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REGULAR ARTICLE

ANTIOXIDATIVE PROPERTIES OF ETHYL ACETATE FRACTION OF UNRIPE PULP OF *CARICA PAPAYA* IN MICE

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ABSTRACT

Unripe *Carica papaya* fruits were extracted with methanol in Soxhlet apparatus and later with a liquid-liquid extraction with the aim of identifying and quantifying secondary metabolite fraction of this plant. Quercetin and β-sitosterol have been isolated from the fruit and the quantities detected were 120.2±0.16 mg/g (dry fruit) and 279.1±0.09mg/g respectively. In addition, the extracts were evaluated *in vivo* for their effects on activities of some antioxidant enzymes which includes Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione transferase (GST), Catalase (CAT) and metabolizing enzyme Glucose-6-phosphate dehydrogenase (G6PDH) in mice treated orally with a daily dose of extracts (100mg/kg) for 7 days. Results showed that ethyl acetate fraction caused significant increase($p<0.05$) in the activities of GR, GPx, GST, and G6PDH. Significant decrease ($p<0.05$) in GPx activity was observed in kidney following administration of ethyl acetate fraction. It is likely that quercetin and β-sitosterol may be responsible for the antioxidant potential demonstrated by the ethyl acetate fraction from unripe fruit.

Keywords: antioxidant, *Carica papaya*, quercetin, β-sitosterol, antioxidant enzymes

INTRODUCTION

Carica papaya also known as pawpaw is widely spread throughout tropical Africa it is a berry developing from syncarpous superior ovary with parietal placentation **(Kochhar 1986; Rice *et al*., 1987)** Fruits have been part of human diet and food supplement over the years. The rise in nutritional importance of fruit has been stimulated by range of degenerative disease prevalent in many part of the world. However, fruits are increasingly becoming popular in Nigerian diet. Pawpaw fruit is one of the most nutritional fruits grown and consumed in Africa. Studies had demonstrated the use of leaf extract of *Carica papaya* as a tonic for the heart, analgesic and treatment for stomach ache **(Giove and Nakazawa, 1996)** and have antioxidant properties (**Rahmat *et al*., 2004)** It has offered some protection against oxidative damage to the gastric mucosa (**Indran *et al*., 2008**). The antioxidant systems present in *Carica papaya* play a protective role against the production of reactive oxygen species and lipid peroxidation by-products. Recent reports have implicated Reactive Oxygen Species (ROS) as the pathogenesis of many human diseases **(Repetto and Llesuy 2002)**. Reactive Oxygen Species (ROS) are free radicals generated as byproducts of normal aerobic metabolism and also from reactions with drugs and toxins **Singh *et al*. (2005**). Excessive production of reactive oxygen species however, results in alteration in the balance between ROS and endogenous antioxidants and creates oxidative stress which is implicated in many pathological conditions such as diabetes **(Choi and Hwang 2005)**, cardio vascular disease (**Singh et al., 2005**), cancer, Alzheimer's disease and ageing **(Surh and Fergusson 2003)**, atherosclerosis **(Landmesser and Drexler 2002; Shah *et al*., 2001)** Some of these medicinal plants used in ethno medicine for the treatment and management of many of these diseases have been investigated for their antioxidative properties **(Aruoma 2003; Semiz and Sen, 2007)**. Many of the metabolites from these medicinal plants especially flavonoids exhibited potent antioxidant activity *in vitro* and *in vivo* **(Usoh *et al*., 2005; Sofidiya *et al*., 2006; Nwanjo, 2007)**.

Thus, the objective of this study is to isolate the active component from ethyl acetate fraction of unripe pulp of *Carica papaya* and investigate the *in vivo* effect on antioxidative indices Glutathione peroxidase (GPx), Glutathione reductase(GR), Glutathione transferase (GST), Catalase (CAT) and metabolizing enzyme Glucose-6-phosphate dehydrogenase (G6PDH) and to corroborate its use in ethno medicine.

MATERIAL AND METHODS

Plant material

Fresh, unripe, mature fruits of *Carica papaya* (Variety Tsolo) were collected and authenticated in National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. The fruits were peeled, seeds removed and the pulp were cut into pieces, air dried and finely powdered with an electric grinder.

Plant extraction and isolation

Seventy grams (70g) of plant material, was macerated at room temperature with 70% ethanol for three days with daily shake. After filtration, the extract was evaporated under reduced pressure to remove the ethanol and after this step, the aqueous extract was partitioned successively with ether and ethyl acetate.

The ethyl acetate fraction (1.0 g) was subjected to column chromatography on silica gel 60 using initially CH_2Cl_2 (700 mL) as mobile phase. Afterward the column was eluted with a binary mixture of increase polarity, starting with CH_2Cl_2:EtOH (9:1 v/v, 700 mL) followed by CH_2Cl_2:EtOH (8:2 v/v, 700 mL), CH_2Cl_2:EtOH (7:3 v/v, 700 mL), CH_2Cl_2:EtOH (6:4 v/v, 700 mL), and CH_2Cl_2:EtOH (5:5 v/v, 600 mL). The resulting fractions, were analyzed by Thin layer chromatography (TLC) and pooled together on the basis of similarities in their chromatographic profile (solvent system: chloroform: ethanol, 70:30 v/v). The separated fractions were observed under UV light (254 and 366 nm) and detection was performed with anisaldehyde-H_2SO_4/100°C for ten minutes. Authentic samples of quercetin, rutin, chlorogenic, caffeic acids, kampferol and β-sitosterol were used as reference standards in order to guide the fractions pool process. Fractions 10 to 18 furnished a subfraction (0.292 g), which was further chromatographed under silica gel 60 and eluted with CH_2Cl_2:EtOH (8:2, v/v), CH_2Cl_2:EtOH (7:3, v/v) and CH_2Cl_2:EtOH (6:4 v/v) to give isolated compounds **1** (0.22 mg). Compounds 2 (0.34 mg) which was obtained from the sub-fraction 39-40 after additional column chromatographic procedures (gradient from CH_2Cl_2: EtOH 5:5 to pure EtOH).

Reagents, standards and apparatus

All chemicals were of analytical grade. Silica Gel 60 for column chromatography, Silica Gel 60 F_{254} coated plates, solvents for the extractions and analytical procedures, ether and ethyl acetate were purchased from Merck (Darmstadt, Germany). Quercetin, rutin, chlorogenic, caffeic acids, and β-sitosterol reference standards were obtained from Sigma

Chemical. Methanol and acetonitrile were of HPLC grade. Deionized water was prepared by a Milli-Q water purification system. High performance liquid chromatography (HPLC) of the samples was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence auto sampler (SIL-20A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, PDA detector SPD-M20A and Software LC solution 1.22 SP1. NMR spectra were carried out on a Bruker AMX 400 spectrometer equipped with a broadband 5-mm probe, using a spectral width of 10 ppm (parts per million). ^{1}HNMR recorded at 400 MHz and ^{13}C NMR at 100 MHz. Chemical shifts were expressed as ppm relative to the TMS. Deutered methanol (methanol-*d*4, 99.8 atom % of deuterium, solvent peaks δH 3.34 and δC 49.0 ppm) was used as solvent for the samples.

Preparation of standard and sample solutions for HPLC quantification

Standard stock solutions of quercetin and β-sitosterol were prepared in mobile phase, at a concentration range of 0.018 to 0.280 mg mL^{-1} for quercetin and 0.030 to 0.240 mg mL^{-1} for β-sitosterol. The ethyl acetate fraction was dissolved in the mobile phase. All solution were filtered through a filter paper and a 0.45 µm membrane filter (Millipore). Triplicate injections were made for each level, and a linear regression was generated.

Chromatographic conditions

Chromatographic analyses were carried out in isocratic conditions using RP-C_{18} column (4.6 mm x 250 mm) packed with 5µm diameter particles. The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and degassed in ultrasonic bath previous to use. Flow rate and injection volume were 0.8mL/min and 20µL, respectively. The chromatographic peaks were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of each peak using the external standard method. All chromatographic operations were carried out at ambient temperature. Quercetin and β-sitosterol reference standards, ethyl acetate fraction from the *Carica papaya* and isolated compounds (**1**-**2**) were quantified at 257 nm.

Animals

All animal procedures were in strict accordance with the NIH Guide for the care and use of Laboratory Animals. Albino mice of both sexes weighing between 24–30 g were used. The animals were kept in separate cages with access to water and food ad libitum, in a room

with controlled temperature (22°C ± 3) and in a 12 h light/ dark cycle with lights on at 7:00 a.m.

Enzyme assays

Glutathione reductase (GR), EC 1.8.1.7, activity was determined according to **Carlberg and Mannervik (1985).** Glutathione peroxidase (GPx), EC 1.11.1.9, activity was measured indirectly by monitoring the consumption of NADPH at 340 nm according to Wendel (1981), using *tert*-butylhydroperoxide as a substrate. Glutathione *S*-transferase (GST), EC 2.5.1.18 activity was assayed by the procedure of **Habig and Jakoby (1981)** using 1-chloro-2,4-dinitrobenzene as a substrate. The activity of glucose-6-phosphate dehydrogenase (G6PDH EC 1.1.1.49) was determined by means of the absorbance increase induced by the reduction of $NADP^+$ to NADPH, at 340 nm **(Glock and Mc, 1953).** Catalase (CAT), EC 1.11.1.6, activity was measured according to **Aebi (1984)**. Enzyme activity is presented as mU/mg of protein or nmol/min/mg of protein.

RESULTS AND DISCUSSION

Successive column chromatographic procedures with ethyl acetate fraction led to the isolation of two compounds (Fig. 1), whose structures were identified based on ^{1}H NMR and ^{13}C NMR spectra and by comparison with literature. The ^{1}H NMR spectrum of compound **1** showed two peaks at 6.12 (1H, *d, J* =2.0 Hz) and 6.33 ppm (1H, *d, J* = 2.0 Hz) consistent with the meta protons H-6 and H-8 on A-ring and an ABX system at δ □7.67 (1H, *d, J* = 2.1 Hz, H-2'), 7.66 (1H, *dd, J* = 8.4, 2.1 Hz, H-6') and 6.83 (1H, d, *J* = 8.4 Hz, H-5') corresponding to the catechol protons on B-ring. The ^{13}C NMR indicated the presence of 15 carbon atoms, the signal at: δ 177.4 was attributed to a carbonyl carbon placed at C-4, the other signals were: 165.6 (C-7), 162.5 (C-5), 158.3 (C-9), 148.8 (C-4'), 148,1 (C-2), 146.2 (C-3'), 137.2 (C-3), 124.2 (C-1'), 121.7 (C-6'), 116.1 (C-5'), 116.0 (C-2'), 104.5 (C-10), 99.2 (C-6), 94.4 (C-8). The spectral data were compatible with those of quercetin **(Slimestad *et al*., 1995; Fossen *et al*., 1998; Lawrence *et al*, 2003; MA *et al*, 2005 Liu *et al*., 2008).**

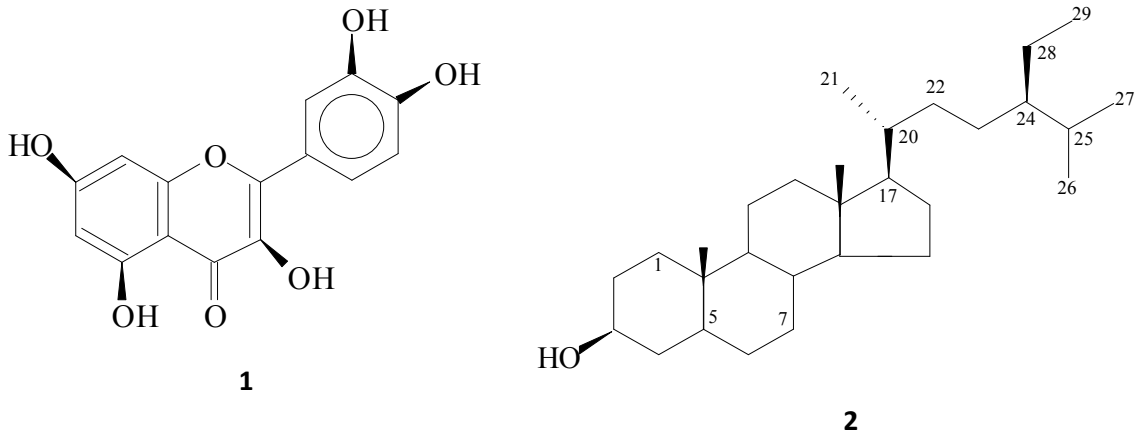

Figure 1 Chemical structures of compounds isolated from *C. papaya*

The ^{1}H NMR spectrum of compound **2** showed two peaks at 3.46 (1H, *m*, H-3), 5.11 (1H, *m*, H-6), 0.62 (3H, *s*, H-18), 0.73 (3H, *s*, H-18), 1.18 (3H, *s*, H-19), 0.84 (3H, *t*, H-29), (**Forgo and Kovér, 2004; Kojima *et al*., 1990**). ^{13}C NMR the signal at: δ 37.26 (C-1), 31.66 (C-2), 71.78 (C-3), 42.30 (C-4), 140.74 (C-5), 121.68 (C-6), 31.91 (C-7), 31.70 (C-8), 51.14 (C-9), 36.51 (C-10), 21.07 (C-11), 39.76 (C-12), 40.44 (C-13), 56.77 (C-14), 25.40 (C-15), 28.22 (C-16), 56.40 (C-17), 11.89 (C-18), 18.78 (C-19), 37.26 (C-20), 18.72 (C-21), 33.94 (C-22), 26.10 (C-23), 45.80 (C-24), 29.16 (C-25), 19.39 (C-26), 19.05 (C-27), 23.08 (C-28), 12.00 (C-29), **(De-eknamkul and Potduang, 2003; Goulart *et al*., 1993; Zanon *et al*., 2008).**

The ethyl acetate fraction from the *Carica papaya* was analyzed by liquid Chromatography. The simple and rapid reversed-phase HPLC method was utilized for the determination of quercetin and β-sitosterol. Figure 2 shows a representative chromatogram obtained for ethyl acetate fraction and the isolated compounds.

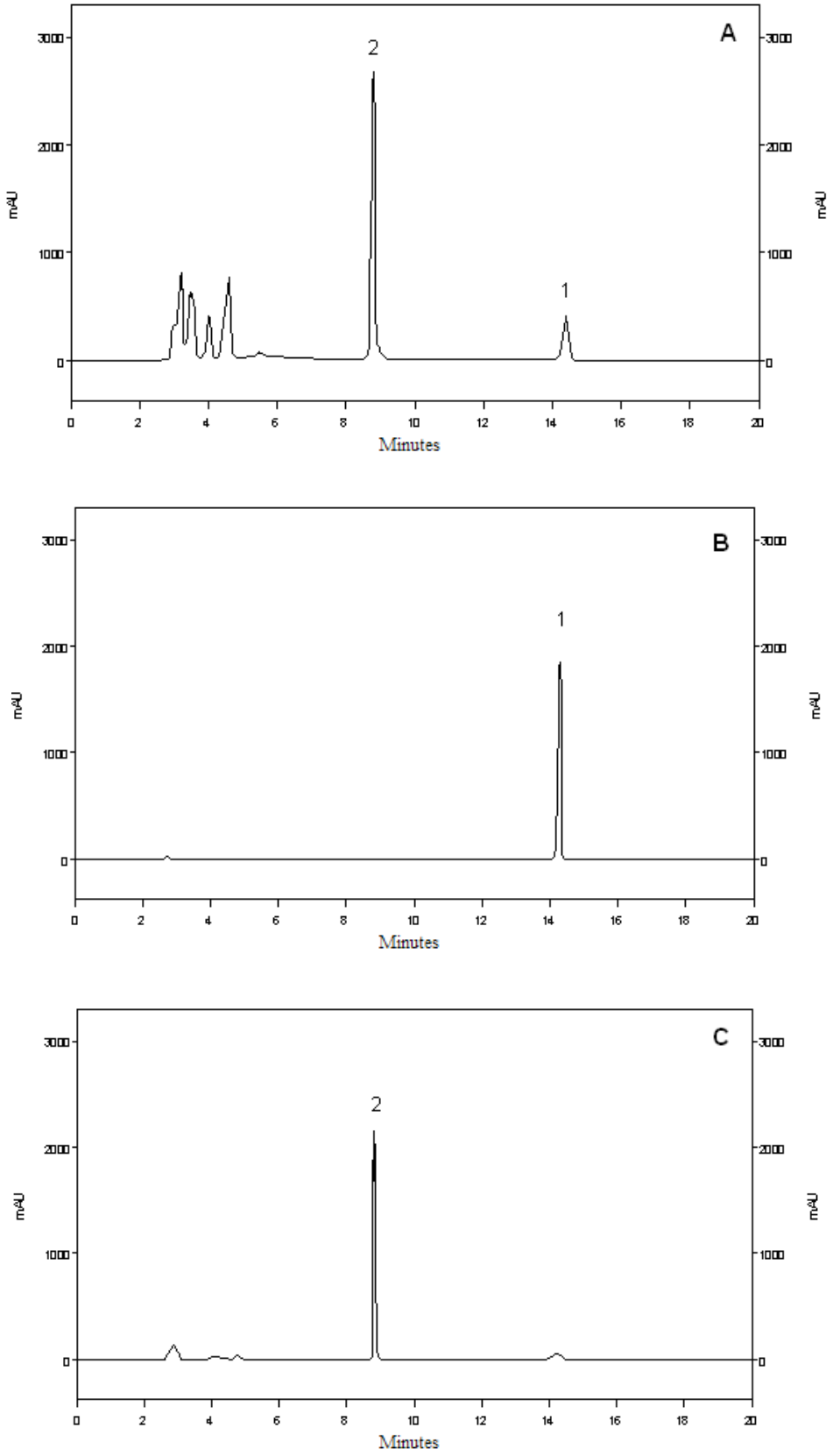

Figure 2 Chromatograms of ethyl acetate sample (**A**), and isolated quercetin (**B**) and β-sitosterol (**C**).

The ethyl acetate contains other minor compounds in addition to quercetin (retention time-Rt 14.2 min, peak 1) and β-sitosterol (Rt = 8.6 min, peak 2). Since extracts of natural origin usually contain a range of chemically diverse constituents occurring in varying concentrations, it is important to use chromatographic methods to analyze these inherently complex mixtures. The HPLC profile of ethyl acetate fraction was acquired, as well the quantification of rutin and β-sitosterol by HPLC-DAD based in the reference rutin and β-sitosterol standards calibration curves. Calibration curve for quercetin: Y = 30153x – 235135 (r = 0.9983), calibration curve for β-sitosterol: Y = 240846x – 5410 (r = 0.9950).

Phenolic compounds and β-sitosterols are important components in vegetable foods, fruits, teas,etc. Fruits have been part of human diet and food supplement over the years. The rise in nutritional importance of fruit has been stimulated by range of degenerative disease prevalent in many part of the world. Plant sterols which are counterparts of cholesterol in animals are present in diet in several forms the most abundant are β-sitosterol and campsterol **(Awad and Fink, 2000)** Studies suggest that phytosterols offer protection from cancers and heart disease in developed countries. Antioxidant activity of β-sitosterol has been reported. β-Sitosterol reduce cellular ROS level through the modulation of antioxidant enzymes. Therefore, the dietary supplement of antioxidants such as vitamins **(Fairfield and Fletcher, 2002)**, flavonoids **(Peluso, 2006)** etc., has been used to prevent the occurrence of many chronic diseases. Quercetin (QU) is a well-known flavonoid distributed ubiquitously in fruits, vegetables, and herbs or related products, e.g. apples, onions **(Hertog *et al.*, 1992),** *Ginkgo biloba* **(Watson and Oliveira 1999)**, and red wine **(Kerem et al. 2004)**, respectively. QU has been extensively investigated for its pharmacological effects that include anti-tumor **(Kanadaswami *et al.*, 2005),** anti-inflammatory **(Comalada *et al.* 2005),** antioxidant **(Inal and Kahraman 2000),** and hepatoprotective **(Lee *et al.* 2003)** activities. Antioxidant enzymes plays a key role in ROS scavenging and the maintenance of cellular redox equilibrium **(Rodriguez *et al.*, 2004).** The presence of Quercetin (120.2 mg/g) and β-sitosterol (279.1 mg/g) in ethyl acetate fraction of unripe pulp of *Carica papaya* could partially explain the pharmacological properties of this plant and demonstrate its importance in diet (Table 1). Since the presence of phenolic compounds (such as flavonoids) in human diet is associated with protective effects against some chronic degenerative disease related to oxidative stress.

Table 1 Quantification of *C. papaya* ethyl acetate fraction

Compounds	**Quantities***	
	mg/g of dry fraction	**Percentage (%)**
Quercetin	120.2 ± 0.16	10.3%
β-sitosterol	279.1 ± 0.09	27.9%

* Results are expressed as mean ± S. E. of three determinations.

Four antioxidative markers Glutathione reductase(GR), Glutathione peroxidase(GPx), Glutathione transferase(GST) and Catalase(CAT) as well as Glucose-6-phosphate dehydrogenase have been established and utilized in the evaluation of oxidative stress in animal model with hypertension, hyperlipidemia, obesity and diabetes (**Saiki, et al., 2007**).

In the present study, effects of ethyl acetate fraction and aqueous extract on the antioxidant markers were compared (Fig 3 and 4). The data showed increase in the activities of GPx, and G6PDH in liver tissue when compared to control following administration of ethyl acetate and aqueous extract(100mg/kg) respectively, while there is a slight reduction in Catalase activity in the liver of animals administered ethyl acetate in comparison to control (Fig 3).

However, the pattern of enzyme activities in the kidney is different (Fig 4). No significant change in activities of GR, GST and CAT were observed in groups of animals administered ethyl acetate (100mg/kg) or Aqueous extract when compared to control that received distilled water only, but renal GPx activity decreased following administration of ethyl acetate fraction.

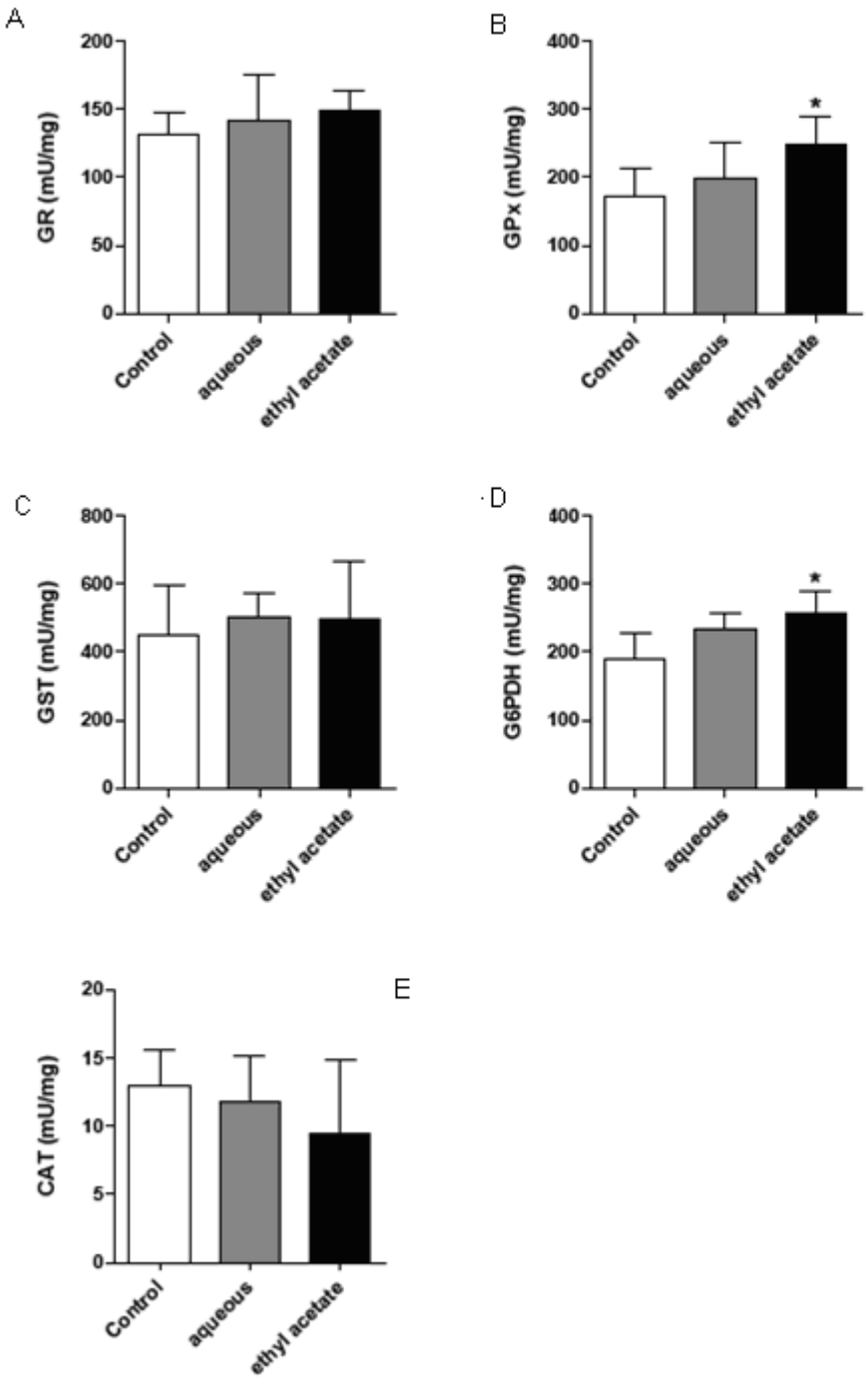

Figure 3 Effect of oral administration of extracts from unripe pulp (100mg/kg on antioxidant enzymes in mice liver. (**A**) Glutathione reductase (GR) activity. (**B**) Glutathione peroxidase (GPx) activity (**C**) Glutathione-s-transferase (GST) activity. (**D**) Glucose-6-phosphate dehydrogenase activity. (**E**) Catalase (CAT) activity.

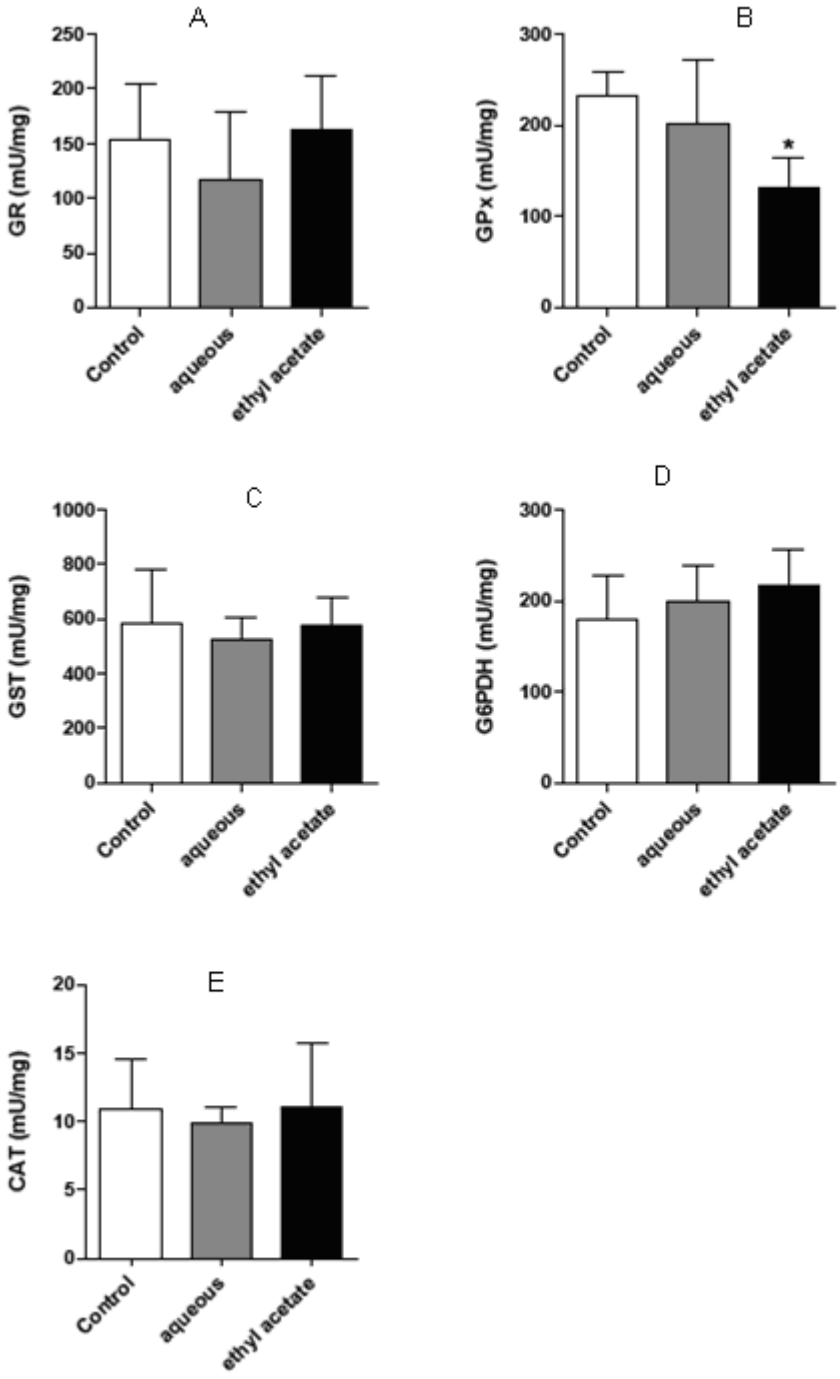

Figure 4 Effect of oral administration of extracts from unripe pulp (100mg/kg on antioxidant enzymes in mice kidney. (A) Glutathione reductase (GR) activity. (B) Glutathione peroxidase (GPx) activity (C) Glutathione-s-transferase (GST) activity. (D) Glucose-6-phosphate dehydrogenase activity. (E) Catalase (CAT) activity.

Both extract increased the activity of G6PDH in tested groups with no significant change when compared to control values. Animals were active, stable and showed no sign of restlessness throughout the period of experiment.

Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. The effects of ROS on living organism depend on many circumstances, depending on the intensity of oxidative stress and efficacy of antioxidant systems and many other factors. The adaptive response is usually realized via synthesis of new molecules of antioxidant enzyme which takes place simultaneously with inactivation of the enzyme by ROS **(Rodriguez *et al*., 2004).** Moreover, the enzymes glutathione peroxidase and catalase participate in the removal of H_2O_2, glutathione peroxidase (GPx) being the main controller of H_2O_2 metabolism.

Glucose-6-phosphate dehydrogenase(G6PDH), the first and rate limiting enzyme of the pentose phosphate pathway, a major cellular reductant central to cell survival, is indispensable to maintenance of the cytosolic pool of NADPH and thus the cellular redox balance. The role of glucose-6-phosphate dehydrogenase has been recognized for a long time. **(Sumathi *et al*., 1996).** In the present study, we observed an increase in the activity of GPx and G6PDH after animals were treated with ethyl acetate fraction of *Carica papaya*. This effect may represent an important mechanism of protection by the fraction, since by having increased ability to remove peroxides, cells may be less susceptible to oxidations stress damage. GPx is an antioxidant enzyme that is highly expressed in the kidney and removes peroxides and peroxynitrite that cause renal damage.Low activities of GPx is one of the early consequences of a disturbance of the pro-oxidant/ antioxidant balance.

It is known that phenolic compounds especially flavonoids in plants, are extractable with ethyl acetate and these compounds have been shown to possess antioxidative properties **(Wu *et al*., 2005).** The elevated levels of antioxidant enzymes (GPx and G6PDH) observed in the ethyl acetate fraction-treated rats may suggest that the components of this fraction might be activating or inducing the synthesis of the enzymes **(Finkel and Holbrook, 2000).**
Furthermore, quercetin and β-sitosterol found in the ethyl acetate fraction of ***Carica papaya*** may be the basis for the antioxidative properties of the plant. It is therefore suggested that the plant may be useful in the management of diseases such as diabetes, sickle cell anaemia and cardiovascular diseases where free radicals are often generated.

CONCLUSION

In conclusion, administration of these extracts invivo provide evidence for the potential free radical scavenger activity and that its regular intake can modulate activity of important antioxidant enzymes which can be of special interest for the management of human diseases associated with oxidative stress.

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