scavenging of ROS by endogenous antioxidant enzymes prior to lipid peroxidation resulting in decreasing lipid peroxidation (24). Moreover, sesquiterpene lactones are among the active components of A. *fragrantissima* such as achillolide A which possessed significant anti-inflammatory action antioxidant activity, as well as preventing the production of NO (26,75,76).

The present study results regarding *A. fragrantissima* ameliorative effect on the DOX-induced cardiotoxicity agree with Hijazi *et al*. 2019 study which demonstrated that DOX-induced tachycardia and QT prolongation were not reversed. It decreased DOX-induced LDH and CK-MB elevations and increased Glutathione concentration in cardiac tissue. It also protected against histopathological alterations caused by DOX (76).

Interestingly, the antitumor properties of *A. fragrantissima* extract as well as its phytoconstituents particularly flavonoids were evidenced (22,25,77). The findings of Alenad et al (2013) showed that *A. fragrantissima* extract; being non-toxic, might be used to treat patients with drug-resistant chronic myelogenous leukaemia (CML) by promoting CML cell differentiation, cell cycle arrest, and apoptosis (25). Moreover, Awad and coworkers (2020) reported that *A. fragrantissima* alcoholic extract as well as its poly methoxylated flavonoids diminished tumor progression in EAC bearing mice (77).

5. Conclusions

DOX has various toxic effects on the structure and function of the heart, as evidenced by elevated cardiac enzymes such as CK, LDH, and CK-MB, altered ECG patterns, and significant cardiac histopathological alterations in male rats. These changes could be explained by the increased oxidative stress, and apoptotic biomarkers. Pre-treatment with *A. fragrantissima* crude extract, its (EtOAc-Bu) fraction, and vitamin E have ameliorated the changes in these parameters in varying degrees and with varying significance. To sum up, Natural products (Vitamin E single compound and *A.fragrantissima* extracts) represent a powerful and alternative strategy to manage chemotherapy-associated cardiotoxicity and to serve as adjuvant therapy.

Author Contributions:

"Conceptualization, E.M.A.M., A.M.A, and M.F.M.; methodology, E.M.A.M., A.M.A, M.F.M., E.E.E, N.M.A, M.K.E.; investigation, E.M.A.M., A.M.A, M.F.M., E.E.E, N.M.A, M.K.E.; formal analysis, A.M.A, M.F.M., E.E.E, N.M.A; Data interpretation, E.M.A.M., A.M.A, M.F.M., E.E.E, N.M.A, M.K.E. Writing—original draft preparation, E.M.A.M.; writing—review and editing E.M.A.M., A.M.A, M.F.M., E.E.E, N.M.A, M.K.E.

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Informed Consent Statement Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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