



The Combination Therapy of Medicinal Plant *Globularia Alypum*, with Adriamycin Limits Free Radical Mediated Cardiac Injury in Rats

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Accepted on: 26-02-2015; Finalized on: 31-12-2015.

ABSTRACT

Adriamycin (ADR) is an efficient chemotherapeutic agent used against several types of tumors; however, its use is limited due to severe cardiotoxicity. Since it is accepted that ADR induced myocardopathy is the consequence of oxidative stress through the mediation of free radicals, antioxidant agents have been used to attenuate its side effects. The aim of this study was to investigate the effect of *Globularia alypum* butanolic extract (GABE) on the acute cardiac toxicity induced by ADR in rats. The phytochemical analysis carried out on the GABE showed the presence of flavonoids. ADR toxicity was evaluated biochemically by measuring the serum concentration of Lactate Dehydrogenase (LDH), Creatinine Phospho Kinase (CPK) and Aspartate Amino Transferase (AST). Oxidative stress in the heart tissue was estimated by measuring the glutathione (GSH) levels and malondialdehyde (MDA) in the homogenate. The pretreatment of rats with the GABE orally at a dose of 100mg/kg for a month resulted in a decrease in the plasma enzymatic cardiac injury (CPK, LDH and AST), reduction in cardiac cytosolic MDA and maintenance of cardiac cytosolic GSH level as compared to ADR treated animals at a dose of 20 mg/kg intraperitoneally. Cardiac histopathological changes were observed in the ADR group as compared to the control group. In contrast, these histopathological changes appeared nearly normal in the group pretreated with GABE. These findings suggest that the butanolic extract of this plant has protective effects against ADR induced cardiotoxicity and may be a useful agent in the combination therapy with ADR to limit free-radical-mediated cardiac injury.

Keywords: Adriamycin, *Globularia alypum*, Cardiotoxicity, Antioxidants, Oxidative stress, Histopathology.

INTRODUCTION

Globularia alypum, L. (*G. alypum*) is a wild plant belonging to *Globulariaceae* family. It is a perennial shrub which is found throughout the Mediterranean area¹. *G. alypum* is commonly used in North African (Tunisia, Morocco, Libya and Algeria) folk medicine². Its leaves are reported to be used in the treatment of diabetes and in renal and cardiovascular diseases. They are also used as laxative, cholagogue, stomachic, purgative, sudorific, antihypertensive, and hypoglycemic³. The infusion of *G. alypum*, exhibiting no toxicological effects, was thus shown to produce a significant hypoglycaemic in rats both by oral and intraperitoneal administration⁴. A significant antileukemic activity of an aqueous extract of *G. alypum* was also reported⁵. Recently, methanol and dichloromethane extracts of *G. alypum* were also shown to reduce histamine and serotonin contraction *in vitro*⁶. The hydro-methanolic extract of *G. alypum* aerial parts showed significant antioxidant effect, based on the scavenging activity of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The antioxidant activity of the *G. alypum* phytochemicals (flavonoids, phenylethanoids, iridoids) were also evaluated for their capacity to scavenge DPPH and some structure–activity relationships were obtained⁷. As a consequence of these properties, *G. alypum* can have immense potential in preventing the oxidative damage to the heart caused by anticancer drugs such as ADR.

ADR, an anthracycline glycoside antibiotic originally

produced by *Streptomyces peucetius* var. *caesius*⁸⁻⁹, is a potent chemotherapeutic agent, often used in the treatment of solid tumors, especially breast and ovarian cancer¹⁰. However, its use is limited by high incidence of cardiac toxicity¹⁰⁻¹¹. Great efforts have been made to understand the mechanism responsible for ADR anti-tumor and cardiotoxic effects. The non-radical-dependent mechanisms such as, blocking the DNA polymerase enzyme, intercalation of the drug between DNA base pairs, DNA topoisomerase II inhibition¹²⁻¹³, and tumor cell apoptosis¹⁴ may explain its anti-tumor action. Several hypotheses have been postulated to explain the mechanism of ADR-induced cardiotoxicity, including the generation of reactive oxygen species (ROS)¹⁵, decrease in calcium channel level¹⁶ and decrease in energy production in myocardium¹⁷⁻¹⁸. Ample evidence is now available supporting the view that increased oxidative stress, because of the ADR induced increase in production of free radicals during its intracellular metabolism play an important role in the development of cardiomyopathy and congestive failure¹⁹⁻²⁰. ADR is known to generate free radicals either by redox cycling between a semiquinone form and a quinone form or by forming a ADR-Fe³⁺ complex (7). In both pathways, molecular oxygen is reduced to superoxide anion (O₂⁻), which is converted to other forms of reactive oxygen species such as hydrogen peroxide (H₂O₂) and hydroxyl radical (OH)²¹. These Free radicals cause diverse oxidative damage on critical cellular components and membranes in heart tissue²². Moreover, the heart is a very sensitive tissue to oxidative stresses



because of its highly oxidative metabolism combined with the fact that it has a lower level of antioxidant defense compared with other metabolic organs such as liver or kidney¹¹⁻²². Due to the successful action of ADR as a chemotherapeutic agent, several strategies have been tried to prevent/attenuate the cardiac toxicity of ADR without affecting their therapeutic efficacy²³.

Assuming that oxidative stress is an important mechanism for explaining the cardiotoxicity caused by ADR, it seems relevant to develop therapeutic strategies using antioxidants to prevent or to attenuate this side effect of the drug²⁴. Different antioxidants such as vitamins A, E, and C, probucol or N-acetylcystein, and S-allylcystein do not interfere with anthracycline activity in tumor cells but their cardioprotective efficacy was limited. The iron chelator IRCF-187 was found to be effective against ADR cardiotoxicity but its use is limited by an hematological toxicity¹⁰.

Over the last few years, considerable attention has been paid to investigating naturally occurring agents for their capability to stimulate defense mechanisms. Plants have limitless ability to synthesize aromatic substances such as polyphenols, mainly flavonoids and phenolic acids, which exhibit antioxidant properties due to their hydrogen donating and metal-chelating capacities²⁵. In the present study butanolic fraction of *G.alypum* was extracted and its efficacy to prevent ADR induced cardiotoxicity was studied.

MATERIALS AND METHODS

Chemicals

The chemicals were purchased from Sigma (USA) and Fluka Chemie (Buchs, Switzerland).

Plant material

Fresh *G.alypum* was collected during February 2008 from the region of El Milia, Algeria. Taxonomic identification was performed by Pr. N.Khallafalah (Laboratory of Genetics, plants biochemistry and Biotechnologies, Faculty of Sciences, University Mentouri - Constantine, Algeria).

Preparation of plant extract

Fresh aerial parts (stems, leaves and flowers) were air-dried in shade at room temperature for a period of 2 weeks. They were then mechanically powdered and sieved. 1779g of the obtained powder were macerated during 48 h at room temperature with mixture of distilled water-methanol (3/7 V/V).

The obtained crude preparation was filtered and concentrated under reduced pressure by rotary evaporator at 47 °C to give the crude methanolic extract. Procedure of extraction and evaporation was repeated three times.

This crude extract was dissolved in water and the obtained aqueous phase was successively extracted with

petroleum ether to remove lipids, ethyl acetate and butanol respectively. All the fractions were dried under reduced pressure²⁶.

Phytochemical analysis

Phytochemical screening was performed on butanol extract (BE) to test the presence of flavonoids. Two methods were used to determine the presence of flavonoids in the GABE²⁷. 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of plant extract followed by addition of concentrated H₂SO₄. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing.

Few drops of 1% aluminium solution were added to a portion of each filtrate. A yellow coloration was observed indicating the presence of flavonoids.

UV-vis spectra of flavonoids in methanol of the GABE

The UV-vis spectra of flavonoids and related glycosides show two strong absorption peaks commonly referred to as band I (300-380 nm) and band II (240-280 nm). Band I is associated with the presence of a B-ring cinnamoyl system of flavonoids. Band II absorption is due to A-ring benzoyl system flavonoids²⁸.

Total flavonoids determination

The flavonoids content in GABE was determined spectrophotometrically using a method based on the formation of a complex flavonoid-aluminium²⁹, having the absorbivity maximum at 430 nm. 1ml of diluted GABE was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 0 to 40 µg /ml in methanol. And the flavonoids content was expressed in mg per g of quercetin equivalent (QE).

Determination of Total polyphenols content

Total polyphenols were measured using Prussian blue assay method described by Price & Bulter and modified by Graham³⁰. Phenolic contents were expressed as gallic acid equivalents. Briefly, 0.1 ml of GABE was dissolved in methanol and 3 ml distilled water were added and mixed up. One ml of K₃Fe (CN)₆ (0.016 M) was added to the sample followed by the addition of 1 ml of FeCl₃ (0.02 M dissolved in 0.1 M HCl) and immediately mixed up using a vortex. After the addition of the reagents to the sample, 5ml stabilizer (30 ml of 1% gum Arabic, 30 ml of 85% H₃PO₄ and 90 ml distilled water) were added to the sample and mixed up.

The absorbance was measured at 700 nm using a spectrophotometer. The amount of total polyphenols in the extract was determined from a standard curve of gallic acid ranging from 0.00 to 200 µg/ml.



Assessment of cardiotoxicity

Experimental animals and treatment protocol

In this investigation, 24 healthy adult male Wistar albino rats (8-week-old weighing 160 –180 g) were used. The animals were obtained from the Pasteur institute, Algeria. The animals were kept under standard laboratory conditions (12 h light :12 h dark and 25±5 °C). The rats were fed standard commercial laboratory chow. The animals were allowed to adapt to the new housing environment for 1 week before the experiment.

To study the effect of chronic administration of GABE on ADR induced cardiotoxicity, four Groups of six animals each were taken and treated as follows:

Group I: Control group received a saline (5ml/Kg b.w), orally for 4 weeks.

Group II: received GABE in dose of 100 mg. kg⁻¹ p.o daily for 4 weeks.

Group III: received a Saline 5 ml/Kg p.o daily for 4 weeks + ADR 20 mg/kg, single Intraperitoneal injection, which is well documented to induce cardiotoxicity³¹⁻³² after 4 weeks.

Group IV: received GABE in dose of 100 mg.kg⁻¹ p.o. + ADR (20 mg/kg) single Intraperitoneal injection after 4 weeks.

Sample collection

Twenty-four hours after the administration of ADR, blood samples were collected by orbital sinus puncture into tubes containing anticoagulant. The samples were centrifuged at 6000t/min for 15 min, and then the plasma was removed immediately and stored at -20 °C until biochemical analyses, then The animals were sacrificed by cervical dislocation and the heart of each animal was rapidly dissected and washed to remove excess blood and cut into two pieces; The first piece was fixed in formalin for histological analysis and the second was homogenized in a Teflon-glass homogenizer with a buffer containing 1.15% KCl to obtain 1:10(w/v) whole homogenate. The homogenates were centrifuged at 4000 t/min (+4 °C) for 10 min to determine malondialdehyde (MDA) and reduced glutathione (GSH) concentrations³³.

Determination of plasma biochemical parameters

Lactate dehydrogenase (LDH; EC: 1.1.1.27), creatine phosphokinase (CK; EC: 2.7.3.2), aspartate aminotransferase (AST; EC: 2.6.1.1) were determined from the plasma that have been obtained by enzymatic colorimetric methods with an auto-analyzer (Technicon RA, opera systems Reference-Number. T01-2801-56).

Determination of reduced glutathione (GSH)

Levels of GSH were assessed only in the cytosol using Ellman assay. Then, 50µl of the cytosol fraction were diluted in 10 ml of phosphate buffer (0.1M, pH 8.0). Twenty microliter of 5,5'-dithiobis 2-nitro-benzoic acid

(0.01M) (DTNB) were added to 3 ml of the mixture dilution. After 15 min, the absorbance of thionitrobenzoic acid (TNB) produced after oxidation of GSH by DTNB was evaluated at 412 nm against a blank prepared by TCA (5%) under the same conditions. The GSH amounts were calculated using a standard curve of GSH, and were expressed in µmole/g³⁴.

Determination of lipid peroxidation (MDA)

The concentrations of MDA as a marker of lipid peroxidation were determined according to the method of Ohkawa and Ohishi³⁵ based on a colorimetric reaction with thiobarbituric acid, and were expressed as nmol /g of heart tissue. Briefly, in a teflon-stoppered test tube, 0.5 ml of 20% trichloroacetic acid solution and 1ml of 1% thiobarbituric acid solution were added to the 0.5 ml of homogenate. The color of thiobarbituric acid pigment was developed in a water bath at 100°C for 15 min. after cooling with tap water to room temperature; 4ml n-butanol was added and shaken vigorously. After centrifugation at 3000 rpm for 15 min, the color of butanol layer was measured at 535 nm. The absorbance values obtained were compared against a standard curve of known concentrations of 1,1,3,3-tetraethoxypropane.

Histopathological examinations

Hearts tissues from all the four treated groups were fixed in 10 % formalin and embedded in paraffin wax. Sections at 5µm were stained with hemotoxylin and eosin. The sections were then viewed under light microscope for histopathological changes³⁶.

Statistical analysis

Results were expressed as mean ± SD of three measurements. The significance difference among the four groups was assessed using one-way ANOVA followed by the Tukey test to identify differences between the groups. Statistical significance was acceptable to a level of p<0.05.

RESULTS

Phytochemical investigation

The extract fraction for each solvent was obtained by concentrating under reduced pressure by using rotary evaporator.

Table 1 provides the yield of each extract fraction. The Phytochemical analysis in the methanolic solution carried out on the butanolic extract of this plant showed the presence of flavonoids.

Table 1: The yield of the organic extracts from *Globularia alypum*

Shade plant weight (g)	Extract fraction	Weight (g)	Yield (%)
1779	Petroleum ether	2	0.11
	Ethyl acetat	5.56	0.31
	butanol	238.31	13.39



UV-vis spectra of flavonoids in methanol of the GABE

The UV-vis absorbance spectrum of GABE (Fig.1) exhibits two absorption peaks at 371 nm corresponding to bands I (Cinnamoyl) and 260 nm corresponding to bands II (Benzoyl) of flavonoids.

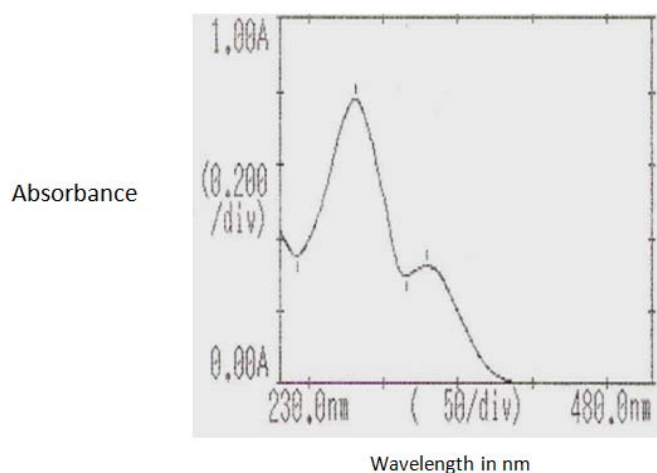


Figure 1: The UV-vis absorbance spectrum of GABE

Total phenol and Flavonoid contents of the GABE

Total phenolic and flavonoid content *in vitro* of butanolic extract of the plant are shown in table 2. The total phenolic content in the extract was estimated by the price and bulter method³⁰. Total phenol content was expressed in mg Gallic acid equivalents per gram of dry extract according to the formula that was obtained from standard Gallic acid graph ($y = 0.004X + 0.108$, $R^2 = 0.971$). Results of the research showed that the phenolic compound of the tested extract was 103.81 ± 3.65 mg of Gallic acid equivalent per g dry extract, while the concentration of flavonoids in GABE was estimated by using spectrophotometric method with aluminum chloride²⁹. The total flavonoid content in the extract was determined as mg of quercetin equivalent per gram of dry extract according to the formula that was obtained from standard quercetin graph ($y = 0.016x + 0.238$, $R^2 = 0.959$). As shown in the Table 2, the flavonoid content was 21.5 ± 1.51 mg of quercetin equivalent per g dry extract. The results of this study show that GABE represents a significant source of phenols and polyphenolic compounds, such as flavonoids.

Table 3: Effect of GABE (100mg kg⁻¹ p.o daily for 4 weeks), on ADR (20mg kg⁻¹, i.p)-induced elevated serum levels of lactic dehydrogenase (LDH), creatine phosphokinase (CPK) and aspartate aminotransferase (AST) activities

Parameters groups	LDH (U.L ⁻¹)	CPK (U.L ⁻¹)	AST (U.L ⁻¹)
Control group	389.66 ± 24.96	361.16 ± 28.35	122 ± 18.80
GABE group	306.83 ± 41.90	336.66 ± 20.33	122.66 ± 31.91
DXR treated group	522.16 ± 35.40**	603.5 ± 48.77***	184 ± 56.88*
GABE + DXR treated group	400.16 ± 43.65##	444.66 ± 53.51##	114.16 ± 7.57#

The results are expressed as mean ± S.E.M. of six rats per group. * P < 0.05 (significant difference), ** P < 0.01 (highly significant difference), *** P < 0.001 (very highly significant difference) compared with control; # P < 0.05 (significant difference), ## P < 0.01 (highly significant difference) compared with ADR treated group.

Table 2: Total polyphenol and flavonoid contents of *Globularia alypum* butanolic extract

<i>G. alypum</i> butanolic extract	Total polyphenol and Flavonoids (mg)	
	mg equivalent Gallic acid / g dry weight	mg equivalent quercetin / g dry weight
	103.81 ± 3.65 mg GAE/ g dry weight	21.5 ± 1.51 mg QE/ g dry weight

Effect of GABE on ADR-induced changes in serum biochemical parameters

Table 3 demonstrates the effect of GABE (100 mg.kg⁻¹, p.o) on the acute toxicity induced by single injection of ADR (20 mg. kg⁻¹, i.p). Intraperitoneal administration of ADR caused cardiotoxicity in all rats. Single i.p. injection of ADR significantly elevated serum LDH, CPK and AST levels reaching 522.16 ± 35.4 U.L⁻¹, 603.5 ± 48.77 U.L⁻¹ and 184 ± 56.88 U.L⁻¹ as compared with the control group. Pretreatment of the animals with GABE (100 mg.kg⁻¹, p.o) reduced significantly the rise in the level of serum LDH, CPK and AST enzyme activities by 400.16 ± 43.65 U.L⁻¹, 444.66 ± 53.51 U.L⁻¹ and 114.16 ± 7.57 U.L⁻¹, respectively, compared with ADR receiving group.

Effect of GABE on ADR -induced changes in GSH contents and lipid peroxides in heart tissues

Table 4 shows the effect of GABE pretreatment on ADR-induced changes in lipid peroxides measured as MDA and reduced GSH levels. MDA in heart tissue was significantly higher in ADR-treated group (146.98 ± 10.57 nmole/g of heart tissue) compared to control (128.95 ± 8.07 nmole/g of heart tissue). Pretreatment of the animals with GABE (100 mg.kg⁻¹, p.o) produced a significant decrease in MDA (130.84 ± 5.12 nmole/g of heart tissue) compared to ADR treated group (146.98 ± 10.57 nmole/g of heart tissue). In addition, there was a high significant decrease in GSH in ADR-treated group (1.63 ± 0.16 μmole/g of heart tissue) compared to control (2.5 ± 0.19 μmole/g of heart tissue). Pretreatment of the animals with GABE (100 mg.kg⁻¹, p.o) ameliorated the depletion of GSH content, there was a high significant increase in GSH in GABE - ADR treated group (2.02 ± 0.13 μmole/g of heart tissue) compared to ADR treated group (1.63 ± 0.16 nmole/g of heart tissue).

Table 4: Effects of GABE (100mg kg⁻¹ p.o daily for 4 weeks) on changes of heart lipid peroxides and reduced glutathione levels induced by ADR (20 mg kg⁻¹, i.p)

Parameters groups	MDA (nmole/g of heart tissue)	GSH (μmole/g of heart tissue)
Control group	128.95 ± 8.07	2.5 ± 0.19
GABE group	116.51 ± 14.30	2.54 ± 0.11
DXR treated group	146.98 ± 10.57*	1.63 ± 0.16**
GABE + DXR treated group	130.84 ± 5.12#	2.02 ± 0.13##

The results are expressed as mean ± S.E.M. of six rats per group. * P < 0.05 (significant difference), ** P < 0.01 (highly significant difference) compared with control; # P < 0.05 (significant difference), ## P < 0.01 (highly significant difference) compared with ADR treated group.

Effects of GABE on myocardial histological change detected by H&E stain

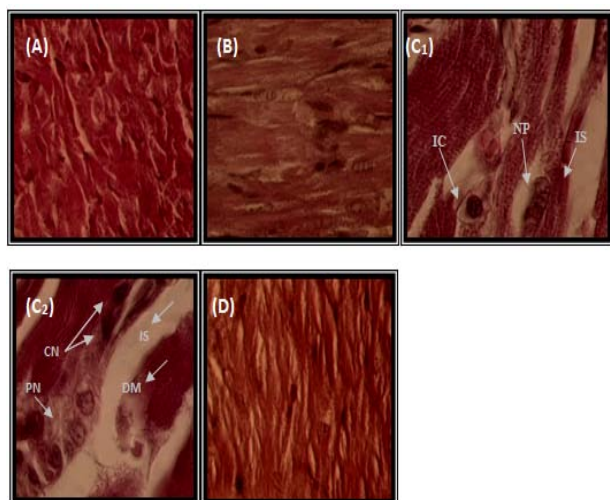


Figure 2: Light photomicrographs of paraffin-embedded H&E-stained rat heart sections. **(A)** Representative rat heart section (100×) from control animals, no pathologic changes present. **Figure 2(B)** Representative heart section (100×) from GABE-treated animals (100 mg/kg, p.o. for 4 weeks) no pathologic features present. **Figure 2 (C₁, C₂)** Representative heart section (100×) from animals treated with ADR (20 mg/kg, i.p. for 48 h) showing myofibril loss - intracellular space- (IS), partial necrosis (PN), condensed nuclei (CN), deformed myocytes (DM), nuclear pyknosis (NP), infiltration of inflammatory cells – mononuclear cell- (IC). **Figure 2 (D)** Representative heart section (100×) from rat treated with GABE (100 mg/kg, p.o. for 4 weeks) and ADR (20 mg/kg, i.p. for 48 h). Near complete protection was observed by GABE on ADR-induced changes.

Histological changes in heart were evaluated as described in section 2 and the results are presented in figure 3. The histology of hearts from control and GABE treated animal's displayed near-normal morphological appearance (figure 2A, 2B). On the other hand, there were histological changes in the ADR group (figure 2C₁, 2C₂). Qualitatively, ADR - induced cardiac damage was recognized by the presence of marked intercellular space, partial necrosis, condensed nuclei, deformed myocyte, nuclear pyknosis and infiltration of inflammatory cells. The lesions were significantly reduced in the group pretreated with GABE compared to ADR group (figure 2D).

DISCUSSION

The present study was undertaken to look for the protective effect of GABE on ADR induced cardiotoxicity in male Wistar rats for the first time. *G.alypum* is a plant, well known for its treatment properties in the traditional North African system of medicine². Its leaves are reported to be benefit in the treatment of cardiovascular diseases³, but there are no reports about *G.alypum* phytochemicals on cardiomyopathy induced by ADR in animal model.

Adriamycin is a broad spectrum antibiotic used as a chemotherapeutic drug for treatment of different forms of human neoplastic disease³⁷. However, the clinical use of this anticancer drug is greatly limited by its dose-dependent cardiotoxicity³⁸. The mechanisms involved in such toxicity have been documented by many investigators³⁹. The generation of free radicals in the heart tissue has been strongly accepted as crucial factors in the pathogenesis of ADR -induced cardiac damage⁴⁰. The semiquinone form, produced by the ADR reduction by several endogenous enzymes, generates superoxide radicals which rapidly generate other ROS species, such as hydroxyl radical and hydrogen peroxide; these radicals are able to induce apoptosis in cardiomyocytes⁴¹.

The tissue of the heart is very sensitive to free radical damage because of its highly oxidative metabolism and because its antioxidant defenses-like superoxide dismutase, GSH and Catalase- are fewer than those of other organs, such as liver⁴² furthermore, ADR also has high affinity for cardiolipin, a phospholipid present mainly in membranes of heart mitochondria leading to accumulation of ADR in the heart tissue⁴³. Previous reports suggest that ADR induces cardiotoxicity by the generation of free radicals⁴⁴.

Administration of ADR in combination with agents that would block its free radical- mediated toxicity without affecting its anti-tumor activity, and prevent its oxidative stress and tissue injury, might serve as a novel combination.

In fact, a number of studies using antioxidant supplements, such as vitamin E and N-acetylcysteine have failed to show a protective effect against chronic cardiomyopathy. Flavonoids are known to be potent antioxidants capable of modulating the activities of various enzyme systems, due to their interaction with biomolecules. Further, flavonoids have been reported for

their chemoprotective potential⁴⁵, render them particularly interesting to investigate them as new protective compounds against ADR cardiotoxicity.

In this study we have investigated the cardioprotective effect of butanolic fraction of *G.alypum* against ADR induced acute cardiotoxicity.

Reported work suggests that the hydro-methanolic extract of *G.alypum* aerial parts showed significant antioxidant effect. It has been previously reported that the major phytochemicals present in the alcoholic extract of *G.alypum* (flavonoids, phenylethanoids, iridoids) are responsible for the potent antioxidant activities⁷.

Most probably, this antioxidant effect of *G.alypum* phytochemicals is responsible for the reduction of ADR - induced toxicity and oxidative stress in our present study.

Our preliminary phytochemical studies in the methanolic solution carried out on the butanolic extract of this plant showed the presence of flavonoids in GABE as revealed by the UV-vis spectra and by the colorimetric method.

Most probably, these bioactive compounds of GABE are responsible for the reduction of ADR-induced toxicity and oxidative stress in our present study.

Increased activity of serum CPK, AST and LDH is a well-known diagnostic marker used to evaluate the existence and extent of myocyte injury. It has been reported that CPK, AST and LDH are released from the heart into blood stream, increasing the concentration in serum⁴⁶. It is widely reported that ADR induced free radical generation triggers membrane peroxidation and disruption of cardiac myocytes, which can lead to increased release of CPK, AST and LDH in the serum⁴⁷.

In the present study, a marked elevation in the activities of CPK, AST and LDH in the serum of ADR-intoxicated rats was observed. Pretreatment with GABE resulted in significant reduction in the activities of CPK, AST and LDH toward near normal as compared with toxic control rats. Pretreatment with GABE led to inhibition of CPK, AST and LDH release. This result could be due to protective effects of GABE on the myocardium, thus restricting the leakage of CPK, AST and LDH in to serum.

Since the role of free radicals in the cardiotoxicity of ADR is widely supported, many researchers have tried to use the MDA and GSH as biological markers of ADR-induced cardiotoxicity⁴⁸⁻⁴⁹.

ADR is capable of generating oxygen radicals, inhibiting GSH synthesis, and increasing MDA levels in humans and experimental animals⁵⁰. In addition, it has been reported that severe GSH depletion is known to associate with lipid peroxidation⁵¹.

GSH plays a major role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by antioxidant enzymes, such as glutathione peroxidase and glutathione S-transferase, resulting in the formation of its oxidized form, disulfide glutathione⁵².

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of free radical mediated lipid peroxidation injury.

Lipid peroxydation plays a very important role in oxidative injury for three reasons. First, it can amplify the number of free radical chain reactions; second, some products of lipid peroxidation are toxic; and third, lipid peroxidation places a demand on the GSH-dependent detoxification system.

In the present study, the relationship between ADR-induced oxidative stress and cardiotoxicity is potent. This is clearly demonstrated by the fact that the increase in lipid peroxides and the decrease in GSH levels are positively correlated with biochemical signs characteristic of ADR cardiotoxicity, elevated serum CPK, AST and LDH.

This observation has been supported by the findings of Lazzarino⁵³ and Gustafson⁵⁴ who reported that, the cardiac content of MDA was increased and GSH content was reduced by administration of ADR acute treatment.

In the present study photomicroscopic evaluation of the rat myocardium revealed that ADR caused myofibril loss - intracellular space, partial necrosis, condensed nuclei, deformed myocytes, nuclear pyknosis and infiltration of inflammatory cells. Similar alterations in ADR treated rats have been reported earlier also⁵⁵.

Pretreatment with GABE showed increase in endogenous antioxidant components GSH. Concomitantly a decrease in extent of lipid peroxidation was also observed. This cardioprotective activity was further supported by lower incidence of fine structural changes in the animals treated with GABE plus ADR.

The results, thus, indicate that GABE possesses marked chemoprotective activity via its antioxidant potential and may have a promising role to play in the treatment of ADR-induced toxicity and oxidative stress.

The application of GABE as an adjuvant or as a food additive during chemotherapy or the treatment of diseases linked to oxidative stress should, therefore, be beneficial.

CONCLUSION

In conclusion, the present result suggests that chronic administration of butanolic extract of *Globularia alypum* has cardioprotective potential against ADR induced cardiotoxicity.

The cardio protective effect of GABE could be due to lipid lowering and decrease the level of serum biomarkers.

The identification of molecules with cardioprotective potential from this fraction of *G.alypum* may provide new directions for identification of cardioprotectives, which could be given concomitantly during ADR treatment.

Further studies are needed to elucidate the exact mechanisms of action of GABE and its clinical application.



REFERENCES

- ES-Safi N, Khlifi S, Kollmann A, Kerhoas L, EL Abbouyia A, Ducot PH, Iridoid Glucosides from the Aerial Parts of *Globularia alypum* L. (Globulariaceae), *Chem Pharm. Bull*, 54(1), 2006, 85-88.
- Elbetieha A, Oran SA, Alkofahi A, Darmani H, Raies AM, Fetotoxic potentials of *Globularia arabica* and *Globularia alypum* (Globulariaceae) in rats, *Journal of Ethnopharmacology*, 72, 2000, 215–219.
- Khlifi S, El Hachimi Y, Khalil A, ES-Safi N, El Abbouyi A, *In vitro* antioxidant effect of *Globularia alypum* L. hydromethanolic extract, *Indian J Pharmacol*, 37(4), 2005, 227-231.
- Skim F, Kaaya A, Jaouhari TJ, Lazrek HB, Jana M, El Amri H, Hypoglycaemic activity of *Globularia alypum* leaves in rats, *Fitoterapia*, 70, 1999, 382-389.
- Caldes G, Prescott B, King JR, A potential antileukemic substance present in *Globularia Alypum*, *Planta Med*, 27(1), 1975, 72-6.
- Bello R, Moreno L, Primo-Yúfera E, Esplugues J, *Globularia alypum* L. extracts reduced histamine and serotonin contraction *in vitro*, *Phytother Res*, 16(4), 2002, 389-92.
- Es-Safi N, Kollmann A, Khlifi S, Ducrot PH, Antioxidative effect of compounds isolated from *Globularia alypum* L. structure–activity relationship, *LWT*, 40, 2007, 1246–1252.
- Zhou Q, Chowbay B, Determination of doxorubicin and its metabolites in rat serum and bile by LC: application to preclinical pharmacokinetic studies, *J. Pharm. Biomed. Anal*, 30, 2002, 1063-1074.
- Bagchi D, Bagchi M, Hassoun EA, Kelly J, Stohs SJ, Adriamycin-induced hepatic and myocardial lipid peroxidation and DNA damage, and enhanced excretion of urinary lipid metabolites in rats, *Toxicology*, 95, 1995, 1-9.
- Beillerot A, JCR Dominguez, Kirsch G, Bagrel D, Synthesis and protective effects of coumarin derivatives against oxidative stress induced by doxorubicin, *Bioorg. Med. Chem Lett*, 2008.
- Kelishomi RB, Ejtemaemehr S, Tavangar SM, Rahimian R, Mobarakeh JI, Dehpour AR, Morphine is protective against doxorubicin-induced cardiotoxicity in rat, *Toxicology*, 243, 2008, 96-104.
- Singal PK, Iliskovic N, Li T, Kummur D, Adriamycin cardiomyopathy: pathophysiology and prevention, *FASEB J*, 11, 1997, 931–936.
- Matos HR, Capelozzi VL, Gomes OF, Di Mascio P, Medeiros MHG, Lycopene inhibits DNA damage and liver necrosis in rats treated with ferric nitrilotriacetate, *Arch. Biochem. Biophys*, 396, 2001, 171–177.
- Skladanowski A, Konopa J, Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells, *Biochem. Pharmacol*, 46, 1993, 375–382.
- Singal PK, Siveski-Iliskovic N, Hill M, Thomas TP, Li T, Combination therapy with probucol prevents adriamycin induced cardiomyopathy, *J. Mol. Cell. Cardiol*, 27, 1995, 1055–1063.
- Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB, Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin, *Cancer Res*, 61, 2001, 771–777.
- Hong YM, Kim HS, Yoon HR, Serum lipid and fatty acid profiles in adriamycin-treated rats after administration of l-carnitine, *Pediatr. Res*, 51, 2002, 249–255.
- Kitagawa K, Takeda K, Saito K, Okamoto S, Makino K, Maeda H, Ichihara T, Differences in fatty acid metabolic disorder between ischemic myocardium and doxorubicin-induced myocardial damage: assessment using BMIPP dynamic SPECT with analysis by the Rutland method, *J. Nucl. Med*, 43, 2002, 1286–1294.
- Li T, Danelisen I, Singal PK, Early changes in myocardial antioxidant enzymes in rats treated with adriamycin, *Mol. Cell. Biochem*, 232, 2002, 19–26.
- Lee V, Randhawa AK, Singal PK, Adriamycin-induced myocardial dysfunction *in vitro* is mediated by free radicals, *Am. J. Physiol*, 261, 1991.
- Liu X, Chen Z, Chua CC, MA YS, Youngberg GA, Hamdy R, Chua BL, Melatonin as an effective protector against doxorubicin-induced cardiotoxicity, *Am J Physiol Heart Circ Physiol*, 283, 2002, 254–263.
- Choi EH, Chang HJ, Cho JY, Chun HS, Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities, *Food and Chemical Toxicology*, 45, 2007, 1873–1881.
- Ferreira ALA, Yeum KJ, Matsubara LS, Matsubara BB, CR Correa, EJ Pereira, Russell RM, Krinsky NI, Tang G, Doxorubicin as an antioxidant: Maintenance of myocardial levels of lycopene under doxorubicin treatment, *Free Radical Biology & Medicine*, 43, 2007, 740–751.
- Ferreira ALA, Salvadori DMF, Nascimento MCMO, Rocha NS, Correa CR, EJ Pereira, LS Matsubara, Matsubara BB, Placido Ladeira MS, Tomato-oleoresin supplement Prevents doxorubicin-induced cardiac myocyte oxidative DNA damage in rats, *Mutation Research*, 631, 2007, 26–35.
- Bommu P, Nanjan CMJ, Joghee NM, Nataraj SM, S Bhojraj, *Phyllanthus maderaspatensis*, a dietary supplement for the amelioration of adriamycin-induced toxicity and oxidative stress in mice, *J Nat Med*, 62, 2008, 149–154.
- Bronner WE, Beeche GR, Extraction and measurement of prominent flavonoids in orange and drape fruits juice concentrates, *J.chromatogr*, 705, 1995, 247-256.
- Edeoga HO, Okwu DE, Mbaebie BO, Phytochemical constituents of some Nigerian medicinal plants, *African Journal of Biotechnology*, 4(7), 2005, 685-688.
- Park yk, matias de Alencar S, Zima de Agenar C, Scamparini RP, Ganzalez M, Maria AA, Comparação Das características fisicoquímicas das propolis produzidas na região subtropical da America Do Sud: Evidencia fitoquímica de sua origem botânica, *Mensagem Doce*, 61, 2001.
- Boharun T, Gressier B, Trotin F, Bruner C, Dine T, Vasseur J, Gazin JC, Pinkas M, Luyckx M, Gazin M, Oxygen species scavenging activity of phenolic extract from hawthorn fresh plant organs and pharmaceutical preparation, *Arzneim Forsh / Drug Res*, 1996, 1-6.



30. Price MP, bulter LG, rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *Journal of agricultural and food chemistry*, 25, 1977, 1268-1273.
31. Singh G, Singh AT, Abraham A, Bhat B, Mukherjee A, Verma R, Agarwal SK, Jha S, Mukherjee R, Burman AC, Protective effects of *Terminalia arjuna* against Doxorubicin induced Cardiotoxicity, *Journal of Ethnopharmacology*, 2008, 1-26.
32. Mohamed HE, El-swefy SE, Hagar HH, The protective effect of glutathione administration on adriamycin-induced acute cardiac toxicity in rats, *Pharmacological Research*, 42, 2000, 115-121.
33. Ray SD, Patel D, Wong V, Bagchi D, In vivo protection of DNA damage associated apoptotic and necrotic cell deaths during acetaminophen-induced nephrotoxicity, amiodarone-induced lung toxicity and doxorubicin-induced cardiotoxicity by a novel IH636 grape seed proanthocyanidin extract, *Res. Commun. Mol. Pathol. Pharmacol*, 107, 2000, 137-166.
34. Ellman GL, Tissue sulfhydryl groups. *Arch biochem biophys*, 2, 1959, 70-7.
35. Ohkawa H, Ohishi N, Yagi K, Assay of lipid peroxides in animal tissue by thiobarbituric reaction, *Analytical biochemistry*, 95(2), 1979, 351-358.
36. Kelishomi RB, Ejtemaemehr S, Tavangar SM, Rahimian R, Mobarakeh JI, Ahmad RD, Morphine is protective against doxorubicin-induced cardiotoxicity in rat, *Toxicology*, 243, 2008, 96-104.
37. Myers CE, Chabner BA, Collins JM, *Cancer chemotherapy: principles and practice*, Philadelphia: J.B. Lippincott Co, 1990, 356-381.
38. Samuel L, Dox-induced cardiotoxicity. *Postgrad Med J*, 1995, 71-318.
39. Mimnaugh EG, Trush MA, Bhatnagar M, Gram TE, Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin, *Biochem Pharmacol*, 34, 1985, 847-56.
40. Abdel-Wahab MH, El-Mahdy MA, Abd-Allah MF, Helal GK, Khalifa F, Hamadaa FMA, Influence of *p*-coumaric acid on doxorubicin-induced oxidative stress in rat's heart, 48, 2003, 461-465.
41. Adeline B, Juan-Carlos RD, Gilbert K, Denyse B, Synthesis and protective effects of coumarin derivatives against oxidative stress induced by doxorubicin. *Bioorganic & Medicinal Chemistry Letters* (2008).
42. Seval Y, Ahmet A, Engin S, Izzet K, Songul O. Protective effect of lycopene on adriamycin-induced cardiotoxicity and nephrotoxicity, *Toxicology*, 218, 2006, 164-171.
43. Ana Lucia AF, Kyung-Jin Y, Luiz Shiguero M, Beatriz BM, Camila RC, Elenize JP, Robert MR, Norman IK, Guangwen T, Doxorubicin as an antioxidant: Maintenance of myocardial levels of lycopene under doxorubicin treatment, *Free Radical Biology & Medicine*, 43, 2007, 740-751.
44. Praveen B, Chandrasekar M, Joghee N, Nanjan MJ, Satishkumar MN, Suresh B, *Phyllanthus maderaspatensis*, a dietary supplement for the amelioration of adriamycin-induced toxicity and oxidative stress in mice, *J Nat Med*, 62, 2008, 149-154.
45. Surh Y, Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances, *Mutat Res*, 428, 1999, 305-327.
46. Deepa PR, Varalakshmi P, Protective effect of low molecular weight heparin on oxidative injury and cellular abnormalities in adriamycin-induced cardiac and hepatic toxicity, *Chem. Biol. Interact*, 146, 2003, 201-210.
47. Liu X, Chen Z, Chua CC, Ma Y, Youngberg GA, Hamdy R, Chua BHL, Melatonin as an effective protector against Doxorubicin induced cardiotoxicity. *American Journal of Physiology: Heart and Circulatory Physiology*, 283, 2002, 254-263.
48. Abdul SN, Shibli JA, Krishna KP, Mohammad A, Aftab A, Abul KN, Protective effects of *Bombyx mori*, quercetin and benazepril against doxorubicin induced cardiotoxicity and nephrotoxicity, *Journal of Saudi Chemical Society*, 2013.
49. Essam AS, Sabah H EIG, Ibrahim S, Osama MA, Ayman AN, Ahmed MM, Antioxidant and cardioprotective activity of *Stachys schimperi* Vatke against doxorubicin-induced cardiotoxicity, *Bulletin of Faculty of Pharmacy, Cairo University*, 50, 2012, 41-47.
50. Singal PK, Siveski-ILiskovic N, Hill M, Thomas T, Li T, Combination therapy with probucol prevents adriamycin-induced cardiomyopathy, *J Mol Cell Cardiol*, 27, 1995, 1055-63.
51. Kimura T, Fujita I, Itoh N, Muto N, Nakanishi T, Takahashi K, Azuma J, Tanaka K, Metallothionein acts as a cytoprotectant against doxorubicin toxicity, *J Pharmacol Exp Ther*, 292, 2000, 299-302.
52. Griffith OW, (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis, *J Nat Med*, 62, 2008, 149-154.
53. Lazzarino G, Vida AR, Mulieri L, Ratitio G, Marvelli I, Prevention by fructos-1.6-bisphosphate of cardiac damage induced in mice by subchronic doxorubicin treatment. *Cancer Res*, 47, 1987, 6511-6.
54. Gustafson DL, Swanson JD, Pritsos CA. Modulation of glutathione and glutathione dependent antioxidant enzymes in mouse heart following doxorubicin therapy. *Free Radic Res Commun*, 19, 1993, 111-20.
55. Debasis B, Chandan KS, Sidhartha DR, Dipak KD, Manashi B, Harry GP, Joe AV, Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract, *Mutation Research*, 523-524, 2003, 87-97.

Source of Support: Nil, **Conflict of Interest:** None.

