Hepatoprotective Activity and Inhibitory Effect of Flavonoid –Rich Extract of Brysocarpus Coccineus Leaves on Mitochondrial Membrane Permeability Transition Pore

Olaniyi T. Adedosu[1], Adejoke T. Oyedeji [2], Temitope Iwaku[3], Adeola F. Ehigie[4], Olufunso O. Olorunsogo[5]

[1, 3, 4] Department of Biochemistry, Ladoke Akintola University of Science and technology,
[2, 5] Biochemistry Department College of Medicine, University of Ibadan, Ibadan, NIGERIA.

[1] Laniyidosu@yahoo.com

ABSTRACT

The hepatoprotective activity of flavonoid –rich extract of Brysocarpus Coccineus leaves, a medicinal plant with anti-tumour ,anti-inflammatory and analgesic property was assessed in sodium arsenite- intoxicated rats,and its effects on Mitochondrial Membrane Permeability Transition (MMPT) Pore which when opened could lead to the release of Cytochrome C, a point of no return for Apoptosis to take place, was investigated in-vitro .Results showed that sodium arsenite intoxication caused significant (P< 0.05) increases in serum cholesterol level and activities of Alanine Aminotransferase (ALT) Aspartate Aminotransferase (AST) and Gamma glutamyl transferase (γGT). The elevation of the levels of these enzymes could be suggestive of an early liver injury. Interestingly, flavonoids –rich extracts, treated with sodium arsenite ameliorated these effects as these parameters were restored significantly (P<0.05) nearly to their control levels thus suggests possible hepatoprotective activity of the extracts. Investigation of the flavonoid –rich extract on MMPT pore in-vitro showed that calcium ions induced the opening of MMPT pore significantly (P<0.05) in rat liver mitochondria while spermine inhibited calcium-induced opening of the pore, indicating that the mitochondria were intact ab-initio. The results further revealed that inhibitory effects of the extract on the pore opening (200, 600,1000,1400, and 1800µg/ml) were 68.00,72.00, 83.20, 84.20 and 87.70% respectively. In this regard the highest degree of inhibition (87.70%) by the extract at 1800µg/ml compared favourably with that of spermine (86.70%), the standard inhibitor. The behaviour of the extract may not be unconnected with its ability to interact with the pore components making it impossible for the mega channel to be assembled. The inhibition of the MMPT pore by the extract may represent an effective therapeutic approach in several pathological conditions which require reduced rate of apoptosis .For chemotherapy further elucidation of the structure of the bioactive agent is required.

Keywords: Hepatoprotective, Mitochondrial Membrane Permeability Transition (MMPT), Flavonoids, Brysocarpus Coccineus inhibition

INTRODUCTION

The past years have witnessed an explosive expansion in the knowledge of the molecular mechanisms that commit a cell to apoptosis (Paolo et al, 2006). Numerous and interacting triggers (genetic programs, plasma membrane receptors, activation, etc.) and regulatory mechanisms (effectors systems, co-factors, inhibitors and modulators) have consequently been identified (Nagata, 1997). The cross-link between molecular actors of apoptosis is today a major research topic, since, on the one hand, defects in apoptosis allow neoplastic and

virally infected cells to escape elimination by the immune system, and on the other inappropriate triggering of apoptosis is a cause of serious neurodegenerative diseases and tissue wastage (Thompson, 1995). However, there is convincing evidence to implicate mitochondria in the execution of the apoptotic pathway. At least 3 mitochondria specific events have been well defined in cells undergoing apoptosis, namely, loss of mitochondrial transmembrane potential (Δ), induction of mitochondrial permeability transition pore (MMPT) opening, and cytosolic translocation of apoptogenic factors, such as cytochrome C and apoptosis-inducing factor (Kluck et al, 1997). Indeed, the event leading to the change of the mitochondrial transmembrane potential is thought to be mediated by the opening of the MMPT pore, a dynamic multiprotein complex located at the contact site between the inner and the outer mitochondrial membranes (Pervaiz et al, 1999), hence mitochondria are now well recognized as central players in modulating cell death in normal development, tissue homeostasis and even in diseases (Kroemer, 2002).

The mitochondrial membrane permeability transition pore is therefore considered to contribute substantially to the regulation of normal mitochondria metabolism and plays an important role in apoptosis (Green et al, 2004). However, various research works are presently geared towards the elucidation of the basic mechanism that regulates apoptosis and its associated mediators which may trigger or inhibit cell death through the modulation of the MMPT pore. Hence, the outcome of these efforts may lay the foundation for chemotherapeutic approaches in the treatment and or prevention of cancer and numerous neurodegenerative diseases associated with apoptosis. Also agents that induce or prevent mitochondrial membrane permeability transition are known to modulate cell survival and cell death or apoptosis (Green, 1999; Brenner and kroemer 2000).

Interestingly, certain bioactive agents of plant origin have been well documented to elicit their chemo-protective and therapeutic effects through the induction or inhibition of the opening of MMPT (Martin, 2006 and Javadov et al, 2007).

Bryosocarpus coccineus, a medicinal plant known for its wide spread distribution in Tropical African countries (Olowokudejo et al, 2008), belongs to the family: *Connaraceae*: Order: *Sapindales* Species: *schum and thonn.*

In Nigeria, its local names include Kasa and Hallilua in Hausa, Oke abolo in Igbo while its numerous names in Yoruba include, Ado, Amuje wewe, Kanti-Kanti, Orikoteni and Yeri eti Omode (Children ear-ring) because of the small size of the leaves (Olowokudejo et al, 2008). However, preliminary photochemical screening of the plant revealed the presence of carbohydrates, tannins, balsams and flavonoids in the leaves extracts [Amos et al, 2002; Oke and Hamburger 2002]. Interestingly, it is not known whether any of the phytochemical constituents of the extracts of the leaves of *Bryosocarpus coccineus* would interfere with the mitochondrial membrane permeability transition (MMPT) pore and hence modulate the intrinsic pathway of apoptosis. This study therefore was aimed at elucidating the effects of flavonoid-rich extracts of the leaves of *Bryosocarpus coccineus* on rat liver MMPT pore in *vitro*, as well as assess the possible hepatoprotective effect of the extract in sodium arsenite intoxicated rats.

MATERIALS AND METHODS

Materials

Mannitol, sucrose, N-(2-hydroxyethyl)pipearizine-N^1-(2-ethanesulfonic acid) (HEPES), rotenone, spermine, bovine serum albumin (BSA) ,sodium arsenite and all other reagents

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were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of highest purity and analytical grade.

Methods

Fresh leaves of *Brysocarpus coccineus* were collected at the Forestry Research Institute of Nigeria (FRIN) Jericho Ibadan Oyo State Nigeria after their identification and confirmation at the herbarium section of the Institute with specimen voucher no 108795 deposited. The leaves were immediately rinsed off of debris and shade-dried for two weeks on laboratory trays. The dried leaves were powdered and weighed while methanol extract were prepared in the cold using various grams of the dried powdered leaves soaked with methanol. The methanol extract was used for flavonoid isolation by column and thin layer chromatographic techniques (Ogundipe et ,.al 2000; Oke and Hamburger 2000).

Normal healthy male wistar strain albino rats were obtained from the animal house of the Biochemistry Department College of Medicine University of Ibadan. The rats weighing between 140-160g were kept in a well-ventilated cage, fed with rat pellet and water ad-libitum.

The animals were allowed to acclimatize for two weeks before the start of the experiment. They were divided into two major groups.The first group were normal rats used for MMPT study while the second were made up of rats used for the hepatoprotective study which were randomly divided into four treatment groups with six rats in each group namely; Group A (control), B (sodium arsenite intoxicated rats) ,C (sodium arsenite and flavonoid extract) and D (flavonoid extract only). Rats from all the groups were sacrificed by cervical dislocation. Low ionic strength rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) and as modified by Olorunsogo and Bababunmi (1985) based on differential centrifugation technique.

The Mitochondria Membrane Permeability Transition Pore (MMPT) opening was determined by the method of Lapidus and Sokolove (1993). This was based on the principle that mitochondria undergoing calcium-induced permeability showed colloidosmotic large amplitude swelling which result in a decrease in photometric absorbance at 450nm taken for 12minutes at thirty seconds interval.

Protein concentrations of the isolated mitochondria were determined by the procedure of Lowry et al, (1951) using BSA as standard. Serum cholesterol, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Gamma glutamyl transferase (γGT) were determined using the method of Trinder, (1969), Wroblwski et al., (1956). Karmen et al., (1955) and Szasz (1969) based on the standard regulation of international federation of clinical chemistry.

RESULTS AND DISCUSSION

The liver performs and regulates a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for cellular homeostasis (Anthea et al., 1993). Many diseases of the liver are accompanied by alterations in the metabolic processes involving the liver as they are frequently exposed to toxic agents. The generation of reactive oxidants during arsenic metabolism has been shown to play an important role in arsenic-induced hepatocyte injury (Chen, 1996). Also, free radicals generated through sodium-arsenite toxicity in cells has been shown to induce DNA and chromosomal damage (Lynn et al., 2000), DNA repair inhibition and apoptosis (Nagakawa et al,, 2002). Several reports have shown that sodium arsenite

treatments results in increased Reactive Oxygen Species (ROS) and Nitric oxide(NO) production in a variety of cells (Lynn et al, 2000; Pei-Chung et al., 2005).

Much of the damage produced stem from the proliferation of oxidative free radicals which when unchecked cause tissue damage by initiating membrane lipid peroxidation which underlines all degenerative diseases (Chen, 1996). However, several thousands of agents are currently being investigated for possible hepatoprotective and chemopreventive activities including herbs and spices as they are thought to contain certain bioactive agents which are mostly phenolic compounds with antioxidant activity.

Interestingly, the Investigation of the possible hepatoprotective activity of the flavonoid –rich extracts of Brysocarpus Coccineus leaves from this study revealed significant increases ($P<0.05$) in the concentration of serum protein, cholesterol and the enzymes activities; ALT, AST and GGT in sodium arsenite-intoxicated rats (Group B) confirming the toxic effect of sodium arsenite in rats model experiments, Figures 1, 2, 3, 4 and 5. The treatment with sodium arsenite and the flavonoids extracts (Group C) however resulted in significant decreases ($P<0.05$) in the concentration of serum cholesterol and the activities of these enzymes in comparison with Group A (Control). The elevation of the levels of these enzymes could be suggestive of an early liver damage. In this investigation, it was observed that flavonoid extracts treated with sodium arsenite ameliorated these effects as these parameters were restored nearly to their control levels. This then suggests the positive modulatory role of the flavonoid extract for possible hepatoprotective ability (Dajas et al, 2005). Furthermore, elevated levels of these enzymes are associated with myocardial infarction, liver cirrhosis hepatitis and neoplasm (Krupp et al, 1987).

One of the major physiological features of mitochondria is the generation of a large transmembrane potential across the mitochondria inner membrane. This is a direct consequence of the biochemical reactions that constitute the respiratory chain (Dunchen, 2004). The mitochondrial membrane permeability transition pore is formed from a complex of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin D, at contact sites between the mitochondria outer and inner membranes. Under the pseudopathological condition of oxidative stress, relatively high Ca^{2+} concentration, and low ATP, the complex flickers into an open-pore state allowing free diffusion of low molecular weight (KDa < 1500) solutes across the inner membrane (Crompton, 1999). These conditions correspond to those that unfold during tissue ischaemia and reperfusion suggesting that pore opening may be an important factor in the pathogenesis of apoptosis. This makes the pore an attractive pharmacological target for cardioprotection (Halestrap, 1999; Duchen, 2004). The inhibitors of the pore have been discovered as compounds capable of producing their effects indirectly by alteration of calcium levels. Examples are Cyclosporin A, ADP, low pH, spermine and bonkreckic acid (Duchen, 2004). Spermine a polyamine derivative and a standard inhibitor of the mitochondrial membrane permeability transition of the heart and the liver mitochondria works majorly by its interaction with the membrane external site where it binds to the anionic sites on the cytosolic site of the inner membrane (Lapidus and Sokolove, 1994). Its ability to compete with Ca^{2+} for binding at low affinity site and interact with negatively charged phospholipids, thus stabilizing the inner membrane have also been suggested as a means of inhibiting the MMPT pore .(Duchen,2004). In this study, results of the effect of the various concentration of the extract on the mitochondria membrane permeability transition pore are presented in Figures 6 and 7 respectively. Figure 6 shows the induction of the MMPT pore opening by calcium and its inhibition by spermine while Figure 7 shows the pattern of inhibition by varying concentrations of the flavonoid-rich extracts on calcium-induced MMPT pore opening in

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normal rat liver mitochondria. The basal change in absorbance in the absence of calcium was $-0.010 \pm 5.67 \times 10^{-3}$ indicating the intactness of the membrane. In the presence of calcium, the opening (swelling) of the MMPT was significantly ($P<0.05$) increased with mean change in absorbance at $\Delta\ 0.27 \pm 1.82 \times 10^{-1}$. However a significant decrease ($P<0.05$) in the opening of the MMPT on the addition of spermine was observed $-0.036 \pm 2.55 \times 10^{-2}$. The percentage inhibition by spermine was 86.66%. Also, it was observed that the flavonoid-rich extracts inhibited calcium induced mitochondrial swelling (Figure 7) in a concentration dependent manner in the order 200µg/ml < 600 µg/ml < 1000 µg/ml < 1400 µg/ml with percentage inhibition of 68.01%, 72.03%, 83.20% and 87.70%, respectively. The extracts inhibited the pore maximally by 87.70% in the presence of calcium compared with spermine, a standard inhibitor of the pore, which inhibited the pore by 86.66%. This suggests that the flavonoid-rich extracts may have the ability to interact with the MMPT components probably acting as an anti-apoptotic agent (Erlejman et al 2004).The anti - oxidative property of the extract were also shown by its hepatoprotective activity as revealed from this study suggestive of the need for further study on the bioactive agents present in the plant as template for drug discovery especially in the prevention ,treatment or management of diseases associated with Apoptosis, neurodegeneration and ageing.

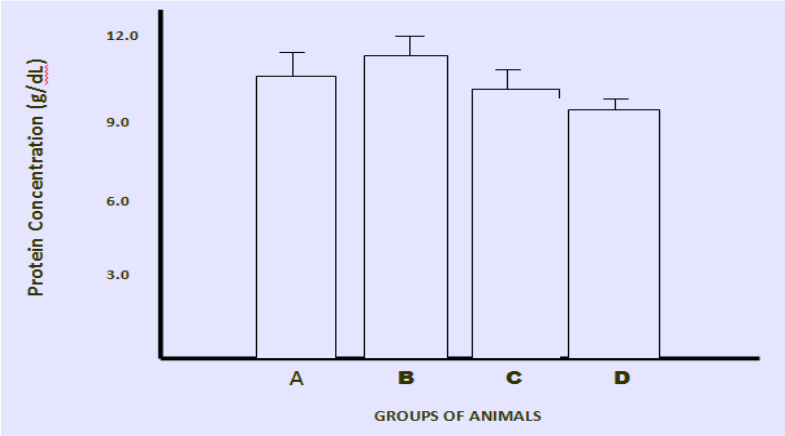

Figure 1. Protein concentration in the serum of various treatment groups. Values are given as mean and standard deviation of six determinations

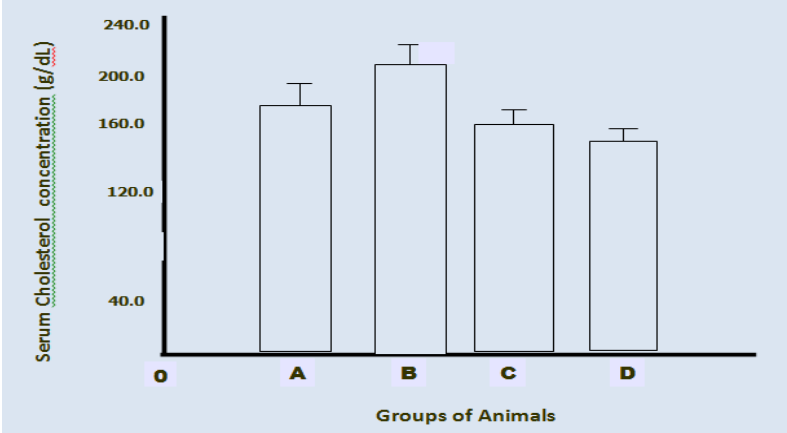

Figure 2. Serum Cholesterol concentration in the various treatment groups. Values are given as mean and standard deviation of six determinations.

*Value significantly different from Control.

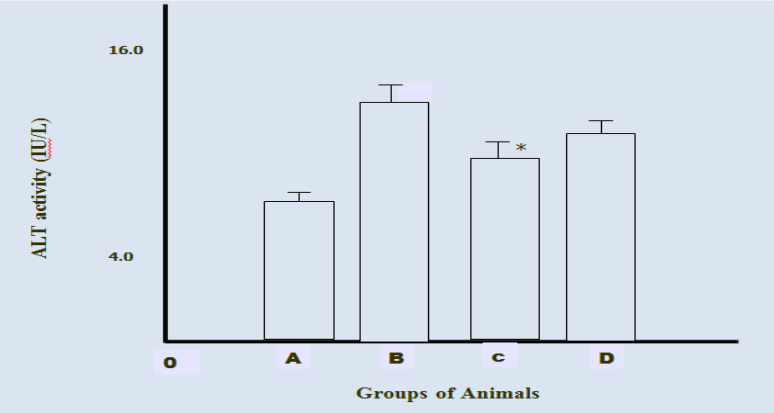

Figure 3. ALT activity in the liver of the various treatment groups.
Values are given as mean and standard deviation of six determinations.
* Value significantly different from control (P<0.05)

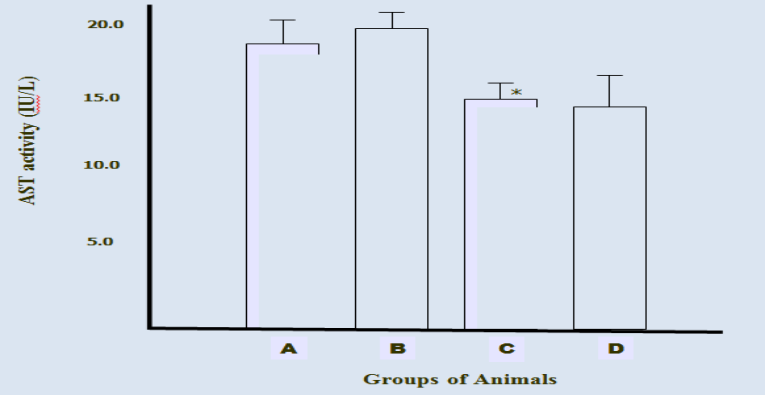

Figure 4. AST activity in the liver of the various treatment groups.
Values are given as mean and standard deviation of six determinations.
Value significantly different from control (P< 0.05)

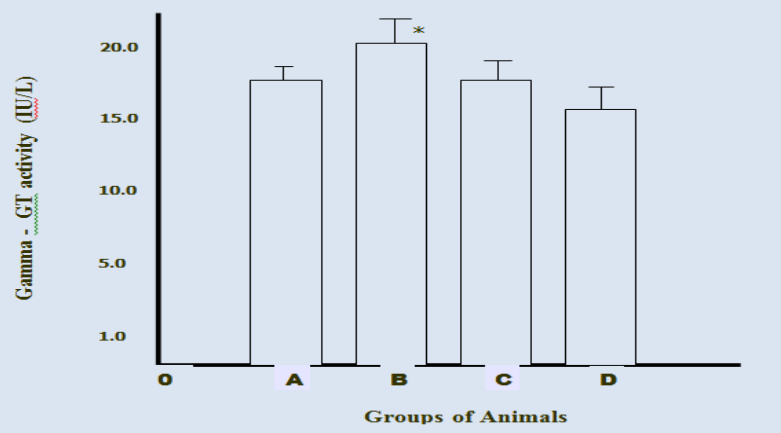

Figure 5. Gamma – GT activity in the liver of the various treatment groups.
Values are given as mean and standard deviation of six determinations.
*Value significantly different form control. (P<0.05)

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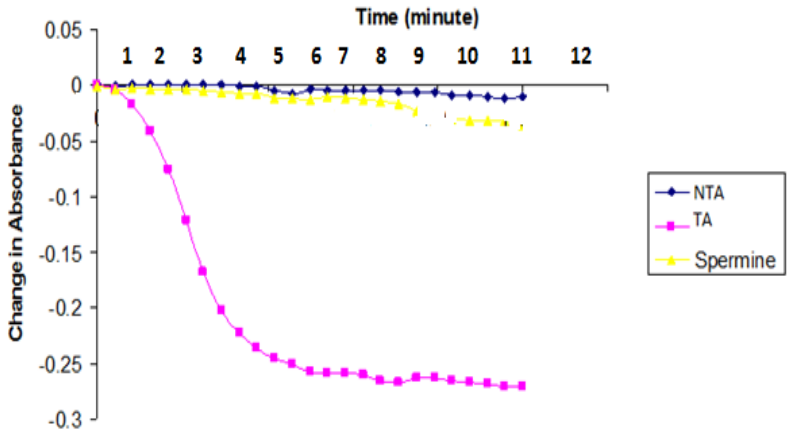

Figure 6. Induction of normal rat liver mitochondrial permeability transition pore opening with calcium and inhibition with spermine

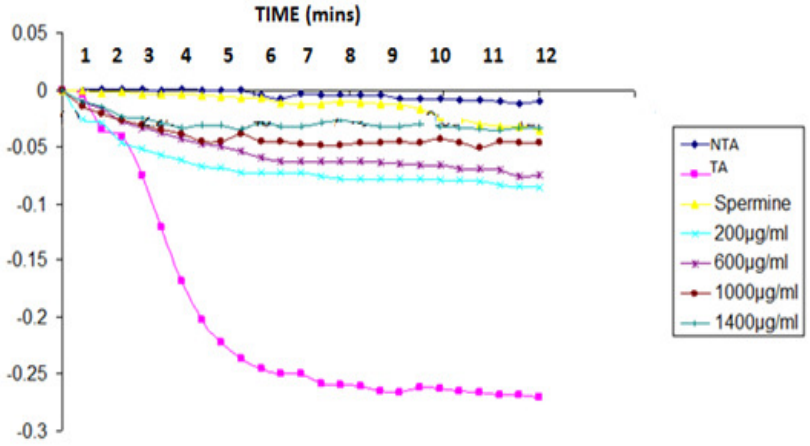

Figure 7. Effect of different concentrations of flavonoid-rich extract of the leaf of *Brysocarpus coccineus* on calcium induced opening of the rat liver mitochondrial permeability transition pore

ACKNOWLEDGEMENT

The authors wish to acknowledge the efforts of Mr Odewor of the Herbarium Unit of the Forestry Research Institute of Nigeria (FRIN) Jericho Ibadan Oyo State for the identification and confirmation of the plant used for this work.

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