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Combination of *Nigella sativa* with *Glycyrrhiza glabra* and *Zingiber officinale* augments their protective effects on doxorubicin-induced toxicity in h9c2 cells

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ABSTRACT

Objective(s): The use of doxorubicin (DOX) is limited by its dose-dependent cardio toxicity in which reactive Oxygen Species (ROS) play an important role in the pathological process. The aim of this study was to evaluate the protective effect of three medicinal plants, *Nigella sativa* (N), *Glycyrrhiza glabra* (G) and *Zingiber officinale* (Z), and their combination (NGZ), against DOX-induced apoptosis and death in H9c2 cells.

Materials and Methods: The cells were incubated with different concentrations of each extract or NGZ for 4 hr which continued in the presence or absence of 5μM doxorubicin for 24 hr. Cell viability and the apoptotic rate were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and propidium iodide (PI) staining assays, respectively. The level of ROS and lipid peroxidation were measured by fluorimetric methods.

Results: Treatment with doxorubicin increased ROS generation, enhanced malondialdehyde (MDA) formation, and induced apoptosis. Co-treatment of the cells with each herb extract increased viability of cells dose-dependently with a maximum protection effect of about 30%, and their potencies were N>G>Z. The combination of the threshold dose of each extract (NGZ) produced a similar effect, which was increased dose-dependently to a maximum protection of 70%. These effects were correlated with the effects of NGZ on ROS and MDA.

Conclusion: All of the extracts have some protective effects against DOX-induced toxicity in cardiomyocytes with similar efficacies, but with different potencies. However, NGZ produced much higher protective effect via reducing oxidative stress and inhibiting of apoptotic induction processes. Further investigations are needed to determine the effects of NGZ on DOX chemotherapy.

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Introduction

Doxorubicin (DOX) is an *important component* of multimodality therapy for several antineoplastic combined *chemotherapy protocols*. It is used to treat a variety of malignacies such as leukemias, Hodgkin and non-Hodgkin lymphoma, and solid tumors (1). However, despite high efficacy, the major side effect of DOX is *cardio-toxicity,* which has dramatically *hindered its* clinical *usage* for a prolonged period of time. The mechanisms of DOX-induced cardio toxicity are not completely understood, but most evidences indicate the generation of reactive oxygen species (ROS) involvement (2). Interestingly, some natural foods have been reported to contain substantial amounts of antioxidants and free radical scavenging agents. These compounds diminish some side effects of chemotherapeutic agents on normal cells by reducing their genotoxicity (2). There are many evidences showing the protective effects for different herbs against oxidative injury-related *cardio toxicity*, and among which *N. sativa, Zingiber officinale* and *Glycyrrhiza glabra* showed the highest protective effect.

N. sativa is used as spice and food preservative (3). Its seeds have long been used in traditional medicine for a wide range of disorders including bronchial asthma, headache, infections, obesity, back pain, hypertension and gastrointestinal problems (4). These effects are mediated via different mechanism such as antioxidant (5), anti-inflammatory (6), anticancer (7), and antihistaminic effects (8). It has been shown that *N. sativa* attenuates isoproterenol induced myocardial infarction (MI) (9). Also, *N. sativa* oil supplementation reduces lead-induced cardio-toxicity by mechanisms related to its ability to decrease the pro-inflammatory cytokines; oxidative stress and cardiac tissue damage, and preserve the activity of antioxidant enzymes (10). Ebru *et al* (2008) showed that pretreatment with *N. sativa* oil decreased the subsequent cyclosporine-A injury in rat heart (11),

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Recent studies revealed a protective effect of thymoquinone against doxorubicin-induced cardio toxicity (12-14).

Z. officinale, commonly known as ginger belongs to the Zingiberaceae family. The rhizome of ginger has an aromatic pungent taste. It is consumed worldwide as a spice and flavoring agent, and is attributed to have many medicinal properties (15). It is used in traditional medicine as carminative, and antipyretic, and in the treatment of pain, rheumatism and bronchitis (15). It has different pharmacological activities such as hepatoprotective (16), antiparasitic (17), antimalarial (18), antimicrobial (19), antidiabetic (20), and radioprotective effects (21). Ginger also is a potential remedy for cardiovascular diseases (22), and can prevent the development of morphine analgesic tolerance and physical dependence in rats (23). It is also used for the treatment of gastrointestinal disorders including gastric ulcerogenesis (24). The plant contains high level of phenolic and flavonoid compounds, responsible for its high antioxidant activities (25).

G. glabra (licorice) is used widely as a flavoring and sweetening agent in tobacco products, chewing gum, candy, toothpaste and beverages. In addition, licorice is frequently prescribed as a treatment in oriental herbal medicine. Licorice root has cancer chemopreventive effect (26).

The therapeutic potential of *G. glabra* as a hypoglycemic (27), hypocholesteremic (28), anti-ulcer (29), anti-inflammatory (30), renoprotective (31), and antiatherogenic (32) have been demonstrated. In traditional medicine, it is useful in the treatment of agent renovascular and cardiovascular diseases, and hence constitutes as the major ingredient of polyherbal formulations indicated for cardiovascular diseases (31). The investigations have shown that *G. glabra* has protective effects against ischemic damages of the several body organs due to its potent antioxidant and free radical scavenging activity (27, 28, 30-32). Also, a recent study has shown that pretreatment with *G. glabra* significantly attenuates ischemic–reperfusion induced myocardial injury by improving antioxidant status in heart (33).

In addition, other species of *G. uralensis,* suppressed doxorubicin-induced apoptosis in H9c2, rat cardiac myoblast cells (34). Treatment with licorice extract significantly protected the mice against DOX-induced cardio toxicity (35), and improved cardiac performance (36).

Based on these findings, we hypothesized that the combination of these medicinal plants may produce a higher protective effect against doxorubicin-induced cardiomyopathy. Therefore, the present study was designed to investigate the potential effects of *Nigella sativa* with *Glycyrrhiza glabra* and *Zingiber officinale* (NGZ) extracts on cell viability, lipid peroxidation level, ROS content and apoptotic induction in H9c2 cardiomyocytes.

Materials and Methods

Reagents and chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Thiobarbituric acid (TBA), 2,7-dichlorofluorescin diacetate (DCFH-DA), Propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagles Medium (DMEM), penicillin-streptomycin and fetal bovine serum were purchased from Gibco. Trichloroacetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany). H9c2 cells were obtained from Pasteur Institute (Tehran, Iran).

Preparation of extracts

N. sativa seeds were collected from Gonabad region (northeast of Iran) and authenticated by *Herbarium* of the *Ferdowsi University* of Mashhad (FUMH) (voucher specimen = 293-0303-1). *G. glabra* rhizomes was purchased from local market and authenticated by FUMH (voucher specimen number = 25947). Whereas *Z. officinale* is not native to Iran, afforded from a local herb *market* in Mashhad and confirmed by plant specialist of FUMH. The seeds of *N. sativa* were washed, dried, and crushed to a powder with an electric micronizer. The *G. glabra and Z. officinale* rhizomes were peeled chopped into tiny bits, air-dried and were ground with a mechanical grinder. Each herbal sample was extracted separately in a Soxhlet extractor with ethanol (70%) and the resulting extract was dried and kept at -20˚C until use.

Cell culture and treatment

H9c2 Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO2. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 100 Units/ml penicillin and 100 µg/ml streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT/ROS and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000 cell/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with each herbal extract alone (6 to 200 µg/ml) for 4 hr and then incubation was continued in the presence of the herbal extract with or without 5 µM doxorubicin for 24 hr. For assaying the effect of three extract in combination, the minimum effective concentration of each extract was chosen (N=12.5, G=25 and Z=25 µg/ml sum=62.5 µg/ml) as the basis and one (62.5 µg/ml), two (125 µg/ml), four (250 µg/ml) and eight (500 µg/ml) folds of this concentration were applied.

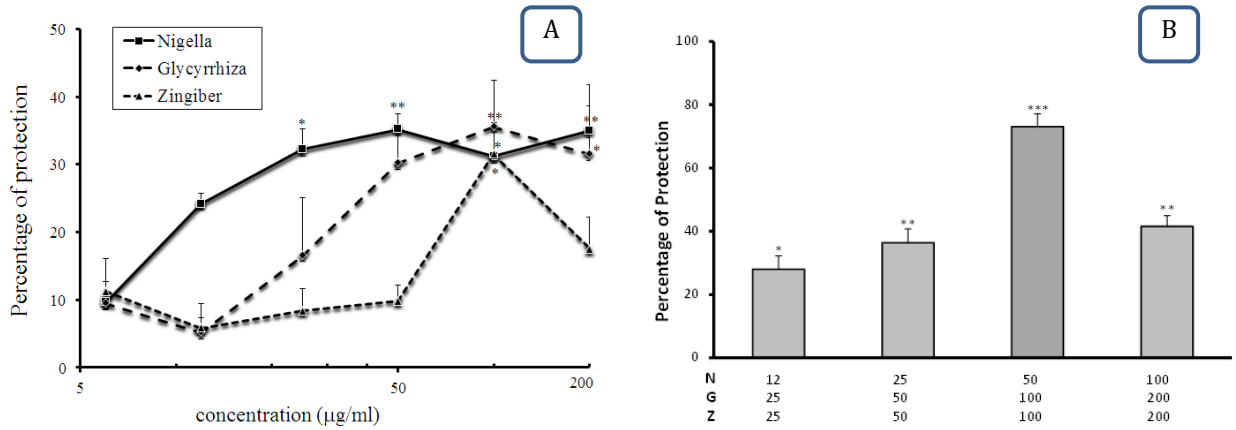

Figure1. Effect of extracts alone (Figure 1A) or NGZ (Figure 1B) on percentage of protection against DOX-induced cytotoxicity in H9c2 cells. Cells were pretreated with different concentrations of NGZ for 4 hr before exposure to 5 µM of DOX for 24 hr. Data are expressed as mean ± SEM of three separate experiments. DOX decreased cell viability to 62.2±2 P<0.001 versus control.$^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 versus DOX

Cell viability assay

Cell viability was determined using a modified MTT assay as described previously (37). Briefly, MTT solution in phosphate-buffered saline (5 mg/ml) was added to each well at final concentration of 0.05%. After 3 hr, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring MDA, which is the end product of lipid peroxidation (38). At the end of incubation, the cells were scraped and centrifuged for 30 min. Then, 400 µl of TCA (15%) and 800 µl of TBA (0.7%) were added to 500 µl of cell samples. The mixture was vortexed and heated for 40 min in a boiling water bath. Then, 200 µl of the sample was transferred to 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

Measurement of reactive oxygen species

Intracellular ROS level was evaluated using a fluorescent probe, DCF-DA. At the end of incubation, the cells were treated (30 min) with DCFH-DA (10µM) at 4˚C in the dark. Then, the fluorescence intensity was detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

PI staining

Apoptotic cells were detected using PI staining of small DNA fragments followed by flow cytometry. It has been reported that a sub-G1 peak that is reflective of DNA fragmentation can be observed following the incubation of cells in a hypotonic phosphate-citrate buffer containing a quantitative DNA-binding dye such as PI. Apoptotic cells that have lost DNA will take up less stain and appear on the left side of the G1 peak in the histogram. Briefly, H9c2 cells were seeded in wells of a 24-well plate and treated according to mentioned protocol. Floating and adherent cells were then harvested and incubated at 4 °C overnight in the dark with 750 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate with 0.1% Triton X-100). Next, flow cytometry was carried out using a FACScan flow cytometer (Becton Dickinson). A total of 104 events were acquired with FACS.

Statistics

All data were expressed as mean ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tamhane's T2 *post-hoc* test. Differences were considered significant at P<0.05.

Results

Effect of extracts alone or in combination (NGZ) on cell viability

Incubation with doxorubicin significantly decreased cell viability to 62±2% compared to control of control (P<0.001). Cell viability was assayed to determine the optimum concentrations necessary for the three extracts (N, G and Z) to protect H9c2 cells against DOX-induced cytotoxicity. The results demonstrated that, in comparison with DOX, *N. sativa* increased cell viability at doses of 25 to 200 µg/ml dose dependently that maximum effect was produced at effect at concentration of 50 µg/ml (75.4±0.84, P<0.01), and *Z. officinale* increased cell viability at doses of 50 to 200 µg/ml, its maximum effect was at dose of 100 µg/ml (75±2.5, P<0.01), while *G. glabra* increased cell viability at dose of 100 µg/ml (73.5±1.5, P<0.05) (Figure 1A).

Results of experiment with combination extract (NGZ) showed that all doses (62.5, 125, 250 and 500 µg/ml) protected H9c2 cells against DOX and

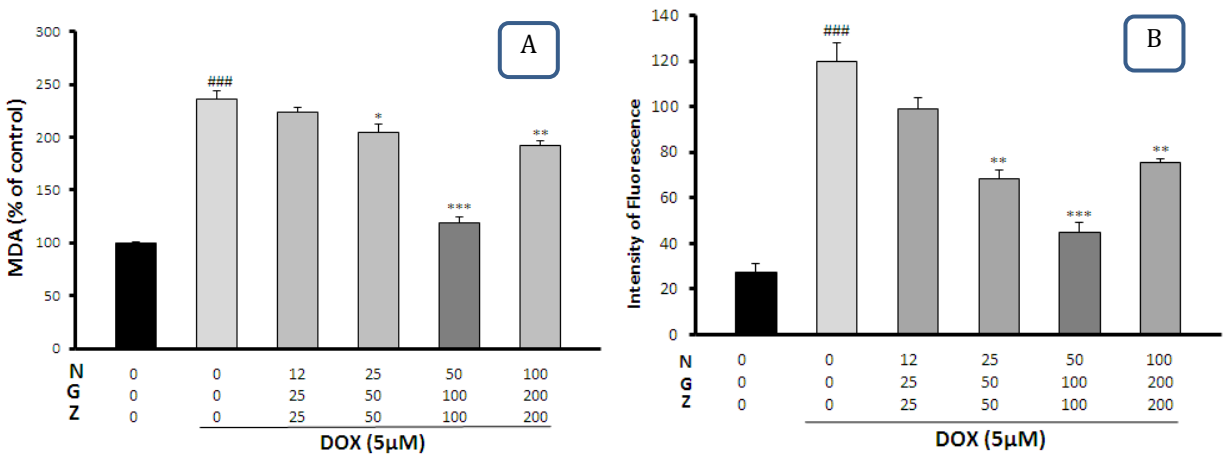

Figure 2. Effect of NGZ on DOX-induced MDA production (Figure 2A) and reactive oxygen species (ROS) generation (Figure 2B) in H9c2 cells. Cells were pretreated with different concentrations of NGZ for 4 hr before exposure to 5 µM of DOX for 24 hr. Data are expressed as mean ± SEM of three separate experiments
###P<0.001 versus control, *P<0.05, **P<0.01 and ***P<0.001 versus DOX

increased cell viability significantly. The best protection was obtained at dose of 250 µg/ml (90.29±1.2, P<0.001, 70% protection).

The effect of NGZ on ROS content and lipid peroxidation

The biochemical determination of malondialdehyde (MDA) represents lipid peroxide formation. The results showed that DOX significantly increased MDA (P<0.001) and ROS (P<0.001) levels in H9c2 compared with control cells. In the presence of DOX, treatment with 125 µg/ml (P<0.05), 250 µg/ml (P<0.001) and 500 µg/ml (P<0.01) doses of NGZ significantly reduced the level of MDA. The most reduction of MDA was observed at the dose of 250 µg/ml (control 100±1.4, DOX 235±7.9 and 250 µg/ml NGZ 119±5.18). Also this combination diminished significantly ROS production in comparison with DOX, at dose of 125 µg/ml (P<0.01), 250 µg/ml (P<0.001) and 500 µg/ml (P<0.01) (Figures 2A and 2B, respectively). The lowest production of ROS was at the dose of 250 µg/ml (control 27±4, DOX 120±8 and 250 µg/ml NGZ 45±4).

The effect of NGZ on apoptosis induction

Apoptosis in H9c2 cell line was detected with flow cytometry using PI staining. Cells were pretreated for 4 hr with various concentrations of the NGZ and exposed to DOX for 24 hr. Analysis of the subG1 peak in flow cytometry histograms revealed the induction of apoptosis in cells treated with DOX (P<0.001). NGZ decreased apoptotic induction significantly at the doses of 62.5 µg/ml (P<0.05), 125 µg/ml (P<0.01), 250 µg/ml (P<0.001) and 500 µg/ml (P<0.01) (Figure 3). The most reduction of apoptotic rate was at the dose of 250 µg/ml (control 12±1.4, DOX 71±4 and 250 µg/ml NGZ 37.5±2.5)

Discussion

Cardiovascular risk factors enhance the production of ROS generated by mitochondrial electron-transport chain, xanthine oxidase, NADPH oxidase and uncoupled nitric oxide synthases in cardiomyocytes (39). Oxidative stress occurs when production of ROS exceeds the capacity of antioxidant defense systems (catalase, SOD and glutathione peroxidase) (40). Because of its deleterious effects, this stress is associated with poor outcomes in cardiovascular diseases (41). Weak antioxidant capacity in cardiomyocytes may be a risk factor responsible for their high sensitivity to oxidative damage (42), and a promising approach to cardioprotection is the use of pharmacological tools to reduce oxidative stress in the heart (37). In the present study, we used H9c2 cells as a pharmacological model to evaluate the potential cardioprotective effects of three different prevalent medicinal plants, *N. sativa, Glycyrrhizin glabra* and *Zingiber officinale* alone and in combination with all three (NGZ). The results showed that the combination of NGZ improves their protective effects against DOX-induced oxidative stress in H9c2 cells.

H9c2 cells are morphologically similar to immature embryonic cardiomyocytes. Considering that these cells preserve electrical and hormonal signal pathways found in adult cardiac cells (43), they are a useful model for studying oxidative stress-induced cardiomyocyte damage (44). In this model, DOX significantly increased the level of ROS and lipid peroxidation and induced the rate of apoptosis. These changes are similar to the DOX-induced deleterious effects on normal cardiac cells, which lead to the loss of cardiomyocytes viability (2).

In this study, pretreatment with each of the three herb extracts alone produced some degree of protection in a concentration dependent manner. These findings are in accordance with previous studies which show the capability of cadioprotective effects of *N. sativa*, *G. glabra* and *Z. officinale* (10-14).

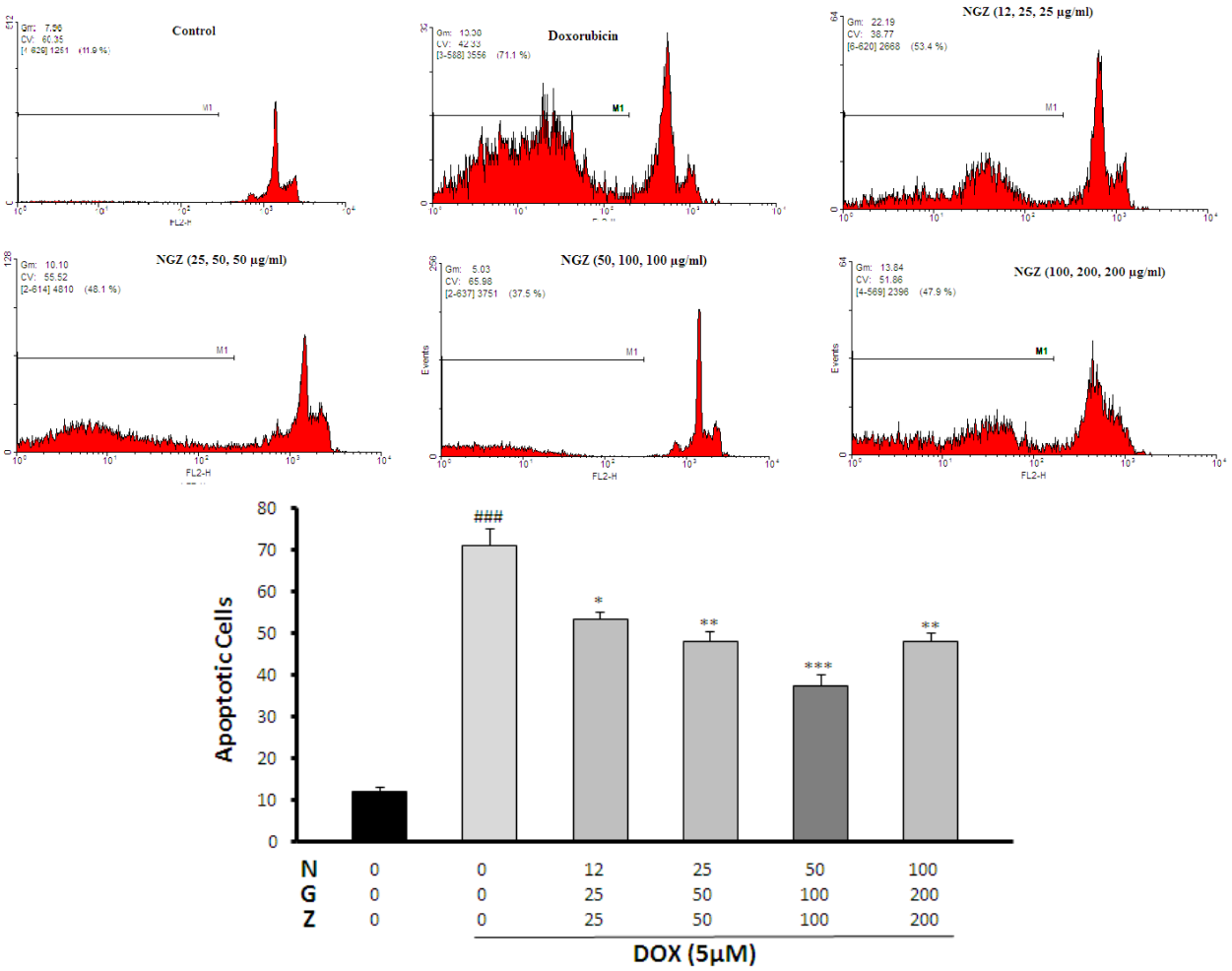

Figure 3. The effects of the NGZ on apoptosis in H9c2 cells using PI staining and flow cytometry. ###P<0.001 versus control, *P<0.05, **P<0.01 and ***P<0.001 versus DOX

In mice, thymoquinone, the pharmacologically active component of *N. sativa*, reduces DOX-induced cardio toxicity without reducing the serum concentration of DOX (12). Also, studies have shown that thymoquinone potentiates cisplatin antitumor activity and protects against cisplatin-induced nephrotoxicity in mice and rats (45). A recent investigation has shown that *N. sativa* oil supplementation attenuates lead-induced cardio-toxicity by mechanisms related to its ability to decrease the pro-inflammatory cytokines; oxidative stress and cardiac tissue damage, and preserve the activity of antioxidant enzymes (10). Ebru *et al* (2008) showed that pretreatment with *N. sativa* oil decreased cyclosporine-A injury in rat heart (11), and later studies revealed a protective effect of thymoquinone against doxorubicin-induced cardio toxicity (12-14). Recently, it was shown that gingerol protected cardiomyocytes against DOX-induced toxicity through its antioxidative effect and modulation of NF-κB as well as apoptosis (46).

Glycyrrhizin, the chief sweet-tasting constituent of *G. glabra* (liquorice) root and its metabolites (glycyrrhizic acid or glycyrrhizinic acid) induce apoptosis and cell growth inhibition in various cancer cells, including human stomach cancer cells, promyelotic leukemia HL-60 cells and hepatoma cells (47, 48). Moreover, experimental results showed that the combination of antibiotic anti-cancer drug with 18β-glycyrrhetinic acid (derivative of the beta-amyrin type obtained from the hydrolysis of glycyrrhizic acid) exhibits a synergistic toxic effect on cancer cell lines (49). The extract of *Glycyrrhiza uralensis* suppresses doxorubicin-induced apoptosis in H9c2 rat cardiac myoblasts (34).

Recent experimental observations reported that *Z. officinale* is an effective anticancer agent (50). Also, in a polyherbal preparation, ginger was one of the components and reported to be effective against DOX-induced cardio toxicity without interfering its antineoplastic activity (51).

Thus, it is of great interest to investigate the effect of combination of these extracts on DOX-induced disorders. In this study, the combination of extracts (NGZ) augmented their protective effects, suggesting

the presence of a positive interaction between components. In this experiment, a maximum effect about 30 percent protection was produced by each extracts. However, the maximum protective effect was achieved with combination of the threshold concentrations of the extracts and the protection increased in concentration dependent manner to a maximum protection of more than 2.5 fold. These observations do not show an additive effect, but implicates the presence of a positive interaction between extracts against DOX-induced apoptosis in H9c2 cells.

In high concentrations, *N. sativa* did not produce any further protective effect, while the effects of the two other extracts were decreased in high concentrations (Figure 1A). The decreasing effect of *G. glabra* or *Z. officinale* at concentration of 200 µg/ml could not be explained by a non-specific effect such as ionic disturbances. The combination at concentration of 250 µg/ml increased their protective effect. However, at higher concentration (500 µg/ml) in mixed extracts, the effect was reduced. This may suggest the presence of some components in crude extracts, which may have toxic effect and reduce the protection.

The molecular mechanisms for protective effect of each extract and augmenting effect of their combination (NGZ) are not clear. However, it has been shown that Glycyrrhizate could ameliorate rabbit myocardial ischemia-reperfusion injury through P38MAPK pathway (52). Also, treatment with *Glycyrrhiza uralensis* extract significantly protected mice from DOX-induced cardio toxicity, by decreasing levels of serum LDH and Creatin Kinase-MB improving heart morphology and increasing GSH-P(X) activity and GSH level (35). Also, results revealed that glycyrrhizin and glycyrrhetinic acid directly affected cardiac performance (36). In this paper, we showed that the protective effects may be mediated by reducing oxidative stress, which could decrease the production of ROS, lipid peroxidation and diminished apoptotic induction.

It is interesting that in comparison with the effect of each extract alone, the combination produced a stronger protective effect. Therefore, it can be concluded that cardioprotective effect of NGZ is mediated at least in part by its active ingredients. However, further investigations are needed to reveal molecular mechanisms to increase the possible positive interactions and diminish negative interactions in combination. These must be included in fractionation of the extracts to assess the effect of each fraction in combination. These may lead to find purified active ingredients, which could be used in a clinical trial.

Conclusion

It was shown that all of three extract produced protective effect on H9c2 cell against DOX-induced toxicity with different potencies, but similar efficacy with a maximum effect of about 30 percent. The protective effect of extracts was increased by using them in combination. This effect is correlated with reducing oxidative stress and inhibition of apoptosis induction. The effects of extracts in combination showed a positive interaction between components of different extracts, which augments their protective effect. These observations could suggest new *experiments* that can be performed to test the roles of each extract components in the combination. However, potential interactions of extracts with DOX chemotherapy should be assessed. These may lead to the development of a new class of drugs to prevent cardio toxicity of DOX and improve its anticancer effect.

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