

Effect of Natural Penicillin Synthesized in Agro-Waste (Sugar Cane Pulp) Media on Wistar Rats

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Abstract

The effect of natural penicillin synthesized in agro-wastes media containing sugar cane pulp was studied. Natural penicillin produced by *P. chrysogenum* (PCL 501) in media substituted with sugar cane pulp was measured after seven days using HPLC analytical method. HPLC analysis of the crude extract, partially purified extract and reference drug revealed the presence of 2 pronounced peaks in the crude extracts, one in the partially purified and one in the reference drug. The retention time of the suspected secondary metabolite in the partially purified extract is similar with that of the reference drug. Penicillin eluted at approximately 2.35 minutes. Toxicity study carried out on mice with the culture extract of *P. Chrysogenum* extract was found to be relatively safe at a dose of 1500 mg/kg body weight of the mouse. *In vivo* sub-acute toxicity studies on biochemical changes in chemical analytes involved in liver function show that there was no significant difference ($p>0.05$) observed in the bilirubin level, liver enzymes activities and albumin fraction of the extract, reference drug and control group seven days after the administration of the extract. Similarly, there was no significant difference ($p>0.05$) observed in the activities of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and malonyldialdehyde (MDA) levels. The result of kidney function showed a significant increase ($p<0.05$) in sodium, urea and creatinine levels in the group administered with reference drug compared to the control group, there was no significant difference ($p>0.05$) in groups where the extract was administered. There were no significant changes

($p > 0.05$) in the PCV level, WBC differentials and total WBC count. However, there was a significant decrease ($p < 0.05$) in Platelets count in rats administered with the extract and reference drug compared to the control. Therefore, natural penicillin synthesized in agro-wastes media containing sugar cane pulp may be safe for therapeutic use.

Keywords: Natural penicillin, sugarcane pulp, toxicity, crude extract, partially purified extract

Introduction

Agro waste contains vast amounts of nutrients (nitrogen, phosphorous and potassium), which on decomposition would be released to influence growth of microorganisms and subsequent production of useful primary and secondary products like enzymes (Vasquez-Alvarez *et al.*, 2004), energy production (Sun and Cheng 2002), biomass production (Tripodo *et al.*, 2004) and production of antithrombotic and anticoagulating metabolites (Onyegeme-Okerenta *et al.*, 2014). Culture extracts of *Penicillium chrysogenum* PL 501 from media supplemented with sugarcane pulp, an agro-wastes, as a carbon source has been shown to yield more penicillin than culture extracts from media containing an equivalent amount of glucose and lactose (Onyegeme-Okerenta *et al.*, 2013).

The potential of a substance to exert a harmful effect on humans or animals, and a description of the effect and the conditions or concentration under which the effect takes place is defined as toxicity (Obidike and Salawu, 2013). The ability of a compound to cause death and do so in half of the animals when the certain dose is administered defines the toxicity of the compound. The purposes of acute toxicity testing are to obtain information on the biologic activity of a chemical and gain insight into its mechanism of action. The information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals. The objective of the sub-acute toxicity tests is to generally evaluate and characterize effects of the compound when they are administered to the experimental animals repeatedly, usually on daily basis over a period and pharmacological effects are particularly evaluated. The route of administration of a test compound is usually limited to the oral route whenever a compound is given on a daily basis for several weeks because repeated administration of the compound does not induce harmful effects in the animals and mice were used because blood samples can be obtained at intervals for chemistry conveniently (Kent and Gallo, 1998; Walum, 1998). When new drugs are subjected to the prolonged toxicity tests, it is not uncommon for the animals to show, either during the test or at autopsy at the end of the test, some altered or even abnormal function of certain organs, blood chemistry and haematology. In these situations it is of considerable value to determine the degree of reversibility and of these effects (Walum 1998). The kidney is the primary excretory organ, followed by the gastrointestinal tract, and the lungs (for

gases). The kidney is highly susceptible to toxicants because the high volume of blood flows through it and it filters large amounts of toxins, which can concentrate in the kidney tubules causing nephrotoxicity (i.e. toxicity to the kidneys). Confirmation of toxicity is enhanced by enzyme assay (Burkitt *et al.*, 2002). In this study, penicillin produced by *P. chrysogenum* (PCL 501) was monitored using HPLC analysis. *In vivo* acute and sub-acute effect of the extract on Wistar albino rats was also carried out.

Methods

Extraction and partial purification of extract

After 7 days of fermentation, the crude extract was obtained by centrifuging the culture filtrates at 4000g for 5 minutes to remove cells. Partial purification and extraction of penicillin were done by adjusting the pH of the cell-free crude extract to 2.5. 20 ml of chloroform and 1 ml of 10% phosphate buffer pH 2.5 was added to 9 ml of the culture filtrate, this was mixed and the layers allowed separating. The chloroform layer was drawn into a second separating funnel. A second 20 ml portion of chloroform was added to re-extract, the extracts were combined and washed with one or more 10 ml portions of 1% phosphate buffer, pH 2.5, the buffer washes were discarded. The penicillin was extracted from the chloroform with 10 ml of 1% phosphate buffer, pH 6.0.

HPLC analysis

HPLC analysis of penicillin from a 7-day culture of *P. chrysogenum* (PCL 501) was carried out with a μ Bondapak C18 column after a careful validation of the method of Laich *et al.*, (2002). The extract was filtered through Whatman No.1 filter paper and left at 4°C for 1 hour. This was further filtered through a Whatman No. 1, 0.45 micron and 0.22-micron filters. This filtrate was divided into 50 ml aliquots and stored in the dark at 4°C until use. Benzyl Penicillin G at a concentration of 0.1 mg/ml was used as the control sample. A C18 gravity column was wetted with 100 ml of HPLC grade methanol and then washed with 50 ml of Milli-Q water. A 50 ml aliquot of the crude extract was added to the column and allowed to adsorb. The flow rate was 1.0 ml/min, and the running conditions were as follows: 1 – 3 min, buffer A (50 mM sodium acetate); 3 – 15 min, gradient buffer B (0 to 60% acetonitrile); 15 – 18 min, gradient buffer B (60 to 80% acetonitrile); 18 – 20 min, buffer B (80% acetonitrile); 20 – 22 min, gradient buffer B (80% to 0% acetonitrile); and 22 – 25 min, buffer A (50 mM sodium acetate). Penicillin eluted at approximately 2.35 minutes.

In vivo study to determine the effect of extract following *E.coli* infection.

A total of 20 Wistar albino rats weighing approximately 200 grams were used for this study. The rats were separated into 4 groups (of five rats each). *E. coli* infection was done by injecting 0.5ml cell suspension of overnight broth culture into the sub cutaneous layer of the right thigh of the rats¹⁹. These animals were observed after 6 – 12hrs.

Group A: Infected with *E. coli*.

B: Infected with *E. coli*

C: Negative control (*E. coli* infected, no treatment was given).

D: Positive control (Normal rats)

Group A: was administered with extracts of *P. chrysogenum* from sugar cane pulps (1500mg/kg body weight in divided doses of 750mg/kg body weight twice daily) for 7 days.

B: was administered with commercial Benzyl penicillin (1500mg/kg body weight in divided doses of 750mg/kg body weight twice daily) for 7 days.

Groups C and D were fed normally and no treatment was given

Blood samples were taken 24hrs before infecting the rats, this served as the control. For *in vivo* biochemical analysis of infected albino rats, blood was collected 24 hrs after bacterial infection with *E.coli*. Extract and reference drug were administered for 7 days. Blood was subsequently collected at 4, 8 and 14 days after commencement of administration of extract and reference drug to respective groups. Liver function tests (AST, ALT, Alkaline phosphatase and bilirubin), kidney function tests, were assessed to evaluate the therapeutic effect of the culture extracts.

Determination of haematological parameters

The determinations of haematological parameters (haemoglobin concentration, packed cell volume, total white blood cell count, platelet count, neutrophils and lymphocytes) were carried out according to the method of Dacie and Lewis (2006).

Determination of Biochemical indices

Total bilirubin concentration, urea and creatinine levels were determined by the method of Tietz (2005). The activity of ALT, AST and ALP were analyzed by kinetic methods kits from Randox (United Kingdom) using a double-beam spectrophotometer. Determination of Na⁺ and K⁺ was by flame photometer method described by Chuang *et al.*, (2005) Chloride was assayed as described by Cheesbrough (2004). The determinations of SOD, Catalase activities, Glutathione and MDA levels were carried out as described by Usuh *et al.*, (2005).

Data Analysis

All assays were done in triplicates and results were expressed as a Mean \pm standard error of the mean (SEM). A significant difference between values was determined by Fisher's projected least significant different t-test with two-tail probabilities of less than 0.05 considered significant. The statistically significant difference in the rate of microbial growth between media types was assessed by a one-way analysis of variance.

Results

HPLC analysis of the crude extract, partially purified extract and reference drug

HPLC analysis of the crude extract (Fig 1), partially purified extract (Fig 2) and Reference drug (Fig 3) revealed the presence of 2 pronounced peaks in the crude extracts. A single peak was observed in the partially purified extract as well as the reference drug. The retention time of the suspected secondary metabolite in the partially purified extract is similar with that of the reference drug. Penicillin eluted at approximately 2.35 minutes.

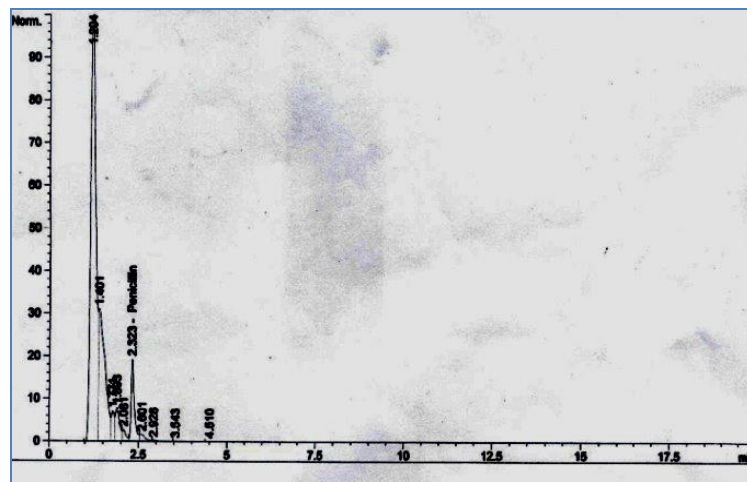


Figure 1: HPLC of crude extract of *P. chrysogenum* cultivated on sugar cane pulp media

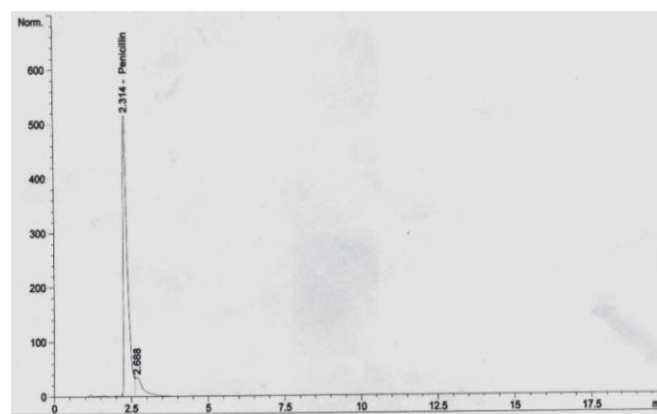


Figure 2: HPLC of a partially purified extract of *P. chrysogenum* cultivated on sugar cane pulp media

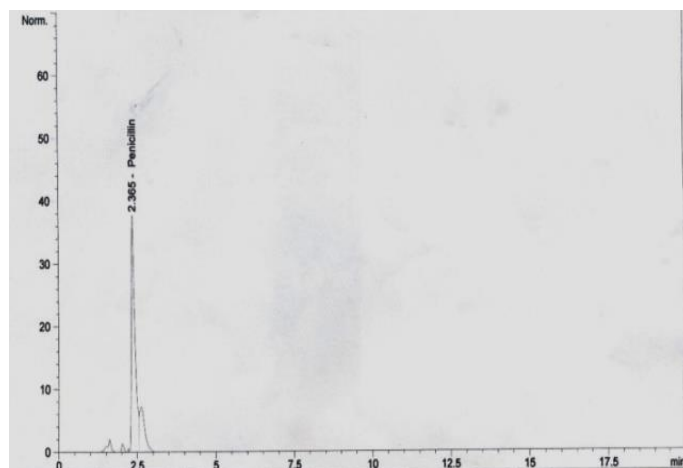


Figure 3: HPLC of Benzyl Penicillin (Reference Drug)

Parameters used to monitor the effect of the extract include the biochemical assay of plasma analytes involved in liver and kidney functions, haematological indices as well as histological studies of the hepatic and renal organs of the animals.

Toxicity Study

Acute toxicity

The result shown in Table 1 represent the toxicity study carried out on mice with the culture extract of *P. chrysogenum*. No significant alterations were observed for the acute toxicity groups. The culture extract was found to be relatively safe at a higher dose of 1500 mg/kg body weight of the mouse since there was no death of the animals recorded in 24 hrs.

Table 1: Acute toxicity results using varying dose range of extract

GROUP	CONTROL	1	2	3	4	5
Dose (mg/kg)	0	500	750	1000	1250	1500
No of Mice	5	5	5	5	5	5
No. of death	0	0	0	0	0	0

Sub-acute toxicity study to evaluate, *in vivo*, the effect of the extract on some biochemical analytes

Biochemical changes in chemical analytes involved in liver function

The result shows graphical representations of data obtained after administration of the extract and reference drug to some experimental animals. It was observed

that the total protein level for the extract is within the normal range, but lower than the reference drug and control (Fig 4). There was no significant difference ($p>0.05$) observed in the albumin fraction of the extract, reference drug and control, while. there were no significant difference ($p>0.05$) in liver enzymes activity (Fig 5) as well as bilirubin level (Fig 6) seven days after the administration of the extract. This finding indicates that the extract as well as the reference drug did not cause any hepatocellular damage.

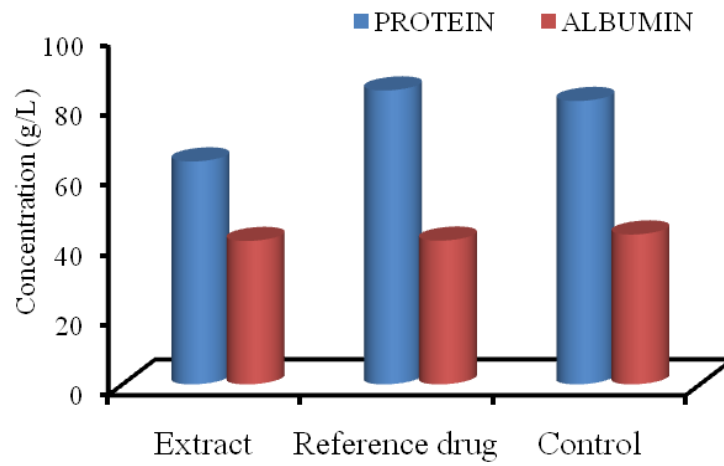


Figure 4: *In vivo* sub-acute toxicity effect of extract and reference drug on Protein and Albumin

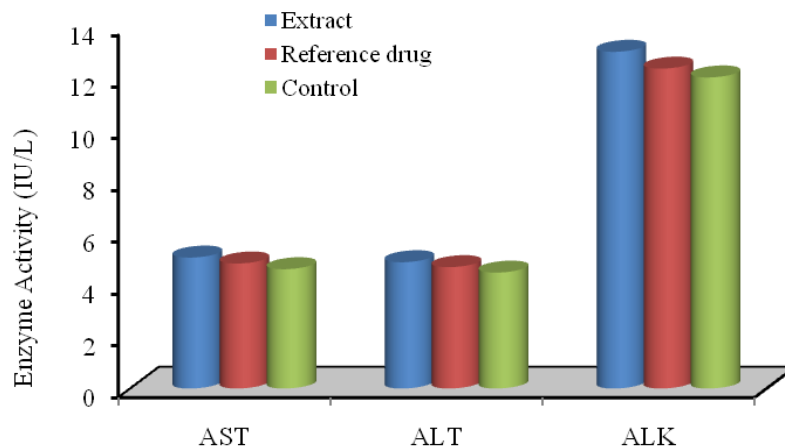


Figure 5: *In vivo* sub-acute toxicity effect of extract and reference drug on liver enzymes

AST – Aspartate amino transferase ALT - Alanine amino transferase ALK - Alkaline Phosphatase

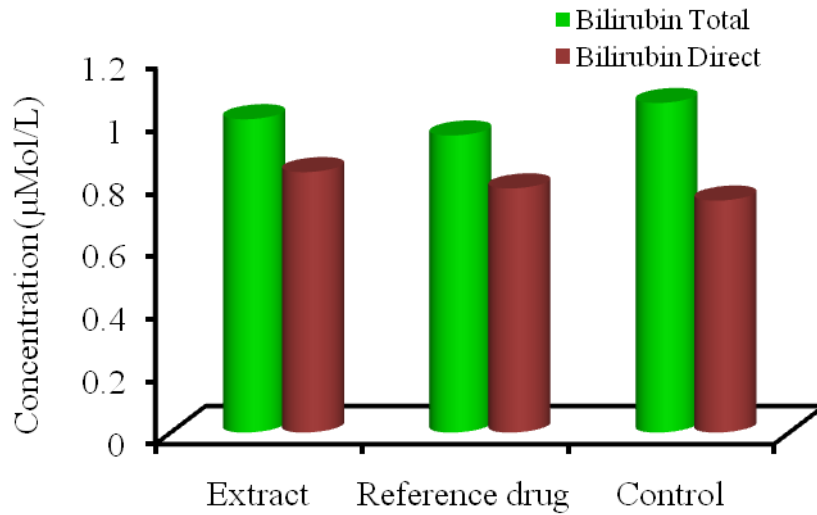


Figure 6: *In vivo* sub-acute toxicity effects of extract and reference drug on Total and Direct Bilirubin

Biochemical changes in analytes involved in kidney function.

In vivo sub-acute toxicity study of chemical analytes involved in kidney function shows a significant increase ($p < 0.05$) in sodium, urea (Fig. 7) and creatinine (Fig. 8) levels in the group administered with reference drug compared to the control group. There were no significant difference ($p > 0.05$) in groups where the extract was administered. This finding indicates that the body handles natural products differently than synthetic drug. There were no changes in chloride concentration for all the groups monitored (Fig. 9).

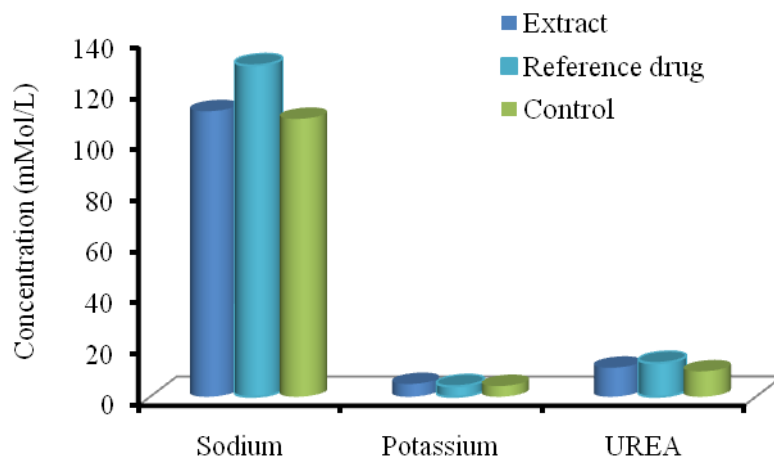


Figure 7: *In vivo* sub-acute toxicity effects of extract and reference drug on renal function

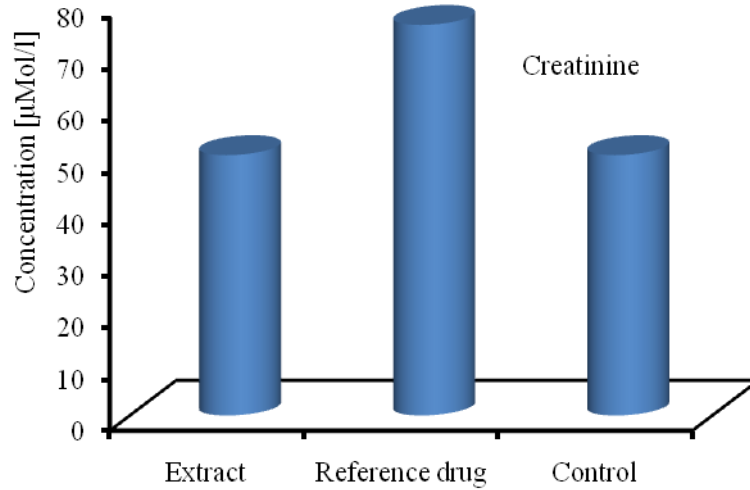


Figure 8: *In vivo* sub-acute toxicity effects of extract and reference drug on creatinine

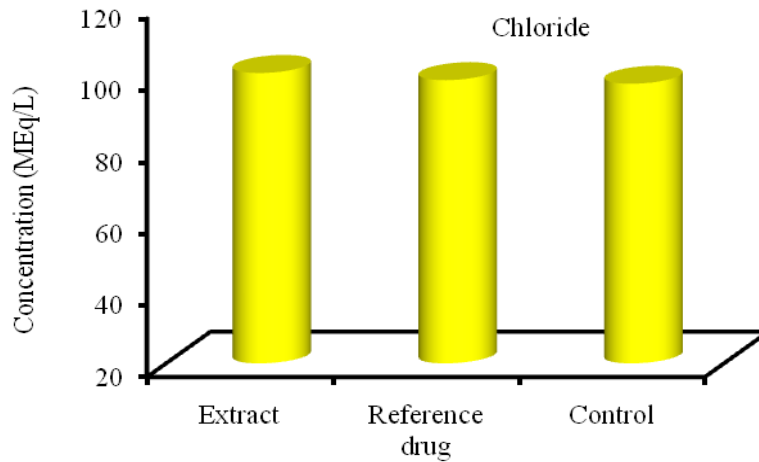


Figure 9: *In vivo* sub-acute toxicity effects of extract and reference drug on chloride

Biochemical changes in chemical analytes involved in oxidative stress enzymes and oxidation products.

Biochemical parameters used in monitoring oxidative stress due to increased generation of reactive oxygen species include the enzymes - catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and oxidation product due to lipid peroxidation - malonyldialdehyde (MDA). From the results in Figures 10, 11&12, there is no significant difference ($p>0.05$) in all the parameters assayed when the extract and reference drug group were compared with the control group.

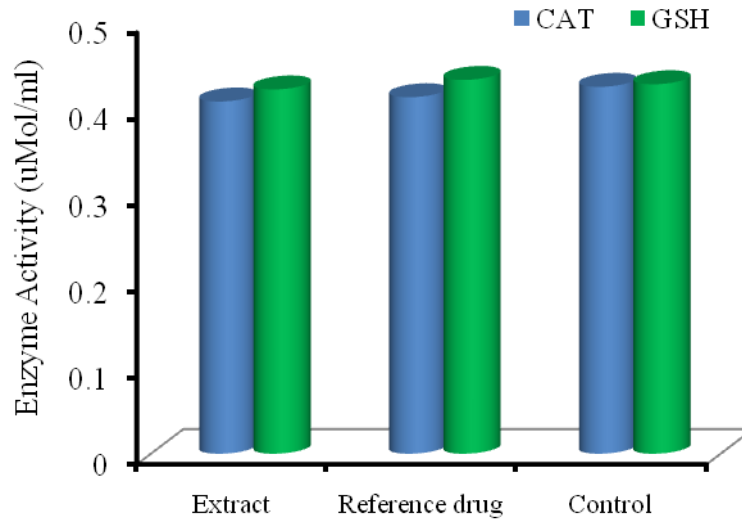


Figure 10: *In vivo* sub-acute toxicity effect of extract and reference drug on CAT, and GSH

GSH – Reduced Glutathione CAT - Catalase

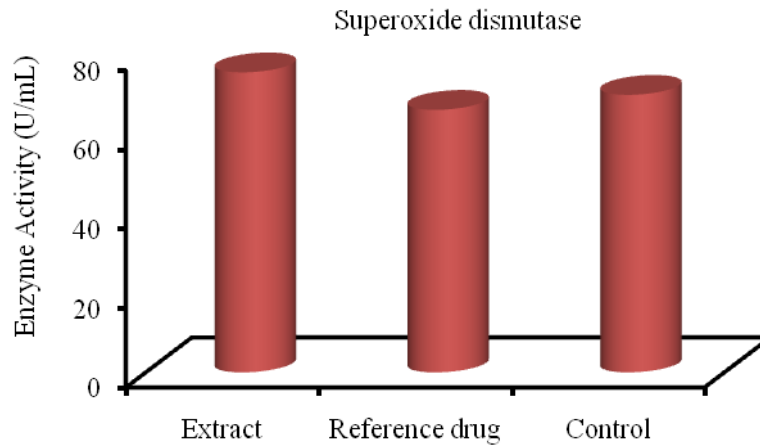


Figure 11: *In vivo* sub-acute toxicity effect of extract and reference drug on superoxide dismutase

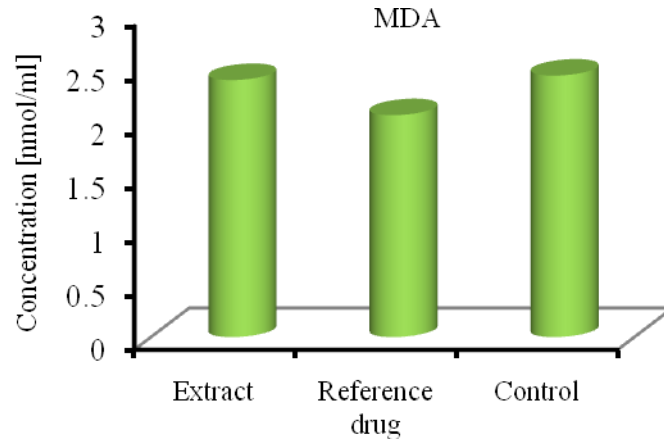


Figure 12: *In vivo* sub-acute toxicity effect of extract and reference drug on malondialdehyde

Changes in haematology indices

Changes in haematological indices involving haemoglobin, packed cell volume, platelet count, total white blood cell count and white cell differentials are represented in Figures 13-15. There were no significant changes ($p > 0.05$) in the PCV level, WBC differentials (Figure 13) and total WBC count (Figure 14). However, in rats administered with the extract and reference drug compared to the control, there was a significant decrease ($p < 0.05$) in Platelets count (Figure 15) indicating thrombocytopenia.

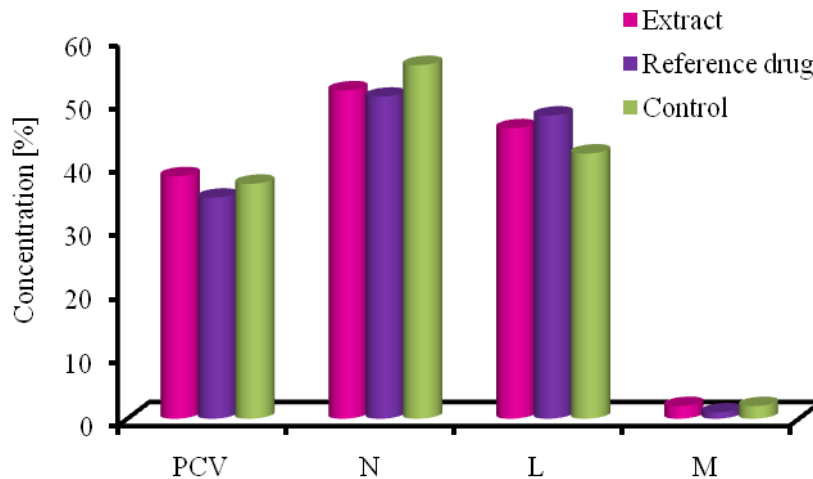


Figure 13: *In vivo* sub-acute toxicity effect of extract and reference drug on haematological indices PCV – Packed Cell Volume N– Neutrophils L – Lymphocytes M - Monocytes

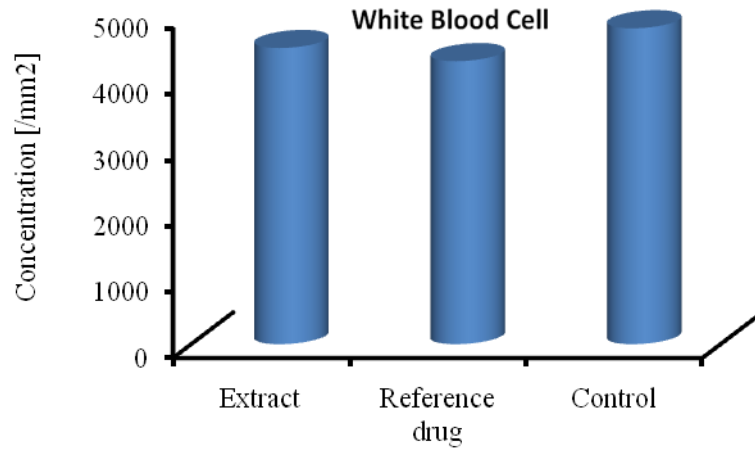


Figure 14: *In vivo* sub-acute toxicity effect of extract and reference drug on WBC

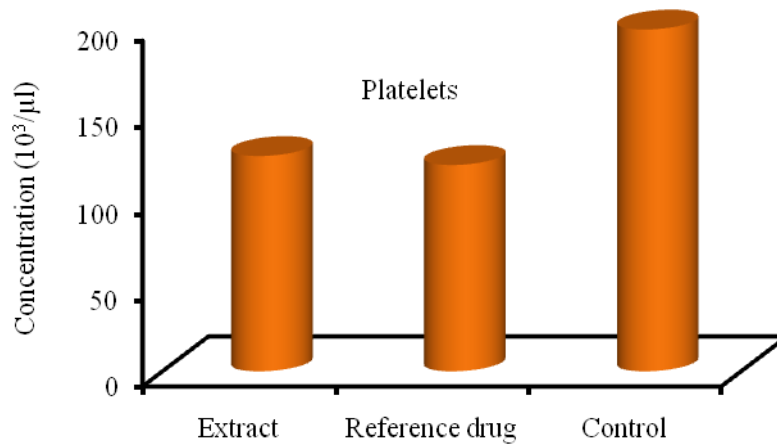


Figure 15: *In vivo* sub-acute toxicity effect of extract and reference drug on Platelets count

Discussion

HPLC analysis of the crude extract, partially purified extract and reference drug revealed the presence of 2 pronounced peaks in the crude extracts, a single peak in the partially purified extract as well as the reference drug. The multiple smaller peaks observed in the crude extract might be due to the presence of hydrolytic enzymes.

Acute toxicity study was carried out by administering the extract on the animals to obtain information useful in choosing doses for repeat-dose studies. It is also aimed at providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity is the effect produced by

a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. The acute toxicity test carried out on mice with the extract provides evidence that the extract is relatively non-toxic at a given dose of 1500 mg/kg body weight; this produced neither side-effect nor the death of the animals. Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. Since there were neither visible side effects nor death in the acute toxicity study, a sub-acute toxicity study was carried out to find out the long-term effect of administration of the extract under normal conditions.

Because the liver is the seat of protein synthesis and drug metabolism, the measurement of the activities of diagnostic enzymes AST, ALT, ALK, in liver tissues plays a significant role in diagnosis and disease investigation and in the assessment of drug or plant extract safety or toxicity. Results obtained from the sub-acute toxicity study showed that there were no alterations in liver enzymes (AST, ALT and ALK) in the group administered with the extract and reference drug when compared to the control group (i.e. group that did not receive the extract or the reference drug). It is well known that an increase in the activity of liver enzymes in the serum is indicative of cellular leakage and loss of functional integrity of cellular membrane of the liver (Iweala and Okeke 2005). Among the different isoenzymes of ALK, the one originating from the liver has been shown to be the main component of the serum (Tietz, 2000). ALT and AST, on the other hand, are localized within the cells of the liver and other organs and are of major importance in monitoring and assessing liver cytolysis (Yakubu *et al.*, 2007). Result obtained therefore, may show an indication of hepatic cellular activity without necrosis. Similarly, the protein and albumin levels were not altered.

The results obtained from bilirubin assay shows that total bilirubin was lower for the extract and reference drug administered groups in the sub-acute toxicity study when compared to the control group. This indicates that the extract had no effect on biochemical processes that should give rise to increase in bilirubin levels in the blood.

Physiological functions of the body are regulated by electrolytes while the excretion of drugs and their metabolite and regulation of electrolyte balance are some of the important functions of the kidney (Tune *et al.*, 1997). The kidney also controls the levels of chloride in the blood and flush out potassium, magnesium as well as sodium. Therefore a shift in physiological levels of these electrolytes may be related to kidney function. In the sub-acute toxicity study, increase in sodium (Na^+), urea, and creatinine levels were observed in the group administered with the reference drug when compared to the control group, while normal Na^+ , urea and creatinine levels were observed in the group administered with the culture extract, with no changes observed in potassium (K^+) and chloride (Cl^-) levels. This finding shows that renal clearance of the natural extract was much faster compared to the reference drug which is a synthetic compound. It also indicates that the body handles natural products (like this extract) differently from synthetic drug. On the other hand increase in Na^+ level in the reference drug group may be as a result of the sodium salts used in the preparation of the drug.

Endothelial cells have a comprehensive array of antioxidant defence mechanisms to reduce free radical formation or limit their damaging effects. These include enzymes such as superoxide dismutase and catalase to degrade superoxide and peroxides respectively, and essential free radical scavengers like ascorbic acid. Superoxide dismutase is a secretory glycoprotein found in blood vessel walls which represent an important vascular enzymatic antioxidant defence system. It dismutates superoxide ions to H₂O₂ and thereby improves endothelial function (Das *et al.*, 2002). In this study, the analysis of free radical enzymes and oxidation product (malonate dialdehyde) (MDA), shows that there were no changes in all the oxidative stress enzymes and oxidation product measured in sub-acute toxicity study. The data obtained provide evidence that the extract does not have the detectable effect that could induce oxidative stress which leads to the generation of free radicals resulting in cell toxicity. This result corroborates the findings of Yogita *et al.*, (2001) where the effect of penicillin treatment on SOD, CAT activity, GSH levels and MDA in islets was studied and found to have no significant change in oxidative stress enzyme levels of the islets.

Haematological indices analysed in the sub-acute toxicity study showed that there were no changes in values of animals treated with the extract and reference drug compared with the control group. However, there was a decrease in platelets counts both in the extract group and in the group treated with reference drug compared with the control. According to Onyegeme-Okerenta *et al.*, (2014) *in vivo* and *in vitro* routes penicillin induce temporary impairment of platelet function. Low platelet values depict thrombocytopenia caused by the extract and reference drug in the host cells as observed in the sub-acute study group.

Conclusion

The present study shows that penicillin extract with antibiotic property was produced in agro-waste media containing sugar cane pulp and that this extract did not produce an irreversible and observable damage to the organs of experimental animals as seen in the acute and sub-acute toxicity studies.

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