Full Length Research Paper

Causes of rice bran extract on immunological and physiological parameters of *Biomphalaria alexandrina*

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The present study was undertaken to evaluate the effect of rice bran extract on immunological and physiological parameters of *Biomphalaria alexandrina* snails and *Schistosoma mansoni* infection. Rice bran methanolic extract showed the strongest antioxidant activity. IC_{50} values for scavenging DPPH was 344.22 µg/ml and reducing power was increased with increasing amounts of the extract. Total phenolic and flavonoid contents were 4.23 mg gallic acid-equivalent (GAE)/g bran and 2.11 mg quercetin-equivalent (QE)/g bran, respectively. The presence of rice bran during infection causes reduction in infection rate by 33.3%. The exposure of snails to infection for 24 h followed by treatment with 500 ppm of rice bran for 24 h caused a reduction in infection rate by 56.5% as compared to the control (80%). The reduction in infection rate of *Schistosoma haematobium* may be due to the increase in immune activity response, activation and vacuolation of haemocytes distributed in hemolymph of snails which represent the important system in immunity defense against any invaders. These results could provide useful information for control of Schistosomiasis in integrated pest management program and environmental protection.

Key words: Schistosomiasis, antioxidant, rice bran, control, immunological and physiological parameters, snail.

INTRODUCTION

Schistosomiasis is still a prime health problem in many tropical and subtropical countries (WHO, 1992). It is affecting about 200 million people infected worldwide with more than 95% of the infections concentrated in Africa (Gryseels et al., 2006; Steinmann et al., 2006). Cur-

rently, almost 600 million people at schistosomiasis risk in 76 endemic countries and an estimated 280,000 deaths deaths are directly or indirectly attributable to the disease annually (van der Werf et al., 2003; Gryseels et al., 2006).

Schistosomiasis is a group of diseases caused by parasitic worms of the genus *Schistosoma*. These blood-dwelling flukes have a complicated life cycle involving freshwater snail intermediate hosts and transmission of

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transmission of the parasite is governed by social-ecological systems and intimately linked with conditions of poverty (King, 2010; Utzinger et al., 2011). In fact, fresh water pulmonate snails of the genus *Biomphalaria* are best known for their role as intermediate hosts of the widely distributed parasite, *Schistosoma mansoni* (Ross et al., 2000). One of the keys to understand the present and future of *S. mansoni* infection in Egypt is to understand more about the snails that play an indispensable role in its transmission (Lotfy et al., 2005).

The defense mechanisms and immunological responses which consist of the immune system have been considered as biomarkers of pollution in aquatic invertebrates (Galloway and Depledge, 2001). Despite the lack of an adaptive immune system, invertebrates are able to survive among potential pathogens and respond to infection by activation of various defense mechanisms (Little et al., 2005). Phagocytosis is one of the various functions of hemocytes, which is a nonspecific immune mechanism against non-self materials (Galloway and Depledge, 2001; Negrao-Correa et al., 2007). The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites (Livingstone et al., 2000). The *Biomphalaria* internal defense system is composed of soluble components of hemolymph and circulating cells, termed hemocytes, which work in association during the snail responses against infectious agents (Van der Knaap and Loker, 1990). In snails, circulating hemocytes, especially the phagocytic cell population, are the principal line of cellular defense involved in destruction of *S. mansoni* larvae inside the intermediate host (Bayne et al., 1980; Noda and Loker, 1989; Negrão-Corrêa et al., 2007).

Histopathological analysis of *S. mansoni* infected *Biomphalaria* showed that hemocyte infiltration around parasite larvae was faster and stronger in snail strains that are more resistant to parasite infection (Negrao-Correa et al., 2007). The effector mechanisms by which hemocytes are able to kill trematode larvae are partially dependent on the capability of these cells to recognize sporocyst tegument molecules, leading to parasite encapsulation and cellular activation, that result in production of highly toxic metabolites of oxygen and nitrogen associated with parasite killing (Hahn et al., 2000).

Particular attention has been directed to immunological changes induced by environmental pollution in aquatic invertebrates. The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites (Livingstone et al., 2000). Furthermore, the development of an infectious disease results from an imbalance between the host and the pathogen due to external factors, like pollutants, and/or internal factors, like susceptibility of the host (Snieszko, 1974).

Rice bran, a byproduct of rice milling, is a constituent (approximately 10%) of the whole rice grain and consists of the bran layers (pericarp, seed coat, nucellus, and aleurone) and the germ (Rohrer and Siebenmorgen, 2004). It is a rich source of natural antioxidants which can be used as free radical scavengers. It is widely recognized that many of the today's diseases are due to the oxidative stress that results from an imbalance between formation and neutralization of free radicals (Arab et al., 2011).

It is hypothesized that antioxidant improved immu-nity system in snails e.g. *Biomphalaria alexandrina* and reduced infection by pathogen e.g. *S. mansoni*. Therefore, this study was undertaken to evaluate the effect of rice bran extract on immunological and physiological parameters of *B. alexandrina* snails and *S. mansoni* infection.

MATERIALS AND METHODS

Preparation of rice bran extract

The rice bran was extracted with 95% methanol overnight with shaking at a bran-to-solvent ratio of 1:10 at room temperature. The next day, the mixture was centrifuged at 3000 rpm for 10 min and then the supernatant was collected. The pellet was extracted with 95% methanol one more time. Both supernatants were pooled and filtered, and then methanol was evaporated under reduced pressure using rotary evaporator. The crude extract residues were stored in clean dry dark vessel till use.

Phytochemicals

Determination of total flavonoid content

The total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to Kähkönen et al. (1999) with same modifications and using quercetin as a standard. Aliquot (250 µl) of the extract (2 mg/ml) was mixed with 250 µl of a 5% $NaNO_2$ solution. The mixture was allowed to stay at room temperature for 6 min, then 200 µl of a 10% $AlCl_3.9H_2O$ solution was added for 6 min followed by the addition of a 2 ml 4% NaOH solution. Distilled water was added to reach a final volume of 5 ml. The solution was mixed and kept at room temperature for 15 min. Absorbance was measured immediately against the prepared blank at 510 nm using a spectrophotometer. Total flavonoid content is expressed as mg of catechin equivalents (CE)/g of extract.

Determination of the total polyphenolic content

The total phenolic content of rice bran methanolic extract was determined using the Folin-Ciocalteu reagent, according to the method of Slinkard and Singleton (1977) with some modifications. Aliquots (100 µl) of the extracts (2 mg/ml) were transferred into test tubes and combined with 100 µl of Folin-Ciocalteu reagent; after 3 min, 100 µl of sodium carbonate solution (2% Na_2CO_3) was added and the volume was adjusted to a final volume of 2.5 ml. After 1 h of incubation in the dark at room temperature, the absorbance was read at 760 nm using a Shimadzu UV-VIS Recording 2401 PC (Japan). The amount of total phenolic compounds was calculated as mg of gallic acid equivalents (mEq gallic acid) from the calibration curve of gallic acid standard solution. The data were presented as the average of five replicate analyses.

Antioxidant activity

Free radical scavenging activity by DPPH (1-1-diphenyl 2-picryl hydrazyl)

The hydrogen atom-or-electron donation ability of the extract was measured from the bleaching of the purple colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2´-diphenylpicrylhydrazyl (DPPH), as a reagent, according to the method of Amarowicz et al. (2004) with some modifications. One milliliter of different concentrations of the methanolic extract of rice bran (32 to 500 µ g/ml) were mixed with 1.0 ml of 0.1 mM DPPH in methanol, and final volume adjusted up to 3.0 ml with MeOH. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min, and then the absorbance was measured at 524 nm using spectrophotometer (Shimadzu UV-VIS Recording 2401 PC, Japan). Methanol was used as blank and ascorbic acid (0.5 to 5 µ g/ml) was used as the reference compound. The absorbance of solvent and DPPH radical without extract was measured as control. The radical-scavenging activities of samples, expressed as percentage inhibition of DPPH, were calculated according to the formula:

$$I (\%) = [(A_C - A_S)/A_C] \times 100$$

where A_C and A_S are the absorbance of the control and sample, respectively.

Reducing power

Total reducing capacity of rice bran extracts was determined according to the method of Oyaizu (1986). One milliliter of extract at different concentrations (300 to 1000 µ g/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3 Fe (CN)_6]$ (1%). The mixture was incubated at 50°C for 20 min, and then a portion (2.5 ml) of trichloroacetic acid (TCA, 10%) was added to mixture, which was centrifuged for 10 min at 1000 × g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml $FeCl_3$ (0.1%). Then the absorbance was measured at 700 nm. Trolox (10 to 160 µ g/ml) was used as the reference compound.

Experimental animals and infection

Miracidia

Schistosoma mansoni miracidia were obtained from The Schistosoma Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Egypt.

Snails

Adult *Biomphalaria alexandrina* snails (5 to 8 mm in height) used in the present study were obtained from the bred stock in Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Egypt.

Infection of snails

Snails were infected by miracidia (10 per snail) at 25°C±1 under ceiling illumination. After exposure, snails were washed and placed in clean plastic aquaria of dechlorinated water and supplied with lettuce leaves till shedding cercarea, then calculate the number of dead snails, total livening snails and number of shedding snails after calculating the percentage of rate infection.

Experimental bioassays

Four groups of *B. alexandrina* snails each group contains 30 adult snails (5 to 8 mm in height).

(1) Normal control group.
(2) *S. mansoni miracidia*-infected group (infected for 24 h then recovery in dechlorinated water for 24 h), then divided into two sub-groups. (A) First sub-group for withdrawal haemolymph for light and electron microscopy investigation. (B) Second sub-group was kept in container includes dechlorinated water till cercarea shedding, calculate the infection rat and then take samples of haemolymph for light and electron microscopy investigations.
(3) Rice bran group (exposed to aqueous solution of rice bran extract 500 mg/L for 3-days), then divided into two sub-groups. (A) First used for haemolmph collections for light and electron microscopy investigations. (B) Second sub-group exposed to *S. mansoni miracidia* for 24 h (5 to 8 miracidia for each snail) then recovery and after that kept adult *B. alexandrina* till shedding cercarea and count number of snails producing cercarea, number of living snails at the same time and number of dead snails to calculate rate of infection in both groups the 2nd sub-group and last groups according to the standard method recorded by WHO (1965).

For each group, glass containers each containing one liter dechlorinated water to which 10 snails were introduced. The glass containers were covered by porous plastic sheets and maintained for shedding at normal laboratory conditions (25 ± 1°C). Dead snails were distinguished and counted and feed on lettuce powder.

Hemolymph collection

Haemolymph samples were collected according to the methods of Abdul-Salam and Michelson (1983) by removing a small portion of the shell situated directly above the heart to insert a capillary tube into it. Two milliliters of hemolymph were pooled from each snail group in a vial tube and kept in ice-bath then centrifuged for 10 min at 3000 rpm at 4°C where the fresh supernatant was used for light microscope and sediment cells for electron microscope investigations.

Hemolymph examination

Light microscopy

Haemocytes staining were prepared according to Mossalem (2008). Monolayer of hemocytes were stained with Giemsa stain for 20 min, according to the methods of Abdul-Salam and Michelson (1983), examined and counted by light microscopy and photographed using Agfa film RSX 100.

Electron microscopy

Sediment cells were fixed in 4% glutaraldhyde with sodium cacodylate. Two hours later, the cells were post fixed in 2% osmium tetraoxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin according to the technique of Grimaud et al. (1980). Semi-thin and ultra-thin sections were cut with a Leika ultra microtome. Ultra-thin sections were contrasted with uranyl acetate and lead citrate stains then examined by Phillips EM 208 Electron Microscope.
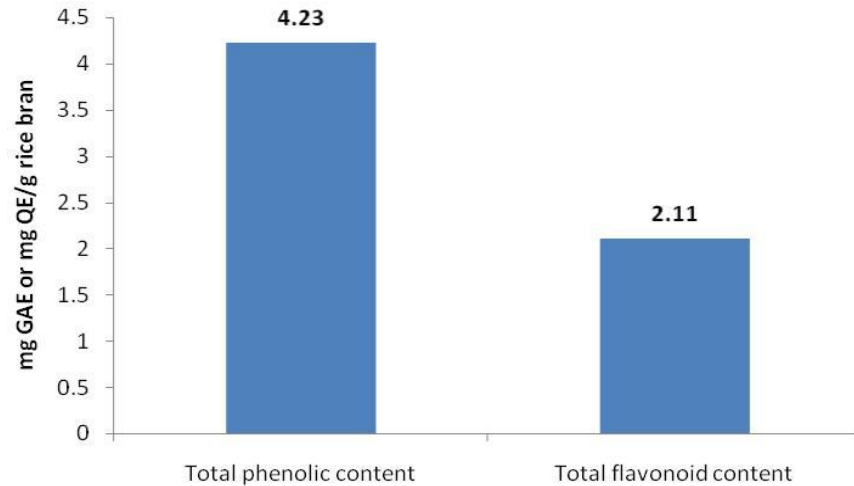
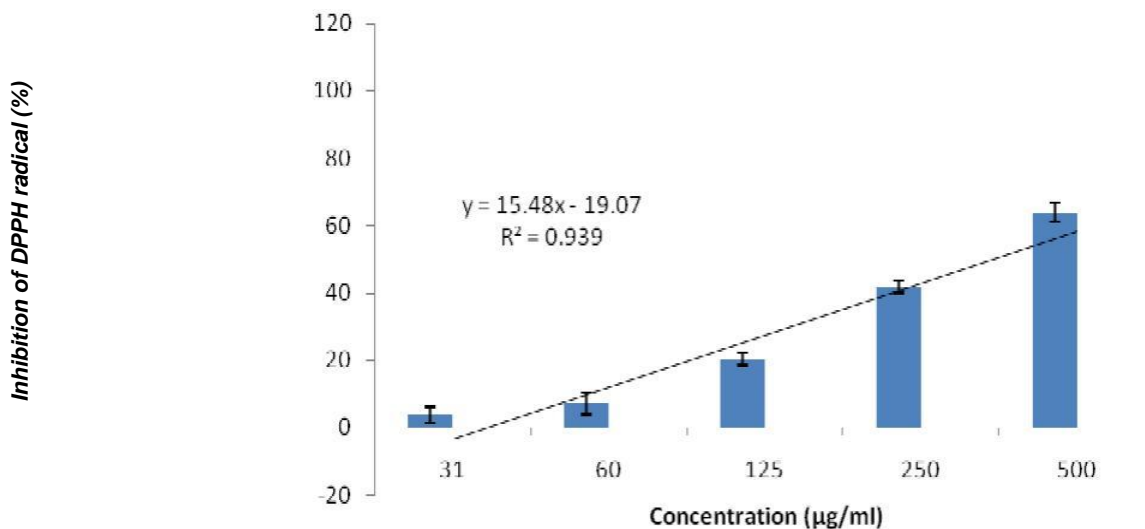**Figure 1.** Total phenolic and flavonoid contents from methanolic rice bran extract.

Figure 2. DPPH scavenging (% of inhibition) activity of methanolic rice bran extract. Bars represented means ± SD.

Estimation of IC_{50} values and statistical analysis

The concentrations of the extract induced 50% inhibition (IC_{50}) were determined by a linear regression analysis between the inhibition percentages against the extract concentrations by log-probit analysis. Results were analyzed by SPSS (version 14.0) for Windows and expressed as mean ± standard division.

RESULTS AND DISCUSSION

The results of the total phenolic content of rice brain extract using Folin-Ciocalteu method, is presented in Figure 1. TPC was expressed as gallic acid equivalents and 4.23 mg GAE/g rice brain was found. In contrast, total flavonoid content of the methanolic extract was 2.11 mg QE/g rice brain (Figure 1).

The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. DPPH radical scavenging is considered a good *in vitro* model widely used to assess antioxidant efficacy within a very short time. It is one of the compounds that possess a proton free radical.

Antioxidant activity on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, is neutrallization its free radical character. However, the scavenging effects were 3.69 and 64.74% for rice bran extract at 31.0 and 500 µg/ml. The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH scavengers. DPPH radical scavenging abilities of the rice bran extract in our study exhibited appre-ciable scavenging activity and the IC_{50} accounted for 344.22 µg/ml (Figure 2).

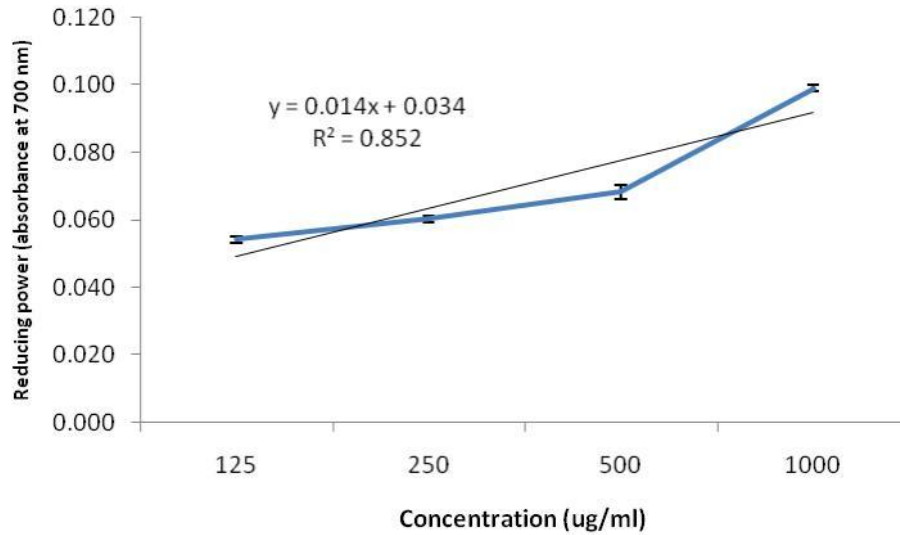Reducing powers of rice bran extract increased rapidly

Figure 3. DPPH scavenging (% of inhibition) activity of methanolic rice bran extract. Bars represented means ± SD.
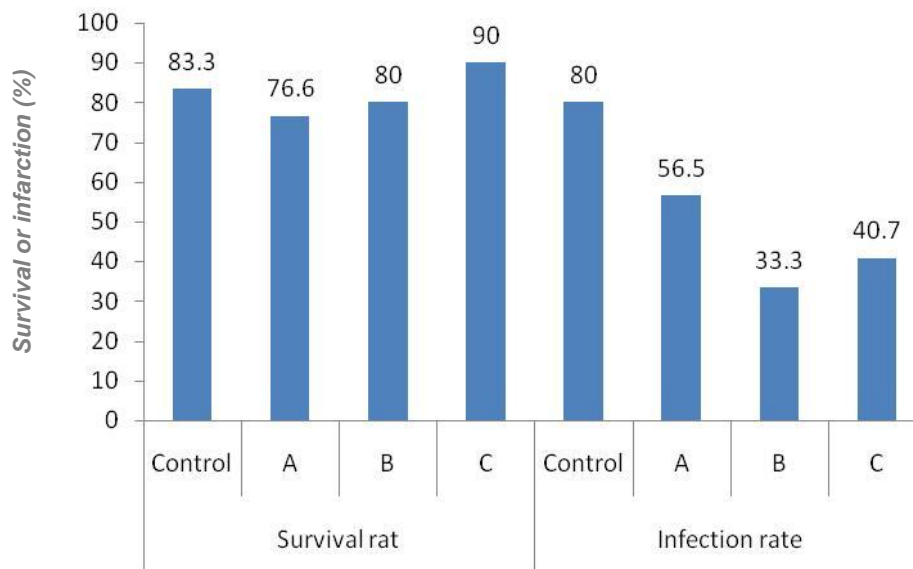

Figure 4. Effect of treatment with rice bran extract before [A], during [B] and after [C] infection with *S. mansoni* on survival and infection rate (%) of *B. alexandrina*.

at low concentrations from 0.125 to 1.00 mg/ml (Figure 3). Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Chanda and Dave, 2009).

The exposure of snails to rice bran for three days then exposed to infection with *S. mansoni* causes reduction in infection rate by 40.70% where the rate of infection in control equal 80.00%. The presence of rice bran during infection causes reduction in infection rate by 33.30%. The exposure of snails to infection for 24 h was then treated with 500 ppm of rice bran for 24 h which caused a reduction in infection rate by 56.5%, with no sig-nificance at survival rate as shown in Figure 4. So the presence of rice bran in all cases before, du-ring, and after infection with *S. mansoni* causes reduction in infec-tion rate, and this may be due to the rice bran increa-sing immune activity response, activation a n d vacuo-lation o f haemocytes which distributed in hemolymph of snails which represent the important system in immunity defense against any invaders as shown in Figures 5 and 6.
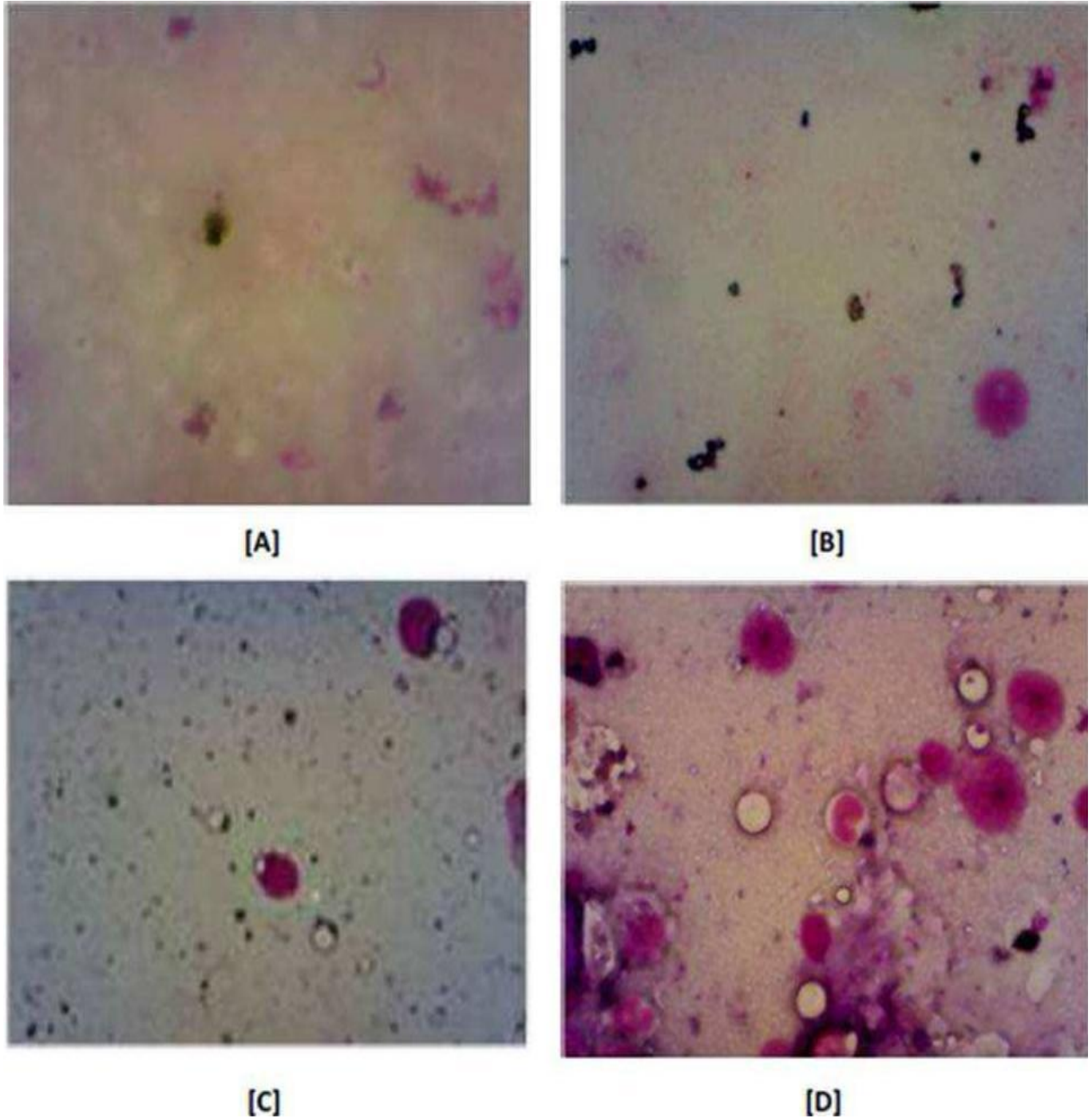
Figure 5. Photomicrograph of snail hemolymph stained by giemsa stain. Control group [A] showing the presence of small distributed cells in the hemolymph of *B. alexandrina* in normal conditions (x100). Haemocytes after exposure to rice bran for 3-days then exposed infected for 24 h [B] showing vacuolation and many pseudopodia for phagosomes (×200). Haemocytes of *B. truncates* infected with *S. mansoni in* the presence of rice bran [C] showing increasing in shape of cells due to activation against infection (×200). Haemocytes after infected for 24 h then exposed to rice bran for 3 days [D] showing haemocytes increased in shape (×200).
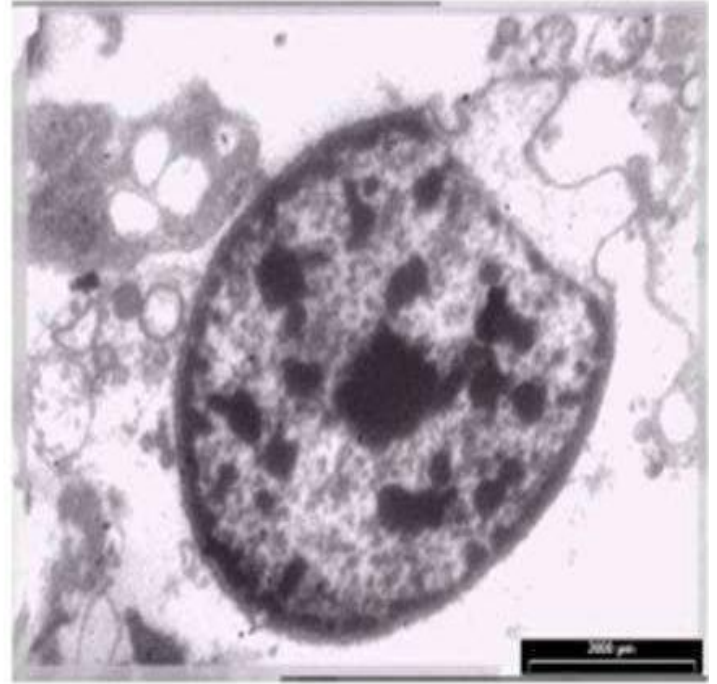
Conclusion

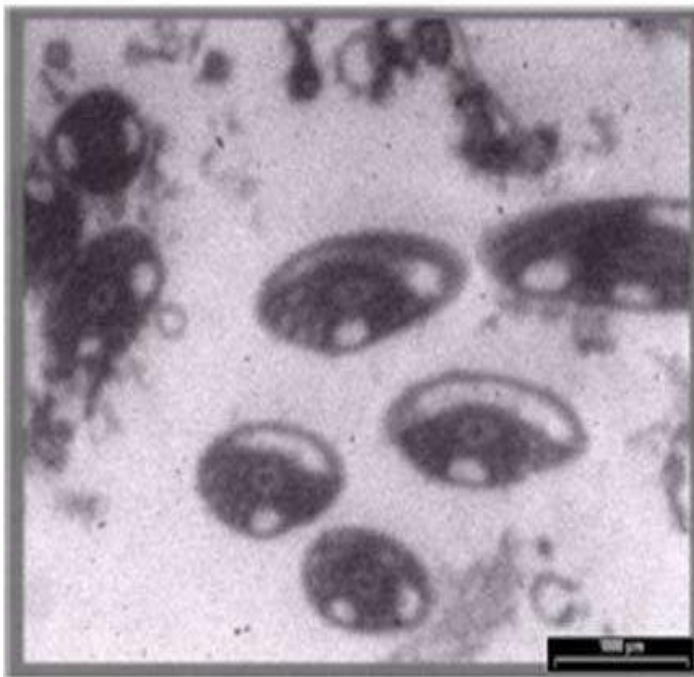Rice bran extract indicated the strongest antioxidant activity. It causes reduction in infection rate of *S. mansoni*, this may be due to the increase in immune activity response, activation and vacuolation of hae-mocytes which distributed in hemolymph of snails which represent the important system in immunity defense
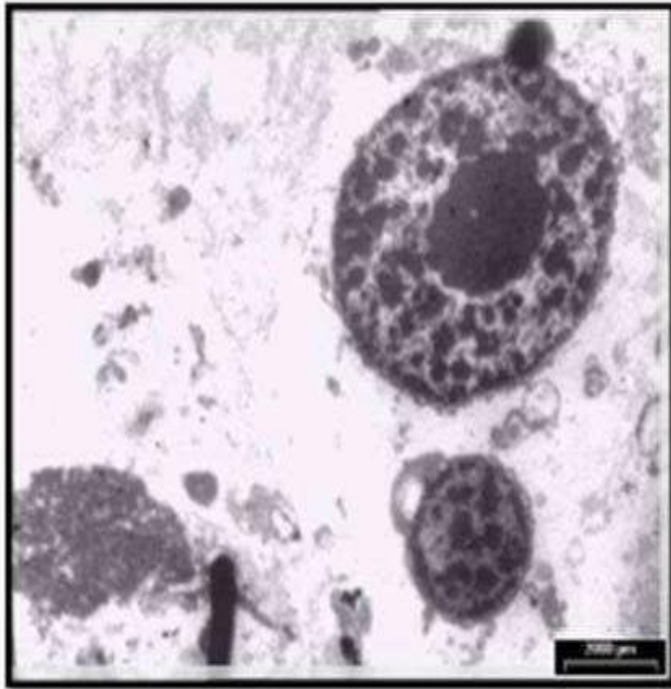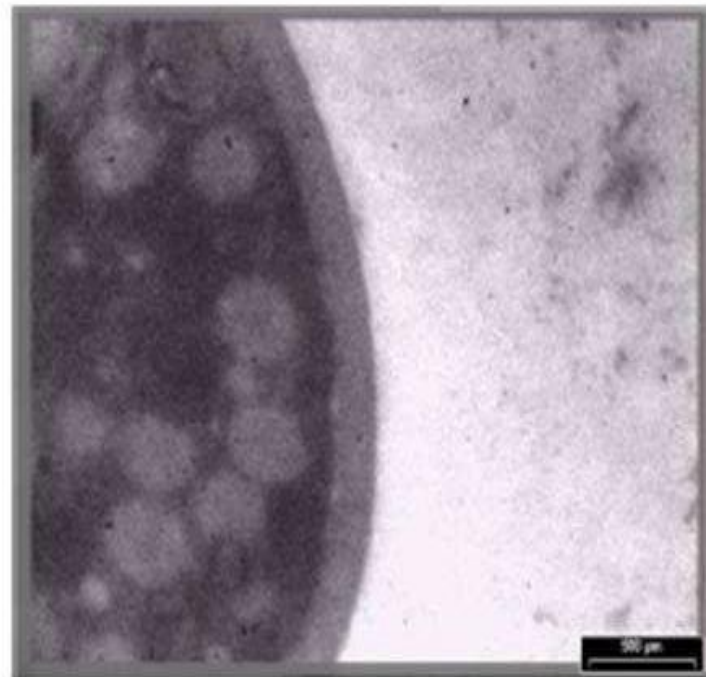
Figure 6. Electron microscope images of normal *B. alexandrina* haemocytes, [A] showing cells with internal organelles e.g. nucleus, mitochondria, endoplasmic reticulum and cell membrane (x2000). Haemocytes after exposed to rice bran for 3-days and infected for 24 h [B] showing vacuolated cytoplasm (V), dense nuclear chromatin (Ch) and pseudopodia forming phagolysosome vacuole (x2000). Haemocytes of *B. alexandrina* infected with *S. mansoni* in the presence of rice bran [C] showing vacuolation of cells and their nucleus become eccentric (x1000). Haemocytes after infected for 24 h then exposed to rice bran for 3 days [D] showing microgram haemocytes with vacuoles and clear cell membrane forming pseudopodia (x500).

against any invaders. These results could provide useful information for control of Schistosomiasis in integrated pest management program and environmental protection.

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