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Hepatoprotective effect of leaf extracts of *Crassocephalum rubens* (Juss. ex Jacq.) S. Moore in rifampicin-induced oxidative stress in Swiss mice

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Abstract
The effect of extracts of *Crassocephalum rubens* leaf on some biochemical indices in rifampicin-induced oxidative stress in mice was investigated. Dried macerated leaf samples were extracted in either ethanol or distilled water. The *in vitro* antioxidant study revealed that the aqueous extract of *C. rubens* contains significantly ($p < 0.05$) higher amount of proanthocyanins (20.0±0.15 mg catechin equivalent/g extract) and flavonoids (66.0±1.44 mg rutin equivalent/g extract) when compared with the ethanol extract (12±0.18 mg catechin and 40±0.26 mg rutin equivalent/g extract, respectively). However, the ethanol extract showed significantly ($p < 0.05$) higher total phenol content (80±3.8 mg gallic acid equivalent/g extract) than the aqueous counterpart (68.0±2.4 mg gallic acid equivalent/g extract). DPPH radical scavenging ability of the aqueous extract (IC_{50} = 60.74 µg/mL) compared favourably with that of vitamin E (IC_{50} = 59.74 µg/mL) but was higher than that of the ethanol extract (IC_{50} = 112.48 µg/mL). Results from the animal study showed increased ($p<0.05$) activities of AST, ALT, ALP, MDA and low GSH levels in the rifampicin (300mg/kg body weight) exposed mice. But co-administration of rifampicin with the extracts (300 mg/kg body weight) or vitamin E (100 mg/kg body weight) resulted in a reversal of these trends. The protective effect of the extracts was more prominent in the aqueous extract treated mice and this may be attributed to the higher polyphenol content, radical scavenging ability and antioxidant activity of the extract. These findings suggest a protective role of *Crassocephalum rubens* extracts against oxidative stress related liver injury.

Keywords: *Crassocephalum rubens*, rifampicin, oxidative stress, antioxidant, liver injury

INTRODUCTION

Rifampicin is a semi synthetic bactericidal antibiotic drug of the rifamycin group, a group of antibacterials produced by *Streptomyces meditterranei* (Prince *et al.*, 2002). It is typically used to treat mycobacterium and viral infections caused by *Staphylococcus aureus*, *Neisseria meningitis and Neisseria gonorrhoea* (Campbell, 2000). In its mode of action, rifampicin inhibits DNA- dependent RNA polymerase in bacteria cells by binding its beta-subunit, thus preventing transcription to RNA and subsequent translation to proteins (Charity, 2007; Tayal *et al*., 2007). Although the mechanism of rifampicin induced toxicity still remains controversial, it is reported that high doses of rifampicin causes a direct toxic injury to the liver cells by promoting the up-regulation of hepatic cytochrome P_{450}

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enzymes (such as CYP2E1 and CYP 3A4) and increase the level of toxic metabolites resulting in increased production of reactive oxygen species that may be deleterious to some important cellular sites (Ramappa and Aithal, 2012; Pandit *et al*., 2012).

In recent years, several orthodox drugs are currently employed in the management of hepatic disorders which in some cases may cause damaging side effects. One of the alternative approaches is the use of medicinal plants in ethno-medicinal practices. Natural products from the plant kingdom have been reported as sources of bioactive substances or phytochemicals (phenolic compounds, terpenoids, alkaloids, flavonoids and tannins) acting as antioxidants. These phytochemicals exhibit a wide range of biological and pharmacological activities such as anti-diabetes, antimicrobial, anti-inflammatory, anti-cancer, and anti-atherosclerotic activities (Olorunnisola *et al*., 2012).

Crassocephalum rubens (Juss. Ex Jacq.) S. Moore, a member of the Asteraceae family, is an erect herb of about 1m high, well distributed in lowlands and mountain situations in Nigeria, Guinea, Republic of Benin, Mali and Cameroon (Adjatin *et al*., 2013a). It is commonly known as *Ebolo* (Yoruba), *Gbolo* (Benin). The leaves are used for soups and sauces in the Southern part of Nigeria and other humid zones of West and Central Africa. The mucilaginous leaves are slightly laxative and are given to women after childbirth for treating stomach ache and liver complaints. An infusion of the leaves is taken against colds, burns, earache, sore (leaf sap) and filarial parasite infected eyes (Gbadamosi and Sulaiman, 2012; Adjatin *et al*., 2013b).

Despite the favorable ethno-pharmacological properties of *Crassocephylum rubens* leaves, there is paucity of scientific information on the validation of some of these claims. The present study therefore evaluates the antioxidant property and protective ability of ethanol and aqueous extracts of *Crassocephylum rubens* leaf against rifampicin-induced oxidative stress in Swiss mice. It is proposed that *Crassocephylum rubens* leaves may have a protective effect on the deteriorated hepatic function that results from free radicals generated by high dose of rifampicin treatment.

EXPERIMENTAL

Collection of plant materials and extraction. Fresh leaves of *Crassocephalum rubens* were collected from a private farm at *Ise Ekiti*, Ekiti State. The plant leaves were identified by a Botanist at the Department of Botany, University of Benin, Benin City, Nigeria and voucher specimen of the sample was deposited at the herbarium of the same Department. The plant leaves were washed; air dried under shade, macerated and soaked in either ethanol for 72 hr to obtain the ethanol extract or distilled water for 48 hr to obtain the aqueous extract. The resulting filtrates were concentrated to dryness by using a rotary evaporator at 40^{o}C. Both extracts were stored at 4^{o}C for subsequent study.

***In vitro* antioxidant study.** The free radical scavenging capacity of the plant extracts against 1,1–diphenyl–2–picrylhydrazyl (DPPH) free radical was determined by a modified method of Szabo *et al.* (2007). Total phenolic content of the leaves was estimated based on the Folin-Ciocalteu's method as modified by Singleton *et al.* (1999). The total phenolic contents were expressed as gallic acid equivalents (GAE). Proanthocyanidin content of the leaves was estimated according to the method of Ayoola *et al.* (2008). The final results were expressed as catechin equivalent. The total flavonoid content of the leaves was determined according to the method of Miliauskas *et al.* (2004). The total flavonoid contents of the extracts were expressed as rutin equivalents.

Animal study. A total of 40 male Swiss mice used for this study were obtained from the animal house, University of Ibadan, Ibadan, Oyo State. The mice weighing between (30-35g) were divided into five (5) groups of eight (8) mice. Animals in group 1 were treated orally with the vehicle - carboxyl methyl cellulose (7%) and this served as the normal control; group 2 animals were exposed to rifampicin (Rif; 300mg/kg body weight); groups 3, 4 and 5 were exposed to rifampicin (300mg/kg body weight) concomitantly with vitamin E (100 mg/kg body weight; Vit E + Rif); ethanol (300 mg/kg body weight; Cr_{alc} + Rif) and aqueous (300 mg/kg body weight; Cr_{H2O} + Rif) extracts of *C. rubens*, respectively. The drug/ extracts were administered orally, once daily and the study lasted for fourteen (14) days. All experimental protocols were performed within internationally accepted guidelines for animals use and care (according to NIN guide for Laboratory Animals Welfare). At the end of the feeding period, the animals were fasted overnight and sacrificed through cervical dislocation. Blood was collected immediately from the heart into plain tubes and the liver was removed and stored for subsequent assays.

Biochemical analyses. The activities of serum aspartate and alanine transaminases (AST and ALT, respectively) were estimated by using the method of Reitman and Frankel, (1957). Alkaline phosphatase (ALP) activity was estimated in the serum by the method of Sood (1999). Reduced glutathione in liver homogenate was determined by reaction with 1, 2-dithio-bis nitro benzoic acid (DTNB) and expressed as µM GSH / g tissue (Jollow *et al*., 1974). Lipid peroxidation level in the liver homogenate was estimated via thiobarbituric acid reactive substances (TBARS), which are indicators of membrane lipid peroxidation. Values for TBARS were reported as malondialdehyde (MDA) and quantified using a Molar extinction coefficient of 1.5×10^5 M $cm^{-}1$ and expressed as µmole MDA g^{-1} of tissue (Gutteridge and Wilkins, 1982).

Statistical analysis. Data were expressed as means ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was performed to test for differences between the groups mean. Significant differences between the means were determined by Duncan's multiple range test and P values < 0.05 were regarded as significant (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

The *in vitro* antioxidant activity and the protective effect of leaf extracts of *Crassocephalum rubens* against rifampicin-induced oxidative stress in Swiss mice were evaluated in this study. The results in Figure 1 revealed that the aqueous extract of *C. rubens* leaf possesses significantly higher ($p < 0.05$) DPPH radical scavenging ability with IC_{50} of 60.74 µg/mL (Table 1) when compared with the ethanol extract (IC_{50} = 112.48 µg/mL). The DPPH radical scavenging capacity of the aqueous extract compared positively with that of the standard radical scavenger, vitamin E (IC_{50} = 59.74 µg/mL). Generally, DPPH radical scavenging assay is based on the reduction of the stable DPPH radical to yellow colored diphenyl picrylhydrazine in the presence of a hydrogen donor (Jothy *et al*., 2013; Omoregie and Oikeh, 2015).

Figure 2 shows the total phenol, flavonoids and proanthocyanins content of *C. rubens* extracts. The aqueous extract contains significantly higher flavonoids and proanthocyanins content in contrast to the ethanol extract. Flavonoids may account for part of the benefits associated with the consumption of fruits and vegetables and have been reported to interfere with the activities of the enzymes involved in reactive oxygen species (ROS) generation, quenching of free radicals, chelating transition metals and rendering them redox inactive in the Fenton reaction (Aiyegoro and Okoh, 2009).

Proanthocyanins are polymeric flavan-3-ols and are the second most abundant group of natural phenolics with effective antioxidants activity (Prior, 2003). As antioxidants, they provide several health benefits, including the prevention of cancer, urinary tract infection and cardiovascular diseases as well as the inhibition of LDL oxidation and platelet aggregation (Bors *et al*., 2000).

The phenolic content of the extracts was found to be considerably high ($P < 0.05$) especially in the ethanol extract in contrast to the aqueous counterpart (Figure 2). Phenolic compounds occur widely in the plant kingdom, especially in fruits and vegetables and constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators (Ebrahimzadeh *et al.*, 2009). The antioxidant capacity of phenolic compounds is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Premanath and Lakshmidevi, 2010).

Several studies have reported that one of the mechanism of rifampicin induced hepatocellular injury occur via induction of CYP2E1-dependent oxidative stress due to the formation of ROS (Shih *et al*., 2013). This may account for the significantly increased ($p<0.05$) level of malonaldehyde (MDA) in the rifampicin treated animals (Figure 3). MDA is the major oxidation product of peroxidized polyunsaturated fatty acids (PUFAs) and increased MDA level is an important indicator of lipid peroxidation. However, co-treatment of the animals with rifampicin and the extracts (Cr_{alc}+Rif and Cr_{H20}+Rif) resulted in significantly reduced ($p<0.05$) MDA levels especially in the aqueous extract treated group. The bioactive principles present in the extracts might have prevented lipid peroxidation by scavenging the electrophilic metabolites and hydroxyl radicals and preventing their interactions with lipids in the membrane (Oliveira *et al*., 2002; Shih *et al*., 2013). This is further supported by the significant reduction in MDA levels observed in animals that were co-treated with vitamin E and rifampicin.

GSH is a highly effective extracellular and intracellular antioxidant compound that neutralizes hydrogen peroxide and hydroperoxides by its scavenging and antioxidant properties. In this study, rifampicin treatment depleted the hepatic GSH contents probably due to an excessive buildup of ROS within the liver tissues leading to increased lipid peroxidation which is evidenced by GSH depletion (Figure 4). On the other hand, the extracts (Cr_{alc}+ Rif, Cr_{H20} +Rif) and rifampicin co-treated groups showed significantly increased ($p < 0.05$) GSH levels suggesting the hepatoprotective effect of the extracts against rifampicin induced oxidative injury. This may be attributed to the phytochemical principles that might have augmented the cellular antioxidative defense system, especially the non-protein thiols such as reduced GSH, earlier compromised by the toxic metabolites of rifampicin induced injury (Rana *et al*., 2006). A similar protective effect was observed in the mice co-treated with standard antioxidant vitamin E and rifampicin (Vit E + Rif).

In hepatocellular injury, the activities of enzymes of liver function (ALT and AST) have been demonstrated to be significantly reduced in the hepatocyte and increased in the plasma (Mayne, 2001). AST and ALT enzymes are released from damaged liver cells either due to increased permeability of the cell membrane or cell necrosis (Hassan *et al*., 2010). Likewise, results from this study showed that mice treated with rifampicin (Rif) developed increased serum activities of AST, ALT and ALP, suggesting hepatocyte injury and loss of functional integrity (Figure 5). Whereas, co-administration of rifampicin with the extracts or vitamin E ameliorated these effects. The hepatoprotective effect of

the extracts was observed to be more prominent in the aqueous extract-treated mice probably due to high levels of phytochemicals as well as antioxidant activity as previously observed in this study. Besides, it is well documented in the literature that *C. rubens* extracts contain some pharmacologically important phytochemicals such as tannins, flavonoids, steroids and coumarins (Adjatin *et al*., 2013a).

The mechanism of hepato-protection by the extracts against rifampicin induced oxidative stress may be attributed to the presence of some phytochemical principles such as saponins, tannins, terpenoids and flavonoids which have been reported to exert antioxidant activities by scavenging reactive oxygen species produced by rifampicin induced liver injury, preventing peroxidation of the membrane thereby stabilizing it (Shih *et al*., 2013). Besides, the extracts may also down-regulate activities of drug-metabolizing cytochrome P_{450} isoforms specifically CYP2E1 and CYP3A4 and thus reduce the production of toxic metabolites of rifampicin (Shih *et al*., 2013). A number of studies have also attested that high doses of bioactive compounds from plant sources such as flavonoids, and is derivatives can induce inhibition of these isoforms in mice (Jang *et al*., 2003; Rana *et al*., 2006; Shih *et al*., 2013).

The overall findings suggest that the aqueous extract of *C. rubens* possess appreciable amount of flavonoids, proanthocyanins and free radical scavenging ability which may partly account for its protective effect against rifampicin–induced oxidative stress in contrast to the ethanol extract. The extracts may provide effective intervention for treatment of oxidative stress induced liver injury.

Table 1: IC_{50} values of extracts of *C. rubens* leaf against vitamin E

Plant	IC_{50} (µg/mL)
Vitamin E	59.74[a]
C. rubens (aqueous extract)	60.74[a]
C. rubens (ethanol extract)	112.48[b]

Data represent mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$.

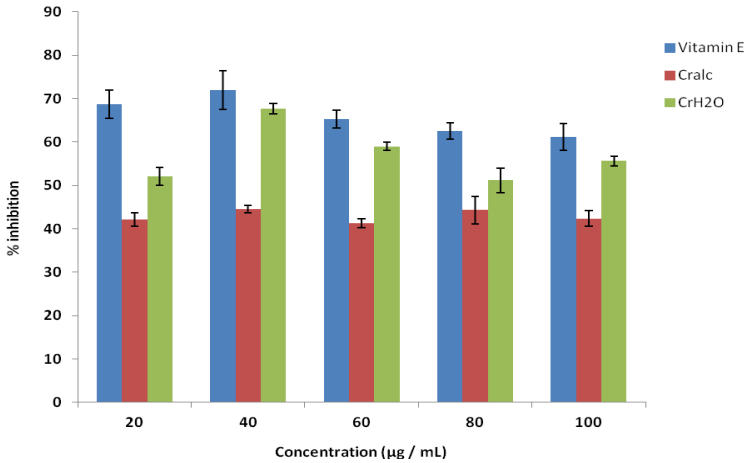

Figure 1: DPPH radical scavenging ability of leaf extracts of *C. rubens*
Values are expressed as mean± SEM (n = 3/group).

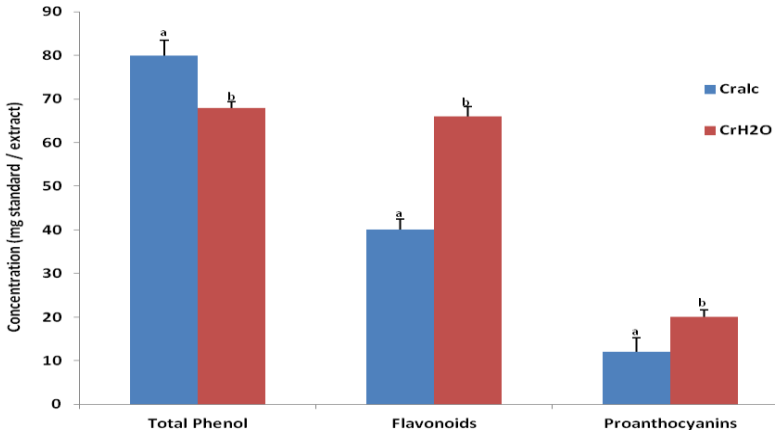

Figure 2: Total phenol, flavonoids and proanthocyanins content of *C. rubens* leaf extract
Data represent mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$. Cralc = Ethanol extract of *C. rubens* leaf; Cr_{H20} = Aqueous extract of *C. rubens* leaf

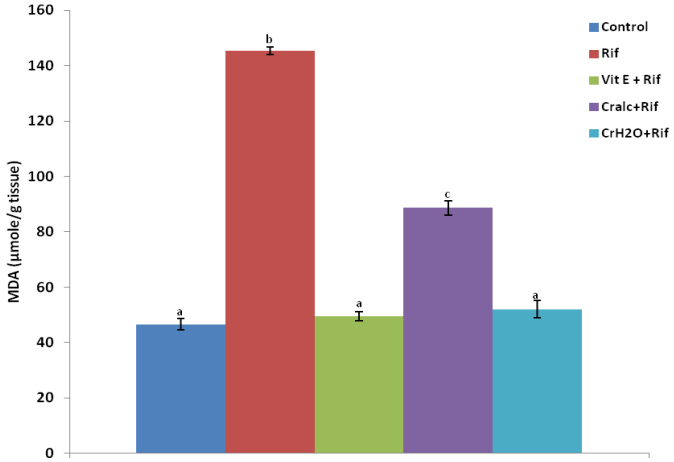

Figure 3: Effect of *C. rubens* extracts on malonaldehyde (MDA) level in rifampicin induced mice
Data represent mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$. Cralc = Ethanol extract of *C. rubens* leaf; Cr_{H20} = Aqueous extract of *C. rubens* leaf

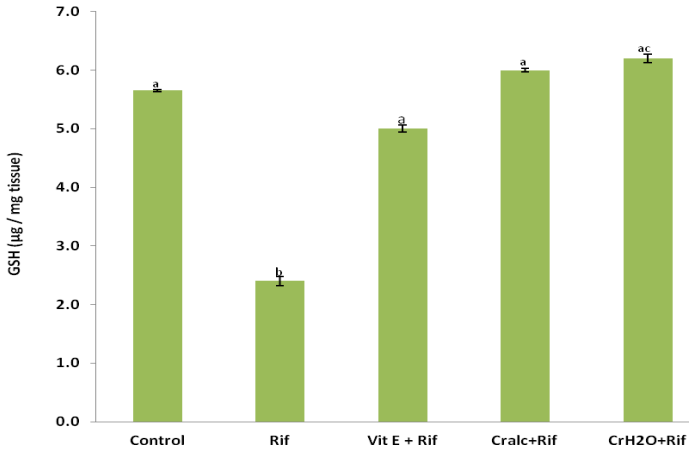

Figure 4: Effect of *C. rubens* extracts on reduced glutathione (GSH) level of mice exposed to rifampicin
Values are mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$. Cralc = Ethanol extract of *C. rubens* leaf; Cr_{H20} = Aqueous extract of *C. rubens* leaf

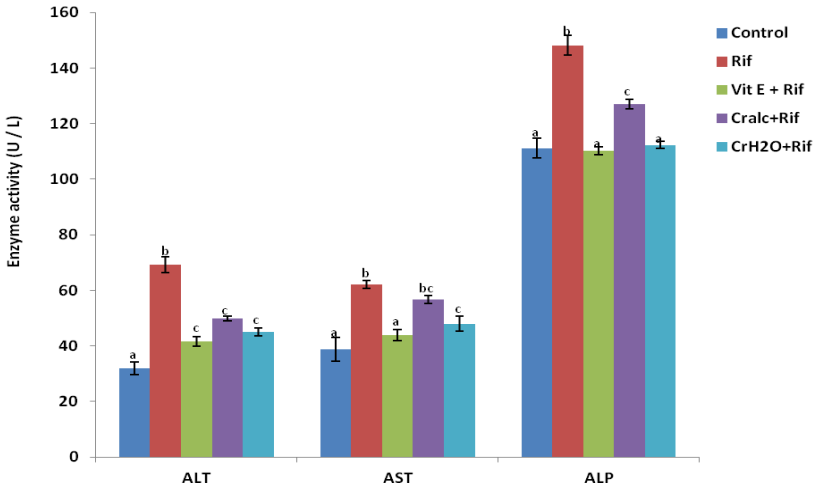

Figure 5: Effect of *C. rubens* extracts on enzymes of liver function of mice exposed to rifampicin
All values represent mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$. Cralc = Ethanol extract of *C. rubens* leaf; Cr_{H20} = Aqueous extract of *C. rubens* leaf

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