Effect of *Cardisoma guanhumi* (land crab) extract on liver function and liver histology of Swiss mice infected with *Bordetella pertussis*

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**Abstract:**

Pertussis also known as whooping cough is an acute human respiratory tract disease caused by *Bordetella pertussis* that is known to be associated with liver pathology. The aim of this study was to investigate the effects of *Bordetella pertussis* infection on the liver function and histology of Swiss mice and to evaluate the mitigating effects of *Cardisoma guanhumi* extract on these changes in comparison to erythromycin treatment. The animals were divided into five groups: group 1 was normal control; group 2 was infected with *Bordetella pertussis* without treatment (negative control); groups 3 and 4 were *Bordetella pertussis* infected and treated with 300mg/kg and 600mg/kg of *Cardisoma guanhumi* extract, respectively; and group 5 was infected and treated with 4000mg/70kg of erythromycin in divided doses. The animals were inoculated with a single infective dose of *Bordetella pertussis* and were consequently treated with the graded doses of the extract and erythromycin for a period of eighteen days, after the animals were confirmed infected. The mice were humanely sacrificed using diethyl ether anesthesia and blood samples taken for evaluation of liver function and liver tissue harvested and processed for histological examination. The results showed that *Cardisoma guanhumi* extract reversed the pathological changes in the liver of mice infected with *B. pertussis* in a dose- and time-dependent manner, suggesting prophylactic and curative potentials of *Cardisoma guanhumi* extract against *B. pertussis*.

**Introduction:**

Bacterial infections are normally associated with hepatic pathology, and although hepatitis pathogenesis is not obvious, liver cell destruction in pertussis-related illness has various mechanisms involving general adverse effects of specific endotoxin and undetermined inflammatory response to gastro-intestinal injury (Abro et al., 2009). For instance, unusual AST and ALT levels suggest hepatocyte disorder and as a result, several researchers have used these enzymes for estimation of hepatic association during typhoid fever. The occurrence of elevated serum enzyme have been reported by Morgenstern and Hayes, 1991 in 52% cases and Mirsadraee *et al*., 2007 in 22% cases, under different conditions while Abro *et al*., (2009) reported elevated levels of alanine transaminase in typhoid patients in 73.3% cases. Furthermore, Momoh *et al*., (2013) noted that infection with *B. pertussis* produced a reduction in Total bilirubin (TB) and an increase in Total protein (TP), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) and Alanine aminotransferase (ALT) while Adeyi *et al*., (2013) reported that infection with *S. typhi* bacteria resulted in an
increase in the levels of ALP, AST, ALT and TP. Several studies in humans have shown that the activities of bacterial infection were similar to observations made in animals as shown by Srikanth and Santhosh (2015) who reported a significant rise in AST, ALT and ALP in a study on the effect of S.typhi infection on hepatic function.

Crabs are decapod crustaceans which belong to the infra order Brachyura. They are mainly covered with thick exoskeleton. Their lower region is wholly concealed under the thoracic cavity. They can be seen in most tropical and subtropical regions of the world as reported by Sammy et al., (2009). Cardisoma guanhumi is a species of land crab that is found in tropical and subtropical estuaries and other maritime areas of land along the Atlantic coast of the Americas (Renata et al., 2012). They are known to be good sources of essential macro and micro minerals such as potassium, phosphorus, calcium, magnesium, copper, iron, manganese, and zinc. Bae (2010) and Sujetha et al., (2015) reported the biomedical and nutritional properties of crabs to include Omega3 (a poly unsaturated acid) contained in crab meat which helps in providing protection against heart diseases. Mahae et al., (2011) noted that the selenium contained in crab meat plays an important role in human antioxidant defense system by preventing cells and tissues from damage and helps in proper functioning of the immune system and metabolism of thyroid hormone while riboflavin present in them helps in the making of steroids and red blood cells, preservation of the skin, support normal growth and iron absorption from the digestive tract and maintain antioxidant activity.

Garry (2015) explained that copper and phosphate content in crab helps in the absorption, storage and metabolism of iron and is concerned in the formation of red blood cells. Ming et al., (2010) and Suneeta (2014) in their separate studies reported that crabs lower blood pressure, protect against heart diseases and possess anti-inflammatory properties. Chitosan derived from crab shell have several properties including anti-microbial and antibacterial properties due to its peculiar characteristics (Mahae et al., 2011). Chitosan fights against numerous pathogenic organisms like fungi, spoilage microorganisms, gram positive and gram negative bacteria (Mahae et al., 2011). However, this is the first attempt at establishing the antibacterial effects of crab extract which was influenced by anecdotal evidence of the curative potential of crabs in whooping cough among the bonny people of Rivers State, Nigeria. This study focused on investigating the effect of Cardisoma guanhumi (land crab) extract on liver function test and liver histology in Swiss mice infected with Bordetella pertussis.

**Material and methods:**

**Sample Collection and Identification:**

Cardisoma guanhumi was caught using a trap in the buguma creek, Rivers State, Nigeria. The samples collected were transported into perforated plastic containers to allow for air during transportation to the Pharmacognosy research laboratory, Department of Pharmacognosy, University of Port Harcourt, Nigeria. The samples were identified by Mr. Otufu Paciya using Food and Agriculture Organization species identification sheets for fresh water and marine crab species.

**Method of Extraction:**

Using the Shahidi and Synowiecki (1991) extraction method, 60 of the freshly collected crabs were sacrificed and the shell separated from the meat and washed with tap water to remove all impurities. The crab shells and meat were then transferred to the oven and dried at 70°C until they were completely dry. Using a laboratory mortar and pestle, the dried crab shells and meat were ground and sieved into the size of 500µm. 40g of the sieved crab was measured using WANT precision electric weighing balance made by want balance instrument company limited, China. into a beaker and 200ml of cod liver oil was added and stirred with magnetic stirrer for 20 minutes, until it was completely mixed. The beaker was then transferred into a water bath at a temperature of 60°C and allowed to stand for 30mins. The mixture was then filtered with a drain off the oil and the residue transferred into a beaker. The residue was treated with 2% potassium hydroxide at a ratio of 1:20 w/v and was stirred.
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Experimental Design:
A total of one hundred and twenty-two (122) animals (swiss mice) were divided into five groups for the curative treatment study. Group 1 (normal) had 10 animals, group 2 (negative control) had 28 animals; groups 3, 4 and 5 consisted of 28 swiss mice each. Group 1 served as the normal control without treatment but was fed with the normal animal feed and water. Group 2 (negative control group) consisted of B. pertussis inoculated mice without treatment. Group 3 consisted of B. pertussis infected mice exposed to low dose (300mg/kg) of Cardisoma guanhumi extract while group 4 consisted of B. pertussis infected mice exposed to high dose (600mg/kg) of Cardisoma guanhumi extract and group 5 consisted of B. pertussis infected mice exposed to 4000mg/70kg of erythromycin. On day 0, at day 6days interval and on day 18, seven animals were sacrificed using diethyl ether anesthesia; samples of blood were collected and the liver removed for assessment of liver function status and histopathological examination, respectively.

Challenging apparently healthy animals with Bordetella pertussis infective dose:
One hundred and twenty-two animals were intraperitonially challenged with the infective dose of Bordetella pertussis which was calculated to be 5 x 10³cfu/ml. After infection had set in (confirmed by physical observation of signs like weakness, non-productive cough, anorexia and the isolation of the organism from the blood of the infected animals on day 0) seven animals were sacrificed and blood samples and liver tissue were collected for preliminary investigation and the rest of the animals from the other treatment groups were given twice daily doses of the various doses of the extract and the standard antibiotic (erythromycin) for 18 days.

Antibiotic and Extract concentration Preparation:
The extract solution for the study was prepared by dissolving 0.5g of the extract in 1ml of di-methyl-sulfoxide (DMSO) solvent to have a stock concentration of 500mg/ml. Since 70kg (70000g) continuously for 2hours at a temperature of 90°C to remove protein from the crab. The sample was filtered and the residues were continuously washed with tap water until the pH became neutral, pH=7. This was done to ensure that all the salt had been removed after removing the protein. The deproteinated crab was transferred into an oven and dried at 60°C until it was completely dry (Shahidi and Synowiecki, 1991). Two point five percent w/v of hydrochloric acid was used at temperature of 20°C for 6 hours to remove the mineral content of the demineralized crab at a ratio of 1:20 w/v. The samples were filtered and washed with tap water until the pH was neutral. The demineralized crab were then transferred to the oven and dried at a temperature of 60°C until completely dried. (Shahidi and Synowiecki, 1991). The demineralized crab was treated with 300ml of acetone for 10mins and dried for 2hrs and the residues were removed to achieve decolourization. The decolourized sample was washed in running water, filtered and dried at 60°C until it was completely dried to obtain crab chitin (Shahidi and Synowiecki, 1991). Deacetylation of chitin was carried out using the method of Yen et al., (2009). The obtained chitin was treated with 40% w/v aqueous sodium hydroxide in the ratio of chitin to the solution 1:15 w/v at 105°C in a water bath for 2hrs. Thereafter, the chitin was filtered with filter pump and washed with deionized water until pH was neutral to obtain the extract. The obtained extract was then dried at 60°C for 2hrs in the oven. The dried extract was preserved in a well labeled bottle and kept in a refrigerator for the experiment.

Isolation of Test Organism:
The test organism Bordetella pertussis (ATCC®9340™) was gotten from the American Type Culture Collection (ATCC), USA. The culture media used for isolation according to ATCC is medium 35: Bordet Gengou/Broth medium from a human clinical specimen at a growth temperature of 37°C in aerobic atmosphere. The product was received freeze-dried at 2°C-8°C and stored at -80°C. The bacterium was reconstituted using Regan-Lowe agar (Charcoal blood Agar) in the...
takes 4000mg of erythromycin daily at severe case of whooping cough, then 25g (average weight of test animal) will take 25gx4000mg/70000g=1.429mg. This means that 25g will take 1.429mg/ml or 2.858mg/0.5ml (1.429x2) or 5.716mg/0.25ml (1.429x4). 5.716mg/0.25ml was prepared from 500mg tablet of erythromycin tablet thus 500mg/Xml=5.716mg/ml. Hence, 500mg tablet of erythromycin was dissolved in 87.47ml distilled water to prepare the erythromycin solution for the study.

**Blood collection:**
Each animal was anaesthetized with diethyl ether in a desiccator and blood was collected by cardiac puncture method and transferred into well labeled sample bottles containing anti-coagulant.

**Liver Function Analysis:**
Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) and Alanine aminotransferase (ALT) determination was carried out using the Randox automated method, Schmidt and Schmidt (1963) and Reitman and Frankel (1957) method, Albumin and Total Bilirubin determination was carried out using the automated Randox technique and the methods of Grant (1987) and Doumas et al., (1971) while the determination of total protein was carried out using the Randox (2015) and Tietz (1995) methods.

**Histopathology studies:**
The study animals (swiss mice) were subjected to diethyl ether anesthesia in a desiccator and dissected aseptically to collect the liver for histopathological studies. The collected tissues were kept in 10% chloroform for preservation and were subsequently trimmed to the size of 3–4mm thickness for fixation. These tissues were fixed, dehydrated, cleared, impregnated, embedded, sectioned and stained with hematoxylin and eosin before mounting according to the method described by Baker (1945).

**Statistical Analysis:**
The results of the measurements are reported as Mean ± Standard Deviation of Mean. The test of significant differences of mean was done by ANOVA and post hoc with least significant difference (LSD) (Mead & Curnow, 1982).

**Results:**
Effect of post inoculation treatment (Post IT) with Cardisoma guanhumi extract on Liver function parameters in Bordetella pertussis infected mice

The results showed that inoculation with an infective dose of B. pertussis caused a significant increase (P<0.05) in Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Alanine aminotransferase (ALT) and Total Protein and a decrease in total bilirubin and albumin level in the mice. However, treatment with Cardisoma guanhumi extract reversed the observed increases, producing a steady decrease in Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Alanine aminotransferase (ALT) and Total protein (TP) levels, with an increase in total bilirubin (TB) and albumin levels with time and increasing doses. The differences in the mean values of all these parameters were significant (ANOVA P<0.05) when compared to normal and negative controls (Tables 1-6). The differences in the effects of the standard drug and extract were however, not statistically significant Tables 1-6).

**Table 1: Effect of Post IT with Cardisoma guanhumi extract on AST (U/L) in B. pertussis infected mice**

|                | DAY 0     | DAY 6     | DAY 12    | DAY 18    |
|----------------|-----------|-----------|-----------|-----------|
| Control        | 89.00±0.000 | 89.00±0.000 | 89.00±0.000 | 89.00±0.000 |
| Negative control | 148.67±2.517 | 156.00±6.245 | 180.00±3.606 | 201.67±3.512 |
| Erythromycin   | 148.67±2.517 | 130.00±2.000 | 107.33±1.155 | 89.00±0.000 |
| Low dose       | 148.67±2.517 | 140.67±1.155<sup>abc</sup> | 126.67±1.155<sup>abc</sup> | 118.00±2.000<sup>abc</sup> |
| High dose      | 148.67±2.517 | 118.67±1.528<sup>abc</sup> | 103.33±1.155<sup>ab</sup> | 92.67±1.528<sup>ab</sup> |
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a= Significant (p<0.05) between test groups and control
b= Significant (p<0.05) between test groups and negative control
c= Significant (p<0.05) between test groups and erythromycin

Control = Animal fed with normal feed and water
Negative control = Animal infected with Bordetella pertussis without treatment
Low dose = 300mg/kg
High dose =600mg/kg
Erythromycin = standard antibiotics drug

Table 2: Effect of Post IT with Cardisoma guanhumi extract on ALP (U/L) in B. pertussis infected mice

|                | DAY 0   | DAY 6   | DAY 12  | DAY 18  |
|----------------|---------|---------|---------|---------|
| Control        | 37.00±0.000 | 37.00±0.000 | 37.00±0.000 | 37.00±0.000 |
| Negative control | 70.67±2.082 | 73.33±1.155 | 78.67±1.528 | 82.67±2.517 |
| Erythromycin   | 70.67±2.082 | 56.00±1.000 | 45.33±1.528 | 37.00±0.000 |
| Low dose       | 70.67±2.082 | 65.33±2.082abc | 56.00±2.000abc | 50.67±1.155abc |
| High dose      | 70.67±2.082 | 61.33±2.309abc | 53.67±1.528abc | 39.00±1.000b |

Table 3: Effect of Post - IT with Cardisoma guanhumi extract on [ALT (U/L)] in B. pertussis infected mice

|                | DAY 0   | DAY 6   | DAY 12  | DAY 18  |
|----------------|---------|---------|---------|---------|
| Control        | 42.00±0.000 | 42.00±0.000 | 42.00±0.000 | 42.00±0.000 |
| Negative control | 81.33±1.528 | 94.00±2.000 | 107.33±2.517 | 124.00±1.000 |
| Erythromycin   | 81.33±1.528 | 68.00±2.000 | 58.67±1.528 | 42.00±0.000 |
| Low dose       | 81.33±1.528 | 76.33±1.528abc | 65.33±1.528abc | 60.00±1.000abc |
| High dose      | 81.33±1.528 | 75.33±1.528abc | 58.00±3.606abc | 45.00±1.000b |

Table 4: Effect of Post-IT with Cardisoma guanhumi extract on [TP (g/dl)] in B. pertussis infected mice

|                | DAY 0   | DAY 6   | DAY 12  | DAY 18  |
|----------------|---------|---------|---------|---------|
| Control        | 46.50±0.000 | 46.50±0.000 | 46.50±0.000 | 46.50±0.000 |
| Negative control | 50.23±0.058 | 55.20±.361 | 59.60±.624 | 64.97±.306 |
| Erythromycin   | 50.23±0.058 | 48.83±.115 | 47.47±.153 | 46.53±.058 |
| Low dose       | 50.23±0.058 | 50.13±.058abc | 50.03±.058abc | 49.40±.458abc |
| High dose      | 50.23±0.058 | 49.30±.361ab | 48.07±.351ab | 47.10±.200b |
Table 5: Effect of Post-IT with *Cardisoma guanhumi* extract on Albumin (g/dl) in *B. pertussis* infected mice

|               | DAY 0       | DAY 6       | DAY 12      | DAY 18      |
|---------------|-------------|-------------|-------------|-------------|
| Control       | 32.60±0.000 | 32.60±0.000 | 32.60±0.000 | 32.60±0.000 |
| Negative control | 21.67±.351  | 18.27±.058  | 14.50±.265  | 11.73±.404  |
| Erythromycin  | 21.67±.351  | 25.00±.436  | 29.17±.839  | 32.33±.379  |
| Low dose      | 21.67±.351  | 21.97±.566<sup>abc</sup> | 22.67±.252<sup>abc</sup> | 23.33±.115<sup>abc</sup> |
| High dose     | 21.67±.351  | 25.57±.252<sup>ab</sup> | 28.00±.265<sup>ab</sup> | 31.53±.153<sup>ab</sup> |

Table 6: Effect of Post - IT with *Cardisoma guanhumi* extract on [TB (µmol/l)] in *B. pertussis* infected mice

|               | DAY 0       | DAY 6       | DAY 12      | DAY 18      |
|---------------|-------------|-------------|-------------|-------------|
| Control       | 1.92±0.000  | 1.92±0.000  | 1.92±0.000  | 1.92±0.000  |
| Negative control | 1.25±.012   | 1.13±.044   | .91±.015    | .44±.020    |
| Erythromycin  | 1.25±.012   | 1.43±.006   | 1.69±.012   | 1.92±.010   |
| Low dose      | 1.25±.012   | 1.31±.006<sup>abc</sup> | 1.37±.006<sup>abc</sup> | 1.40±.010<sup>abc</sup> |
| High dose     | 1.25±.012   | 1.29±.010<sup>abc</sup> | 1.52±.040<sup>abc</sup> | 1.80±.056<sup>abc</sup> |

Effects of *Cardisoma guanhumi* extract on the Liver Histo-architecture in *B. Pertussis* Infected Mice

Histological examination of the liver tissue of the animals in control shows a normal structure of the liver tissue with clear portal tract, central vein, sinusoids. However, liver tissues from mice infected with *B. Pertussis* with no treatment at day 0 (when infection have been established) showed fatty change, vacuolar change, inflammation and hepatocytes. When administered with low (300mg/kg) and high dose (600mg/kg) of *Cardisoma guanhumi* extract for 6 days and 12 days, there were still fatty change, hepatocytes, and inflammation but was reduced at day 12. A close examination of the liver tissue on day 18 showed no histologic change when administered with high dose while those infected with *B. pertussis* and not treated showed increased inflammation and vascular change. Similarly, those treated with erythromycin for 6 days showed mild inflammation and fatty change. However, there was no histologic change on day 12 and 18 as the liver tissue appeared normal. This is shown in plates 1-14

**Histological Plates:**

Plate 1: Photomicrograph of liver tissue of normal mice showing portal tract, central vein and sinusoids with no histologic change. Hepatocytes fatty change inflammation and vacuolar change.
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- **Plate 2:** Photomicrograph of liver of mice infected with *B. pertussis* showing fatty change, inflammation and hepatocytes vacuolations (Day 0)
- **Plate 5:** Photomicrograph of liver of mice infected with *B. pertussis* and treated with 600mg/kg of *Cardisoma guanhumi* extract for 6 days showing fatty change, inflammation and hepatocytes vacuolations
- **Plate 3:** Photomicrograph of liver of mice infected with *B. pertussis* without treatment on day 6 showing fatty change inflammation and hepatocytes vacuolations
- **Plate 6:** Photomicrograph of liver of mice infected with *B. pertussis* and treated with 4000mg/70kg of erythromycin for 6 days showing fatty change, mild inflammation and hepatocytes vacuolations
- **Plate 4:** Photomicrograph of liver of mice infected with *B. pertussis* and treated with 300mg/kg of *Cardisoma guanhumi* extract for 6 days showing fatty change inflammation and hepatocytes vacuolations
- **Plate 7:** Photomicrograph of liver of mice infected with *B. pertussis* without treatment 12 days showing fatty change, elevated inflammation and hepatocytes vacuolations
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Plate 8: Photomicrograph of liver of mice infected with B. pertussis and treated with 4000mg/70kg of erythromycin for 12 days showing no histologic change.

Plate 9: Photomicrograph of B. pertussis infected liver treated with 300mg/kg of Cardisoma guanhumi extract for 12 days showing fatty change, inflammation and hepatocytes vacuolations.

Plate 10: Photomicrograph of liver of mice infected with B. pertussis and treated with 600mg/kg of Cardisoma guanhumi extract for 12 days showing mild inflammation and hepatocytes vacuolations.

Plate 11: Photomicrograph of liver of mice infected with B. pertussis without treatment for 18 days showing fatty change, inflammation and hepatocytes vacuolations.

Plate 12: Photomicrograph of liver of mice infected with B. pertussis and treated with 4000mg/70kg of erythromycin for 18 days showing no histologic change with clear portal tract, sinusoids and central vein.

Plate 13: Photomicrograph of B. pertussis infected liver treated with 300mg/kg of Cardisoma guanhumi extract for 18 days showing no histologic change with clear portal tract, central vein and sinusoids.
Table 14: Photomicrograph B. pertussis infected liver treated with 600mg/kg of Cardisoma guanhumi extract for 18 days showing no histologic change with clear portal trac, central vein and sinusoids

Discussion:

An increase in the levels of circulating liver enzyme is a major reason for suspecting liver disease (Mohd et al. 2013), for an injury to the hepatic cells causes a distortion of metabolic function. The results of this study showed that inoculation with an infective dose of B. pertussis caused a significant increase (P<0.05) in Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Alanine aminotransferase (ALT), Total protein (TP), Total bilirubin (TB) and Albumin levels in the mice but treatment with Cardisoma guanhumi extract and the standard drug (Erythromycin) reversed the observed increases, producing a steady decrease in all the parameters. Bordetella pertussis infection is reported to cause hepatic pathology which results in a significant rise (P<0.05) in Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Alanine aminotransferase (ALT) and Total protein (TP) levels (Abro et al., 2009: Srikanth and Santhosh, 2015).

The results of this study agree with this report. This is possible because the occurrence of hepatic disease causes an increase of the liver cell resulting in a leakage and increase in blood levels of transaminases (Nwachukwu et al., 2015). When hepatic damage occurs, there is an increase in the serum liver enzymes which is caused by the pathologic effects of broad inflammatory reaction in response to the gastrointestinal perforation, cytotoxins and some specific endotoxins that have been synthesized and freed by B. pertussis that have infected the Kuffer cells (Khosla et al., 1988).

The administration of Cardisoma guanhumi extract caused a reversal in the significant rise (P<0.05) in the liver enzymes associated with B. pertussis infection. From the results of this study, it is clear that post inoculation treatment of test animals with Cardisoma guanhumi extract reversed the ability of B. pertussis to destroy and elevate permeability of the hepatocytes, cause the release of some target endotoxins, gastrointestinal perforations and the synthesis of cytotoxins by the liver. These findings suggest hepato-protective and anti-bacterial effects of Cardisoma guanhumi extract on the Kuffer Cells (Lovet and Douye, 2013). These effects were also time and dose dependent. Treatment of infected animals with the different doses of Cardisoma guanhumi extract caused a restoration to usual liver histo-architecture in mice by day 18, with the low dose of the extract also effective in reversing these pathological changes in the liver.

Conclusion:

It is clear from the results that Cardisoma guanhumi extract reversed the adverse pathological changes in the liver induced by B. pertussis infection in a dose and time dependent manner.

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