



Mitigating effects of walnut oil on doxorubicin-induced Cardiotoxicity in mice

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Abstract

The use of medicinal plants increased considerably as it is necessary for producing more effective drugs with fewer side effects. Walnut has different properties with therapeutic potential in traditional medicine. The aim of this study is to determine the *in vitro* antioxidant activity of walnut oil and its cardioprotective effect against doxorubicin induced cardiotoxicity in mice. The *in vitro* antioxidant activity of the oil was analyzed using ferric reducing antioxidant power (FRAP) and 1,1--diphenyl-β-picrylhydrazyl (DPPH) assay. The FRAP of the oil increases with increase in concentration. The DPPH scavenging activity of the oil increases with increase in concentration but has a low activity when compared with ascorbic acid.

Twenty-five mice were divided into five with group A being the control given 0.3ml of 0.9% normal saline; group B was given a cumulative dose of 45 mg/kg body weight of Doxorubicin; group C was given 15 mg/kg body weight of walnut oil only; group D was given 20 mg/kg body weight of walnut oil and cumulative dose of 45 mg/kg body weight of Doxorubicin; and group E was given 10mg/kg body weight of oil and cumulative dose of 45 mg/kg body weight of Doxorubicin. The walnut oil was administered orally daily while Doxorubicin was administered intraperitoneally. At the end of three weeks, the lipid profile was assessed and the histopathological examination of the heart was assessed.

In group B, total cholesterol, triglyceride (TG) and high-density lipoprotein (HDL) increases as compared with the control (group A). Group C, D, E showed a decrease in cholesterol and TG. HDL level increased in group C and D while a reduction was noticed in group E. The LDL level of group D and E decreased while that of group C increased. The heart histopathology indicated the toxicity of Doxorubicin as seen in group B however, the toxicity level reduced in group D and E showing moderate multiple foci of myofiber coagulation. There were no observable changes in the heart morphology of group C animal when compared to group A. It can be concluded from our results that walnut oil can be used to reduce toxicity level of Doxorubicin and hence possessed a cardioprotective role

Keywords: cardioprotective, doxorubicin, toxicity, lipid profile, walnut oil, *in vitro* antioxidant

Introduction

Consumption of walnut oil has been associated with a decrease risk of lipid disorder. Walnut in particular have a unique profile, they are rich in polyunsaturated fatty acids, which may improve blood lipids and other cardiovascular risk factors (Pein *et al.*, 2004; Shenoy *et al.*, 2011) [13]. Cardiovascular complications have emerged as a major concern for cancer patients. Many chemotherapy agents are cardiotoxic and some appear to also alter lipid profiles, although the mechanism for this is unknown. Indeed, cardiovascular disease (CVD) is the most common cause of death, next to the cancer itself, in many forms of solid tumours including breast cancer (Pein *et al.*, 2004; Shenoy *et al.*, 2011) [13].

A common chemotherapy regimen for breast cancer patients is doxorubicin. Doxorubicin, an anthracycline is a widely used chemotherapeutic agent and now part of standard therapeutic regimens for a variety of cancers (e.g, hematopoietic malignancies and advanced solid tumors of the breast, ovary, thyroid, and bone (Carter, 1975).

It is undisputed that dietary habits affect coronary risk factors and hence the risk of a coronary event. In the search for bioactive components in foods that favorably affect cardiovascular disease (CVD) risk, nuts have begun to attract attention (Kris-Etherton *et al.*, 1999) [7]. Epidemiologic studies have consistently demonstrated an association between nut consumption and coronary heart disease (CHD) morbidity and mortality in different population groups (Kris-Etherton *et al.*, 2001) [8]. These health promoting properties have been attributed mainly to the presence of unsaturated fatty acids and other bioactive nutrients, including phenolic compounds, tocopherols, phytosterols, high-quality protein, fiber, and minerals (Ros, 2006; Segura *et al.*, 2006; Brufau *et al.*, 2006; Oliveira *et al.*, 2008) [14, 16, 3, 11].

The unfavorable lipid profiles produced by some chemotherapy agents may be detrimental in the long term to cancer patients, especially those already at risk of cardiovascular disease (CVD). Also, the effects of these chemotherapeutic agents on lipid metabolism and organ antioxidant enzymes still remain largely enigmatic. Therefore, this study may be useful in tailoring effective follow-up care plans for cancer survivors.

Material and Methods

Chemicals and drugs

Ferrous sulphate, ascorbic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich Co MO, USA. All other chemicals used were of analytical grade.

Doxorubicin was purchased from Kunle Ara pharmacy, Orita-mefa, Ibadan, Southwestern, Nigeria.

Experimental Animals

Twenty-Five male mice weighing between 30-35g were used for the study. The animals were purchased from the animal house, College of Medicine, University of Ibadan, Oyo state, Nigeria. The mice were divided into 5 groups each and were kept in separate cages in the Animal house, The Polytechnic, Ibadan. The animals were kept for 2 weeks prior to starting the experimental protocol for proper acclimatization. The mice were given free access to water and diet during the whole experimental period.

Preparation of Walnut Oil

The walnuts were purchased from Bodija market, Ibadan and authenticated with voucher number FHI.112608 at Forest Herbarium, Forest Research Institute of Nigeria (FRIN).

Walnuts were separated from their shells, air-dried and milled. The oil was extracted with n-hexane using a Soxhlet apparatus. After extraction the solvent was removed by gentle evaporation over a rotary evaporator. The oil was stored in the fridge at about 4°C until used.

In Vitro Antioxidant Assay

Ferric Reducing Antioxidant Power (Frap) Assay

Ferric ion reducing antioxidant power assay (FRAP) Ferric ions reducing power was measured according to the method of Oyaizu with a slightest modification.

Procedures

Hydroalcoholic extract of walnut oil in different concentration ranging from 100 to 500 were mixed with 2.5 ml of 20 mM phosphate buffer and 2.5 ml 1%, w/v potassium ferricyanide, and then the mixture was incubated at 50 °C for 30 minutes. Afterwards, 2.5 ml of 10%, w/v trichloroacetic acid and 0.5 ml of 0.1% w/v ferric chloride were added to the mixture, which was kept aside for 10 minutes.

The absorbance was measured at 700 nm while ascorbic acid was used as positive reference standard. All assays were run in triplicate.

DPPH Radical Scavenging Assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) was measured by diluting the oil sample (1 mL) with 1mL of DPPH solution (0.3 mM). After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated using the equation as given below.

% inhibition of DPPH radical $\text{Abr-Aar/Abr} \times 100$

Where: Abr was the absorbance control and Aar was the absorbance of the sample reaction has taken place.

Physico-Chemical Parameters

The refractive index of the oil sample was measured using a refractometer.

The peroxide, saponification, iodine and acid value of the oil were measured following standard procedures respectively.

Experimental Protocol

The five groups of mice were treated according to the assay protocol summarized below. The walnut oil was administered orally.

Group A – Rats administered with normal saline for 21 days

Group B - Rats administered with 15mg/kg bodyweight of Doxorubicin intraperitoneally on 7th, 14th and 21st day respectively.

Group C - Rats administered with 15mg/kg bodyweight of walnut oil daily for 21days

Group D- Rats administered with 20mg/kg bodyweight of walnut oil daily for 21days and Doxorubicin (15mg/kg) on 7th, 14th and 21st day respectively.

Group E - Rats administered with 10mg/kg body weight of walnut oil daily for 21days and Doxorubicin (15mg/kg) on 7th, 14th and 21st day respectively.

Collection and Analysis of Samples

At the end of three weeks, blood was collected from the animals into heparinized tubes by ocular puncture under light ether anaesthesia after an overnight fast and the animals were dissected to remove the heart. The heart was preserved with 10% formalin for histopathology examination and the lipid profile of the blood samples was assessed following standard procedures.

Statistical Analysis

The results were analyzed with one way ANOVA and student T-test using graph prism 5 and Microsoft excel with $p \leq 0.05$ considered significant.

Results

Table 1: Frap Assay

| Concentration (AAE mg/g) | Oil Sample |
|--------------------------|-----------------|
| 100 $\mu\text{g/ml}$ | 0.68 \pm 0.01 |
| 200 $\mu\text{g/ml}$ | 0.91 \pm 0.05 |
| 300 $\mu\text{g/ml}$ | 1.19 \pm 0.06 |
| 400 $\mu\text{g/ml}$ | 1.64 \pm 0.01 |
| 500 $\mu\text{g/ml}$ | 2.44 \pm 0.02 |

Values are Mean \pm SD (n = 3) of triplicate determinations

Table 2: DPPH Radical Scavenging Assay: Percentage (%) inhibition

| Conc. ($\mu\text{g/mL}$) | Oil sample (%) | Ascorbic acid (%) |
|----------------------------|-------------------|-------------------|
| 100.00 | 51.75 \pm 1.25 | 77.70 \pm 4.40 |
| 200.00 | 69.69 \pm 3.05 | 92.76 \pm 8.75 |
| 300.00 | 73.09 \pm 5.08 | 93.55 \pm 9.11 |
| 400.00 | 77.73 \pm 11.01 | 93.95 \pm 15.00 |
| 500.00 | 83.19 \pm 7.90 | 94.55 \pm 8.01 |

Values are Mean \pm SD (n=3) of triplicate determinations

Table 3: Physico-Chemical Analysis

| Parameter | Oil Value |
|--------------------------------|------------------|
| Acid value (mgKOH/g) | 8.72 \pm 0.11 |
| Peroxide Value (mg/kg) | 92.01 \pm 0.18 |
| Iodine Value (g/100g) | 65.27 \pm 1.05 |
| Saponification value (mgKOH/g) | 76.30 \pm 3.08 |
| Refractive Index | 1.307 \pm 0.01 |

Values are Mean \pm SD (n = 3) of triplicate determinations

Table 4: Lipid Profile Analysis

| Treatments Group | Cholesterol | TG | HDL | LDL |
|--|--------------------|--------------------|--------------------|-------------------|
| Mice administered with normal saline | 51.00 \pm 1.00 | 39.00 \pm 1.00 | 24.00 \pm 1.00 | 17.70 \pm 0.30 |
| Mice administered with doxorubicin only | 64.50 \pm 1.50* | 53.50 \pm 3.50* | 11.00 \pm 2.00* | 28.00 \pm 2.00* |
| Mice administered with walnut oil only 15mg/Kg | 45.00 \pm 10.0 | 32.50 \pm 2.50 | 28.00 \pm 1.00 | 18.00 \pm 2.00 |
| Mice administered with 20mg/Kg walnut oil +Dox | 52.00 \pm 5.50** | 45.00 \pm 1.00** | 18.50 \pm 4.50** | 11.00 \pm 0.80 |
| Mice administered with 10mg/Kg walnut oil+ Dox | 55.00 \pm 2.50** | 43.50 \pm 0.50** | 14.00 \pm 1.00** | 21.30 \pm 1.90 |

*=significant when compared with the control group

**= significant when compared with DOX group

Values are expressed as Mean \pm SD, n=5

Photomicrographs of Heart Histopathology (HE x400)

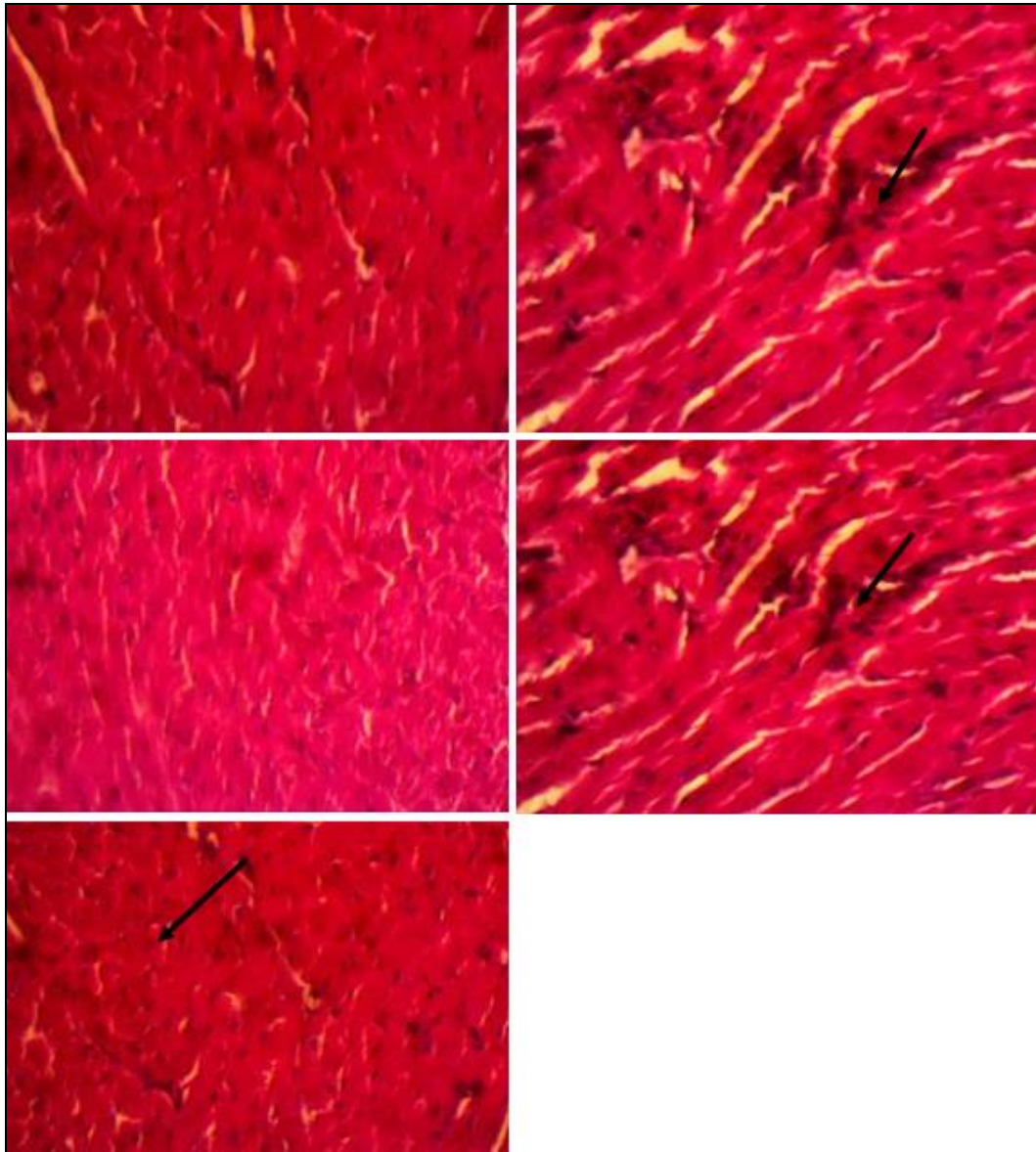


Fig 1: A. The heart muscle showing no observable lesion. B. The heart muscle showing multiple foci of myofibre coagulation necrosis and inflammation. C. The heart muscle of group C showing no observable lesion D. Heart muscle of showing severe multiple foci of myofibre coagulation necrosis and inflammation. E. Heart muscle showing moderate multiple foci of myofibre coagulation necrosis and inflammation.

Discussion

Walnut have been reported to contain alpha-linolenic and linoleic acid (Crews *et al.*, 2005) [4]. Ros *et al.*, and Zhao *et al.*, have shown that diet containing alpha-linolenic and linoleic acid such as walnut significantly lower total cholesterol, LDL and TG (Mushtaq and Khan, 2009) [10]. Doxorubicin is an important antineoplastic drug used for the treatment of several malignancies either alone or in combination with other chemotherapy agents. The main hindrance in the optimum utilization of DOX in cancer therapy is induction of cardio toxicity, hepato and nephrotoxicity.

The Ferric Reducing Antioxidant power assay is a spectrophotometric method used to analyze the Ferric reducing ability of a test sample. It is based on electron transfer reactions, which usually result on the reduction of a colored oxidant. The colorless oxidized Fe^{3+} form of iron is converted to a blue colored Fe^{2+} tri-pyridyl triazine (TPTZ) - reduced form. Free radicals are inevitably produced in biological systems and also encountered exogenously, and are known to cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and aging (Singh and Singh, 2008). FRAP is to assess the reduced concentration of ferric FRAP and it include the simultaneous use of ferricyanide and ferric ions as chromogenic oxidants supplied more favorable redox conditions for a greater variety of antioxidants (Apak, 2004) [1].

From table 1 above, it can be deduced that the Ferric reducing antioxidant activity of the walnut oil increases with increase in concentration. Also 1,1--diphenyl- β -picrylhydrazyl (DPPH) free radical scavenging method offers an approach for evaluating the antioxidant potential the walnut oil. As shown in table 2, the DPPH radical scavenging analysis was used to evaluate the free radical scavenging capacity of the oil. The result showed that

the scavenging capacity is directly proportional to the concentration. However, the radical scavenging activity of the oil is lower when compared to that of ascorbic acid.

Saponification value is an indication of the size or nature of fatty acid chains esterified to glycerol. In combination with acid values, saponification values are useful in providing information as to the quantity, type of glycerides and mean weight of the acids in a given sample of oil. Saponification is only of interest if the oil is for industrial purposes, as it has no nutritional significance. But due to the fact that each fat has within the limits of biological variation, a constant fatty acid composition, determination of the saponification value is a reasonable means of characterizing the fat (Derise, 1974) ^[5]. As shown in table 3, the oil is characterized by the saponification value of 76.30 mg KOH/g and can thus be used industrially for soap making and other cosmetics. A high saponification value indicates a high content of triacylglycerol, consistent with high ester value, and also an indication that the oil can be used in cosmetic industry (Bello, 2011) ^[2].

Iodine value is a measure of the unsaturation of fats and oils, and is expressed in terms of the number of centigrammes of iodine absorbed per gramme of the sample (i.e. percent iodine absorbed) during oxidation, which consumes the double bonds resulting in a reduction in iodine. It is an indicator for double bindings in the molecular structure, which influences the long term stability properties of the oil (i.e. important for storage). Oils having high iodine number are polyunsaturated indicating the degree of unsaturation and are desired by oil processors, while a lower iodine number is indicative of lower quality (Bello, 2011) ^[2].

The high iodine value indicates higher degree of unsaturation of oil. This reflects that the oil with iodine values of 65.27g/100g can be effective industrial processes requiring high iodine value.

Acid value determination is often used as a general indication of the condition and edibility of the oil. This is because an increase in acid value is accompanied by development of objectionable flavours and odours (Hariod, 1990) ^[6]. Acid value represents the milligram KOH required to neutralize the free fatty acid in 1g of oil.

The walnut oil has a low peroxide value of 92.01mg/kg, indicating a good preservation status. The acid value indicates the oxidative rancidity of oil. The acid value of walnut oil estimated as 8.72mg/g indicates that the oxidation of the oil is low which show the good qualitative nature of the oil. The refractive index was estimated to be 1.307.

Table 4 shows the lipid profile results of the experimental mice. The level of total cholesterol was significantly increased in the doxorubicin only group when compared to the control group and the walnut only group. Furthermore, the cholesterol level was reduced in group C and D with significant difference of $p \leq 0.05$ while E had a slight significance of $p \leq 0.01$.

Also it was observed that there was a significant $p \leq 0.05$ increase in the level of triglycerides caused by DOX treatment which may increase the risk of heart disorder and also pose a threat to diabetes. This is similar to the studies reported by Zakari, 1982 ^[18]. Our results showed that the walnut oil decreased the triglycerides level and thus could lead to a decrease in risk of heart disease.

It was observed that the HDL decreased in DOX treated mice when compared with normal group indicating that Dox can cause a reduction in high density lipoprotein.

There was a significant difference in LDL in Group C and B with $p \leq 0.05$ and Group D with slight a difference with $p \leq 0.01$.

This study therefore shows that walnut significantly reduced TC, TG and LDL and also increase HDL in comparison with DOX treated mice.

Histological analysis of the heart muscles further showed that the findings made with the lipid profile where the DOX treated mice resulted in cardiotoxicity manifested by occurrence of multiple foci of myofibre coagulation necrosis and inflammation. The Histopathological changes observed in DOX treated mice were similar to those of previously reported (Koti *et al*; 2009). But pretreatment with walnut oil showed no morphological changes indicating the protective effect of walnut oil.

Hence it can be concluded that walnut oil treatment proved to be cardioprotective as it reduces the toxicity level of doxorubicin as observed by a decrease in serum lipid profile.

Conclusion

The result indicates that walnut oil deserves attention as a supplement in combination with doxorubicin so as to reduce its toxicity level.

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