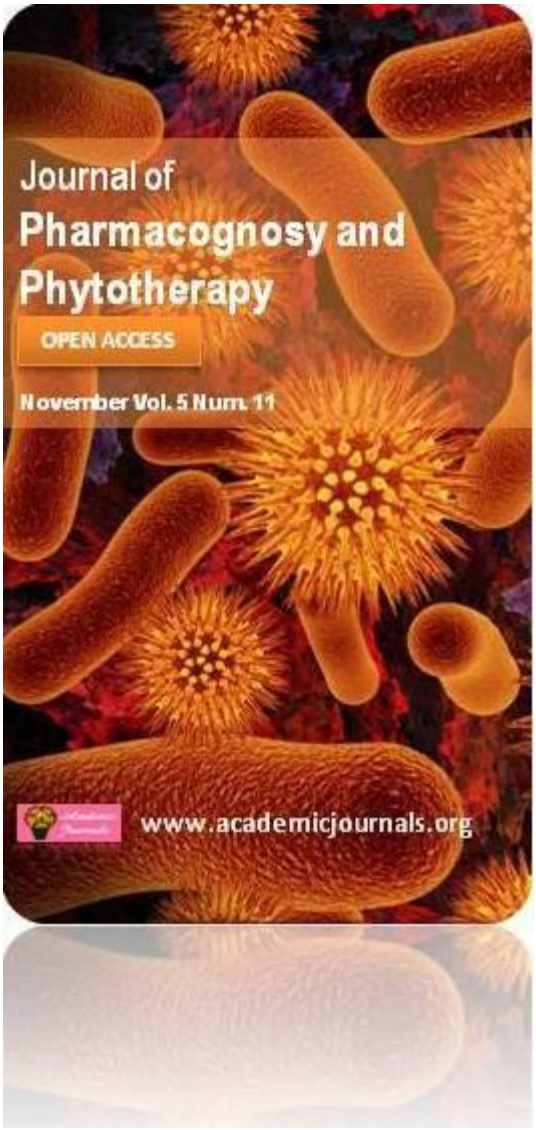

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Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

In vivo and *in vitro* antibacterial activities of *Momordica charantia* on *Salmonella typhi* and its effect on liver function in typhoid-infected rats

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Typhoid fever is a disease prevalent in the tropics. In spite of the availability of various therapies, treatment of patients with the disease has been quite challenging in the face of resistance to drugs used. *Momordica charantia* has been used locally to treat typhoid. This study investigated the antimicrobial potency of methanolic extract of *M. charantia* leaves on *Salmonella typhi* in male albino rats (Sprague dawley) and the effects of treatment on liver function. There were 5 groups of 10 rats each. 1 ml aliquot of the 4th dilution of *S. typhi* was administered orally to rats in four of the groups to be infected with typhoid, while the last group served as the control. Infected groups were thereafter treated with 100 and 200mg/kg of *M. charantia* and 10mg/kg of chloramphenicol, respectively for seven days, while the remaining group was not treated after infection. The effect of treatment on infection level, body weight and liver enzymes were thereafter investigated. Marked reduction in infection level was observed in all treated rats. Rats treated with 200 mg/kg of the plant extract had total clearance by the sixth day, while significantly lower ($p < 0.05$) infection level was recorded in rats treated with the plant extract than those treated with the standard drug. Mean body weight of all treated rat groups increased during treatment. Concentrations of total and direct bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) were higher ($p < 0.05$) in untreated rats than the treated rats. In conclusion, these results suggest that leaf extract of *M. charantia* is a potent antimicrobial drug against *S. typhi* with hepatoameliorative potentials.

Key words: Typhoid, *Momordica charantia*, liver, *Salmonella typhi*.

INTRODUCTION

Typhoid fever remains an important cause of illness globally with the annual incidence at 21 million cases of which 1 to 4% end fatally (Ivanoff, 1995). Most of the disease burden occurs in developing countries due to poor sanitary conditions (Brown et al., 1996). The disease at its early stages is characterized by high fever, colic pain, anorexia, lethargy, malaise, dull continuous headache and diarrhea. At advanced stages there is

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often a protracted fever and mental dullness, other symptoms may include intestinal bleeding, slight deafness and parotitis paratyphoid (Ackers, 200).

Typhoid is a systemic infection caused by salmonella enteric serotype typhi. This is a highly adapted human pathogen and possesses remarkable mechanism for persistence in host. *Salmonella typhi* is transmitted via the faecal-oral route, either directly from person to person or by ingestion of food or water contaminated with faeces (Ivanoff, 1995). The drugs for the treatment of typhoid fever are antibiotics such as ampicillin, chloramphenicol, trimethoprim, sulfamethoxazole and streptomycin, however, resistance to these drugs is now common (Threfall et al., 2001). In view of the increasing resistance to antibiotics and limited scope of vaccines, the need of the hour is to evaluate the efficacy of natural plant products for the treatment of this infectious disease. The usage of plant products as traditional health remedies is the most popular for about 80% of world's population and is reported to have minimal side effects (Grover and Yadav, 2004; Threfall et al., 1999; Threfall et al., 2001; Wain et al., 1997).

Bitter melon, also known as balsam pear is a tropical plant, widely cultivated in Asia, Africa and South America, and has been used extensively in folk medicine as remedy for eczema, hemorrhoids, scabies, skin conditions, infections, infestations of ticks and chiggers and stubborn sores and wounds, diabetes and enteric fevers (Girron et al., 1991). A decoction of leaf of bitter melon was also observed to inhibit the growth of *Bacillus substilis, Salmonella* spp and *Escherichia coli* (Day et al., 1990). Bitter melon is composed of several compounds with confirmed multitherapeutic properties. In many Nigerian communities, traditional healers have recommended the use of leaf of *Momordica charantia* for the treatment of typhoid fever; however, there have been no scientific study on the efficacy of this plant for the treatment of the disease.

This study therefore evaluated the *in vitro* and *in vivo* antibacterial potency of *M. charantia* on *S. typhi* and also investigated the effects of the treatment on liver function in albino rats.

MATERIALS AND METHODS

Animals

Adult male albino rats of Wistar strain (Sprague dawley) weighing between 150 to 180 g were obtained from the animal house of the Department of Biological Sciences, University of Agriculture, Abeokuta for the study. They were kept in rat cages at room temperature (27 ± 2°C) and humidity (55 ± 5%) and a 12 h cycle of light and dark. They were given free access to rat pellet and water *ad labitum*. The experiment was performed in accordance with the National Institute of Health guidelines of care and use of laboratory animals.

Source of specimen and induction

S. typhi was obtained from the Microbiology laboratory of Nigeria Institute for Medical Research (NIMR) Lagos, Nigeria on already prepared *Salmonella shigella* Agar (SSA). The collected samples were immediately taken to the laboratory at Leadcity University, Ibadan, where they were incubated within 2 to 3 h of collection at 37°C for 24 h. Emerged colonies were stripped from the plate into normal saline. 1 ml aliquot of the 4th dilution of the sample was administered orally to the animals to induce typhoid. 1 ml of blood from the inoculated animals were drawn from the vein 24 h after and inoculated on prepared SSA on petri dishes. The plates were incubated at 37°C for 24 h. Emergence of colonies of *S. typhi* confirmed the induction of typhoid in the animals.

Plants, extracts preparation and treatment

Fresh leaves of *M. charantia* were collected from the campus of University of Agriculture, Abeokuta. The plants were authenticated at the Department of Forestry and Wildlife of the University of Agriculture, Abeokuta where the voucher specimen (MC20) was deposited. The leaves were air dried and blended using an electric blender. 200 g of the powdered leaves was soaked in one litre of absolute methanol and allowed to stand in the menstrum for 3 days. The extract was then filtered using Whatman filter paper No 1. The filterate was poured into an evaporating dish and allowed to evaporate at room temperature to constant weight. The yield was about 4.6% w/w. Dilution to desired concentration was done when needed. Diluted extract was administered orally to the rats at 10:00 GMT for 6 days.

Experimental design

The animals were divided into 5 groups of 10 rats each.

1. Group A: This group served as the control. They were not treated throughout the experiment, but were given free access to normal animal pellet and water *ad labitum*.
2. Group M1: This group contained typhoid-induced rats treated with 100 mg/kg of *M. charantia*.
3. Group M2: This group contained typhoid-induced rats treated with 200 mg/kg of *M. charantia*.
4. Group C: This group contained typhoid-induced rats treated with 10 mg/kg of a standard antibiotic drug chloramphenicol.
5. Group U: This group contained typhoid-induced rats. They were not treated after typhoid induction but served as the positive control group.

The body weight of the animals was measured at pre and post treatment and recorded as mean weight per group.

Confirmation after treatment

1 ml of blood from the treated animals was drawn fron the vein of treated animals 24 h after treatment each day and inoculated on already prepared SSA on petri dishes. The inoculated plates were incubated at 37°C for 24 h. The counts of emerged colonies were used to evaluate the efficacy of treatment.

Blood collection and dissection

At the end of the experiment, blood was collected from each rat by

cardiac puncture method. The blood was immediately transferred into appropraitely labelled blood sample bottles containing anticougulant.

In vitro sensitivity test using antibiotic disc

2.8 g of nutrient agar powder was weighed and added to 100 ml of sterile water. The mixture was sterilised (using the autoclave) at 121°C for 15 mins. The sterile nutrient agar was allowed to cool to 45°C and then poured into sterile petri-dishes containing the test organism. The petri-dishes were rotated anti-clockwise to allow even distribution of the agar and then left to solidify. Thereafter, a ring of disks of each (Mast Diagnostics, UK) containing single concentrations of each antimicrobial agent was then placed onto the inoculated surface. After 24 h incubation at 37°C, clear zones produced by antimicrobial inhibition of bacterial growth were measured in mm using a straight line ruler.

Estimation of liver function

Adopting the methods described by Tietz (1994), the levels of total and direct bilirubin, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate animotransferase (AST) were determined in the serum using assay kits from Roche Diagnostics on Roche modular (model P800) Mannhein, Germany.

Histological studies

The rat livers were collected and fixed in 10% formalin. The organs were processed routinely for histopathological evaluations.

Statistical analysis

Data obtained were expressed as mean ± SEM. Significant difference between test and control groups was carried out using analysis of variance (ANOVA) of the statistical package for social sciences (SPSS) computer software, version 16.0 at 95% confidence intervals.

RESULTS

Colony counts of *S. Typhi* during treatment

Colony counts of *S. typhi* after induction ranged from 8.5 to 9.1 CFU (Table 1). Treatment of infected rats with the plant extract and chloramphenicol significantly decreased ($p < 0.05$) colony counts of *S. typhi* compared to the pretreatment values. Groups treated with the plant extract recorded significantly lower ($p < 0.05$) colony counts than rats treated with chloramphenicol, while rats treated with 200 mg/kg of the plant extract (M2) had total clearance by the 6th day of treatment.

Body weight of treated rats

The mean body weight of all typhoid-infected groups reduced significantly ($p < 0.05$) after induction of typhoid (Figure 1). During treatment however, all treated groups recorded steady increase in body weight. Rats treated with the extract had higher weight gain than rats treated with chloramphenicol, while rats treated with 200 mg/kg of the extract also gained more weight than the group treated with 100 mg/kg.

In vitro sensitivity test of *S. typhi*

The efficacy of antibiotic sensitivity disc on *S. typhi* showed that ciprofloxacin had the highest zone of inhibition (16 mm) while cefuroxime, nitrofurantoin, tetracycline and ampicillin had the least (4 mm). However, methanolic extract of *M. charantia* had an inhibition zone of 14 mm (Table 2).

Liver function of treated typhoid rats

The concentrations of ALT and AST were highest in the untreated group (U) and lowest in groups M2 and C, respectively (Table 3). The concentrations of ALP and GGT were highest in groups C and A and lowest in groups U and C. Total and direct bilirubin were lowest in groups M2 and C and highest in group U.

Histology of the Liver

Liver of the control rats showed normal appearance of the hepatocytes with no visible lession (Figure 2A). However, marked vacuolations of the hepatocytes was observed in liver of the untreated typhoid-infected rat (Figure 2D). The degree of degenerations were milder in the typhoid-infected group treated with 100 mg/kg of *M. charantia* while, rats treated with 200 mg/kg of the extract had normal hepatocytes with no visible lession (Figure 2B and C).

DISCUSSION

The marked reduction in colony counts of *S. typhi* in blood of typhoid rats treated with various doses of *M. charantia* confirmed the antimicrobial potency of the plant and therefore suggests its efficacy in the treatment of typhoid fever. Result of this study also suggests that the plant is a stronger antimicrobial agent compared to chloramphenicol as rats treated with 200 mg/kg of the extract had total clearance of *S. typhi* by the 6th day of treatment, while rats treated with 100 mg/kg of the extract recorded lower colony counts than those treated with chloramphenicol at the doses tested. Increase in body

Table 1. Counts of *S. typhi* (10^4 cfu^{-1} ml) in blood of typhoid-induced rats treated with *M. charantia.*

Group code	Confirmation Level	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
A	n.d	n.d	n.d	n.d	n.d	n.d	n.d
M1	8.6 ± 2.3^{a}	7.5 ± 3.8^{ab}	6.1 ± 2.4^{b}	5.4 ± 2.4^{b}	5.1 ± 1.8^{b}	3.2 ± 2.1^{a}	2.4 ± 1.1^{a}
M2	9.1 ± 2.5^{ab}	6.8 ± 1.5^{a}	5.2 ± 2.3^{a}	3.4 ± 1.7^{a}	2.1 ± 2.3^{a}	n.d	n.d
C	8.5 ± 1.3^{a}	8.1 ± 2.3^{b}	7.2 ± 2.8^{c}	6.5 ± 1.9^{bc}	6.1 ± 1.4^{c}	4.2 ± 2.5^{b}	3.7 ± 1.5^{b}
U	8.8 ± 1.5^{a}	8.5 ± 2.1^{bc}	8.7 ± 3.0^{d}	7.9 ± 2.6^{c}	8.2 ± 1.4^{d}	8.1 ± 2.0^{c}	8.5 ± 2.9^{c}

Values are mean ± SE. $n \leq 10$. Values within a column having different superscripts are significantly different at $p < 0.05$.
n.d = not detected. A: Control rats; M1: Typhoid-infected rats treated with 100mg/kg methanol extract of *Momordica charantia*; M2: Typhoid-infected rats treated with 200mg/kg methanol extract of *Momordica charantia*; C: Typhoid-infected rats treated with 10 mg/kg Chloramphenicol; U: Untreated Typhoid-infected rats.

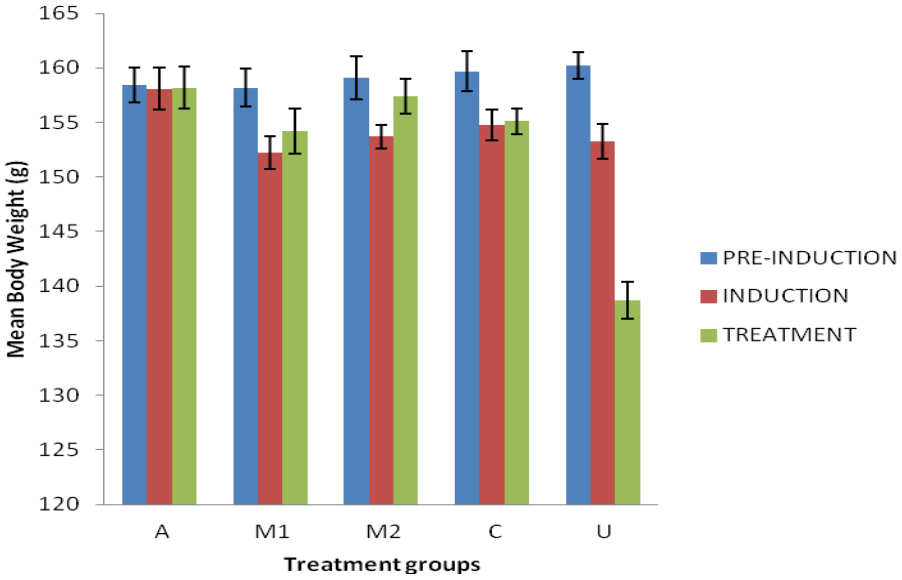

Figure 1. Body weight of treated typhoid-infected rats. A: Control rats; M1: Typhoid-infected rats treated with 100mg/kg methanol extract of *Momordica charantia*; M2: Typhoid-infected rats treated with 200mg/kg methanol extract of *Momordica charantia*; C: Typhoid-infected rats treated with 10mg/kg Chloramphenicol; U: Untreated Typhoid-infected rats.

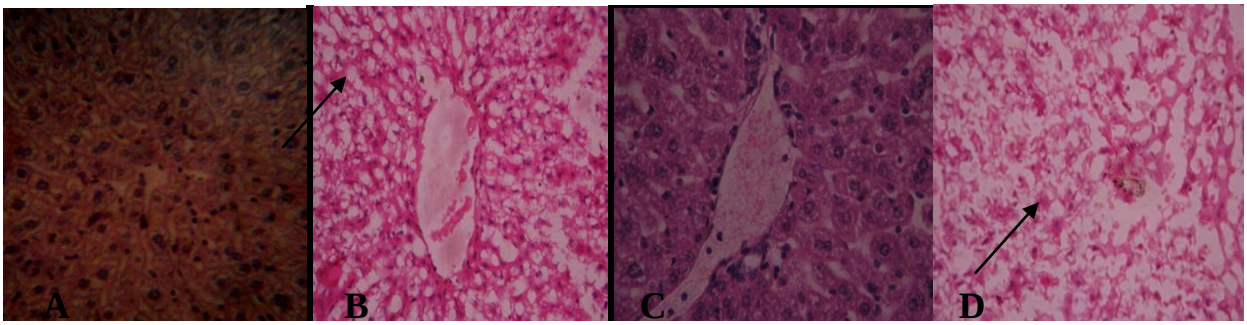

Figure 2. Histology of the liver. A. Liver of control rat show normal arrangement of the hepatocytes with no visible lesion. B. Liver of typhoid-infected rat treated with 100mg/kg of *M. charantia* show mild vacoulations of the hepatocytes (HP). C. Liver of typhoid-infected rat treated with 200mg/kg of *M. charantia* show normal hepatocytes. D. Liver of untreated typhoid-infected rat show markedly vacoulated hepatocytes. All panels were stained with H & E, magnification ×300.

Table 2. Zones of inhibition of *Salmonella typhi* using antibiotic sensitivity disc.

Antibiotic disc	Diameter of zone of inhibition (mm)
Cefuroxime	4
Nitrofurantoin	4
Augmentin	7
Norfloxacin	11
Tetracycline	4
Gentamicin	9
Ciprofloxacin	16
Chloramphenicol	8
Ampicillin	4
Nalidixic Acid	5
Momordica charantia	14

Table 3. Concentration of liver enzymes of treated typhoid rats.

Group code	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Bilirubin (mmol/L)	
					Total	Direct
A	14.88 ± 0.33^{ab}	29.14 ± 0.45^{b}	13.80 ± 0.43^{bc}	12.32 ± 0.23^{d}	12.24 ± 0.49^{c}	15.81 ± 0.23^{d}
M1	14.30 ± 0.32^{a}	29.47 ± 0.55^{b}	11.04 ± 0.13^{a}	10.65 ± 0.32^{b}	11.83 ± 0.16^{b}	12.69 ± 0.43^{c}
M2	14.02 ± 0.45^{a}	27.97 ± 0.52^{a}	11.96 ± 0.43^{b}	11.81 ± 0.25^{c}	10.11 ± 0.17^{a}	11.06 ± 0.22^{b}
C	15.73 ± 0.54^{c}	29.37 ± 0.23^{b}	17.36 ± 0.43^{c}	6.56 ± 0.56^{a}	13.16 ± 0.39^{cd}	7.87 ± 0.48^{a}
U	16.71 ± 0.12^{d}	30.94 ± 0.24^{c}	19.32 ± 0.22^{d}	18.49 ± 0.24^{e}	15.85 ± 0.26^{d}	20.07 ± 0.26^{e}

Values are mean±SE. n ≤ 10. Values within a column having different superscripts are significantly different at $p < 0.05$. A: Control rats. M1: Typhoid-infected rats treated with 100mg/kg methanol extract of *Momordica charantia*; M2: Typhoid-infected rats treated with 200mg/kg methanol extract of *Momordica charantia*; C: Typhoid-infected rats treated with 10mg/kg Chloramphenicol; U: Untreated Typhoid-infected rats; ALT: Alanine aminotranferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase.

weight observed in the rats during treatment also indicates the therapeutic potential of the plant as treated rats were observed to consume more food during treatment.

In vitro investigation of various antibiotics and the plant extract also confirmed a stronger antimicrobial potency of the extract on *S. typhi* than most of the antibiotics tested. Studies by Omoregbe (1996) had earlier reported that aqeous, ethanolic and methanolic extracts of *M. charantia* leaves presented antimicrobial potency against *E. coli, S. paratyphi, Shigella dysenterae, Streptomyces griseus* and *Mycobacterium tubercolosis*. Fresh leaves extract of *M. charantia* had been reported to contain many secondary metabolites such as tannins, flavanoids and alkaloids which have been reported to have many biological activities including antimicrobial (Sankaranarayanan and Jolly, 1993).

The rise in serum levels of AST, ALT, ALP, GGT and total and direct bilirubin in serum of untreated rats can be attributed to the damage to the structural integrity of the liver cells, because these enzymes are cytoplasmic in location and are released into the blood circulation upon cellular damages (Sallie et al., 1991). Histological observation of the liver of untreated rats revealed marked vacuolations of the hepatocytes which confirmed structural damage of the liver cells. Treatment of infected rats with the plant extract however, revealed gradual recovery of the injured liver cells, as all enzymes analysed were almost normal in the treated groups compared to the control and mild degeneration of the hepatocytes were also observed. This observation indicates the restorative effect of *M. charantia* on the liver cells.

The results of this study indicated that the methanol extract of the leaf of *M. charantia* contains potent antimicrobial agents(s) against *S. typhi* with restorative (and/or protective) influence against the biochemical and histological defects caused by the disease on the liver of the rats. Further studies are necessary to isolate and characterize the component of the leaf that is responsible for these observed effects and to elucidate its mechanism of action.

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Hepatoprotective effects of natural *Calculus Bovis* against diethylnitrosamine induced hepatic injury in rats

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Natural *Calculus Bovis* (NCB) is an important Chinese medical material commonly referred to as pigment gallstones of cows, which has been applied in many effective traditionally preparations of Chinese medicine for many years. The purpose of the study was to investigate effects of NCB on diethylnitrosamine induced hepatic injury in rats. Hepatoprotective and antioxidant effect of NCB on liver injury were also analyzed. Male, six-week old Wistar rats were treated with a single dose of diethylnitrosamine (200 mg/kg b.w., i.p.) and left for four weeks. For hepatoprotective and antioxidant studies, the NCB group was treated daily for four weeks. Hepatoprotective markers of aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase were determined in serum of rats. All the experimental animals administered with diethylnitrosamine were obviously elevated serum hepatoprotective markers. The treatment of the NCB prevented the diethylnitrosamine induced hepatic damage and oxidative stress. The experimental rats administered with the NCB protected the liver against diethylnitrosamine induced hepatic injury.

Key words: *Calculus bovis,* diethylnitrosamine, hepatic injury, hepatoprotective, rats.

INTRODUCTION

Calculus Bovis, an animal byproduct in slaughterhouses, is an important Chinese medical material commonly referred to as cow bezoar. *C. Bovis* known an effective traditionally Chinese medicine has been applied in many preparations of Chinese medicine for a long time (Wan et al., 2008). The components of NCB were rich in bilirubin and biliverdin and had higher content of essential amino acids (Wan et al., 2009). NCB has also been used as an important component of many effective Chinese medical preparations (Tian et al., 2005). These *C. bovis* products are shown to have many beneficial effects in treating convulsions, epilepsy and mental illness, according to the practical utilities of traditional Chinese medicine (Yen, 1984). NCB also has sedative, fever reduction and anti-inflammatory effects (Hu et al., 2006).

Diethylnitrosamine is a well known hepatotoxin and hepatocarcinogen (Shu and Hollenberg, 1997). A possible involvement of oxidative stress induced hepatocarcinogenesis of rats is shown as follows. The hepatic metabolism of diethylnitrosamine produces reactive oxygen species resulting in oxidative stress and liver cellular injury (Nishimura et al., 2008). The aspartate aminotransferase values of rats intoxicated with diethylnitrosamine are evaluated in a preventive and curative model. The aspartate aminotransferase, cytoplasmic in nature, enters into the circulatory system because of the altered membrane permeability of liver injury (Wills et al., 2006). The evaluation of traditional

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Table 1. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum aspartate aminotransferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	108.40	±6.47^{a,x}	114.80	±26.78^{a,z}	111.40	±16.99^{a,x}
1	115.20	±13.50^{b,x}	136.25	±12.50^{a,yz}	132.50	±8.66^{a,x}
2	113.40	±20.13^{a,x}	145.25	±28.06^{a,xy}	133.75	±21.22^{a,x}
3	112.80	±15.12^{b,x}	163.00	±19.37^{a,x}	126.50	±12.61^{b,x}
4	117.50	±18.50^{b,x}	166.75	±16.40^{a,x}	128.75	±10.59^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

drugs has revealed a chemopreventive function of hepatoprotection (Sultana et al., 2008). Natural occurrence substances in ruminant products might have important physiological effects, including anticarcinogenic effects (Tanaka, 2005). However, the literature describing the formation of hepatoprotection of the NCB against experimental animals induced by diethylnitrosamine is limited.

These experimental animals were treated with NCB to increase the antioxidant function and to withstand diethylnitrosamine induced oxidative injury in the study. Thus, the objectives of the present study were to investigate protective roles of the NCB treatments on diethylnitrosamine induced serum liver enzymes in a time-dependent manner over the study period.

MATERIALS AND METHODS

Experimental animals

The animal model of the study was according to the method of Pradeep et al. (2007). Male six-week old Wistar rats purchased from Taiwan BioLASCO Co. (Taipei, Taiwan) were used in this study. The experimental rats were housed in polypropylene cages with 12 h light and dark cycle in an environment controlled room (25°C, 85% humidity). The experimental animals were fed standard pellet feed and tap water ad libitum. These animals were balanced for one week prior to the start of the experiment. Procedures involving experimental animals and their care were executed in conformity with the institutional guideline of Council of Agriculture (Taiwan) for the care and use of laboratory animals.

Experimental design and materials

Wistar rats were divided into three groups with five animals in each group. The experimental design was as follows: Group I rats were served as the control group and were treated with saline water orally for four weeks; Group II rats were administered a single dose of diethylnitrosamine (200 mg/kg b.w., i.p.) at the initial start of the experiment and left for four weeks. The diethylnitrosamine was purchased from Sigma-Aldrich (St. Louis, MO, USA); Group III rats were treated with diethylnitrosamine (200 mg/kg b.w., i.p.) at the initial start of the experiment followed by NCB (100 mg/kg b.w., p.o.) from day 1 till day 28. The doses of NCB of the experimental animals were selected by performing an effective dose according to Yu (2004). The NCB samples of the study were purchased from local drugstores.

Biological assays

Animals were subjected to ether anesthesia over the experimental period. Blood samples were removed from the lateral tail vein with a 26-gauge needle at week 0, 1, 2, 3 and 4, respectively. The blood samples were collected and kept for 30 min at 4°C. Then, samples after clotting were centrifuged at 2,500 rpm at 4°C for 15 min. The supernatant serum of the blood samples was immediately isolated to avoid hemolytic and then frozen at –80°C for further analysis.

Serum liver enzymes, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase activities were carried out by the usual routine clinical methods with a Roche automatic biochemical analyzer (Roche, Mannheim, Germany) using commercially available kits.

Statistical analysis

The study was executed in triplicate. Data were analyzed using a general linear model in the SAS system (SAS, 2006). The significant differences among samples were analyzed by using Duncan's multiple range tests. Significance is reported at the $p<0.05$ level.

RESULTS AND DISCUSSION

Aspartate aminotransferase

The aspartate aminotransferase values of the control group (Group I) were not significantly different during the study period (Table 1). The experimental animals were administrated with a single dose of diethylnitrosamine to have higher values in the activities of the aspartate aminotransferase, the increase values of the diethylnitrosamine treatment being near 45% for aspartate aminotransferase when compared with the initial group. The groups (Group II and III) showed an increase of aspartate aminotransferase after the treatment with diethylnitrosamine.

The aspartate aminotransferase values of the initial

Table 2. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum alanine aminotransferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	38.60	±5.18^{a,y}	38.60	±2.88^{a,y}	41.40	±7.70^{a,y}
1	43.60	±6.40^{b,xy}	69.00	±23.82^{a,x}	55.75	±7.59^{ab,x}
2	46.20	±6.38^{b,x}	78.00	±6.73^{a,x}	53.50	±14.25^{b,x}
3	44.00	±6.28^{c,xy}	82.00	±8.49^{a,x}	56.75	±11.56^{b,x}
4	41.50	±7.14^{c,xy}	80.00	±6.22^{a,x}	57.00	±10.71^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

groups did not show significant difference. The diethylnitrosamine group significantly increased the activities of aspartate aminotransferase after two weeks compared to the initial group ($p<0.05$). The treatment of NCB significantly decreased the activities of aspartate aminotransferase at weeks 3 and 4 during the study period compared to the diethylnitrosamine group ($p<0.05$).

Levels of aspartate aminotransferase were increased in the diethylnitrosamine treated rats. The liver function was restored to its normal activity by the hepatoprotective action of NCB. The elevated levels of aspartate aminotransferase are known to illustrate liver damage (Balamurugan et al., 2008). The increased activities of aspartate aminotransferase in the rats administered with diethylnitrosamine were due to extensive liver damage. The treatment with NCB decreased the serum aspartate aminotransferase activities. The results indicated the stabilization of plasma membrane and the repairing of the hepatic tissue damaged by diethylnitrosamine. The observation correlated with the results of Wills et al. (2006) who indicated that aspartate aminotransferase activities were significantly increased in the study because of diethylnitrosamine induced hepatotoxicity in Wistar rats. The aspartate aminotransferase values of Wistar rats are increased by the diethylnitrosamine treatment (Pradeep et al. 2007).

The experimental rats showed lower values of aspartate aminotransferase showing the protective role of NCB. The antioxidative capacity of NCB might have potent antioxidative capacities. Therefore, the natural *C. Bovis* might be counteracting the diethylnitrosamine induced liver injury although diethylnitrosamine is a hepatotoxin and hepatocarcinogen (Shu and Hollenberg, 1997). There have been reports on the effect of natural substances on serum biochemical parameters related to hepatic functions (ALP, AST, ALT and γ-GT) (Olorunnisola et al., 2012). The NCB components, strong antioxidants, are rich in bilirubin and biliverdin which might be correlated to the hepatoprotection in the study (Wan et al., 2009). A possible involvement of oxidative stress induces hepatocarcinogenesis of rats. The hepatic metabolism of diethylnitrosamine produces reactive oxygen species resulting in oxidative stress and liver cellular injury (Nishimura et al., 2008). The aspartate aminotransferase values of rats intoxicate with diethylnitrosamine in a preventive and curative model. The enzyme is cytoplasmic in nature. The aspartate aminotransferase enters into the circulatory system because of the altered membrane permeability of liver injury (Wills et al., 2006).

Alanine aminotransferase

The alanine aminotransferase values of the control group varied in a narrow range during the study period (Table 2). The results of rats administered with diethylnitrosamine showed a significant increase in the activities of the alanine aminotransferase after one week ($p<0.05$). The increased values of the diethylnitrosamine treatment at week 4 were over two-fold for alanine aminotransferase when compared to the initial group. To clarify the direct effect of *C. Bovis* on serum alanine aminotransferase, the experimental rats were fed with natural *C. Bovis*. The rats were given an injection of diethylnitrosamine and fed with natural *C. Bovis.*

The results of the various initial groups of alanine aminotransferase were not significantly different. The alanine aminotransferase values of the diethylnitrosamine treated group were higher than those of other groups until at the end of the study. The values of the natural *C. Bovis* group were significantly lower than those of the diethylnitrosamine treatment after two weeks ($p<0.05$).

The initial activities of alanine aminotransferase were not shown to be significantly different among treatments. The activities of alanine aminotransferase of rats treated with diethylnitrosamine were significantly higher than those of other groups after two weeks. The values of alanine aminotransferase of the diethylnitrosamine group at the end of the study were shown over two-fold when compared to the control group. The alanine aminotransferase enzyme of liver injury is indicated. The

Table 3. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum γ-glutamyl transferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	3.60	±1.14^{a,x}	4.00	±1.22^{a,y}	5.00	±2.55^{a,x}
1	4.00	±0.70^{b,x}	7.84	±1.85^{a,x}	5.25	±2.22^{b,x}
2	3.40	±1.34^{b,x}	7.26	±2.03^{a,x}	5.00	±0.82^{b,x}
3	3.66	±1.30^{b,x}	7.38	±2.02^{a,x}	5.13	±0.70^{b,x}
4	3.98	±0.94^{b,x}	7.95	±2.15^{a,x}	5.18	±0.74^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

alanine aminotransferase catalyzes the conversion of alanine to pyruvate and glutamate. The enzyme, an indicator of liver injury, could be released from injured liver cells. The alanine aminotransferase levels conversely are related to the function of the hepatic cells (Balamurugan et al., 2008). A significant increase of alanine aminotransferase levels is illustrated due to diethylnitrosamine induced hepatotoxicity in experimental rats (Wills et al., 2006; Pradeep et al., 2007). The antioxidant of natural *C. Bovis* might be bile pigments, amino acids and bile salts. The bilirubin content is rich in the natural *C. Bovis*. The bile pigment might play an important role as an antioxidant substance (Lin et al., 2007). The antioxidative activities of bilirubin and biliverdin are studied. The bilirubin and its metabolic precursor biliverdin are strong antioxidant agents (Farhan et al., 2001). The bilirubin of natural *C. Bovis* is an effective antioxidant of peroxynitrite mediated protein oxidation. The bilirubin is a bile pigment having an important role as an antioxidant. Thus, the potent antioxidant might decrease the injury of the liver induced by diethylnitrosamine (Minetti et al., 1998). Antioxidants are essential for intracellaur free radical scavenging. The bilirubin of natural *C. Bovis* manifested as a prooxidant showing its cytopathic effect (Rao et al., 2006).

Gamma glutamyl transferase

The rats treated with diethylnitrosamine with or without NCB showed an increase in γ-glutamyl transferase activities at weeks 1, 2, 3 and 4 as compared to control animals (Table 3). The γ-glutamyl transferase activities of rats administered with diethylnitrosamine after one week were significantly higher than those of the initial group ($p<0.05$). The values of γ-glutamyl transferase of the diethylnitrosamine treatment at week 4 were near two-fold higher than those of the initial group. The activities of γ-glutamyl transferase of the rats treated with NCB were not significantly different during the study period. The activities of γ-glutamyl transferase were significantly increased in the diethylnitrosamine group.

The results of γ-glutamyl transferase of experimental animals did not appear significantly different among the various initial groups. The γ-glutamyl transferase values were significantly increased in diethylnitrosamine treated rats as compared to the other groups at week 1 ($p<0.05$). The experimental rats treated with the NCB group for one week significantly reduced the values of the γ-glutamyl transferase. These results showed natural *C. Bovis* tended to prevent liver damage by suppressing the leakage of enzymes through cellular membranes by preserving the integrity of the plasma membranes.

The hepatoprotective and antioxidant effects of rats treated with diethylnitrosamine are studied. The serum γ-glutamyl transferase values of rats were elevated after the administration of diethylnitrosamine (Pradeep et al., 2007). The antioxidants might have the protective effects in diethylnitrosamine induced oxidative stress. Several compounds, which possessed antioxidant potential, such as ellagic acid, curcumin and garlic powder, are shown to counteract diethylnitrosamine induced oxidative damage (Bansal et al., 2005). Natural *C. Bovis* might have a higher content of bile acids and ursodeoxycholic acid (Lin et al., 2007). The reducing power of carboxyl acids, ursodeoxycholic acid, is shown. The antioxidant mechanism of carboxyl acids is due to its carboxylic groups (Campo et al., 2004). NCB has higher content of bilirubin which might be related to the better reducing capacity because bilirubin might be proton donors. NCB has higher content of bile acids which has a carboxyl group and are good sources of proton donors. The proton donors might stop the reaction chain of free radicals to form stable products (Lin et al., 2007). The function of ursodeoxycholic acid having protective effects causes by oxidative stress and apoptosis (Perez et al., 2006).

Alkaline phosphatase

The results of alkaline phosphatase of the control group varied in a narrow range during the study period (Table 4). The alkaline phosphatase activities of the rats treated with diethylnitrosamine showed a significant increase of

Table 4. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum alkaline phosphatase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	141.00	±24.58^{a,x}	144.60	±28.69^{a,z}	159.80	±6.14^{a,z}
1	142.80	±19.10^{b,x}	175.00	±7.85^{a,y}	166.75	±9.25^{a,yz}
2	140.20	±17.34^{b,x}	180.75	±7.63^{a,xy}	168.25	±7.80^{a,xyz}
3	133.80	±12.32^{c,x}	190.75	±7.54^{a,xy}	173.25	±8.22^{b,xy}
4	137.20	±11.90^{c,x}	199.00	±9.63^{a,x}	177.75	±8.38^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

Table 5. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum lactate dehydrogenase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	140.74	±20.16^{a,y}	144.40	±17.21^{a,z}	148.60	±3.78^{a,z}
1	143.40	±18.60^{b,xy}	174.75	±13.77^{a,y}	167.25	±6.34^{a,y}
2	145.40	±16.53^{c,xy}	199.00	±11.46^{a,x}	166.50	±6.54^{b,y}
3	144.60	±12.12^{c,xy}	208.25	±15.78^{a,wx}	177.25	±4.03^{b,x}
4	148.75	±3.50^{c,x}	216.25	±14.66^{a,w}	187.50	±4.04^{b,w}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

alkaline phosphatase after one wk when compared to the control group ($p<0.05$). The values of the diethylnitrosamine administrated rats were significantly higher than those of the initial group at week 1, 2, 3 and 4. The alkaline phosphatase activities of rats treated with the diethylnitrosamine groups were significantly higher than those of the NCB group at week 3 and 4 ($p<0.05$).

The initial alkaline phosphatase values of various groups were not significantly different. The alkaline phosphatase values of the control group were significantly lower than those of the diethylnitrosamine treatment after one week ($p<0.05$). The alkaline phosphatase activities of the diethylnitrosamine with the NCB group were significantly lower than those of the diethylnitrosamine treatment along after three weeks ($p<0.05$). An increase in the activities of the alkaline phosphatase was observed in the treated rats after administration of diethylnitrosamine in the study period. The formation of reactive oxygen species of the diethylnitrosamine treatment might alter the antioxidant system, while the presence of NCB might counteract diethylnitrosamine induced oxidative stress.

Lactate dehydrogenase

The lactate dehydrogenase values of the control group are shown in Table 5. An administration of a single dose of diethylnitrosamine to rats produced a significant increase in lactate dehydrogenase after one week ($p<0.05$) compared to the initial group. The values of lactate dehydrogenase of the diethylnitrosamine treated groups were increased throughout the study period. The lactate dehydrogenase activities of the diethylnitrosamine and the NCB groups treated rats were increased after one week.

The values of lactate dehydrogenase of the initial groups with various treatments were not shown to be significantly different. The lactate dehydrogenase values of the NCB group were significantly lower than those of the diethylnitrosamine treated group after two weeks ($p<0.05$). The treatment of NCB decreased the lactate dehydrogenase values in the study period compared to the diethylnitrosamine treatment. The lactate dehydrogenase enzyme might be released from the cytoplasm into the blood circulation immediately after

rupture of the plasma membrane and cellular injury. Reactive oxygen species released by the metabolism of diethylnitrosamine might have caused damage to the hepatocellular membranes. The reactive oxygen species might cause the oxidative stress and cellular damage. The cytosolic contents were released into the systemic circulation.

The inhibition of hepatocellular carcinoma by natural antioxidants in experimental animals administrated with diethylnitrosamine is studied. The alkaline phosphatase activities are significantly increased after the treatment of diethylnitrosamine (Shiota et al., 1999). The protective role of an antioxidant treatment (vitamin E) on diethylnitrosamine induced oxidative stress in rats is illustrated. There is evidence of the formation of reactive oxygen species resulting in oxidative stress which might be one of the factors in the etiology of cancer. The alkaline phosphatase activities are significantly increased following the diethylnitrosamine treatment to rats (Bansal et al., 2005).

The increased lactate dehydrogenase activities are caused by the hepatocellular necrosis. The enzyme of the rats is significantly increased after the administration of diethylnitrosamine (Arai et al., 2002). The lactate dehydrogenase values of experimental animals treat with diethylnitrosamine showed a significant increase over the study period (Bansal et al., 2005). The utilities of ursodeoxycholic acid in liver diseases are studied. Ursodeoxycholic acid having cytoprotective, membrane stabilizing and antioxidative effects is currently the only drug for the treatment of chronic liver diseases (Kumar and Tandon, 2001).

CONCLUSION

The effects of natural *C. Bovis* on diethylnitrosamine induced hepatic injury in rats were analyzed in the study. The results of the present study showed natural *C. Bovis* to be effective in reducing the aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase activities. In conclusion, natural *C. Bovis* exhibited good hepatoprotective properties by reversing the oxidant-antioxidant imbalance during rats treated diethylnitrosamine induced oxidative stress in the study.

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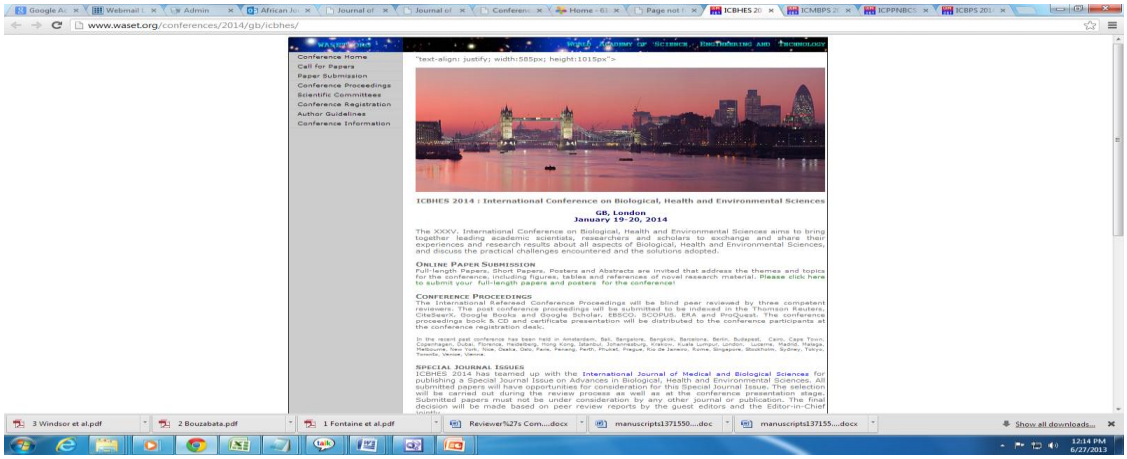

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