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Article *in* Phytotherapy Research · April 2013

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PHYTOTHERAPY RESEARCH
Phytother. Res. (2012)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.4758

Biochemical and Haematological Evaluation of Repeated Dose Exposure of Male Wistar Rats to an Ethanolic Extract of *Artemisia annua*

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***Artemisia annua* is widely used for the treatment of malaria and other disorders. In a previous study, the artemisinin concentration in the dry leaves of *A. annua* grown under humid tropical conditions was determined to be 1.098% using reversed phase high performance liquid chromatography. In the current study, biochemical and haematological evaluations of ethanolic leaf extracts derived from such plants (EAA) were carried out in 20 male Wistar rats. Rats were divided into four study groups of saline-treated (control) and test groups exposed orally to graded doses of EAA for 28 days. The results showed that the liver function and haematological indices, and testosterone levels were not adversely affected. High density lipoprotein -cholesterol was reduced at 100 mg/kg of EAA, atherogenic index as well as low density lipoprotein -cholesterol was raised, and glucose concentration was reduced significantly at the 100 and 200 mg/kg of EAA ($p < 0.05$). In addition to serving as a possible antidiabetic agent, EAA may not predispose users to hepatotoxicity, haematotoxicity and testicular toxicity. However, due to the possible risk of atherosclerosis, we advise that the plant extract should be taken with caution in people with atherosclerotic condition. Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: Artemisia annua; hepatotoxicity; haematotoxicity; testicular toxicity; antidiabetic agent.

INTRODUCTION

Artemisia annua L. (Asteraceae) is a fragrant annual antimalarial Chinese herb recorded before 168 BC and widely distributed in Asia, North America and Europe. It has been cultivated in many parts of the world including Nigeria (Brisibe *et al*. 2009; 2012a). It is commonly called sweet wormwood, sweet Annie, sweet sagewort, annual wormwood or qinghao (in Chinese). The active antimalarial constituent of *A. annua*, artemisinin, was isolated and characterised in 1971 (Klayman, 1985). Other main sesquiterpenoids found in the aerial portions of the plant include artemisinic acid, artemisilactone, artemisinol and epoxyarteannuinic acid (WHO, 2006).

The aerial parts of *A. annua* also contain a diverse and extensive portfolio of several biologically active chemicals including monoterpenoids, flavonoids (such as luteolin, apigenin and peduletin), coumarins (such as scopoletin and tomentin), steroids, phenolics, purines, terpenes (such as costunolide), lipids and aliphatic compounds (branched, unbranched, saturated or unsaturated), with varying levels of oxidation at C-1 (alcohol, aldehyde, ketone, acid or ester) (Bhakuni *et al*., 2001; 2002). Some of the methoxylated flavonoids from *A. annua* that may potentiate the antimalarial activity of artemisinin in the crude extracts of this plant include casticin, artemetin (Elford *et al*., 1987), chrysosplenol D and chrysoplenetin (Stermitz *et al*., 2002). Acyclic monoterpenoids such as artemisia ketone (Brown, 2010) are the major constituents of the essential oil in *A. annua* (Holm *et al*., 1997; Lari *et al*., 2002). Other phytochemicals found in the essential oil derived from the plant include linalool, 1,8-cineol, *p*-cymene, thujone, and camphor (Carnat *et al*., 1985). Camphor has been reported to induce excitation on the central nervous system (CNS), while others can result in depression and reduce spontaneous activity. These compounds are able to cross biological membranes due to their high liposolubility. Although *A. annua* is regarded as safe for the treatment of malaria and fever (Klayman, 1985), the essential oil presented above might affect the CNS (Perazzo *et al*., 2003).

A. annua shows anti-inflammatory, antipyretic (Huang *et al*., 1993), anti-cancer (Zheng *et al*., 1996), antifungal (Liu *et al*., 2001), antiulcerogenic (Foglio *et al*., 2001), antiparasitic (Kim *et al*., 2002) and cytotoxic (Nibret and Wink, 2010) activities, in addition to its antimalarial activity.

Malaria is an ancient disease that has had influence on the economies and development of many nations. During wars, malaria contributed to defeats in malaria endemic areas because the number of soldiers killed by malaria was more than during battle (Ridder *et al*., 2008). Malaria

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Received 13 March 2012
Revised 20 May 2012
Accepted 20 May 2012

occurs in many parts of the world, but mortalities steadily decreased from 1900 to 1977 all over the world except in sub-Saharan Africa where there was significant increase in mortalities (Snow *et al.*, 2001; Carter and Mendis, 2002; Barnes and White, 2005).

Recently, the WHO approved the use of artemisinin-based combination therapies (ACTs) to overcome development of malaria parasite resistance. Unfortunately, these drugs are still too expensive and not affordable to the poor population of the developing world due to an increase in demand that resulted in the hike in cost of the drugs (Cyranoski, 2004). As a result of this, therefore, natural products such as *Artemisia annua* are used in malaria endemic regions. In recent years, attention to natural products for the treatment of many disorders like malaria, anaemia, hypertension and neurodegenerative diseases has increased. In fact, about 80% of the population of developing countries still use plant-based traditional medicines (Willcox *et al.*, 2011). *Artemisia annua* is one of the widely used antimalarial plants. The use of herbal teas prepared from the dried leaves of *A. annua* is being promoted as an alternative treatment regimen for malaria in endemic regions where people do not have access to or cannot afford ACTs. *Artemisia annua* prepared with milk was reported to be more effective when compared with the water extract due to the presence of fat content in milk. In addition, the presence of oil increases the potency of ethanolic extract of *A. annua*. As a result of this, ethanolic extract as well as whole leaf powdered tablets of *A. annua* are considered as likely candidates that will provide inexpensive, safe and effective herbal product when used in combination with other antimalarial agents as part of ACT (Rezelman and Goris, 2008). The limitations to the use of this plant, however, are that it is difficult to ensure that *A. annua* tea can be safely used without promoting the emergence of resistant strains of *Plasmodium* and to certify that patients receive adequate doses (Willcox *et al.*, 2007). Although, *A. annua* herbal preparations were reported to be safe in adults (Klayman, 1985), scientific studies designed to evaluate the biochemical and haematological toxicity effects of this plant are scarce, and hence, the need for the current study.

MATERIALS AND METHODS

Plant material and tissue culture conditions. Plant tissue culture was carried out at Molecular Bio/Sciences Ltd., 124 MCC Road, Calabar, Cross River State, Nigeria. Seeds of an *A. annua* hybrid line (3 M) identified in a previous study possessing enhanced agronomic performance and high artemisinin content under lowland humid tropical conditions were used in this study (Abolaji *et al.*, 2010; Brisibe *et al.* 2012a). The seeds were surface sterilized, rinsed and subjected to aseptic culture conditions in 500-ml vessels containing 100 ml of half-strength (Murashige and Skoog, 1962) basal medium as outlined earlier (Brisibe *et al.*, 2009). Fully developed plants grown in 20-L plastic containers of 30-cm diameter, filled with sandy loam to soil that was mixed properly with poultry droppings, were maintained in an air-conditioned glasshouse using fluorescent lamps (with a light intensity of 3000 lux) at a temperature of $28 \pm 2\,^{\circ}C$ and a relative humidity of 65%. Leaves were harvested from these plants, dried under natural conditions in a shade and stored in air-tight plastic containers until required.

Plant identification. The plant was carefully identified at the Department of Botany, University of Calabar, Calabar, Cross River State, Nigeria, where a voucher specimen (U. Cal 01/110) was preserved.

Preparation of extract for animal administration. Air-dried *A. annua* leaves (400 g) previously analysed using reversed phase high performance liquid chromatography and confirmed to contain 1.098% of artemisinin (Abolaji *et al.*, 2010) were pulverized to coarse powder and subjected to Soxhlet extraction at 17–20 °C for 48 h using 98% ethanol. After filtration, and evaporation under vacuum, the extract was left in the fume hood until the solvent was completely evaporated yielding 56 g of a greenish sticky extract representing 14% yields.

Animals and animal care. Animal studies were carried out in accordance with the declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals through the clearance of institutional animal care and use committee. Male Wistar rats weighing between 220 and 250 g were obtained from the Animal House Facility of the International Center for Chemical and Biological Sciences, University of Karachi, Pakistan, where this research was conducted. The animals were housed for one week prior to experiment under controlled conditions with 12 h light/dark cycle, temperature $22 \pm 2\,^{\circ}C$ and free access to feed and water.

Animal treatment. Animals were randomly assigned five per group to control (saline), and test groups administered orally with 100, 200 and 300 mg/kg for 28 days, approximately equivalent to 5, 10 and 15 times higher than the therapeutic dose of 9g *A. annua*/day, respectively, and using a yield of 14% with average adult weight of 65 kg (Rath *et al.*, 2004). The 9g *A. annua*/day was chosen as a reference dose because ethanol was only used to facilitate extraction, and all ethanol was removed from the final concentration. Since the yield of 80–95% of plant content of artemisinin is present in ethanolic extract of *A. annua* (Rezelman and Goris, 2008), the artemisinin content of EAA used in this study is between 0.88 and 1.04%, because we obtained 1.098% artemisinin in the dried leaves of *A. annua* in our previous study (Abolaji *et al.*, 2010). Following last dose administration, the rats were fasted for 18 h, anaesthetized using *i.p.* pentothal sodium (60 mg/kg, Abbot Laboratory, Pakistan) and sacrificed. The blood and various organs were collected for subsequent studies.

Blood and organ collection. Blood was collected by cardiac puncture using sterile syringes, and needles into anti-coagulant free serum separator tubes, allowed to clot for 1 h and centrifuged at 3000 rpm for 10 min using Eppendorf 5810R centrifuge. Serum was transferred into plastic tubes, and stored at $-20\,^{\circ}C$ till further use. The blood for the glucose determination was collected into tubes containing anti-coagulant. The following organs of the rats were carefully removed and weighed: testes, liver, kidneys, spleen, hearts and lungs.

The liver and testes were stored in formalin for histopathological study.

Biochemical analysis. Serum was used to determine the levels of creatinine, total bilirubin, direct bilirubin, total protein, albumin, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase, total cholesterol, triacylglycerol, high density lipoprotein (HDL-c), low density lipoprotein (LDL-c), sodium (Na^+), potassium (K^+), chloride (Cl^-), and bicarbonate (HCO_3^-) ions, while the glucose levels were determined in the plasma collected using Hitachi 902 automated analyser (Roche Diagnostics, Germany).

Hormonal analysis. The levels of testosterone were determined in the serum using Elecsys 2010 automated analyser (Roche Diagnostics, Germany).

Haematological analysis. The following haematological parameters were determined in the whole blood of male rats collected into EDTA bottles using Beckman Coulter HMX analyser, (USA): haemoglobin (Hb), red blood cell (RBC) count, packed cell volume (PCV), mean corpuscular volume, mean corpuscular haemoglobin (MCH), MCH concentration (MCHC) and white blood cell (WBC) counts.

Histopathology of tissues. Tissue samples of testes and liver were removed from experimental animals, blotted dry and fixed in 10% formaldehyde (pH 7.2 to 7.4). The fixed tissue was embedded in paraffin, sliced into 10-μm-thick sections, stained with Mayer's haematoxylin & eosin and examined with light microscope (Wu *et al.*, 2006).

Statistical analysis. The data were evaluated by using one-way analysis of variance with a post hoc Dunnett's test. Values were presented as means ± standard error. Statistical analysis was performed using SPSS10 software (SPSS Inc., Chicago, IL, USA). Probability value (p) < 0.05 was considered to be significant.

RESULTS

Animals treated with EAA did not show any sign of toxicity. As shown in Table 1, expected weight gains occurred in all the groups. In Table 2, organ weights of rats are displayed. Groups treated with 200 and 300 mg/kg, respectively, of EAA tended to increase significantly in heart and kidney weights while there were significant ($p < 0.05$) increases in the weights of the epididymis in all the EAA treated groups compared to the control group. There was no change in the weights of the testes, seminal vesicle, lungs and the spleen in the EAA-treated groups compared to the control group.

It was observed in this study that apart from total protein that was significantly ($p < 0.05$) elevated at the 200 mg/kg of EAA, there was no change in the levels of ALT, AST, albumin and total and direct bilirubin in all the EAA-treated groups compared to the control group (Fig. 1a–d).

The results of the effect of EAA on the renal function markers such as the electrolytes and creatinine are shown in Fig. 2. Apart from the observed significant ($p < 0.05$) elevation of bicarbonate ions in all the EAA-treated groups, there was no significant change in the levels of the other renal function markers in relation to the control group.

The results of the effect of EAA on serum lipid profile and atherogenic index are shown in Figs. 3a and 3b respectively. EAA did not appear to have any effect on the total cholesterol and triacylglycerol levels. However, HDL-cholesterol decreased ($p < 0.05$) significantly in the 100 mg/kg EAA group. The reductions at the 200 and 300 mg/kg of EAA were not significant. There were noticeable elevations of LDL-cholesterol, and the atherogenic index at 100 and 200 mg/kg of EAA ($p < 0.05$).

It was observed further in Fig. 3c, that glucose concentrations decreased in all the EAA-treated groups compared to the control group. The levels of testosterone evaluated were not reduced in all the EAA-treated groups (Fig. 3d). The results of the histopathology shown in Fig. 4 indicated that the liver and the testes were not adversely affected by EAA.

The results of the haematological parameters showed that, apart from the WBC counts observed to be elevated at the 200 and 300 mg/kg of EAA, all the other haematological indices evaluated did not change significantly compared to the control group (Table 3).

DISCUSSION

Artemisia annua has been used in traditional Chinese medicine for the treatment of fever associated with malaria. The artemisinin concentration of the plant cultivated in most parts of the world varies from 0.02% to 1.1% of the dry weight, depending on geographical location and cultivation conditions (Mueller *et al.*, 2000). With breeding techniques, artemisinin yields of 1.4% of the dry weight have been achieved (Delabays *et al.*, 2001). In a previous study, we obtained a yield of 1.098% of artemisinin in *Artemisia annua*

Table 1. Change in body weight following repeated dose exposure of male Wistar rats to EAA for 28 days

Treatment *A. annua*	Initial Weights	Final Weights	Change in Weights (%)
Control	233.40 ± 17.08	273.80 ± 26.48	17.41 ± 9.22
100 mg/kg	233.60 ± 24.18	278.60 ± 23.78	19.26 ± 18.81
200 mg/kg	250.40 ± 15.77	281.80 ± 28.73	12.53 ± 6.97
300 mg/kg	233.60 ± 13.79	259.80 ± 14.34	11.21 ± 6.90

Data presented as Mean ± SEM, $n = 5$.

Table 2. Organ weights of male Wistar rats following repeated dose exposure to EAA for 28 days

Treatment *A. annua*	Testes (g)	Seminal Vesicle (g)	Epididymis (g)	Liver (g)	Heart (g)	Lungs (g)	Spleen (g)	Kidneys (g)
Control	2.98 ± 0.04	1.72 ± 0.17	1.18 ± 0.07	7.54 ± 0.23	0.78 ± 0.04	1.50 ± 0.12	0.54 ± 0.02	1.76 ± 0.02
100 mg/kg	2.24 ± 0.36	1.94 ± 0.19	1.40 ± 0.03*	8.04 ± 0.17	0.86 ± 0.24	1.52 ± 0.06	0.60 ± 0.04	2.16 ± 0.02
200 mg/kg	3.42 ± 0.27	1.48 ± 0.09	2.46 ± 0.11*	7.62 ± 0.55	1.12 ± 0.06*	1.68 ± 0.08	0.56 ± 0.02	2.22 ± 0.17*
300 mg/kg	2.82 ± 0.07	1.56 ± 0.02	2.04 ± 0.02*	6.4 ± 0.12*	0.94 ± 0.07*	1.60 ± 0.03	0.56 ± 0.02	1.90 ± 0.04*

Data presented as Mean ± SEM, $n = 5$. P
* < 0.05.

using reversed phase high perfomance liquid chromatography with hexane as an extracting solvent (Abolaji *et al*., 2010). We quantified artemisinin level in the plant to be sure that the plant is therapeutically active. In this study, we used ethanolic extract of *A. annua* because it is preferred if the drug is to be administered as an extract. If the purpose is to isolate the pure compound, hexane is better as a solvent (Rezelman and Goris, 2008). Artemisinin is not the only active ingredient in *A. annua* (Carbonara *et al*., 2012), which implies that the presence of other active ingredients may suggest that *A. annua* is a natural ACT (Willcox *et al*., 2004).

In the present study, experimental groups were exposed to different dosages of EAA. We investigated the biochemical and haematological changes after repeated dose exposure to EAA for 28 days in male Wistar rats. This was necessary in order to determine the safety assessment of the plant in people who use the plant for the treatment of malaria and other disorders. Biochemical parameters were used to assess liver and renal function biomarkers as well as atherosclerotic risk. In addition, haematological constituents were used as important biomarkers for assessment of haematotoxic effect of EAA. At present, detailed *in vivo* reports on the biochemical and haematological assessment of EAA in a mammalian model are not available. Because of this reason, we could not compare our findings with previous results.

Serum hepatic biomarkers such as protein, albumin, bilirubin, ALT and AST were evaluated for hepatotoxic effect of EAA. Because the liver is a key organ in the metabolism and detoxification of xenobiotics, it is vulnerable to damage induced by a variety of chemicals (Tennant, 1997). Since there was no change in the levels of ALT, AST, albumin and total and direct bilirubin in all the EAA-treated groups, it implies that EAA may not predispose the users of this plant to hepatotoxicity.

The observed non-significant change in the levels of the renal function markers, apart from the bicarbonate ions, means that EAA does not predispose users to renal dysfunction. We did not carry out histopathology of the kidney; this would have helped to confirm this finding. However, there may be a metabolic disturbance of the acid–base balance due to reduction in bicarbonate ion concentrations in all the EAA-treated groups.

The lipid profile results indicate a risk of cardiovascular disease in the users of *A. annua*. The clinical significance of lipids is primarily associated with their contribution to coronary heart disease (CHD) and various lipoprotein disorders. Increased LDL-cholesterol is a risk factor in the cause of atherosclerotic diseases. Numerous studies have established that, when total-cholesterol and LDL-cholesterol concentrations are high, the incidence and prevalence of CHD are also high (Nader *et al*., 2008). LDLs are responsible for transporting cholesterol from the liver to the various cells and tissues of the body, where cholesterol is separated from the lipoprotein and utilised. When LDLs pass through the wall of the artery, they react with reactive oxygen species, and thereby get oxidised. Because the oxidised LDL formed cannot be processed by the WBC, they get deposited in the artery leading to the hardening of the arteries which eventually results in heart attack (Kunitomo, 2007; Adebayo *et al*., 2011). Thus, the increase in serum LDL-cholesterol concentration, despite the fact that there was no change in the

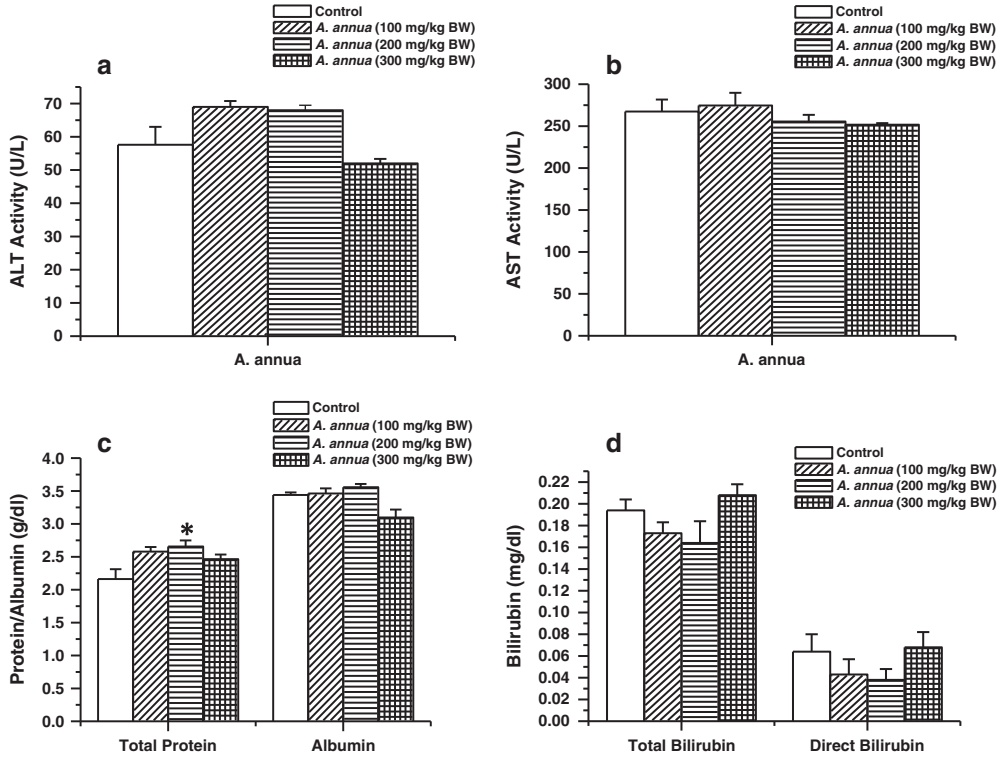

Figure 1. Effect of EAA on ALT (**a**), AST (**b**), total protein and albumin (**c**), and total and direct bilirubin (**d**) levels following repeated dose exposure of male Wistar rats to EAA for 28 days. Data presented as Mean + SEM, $n = 5$.

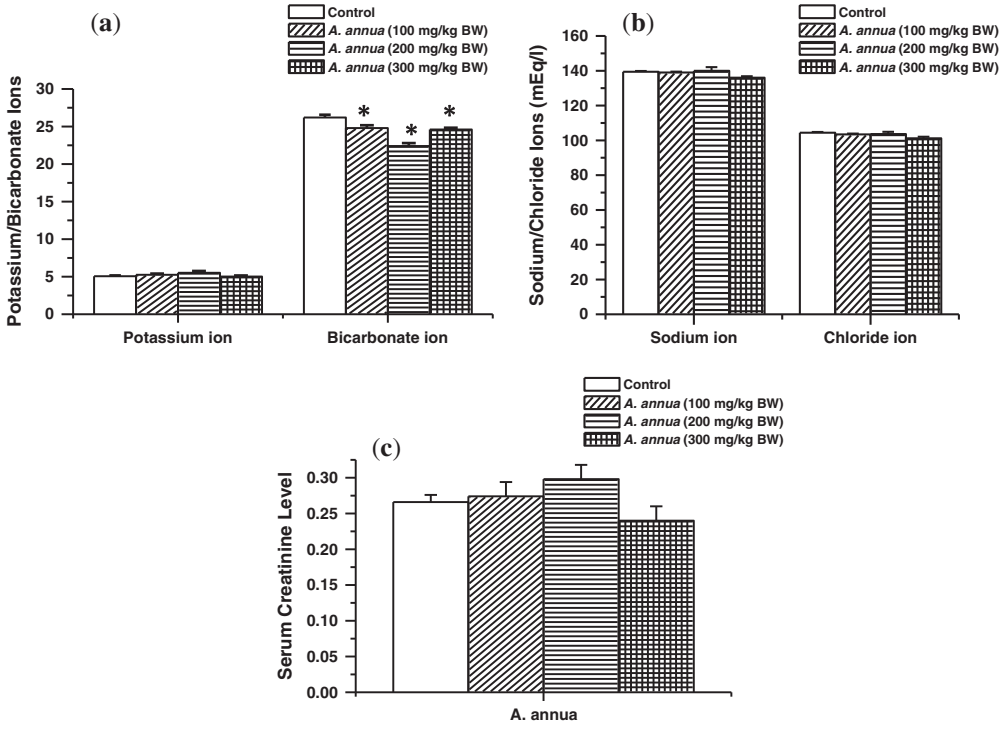

Figure 2. Effect of EAA on serum potassium and bicarbonate (**a**), sodium and chloride (**b**) ions, and creatinine (**c**) levels following repeated dose exposure of male Wistar rats to EAA for 28 days. Data presented as Mean + SEM, $n = 5$. *$p < 0.05$

levels of total cholesterol and triacylglycerol, suggests that EAA may still predispose users to cardiovascular diseases.

HDLs on the other hand, are involved in the transportation of excess and unused cholesterol from the tissues of the body back to the liver. From here, cholesterol is catabolised to bile acids and excreted. This process makes HDL beneficial to health because it retards atherosclerotic build-up (Gordon *et al.*, 1977). The observed reduction in the HDL-cholesterol by EAA may further predispose users to cardiovascular diseases.

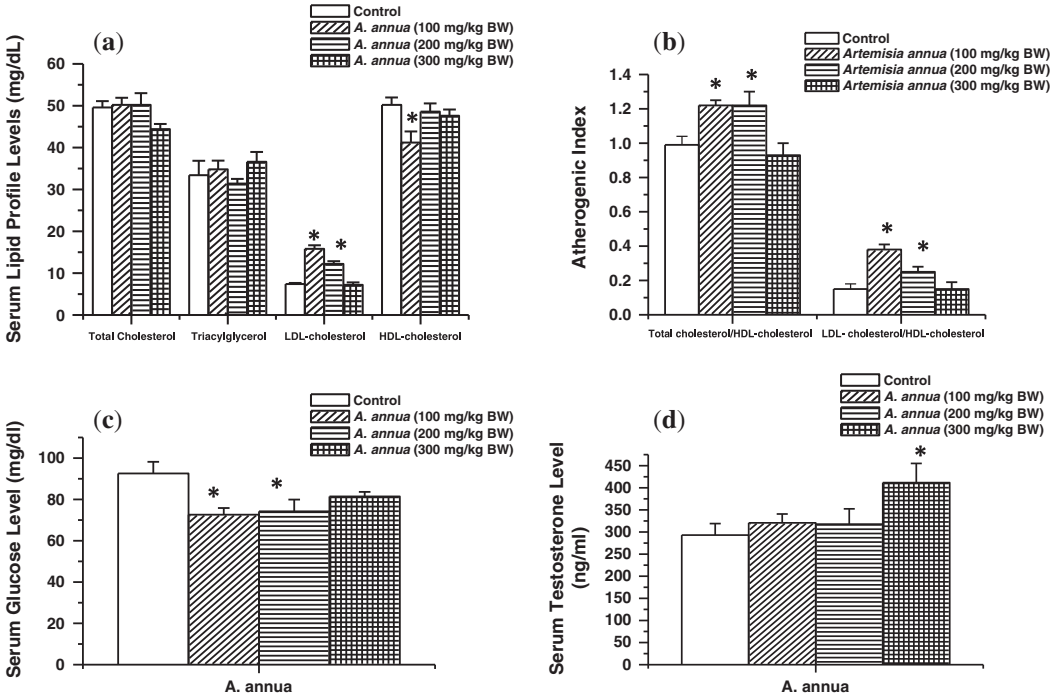

Figure 3. Effect of EAA on serum lipid profile levels (**a**), atherogenic index (**b**), serum glucose **(c)**, and testosterone (**d**) levels following repeated dose exposure of male Wistar rats to EAA for 28 days. Data presented as Mean + SEM, $n = 5$. $^*p < 0.05$

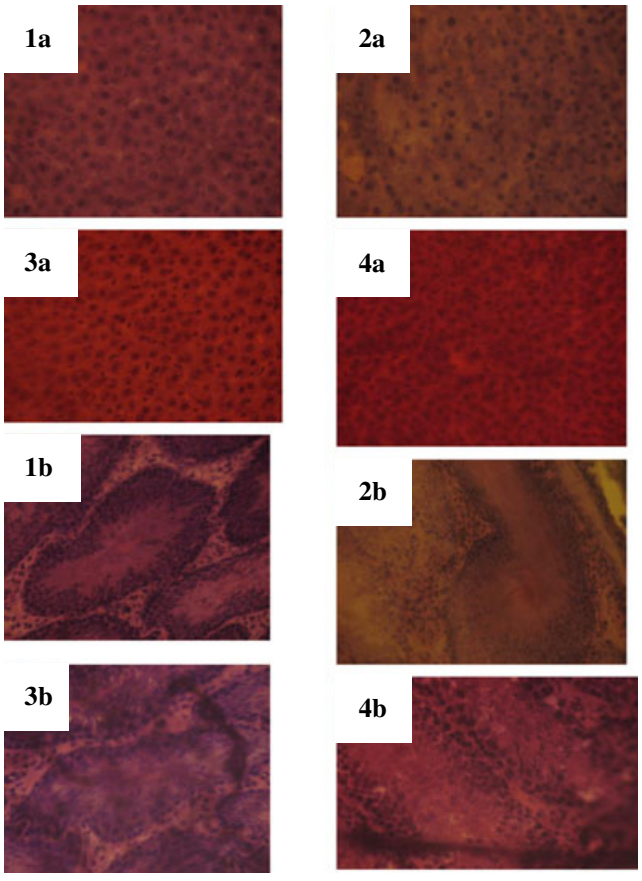

Figure 4. (1a-4a) are representative photomicrographs of liver slides from control **(1a)** and EAA-treated rats. **1a** received normal saline, **2a**, **3a**, and **4a** received 100, 200, and 300 mg/kg EAA, respectively. (X40) haematoxylin and eosin (H&E). (**1b-4b**) are representative photomicrographs of testes slides from control (**1b**) and *Annua* - treated rats. **1b** received normal saline, **2b**, **3b**, and **4b** received 100, 200, and 300 mg/kg *A. annua*, respectively. (X40) haematoxylin and eosin (H&E). There was no detectable toxicity in the liver and testes of *A. Annua*-treated groups compared with control. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

The ratios LDL-cholesterol/HDL-cholesterol and total cholesterol/HDL-cholesterol are used to calculate atherogenic index, since they usually correlate each other (Glueck and Segal, 1986; Kazi-Aoul and Benmiloud, 1987; Adebayo *et al*., 2011). The results obtained by using these two ratios, showed that the atherogenic index in the male rats increased significantly at the 100 and 200 mg/kg of EAA. This further confirms that EAA may subject users to some level of cardiovascular diseases.

The decrease in glucose concentrations observed in this study indicates its blood glucose lowering effect, suggesting that *A. annua* could serve as a candidate for antihyperglycaemic drug in addition to its antimalarial activity. Some Artemisia species have been reported for the treatment of diabetic condition (Ribnicky *et al*., 2006; Nofal *et al*., 2009). For example, *A. herba-alba, A. santonicum* and *A. dracunculus* were used in different investigations for the treatment of diabetic conditions (Al-Waili, 1986; Swanston-Flatt *et al*., 1989, 1991; Korkmaz and Gurdal, 2002). Recently, Brisibe *et al*. (2012b) reported that powdered dried leaves of *A. annua* used as dietary supplements possess antidiabetic properties in rats.

Furthermore, the fact that testosterone levels evaluated were not reduced in all the EAA-treated groups suggests that EAA may not be toxic to the testes.

The results of the histopathology indicating that the liver and the testes were not adversely affected by EAA further confirmed our earlier statements that EAA may not be toxic to the liver and the testes.

The haematological profile reflects the general health status of an individual (WHO, 2004). Due to the fact that the different doses of EAA administered did not cause any significant change in the levels of Hb, RBCs, PCV, MCH, and MCHC, it implies that EAA does not result in haematotoxicity. The significant elevations of WBCs may only be due to an immune system that was

Table 3. Effect of EAA on haematological indices following repeated dose exposure of male Wistar rats to EAA for 28 days

Treatment *A. annua*	Hb (g/dl)	RBC (million/μl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	WBC (10^9/L)
Control	14.80 ± 0.25	7.66 ± 0.19	42.74 ± 1.07	56.94 ± 0.75	19.28 ± 0.30	33.68 ± 0.49	5.12 ± 1.10
100 mg/kg	14.98 ± 0.15	7.50 ± 0.10	44.40 ± 0.47	58.08 ± 0.55	19.50 ± 0.11	33.92 ± 0.33	6.34 ± 0.15
200 mg/kg	14.54 ± 0.08	7.48 ± 0.12	43.16 ± 0.38	57.04 ± 0.44	19.00 ± 0.31	34.14 ± 0.29	7.78 ± 0.87*
300 mg/kg	14.78 ± 0.16	7.44 ± 0.15	43.44 ± 0.52	57.62 ± 0.65	19.76 ± 0.26	34.02 ± 0.25	8.52 ± 0.84*

Data presented as Mean ± SEM, $n = 5$. P
*< 0.05.

triggered due to the presence of EAA in the system (Celik and Suzek, 2008).

CONCLUSION

We conclude that in addition to the fact that EAA may not predispose users to hepatotoxicity, haematotoxicity and testicular toxicity, it could be a potent antidiabetic agent in addition to its antimalarial effect.

Conflict of Interest

The authors have no conflicting interests to declare.

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