

Full Length Research Paper

Toxicity studies in rats fed nature cure bitters

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Graded doses of Nature Cure Bitters (NCB) were administered daily (100, 200 and 400 mg/kg p.o) to rats for 28 days and the effects on body weight, organ weight, clinical signs, gross pathology, haematology, histology and serum biochemical parameters were evaluated. The relative weights of the heart, liver and testes of treated rats were unaffected in contrast to a significant increase in the relative weights of the lungs, kidneys and spleen. The packed cell volume and haemoglobin concentrations were significantly reduced whereas total leucocyte counts and glucose levels were remarkably increased. A significant decrease in alkaline phosphatase occurred in all the groups but alanine aminotransferase and albumin levels were significantly elevated. NCB elicited hypo-cholesterolaemic effects in addition to lowering urea, uric acid, BUN and total protein concentrations. Histological findings did not reveal any treatment-related effects. The calculated therapeutic index was >37.5. These preliminary results suggest that NCB was not likely to produce severe toxicological effects on organ weights, haematological and biochemical indices when given at normal therapeutic doses.

Key words: Nature Cure Bitters, organ weight; pathology, haematology; serum biochemistry.

INTRODUCTION

Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases, this practice being an alternative way to compensate for some perceived deficiencies in orthodox pharmacotherapy (Sofowora, 1989; Zhu, 2002). Unfortunately, there is limited scientific evidence regarding safety and efficacy to back up the continued therapeutic application of these remedies. The rationale for their utilisation has rested largely on long-term clinical experience (Zhu, 2002). But now, with the upsurge in the use of herbal medicines, a thorough scientific investiga-

tion of these plants will go a long way in validating their folkloric usage (Sofowora, 1989).

Nature Cure Bitters (NCB) is one of such polyherbal formulations used for various ethnomedicinal purposes in Nigeria. The constituents of Nature Cure Bitters include *Hillieria latifolia* H. Walt (Phytolaccaceae), *Citrus aurantifolia* Swingle (Rutaceae) and *Xylopiya aethiopica* A Rich (Anonaceae) (Table 1). Ethnomedicinally, 140 mls of the liquid product (80 mg/kg/day) is taken daily in two divided doses for seven days for the treatment of liver cirrhosis, kidney failure, diabetes mellitus, waist pain, arthritis, rheumatism, infertility problems as well as typhoid fever and haemorrhoids. It is also given for the relief of menstrual pain, convulsions, constipation and stomach-ache (Nwaneri, Nwaneri Botanic Centre Ltd., Enugu, Nigeria, Personal Communication).

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Table 1. Approximate percentage composition of nature cure bitters (g/100 ml).

Component	Family	Quantity Used (%)
<i>Hillieria latifolia</i> (root)	Phytolaccaceae	15
<i>Citrus aurantifolia</i> (fruit)	Rutaceae	10
<i>Xylopi aethiopia</i> (root)	Anonaceae	0.8
Distilled water to		100 ml

The medicinal uses of *Hillieria latifolia*, *Citrus aurantifolia* *Xylopi aethiopia* are well documented in the literature (Dalziel, 1937). But to our knowledge, there is yet no record in the literature of the toxicity profile of all these plants used in combination. Such sub-acute or sub-chronic toxicity data may be required to predict the safety or otherwise of long term low dose exposure to a particular medicinal product (McNamara, 1976).

The present investigations were therefore carried out in our laboratory to determine the acute and sub-acute toxicity profile of NCB in rodents.

MATERIALS AND METHODS

Test Material

Nature Cure Bitters was supplied by Dr. E. P. C. Nwaneri, Nwaneri Botanic Centre Ltd., Enugu, Nigeria as a liquid formulation. The material was stored in a refrigerator at 4°C and protected from light until time of drug administration when it was allowed to warm up to room temperature. Upon the attainment of room temperature (27 ± 2°C), the appropriate volumes of the preparation were administered directly to the experimental animals i.p or via a gastric tube.

Animals

Adult Wistar rats (180 – 240 g) and Swiss albino mice (20 – 30 g) of either sex were used for the acute toxicity studies. But only male rats (200 -250 g) were used for the sub-acute toxicity profiling. The animals were supplied by the Animal Facility Centre (AFC), National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. They were fed *ad libitum* with standard feed (Ladokun Feeds, Ibadan, Nigeria) and had free access to water (Abuja Municipal water supply). They were also maintained under standard conditions of humidity, temperature and 12 h light/darkness cycle. The animals were acclimatised for a week before the commencement of the study. A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) Regulations of the WHO (1998). The “principles of laboratory animal care” [NIH Publication #85-23, 1985] were also followed in this study.

Chemicals

Kits for glutamate oxaloacetate transaminase (GOT, AST), glutamate pyruvate transaminase (GPT, ALT), alkaline phosphatase (ALP), total proteins, albumin, total bilirubin, glucose, urea, uric acid, Blood Urea Nitrogen (BUN), total cholesterol and triglycerides used

for the biochemical studies were supplied by Human Gesellschaft für Biochemica and Diagnostica MBH, Germany.

Acute toxicity studies

The acute toxicity (LD₅₀) was estimated i.p. and p.o. in mice and rats (n = 17 in each case) following Lorke’s method (1983). Dose levels used ranged from 500 to 3000 mg/kg. The number of deaths in each group within 24 h (for i.p.) or 72 h (for p.o.) was recorded. The acute toxicity LD₅₀ was calculated as the geometric mean of the dose that resulted in 100% mortality and that which caused no lethality at all. If any inconsistencies were observed in the mortality patterns, then an estimation of the LD₅₀ was carried out using the probit-log analysis.

Sub-acute toxicity studies

Twenty four (24) rats were selected by stratified randomisation and then divided into four groups of six each. The first group served as control while the remaining three groups were given 100, 200 and 400 mg/kg of Nature Cure Bitters orally for 28 days. The first day of dosing was taken as D0 whereas the day of sacrifice was designated as D28.

Weekly body weight

The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing, once weekly during the dosing period and once on the day of sacrifice.

Mortality and clinical signs

During the four-week dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing.

Relative Organ Weight

On day 28 of the dosing period, all the animals were euthanised by exsanguination under chloroform anesthesia. Different organs namely the heart, liver, lungs, spleen, kidneys and testes were carefully dissected out and weighed in grams (absolute organ weight). The relative organ weight of each animal was then calculated as follows:

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

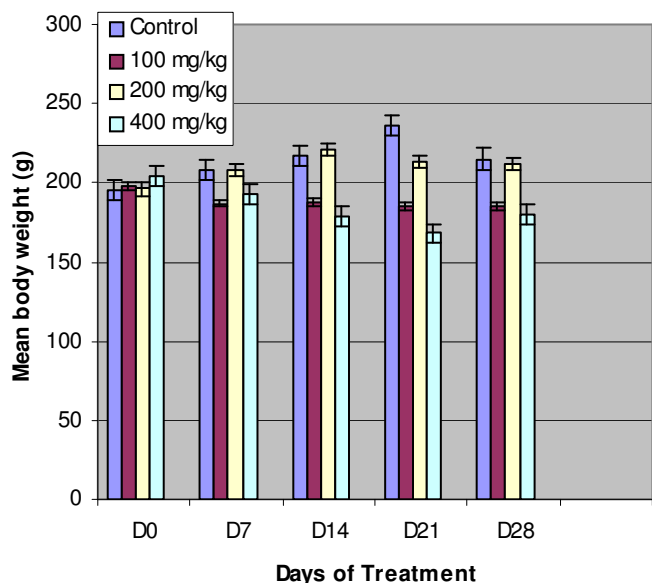


Figure 1. Mean Body Weight Changes in Rats fed Nature Cure Bitters for 28 days. The control rats gained weight throughout the duration of treatment whereas weight loss was observed in rats fed 100 and 400 mg/kg of NCB. The 200 mg/kg group also gained weight in a manner similar to that of the control rats. However, these body weights were not significantly different from control.

Gross pathology and microscopic examination

Dead animals were weighed and necropsied soon after death (or as soon as possible if death occurred overnight) and macroscopic examination of the organs carried out. Tissue biopsies from the heart, liver, kidneys, lungs, spleen and testes were fixed in 10% formal saline. These were processed with an automatic tissue processor (Shandon Citadel Model 2000). Following dehydration and embedding, sections were cut at 4-5 μ with the rotary microtome, stained with hematoxylin and eosin and examined microscopically. Additional thin sections of the kidneys were cut at 3 μ and stained for the periodic acid Schiff (PAS) reaction (Luna, 1968).

Haematology

The packed cell volume (PCV) and haemoglobin (Hb) estimation were carried out using the microhaematocrit and cyanmethaemoglobin methods of Baker and Silverton (1985). The methods of Baker and Silverton (1985) and Jain (1986) were employed to determine the total leucocyte counts (TLC) whereas the longitudinal method of Baker and Silverton (1985) provided a good assay for the differential leucocyte Count (DLC).

Preparation of sera samples

On Day 28 of the dosing period, all the animals were exsanguinated under chloroform anaesthesia and blood samples were drawn from the heart of each sacrificed animal. The samples were collected in plastic test tubes and allowed to stand for 3 h to ensure complete

clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 min and clear serum samples were aspirated off and stored frozen.

Serum biochemistry

The following parameters were determined colorimetrically by employing the standard ready-to-use kits and methods of Human (Human Gesellschaft für Biochemica and Diagnostica MBH, Germany): Glutamate oxaloacetate transaminase (GOT, AST), glutamate pyruvate transaminase (GPT, ALT), alkaline phosphatase (ALP), total proteins, albumin, total bilirubin, glucose, urea, blood urea nitrogen (BUN), uric acid, total cholesterol and triglycerides. The manufacturer's instructions for each biochemical parameter were strictly followed in the course of the investigations.

Statistical analysis

The results are expressed as means \pm standard error of the mean (SEM). Some of the data were analysed as a completely randomised design using one-way analysis of variance (ANOVA) whereas the remaining results were amenable to two-way ANOVA (Petersen, 1985; Scheffe, 1993). Any significant difference between means was assessed by both the Student's t-test and Dunnett's post hoc test (Dunnett, 1993). 95% level of significance ($P < 0.05$) was used for the statistical analysis.

RESULTS

Acute toxicity studies

At the dose levels tested, no untoward clinical signs were observed in the surviving mice and rats. There were no changes in the nature of stool, urine and eye colour of all the animals. No mortality was observed in the different groups of mice and rats that received NCB orally after 72 h. 100% mortality occurred in mice that were given 1600 mg/kg of NCB i.p whereas no lethality was observed in those given 1200 mg/kg (i.p) after 24 h. In rats, i.p administration of NCB produced no deaths at 1600 mg/kg post 24 h. Similarly, 3000 mg/kg of NCB was well tolerated in rats even after 72 h. Hence the LD₅₀ values were estimated to be as follows: Rats: > 1600 mg/kg (i.p) and > 3000 mg/kg (p.o); Mice: 1,386 mg/kg (i.p) and > 2,500 mg/kg (p.o).

Weekly body weight

From D0 to D28, there were variable changes in the body weight of rats in all the groups. The control rats gained weight throughout the duration of treatment whereas weight loss was observed in rats fed 100 and 400 mg/kg of NCB. Surprisingly, the 200mg/kg group also gained weight in a similar fashion as the control rats. However, these changes in the body weights of treated rats were

Table 2. Effects of nature cure bitters on the relative organ weights of rats (g/100g body weight).

Treatment (mg/kg/day)	Heart	Lungs	Liver	Spleen	Kidneys	Testes
Control	0.3±0.06	0.72± 0.02	3.88± 0.24	0.42 ±0.10	0.63 ± 0.08	2.49± 0.02
NCB (100)	0.41± 0.03	0.89±0.07*	3.75± 0.12	0.46 ±0.03	0.73±0.03	2.35 ±0.07
NCB (200)	0.43± 0.07	0.91± 0.09*	3.46± 0.16	0.52± 0.10	0.69 ± 0.03	2.20 ±0.11
NCB (400)	0.45± 0.05	0.83± 0.06	4.3 ± 0.13	0.76±0.07*	0.89 ±0.05*	2.51±0.09

NCB = Nature Cure Bitters, n = 6,

*P < 0.05 significantly different from control

Table 3. Haematological profile in male rats given 100-400mg/kg of NCB for 28 days.

Treatment (mg/kg/day)	PCV (%)	Hb (g/dL)	TLC (X 10 ⁹ /L)	DLC (%)	
				N	L
Control	40.0 ± 1.2	13.7 ± 0.4	3.8 ± 0.5	31.0 ± 2.5	69.0 ± 2.5
NCB (100)	34.5 ± 0.7*	11.8 ± 0.2*	7.0 ± 0.7*	33.5 ± 3.2	66.5 ± 3.2
NCB (200)	39.0 ± 1.6	13.4 ± 0.5	9.70 ± 2.2*	31.3 ± 4.8	68.7 ± 4.8
NCB (400)	33.0 ± 1.1*	11.3 ± 0.3*	12.60 ± 3.4*	38.0 ± 5.1	62.0 ± 5.1

PCV = Packed cell volume, Hb = Haemoglobin concentration

TLC = Total leucocyte count, DLC = Differential leucocyte counts

N = Neutrophils, L = Lymphocytes, n = 6

*P < 0.05 significantly different from control

not significantly different from control (Figure 1).

Clinical signs and mortality patterns

During the four weeks of treatment, dose-dependent mortalities of 16.7, 33.4 and 50% occurred in the 100, 200 and 400 mg/kg groups respectively. Mild sedative effects were clearly perceptible but no other adverse clinical manifestations (e.g. diarrhoea, haematuria, restlessness, unco-ordinated muscle movements etc) were seen in the experimental animals during the dosing period. Most deaths observed were due to respiratory distress.

Relative organ weight

There were no significant changes in the relative weights of the heart, liver and testes of treated rats in relation to control groups. However, 100 and 200 mg/kg of NCB produced a significant increase in the relative weight of the lungs. At 400 mg/kg, a significant increase in the spleen and kidney weights occurred but the elevations at 100 and 200 mg/kg were not statistically remarkable (Table 2).

Gross and histological pathology

No treatment-related gross pathological changes were found in the heart, liver, lungs, spleen, kidneys, and testes of the rats at the dose levels tested. The incidence of histopathological findings was similar in both control and treated rats (data not shown).

Haematology

The packed cell volume (PCV) and haemoglobin concentrations (Hb) were reduced at all the dose levels tested and these reductions were significantly different from control at 100 and 400 mg/kg. Similarly, there was a significant, dose-dependent increase in the total leucocyte count (TLC) in all the groups. No significant effects were observed in the differential leucocyte counts at the experimental doses (Table 3).

Effects on hepatic function indices

The effects of Nature Cure Bitters on liver function indices are presented in Table 4. The alkaline phosphatase (ALP)

Table 4. Effects of nature cure bitters on hepatic function indices in male rats.

Treatment (mg/kg/day)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	T.BIL (μ mol/L)	ALB (g/dL)	Glu (mg/dl)	T. PROT (g/dL)
Control	179 \pm 17	93 \pm 16	601 \pm 56	5.0 \pm 1.0	5.8 \pm 0.6	87 \pm 5	10.2 \pm 2.5
NCB (100)	226 \pm 28	114 \pm 14	359 \pm 66*	5.8 \pm 0.9	8.0 \pm 1.1	119 \pm 11*	8.3 \pm 0.2
NCB (200)	149 \pm 7	121 \pm 11	364 \pm 46*	3.7 \pm 2.9	6.8 \pm 0.6	152 \pm 26*	8.6 \pm 1.8
NCB (400)	220 \pm 19	168 \pm 15*	406 \pm 54*	4.5 \pm 1.3	8. \pm 0.7*	199 \pm 48*	6.2 \pm 0.5

AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase

ALP = Alkaline Phosphatase, T.BIL = Total Bilirubin

ALB = Albumin, Glu = Glucose, T.PROT = Total Proteins ;

* P < 0.05 significantly different from control; n = 6

and total protein levels were decreased at all the dose levels tested although this decrease did not appear to be dose-dependent. While the decrease in total protein levels was not significantly different from control, that of ALP was significant at all the dose levels tested.

There was a dose dependent increase in serum ALT concentration, this elevation being significant at only 400 mg/kg. Similarly, albumin levels were apparently increased at all the dose levels tested and this effect was remarkable at 400 mg/kg. Variable but non-significant responses were obtained in the serum AST and total bilirubin concentrations. Total bilirubin levels increased initially at 100 mg/kg but were subsequently reduced at 200 and 400 mg/kg whereas AST concentrations increased at 100 and 400 mg/kg with a reduction occurring only at 200 mg/kg. NCB caused a significant, dose-dependent increase in serum glucose levels at all the doses tested.

Effects on renal function parameters

Once daily administration of Nature Cure Bitters for four weeks resulted in decreased levels of urea, uric acid and blood urea nitrogen (BUN) at all the dose levels tested. The reduction in uric acid was statistically significant at 100 and 400 mg/kg (Table 3).

Effects on serum lipid profile

Serum triglyceride concentrations were decreased dose-dependently at 100 and 200 g/kg whereas at 400 mg/kg, an elevation occurred. The decrease at 200 mg/kg was remarkably different from control. On the other hand, NCB caused a dose-dependent decrease in total cholesterol levels. This hypocholesterolaemic effect of NCB in rats was significant at 200 and 400 mg/kg (Table 4).

DISCUSSION

Even though the acute toxicity (LD_{50}) test has been widely criticized as a parameter for assessing toxicity (Lorke, 1983; Klaasen, 2001; Timbrel, 2002), there are still certain occasions when some useful information could be obtained from such studies. Apart from giving a clue on the range of doses that could be used in subsequent toxicity testing, it could equally reveal the possible clinical signs elicited by the substance under investigation. It's also a useful parameter for estimating the Therapeutic Index (ie LD_{50} / ED_{50}) of drugs and xenobiotics (Rang et al., 2001).

The present study has shown that NCB possesses fairly high oral LD_{50} values (> 2500 and > 3000 mg/kg in mice and rats, respectively) in relation to its folkloric therapeutic dose (80 mg/kg/day). Thus its high oral Therapeutic Index (> 37.5) might be used as a rough indication of a wide margin between the effective and toxic doses.

But such acute toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses. Hence sub-acute and chronic toxicity studies are almost always invaluable in evaluating the safety profile of phytomedicines. This probably explains why some authors have suggested that sub-chronic toxicity data may be needed to predict the hazard of long-term, low-dose exposure to a particular compound (McNamara, 1976).

The high mortality rate obtained at 400 mg/kg is probably an indication that this dose level is too toxic to the experimental animals. But 400 mg/kg is about five times the therapeutic dose and this high dose is rarely used ethnomedically. This may be an important point in assessing the suitability of NCB for therapeutic use (Gathumbi et al., 2002).

Ordinarily, liver cell damage is characterized by a rise in plasma enzymes (AST, ALT, LDH etc). From our findings, AST concentrations were consistently higher

than ALT levels which are expected since body cells contain more AST than ALT (Mayne, 1996). Usually, about 80% of AST is found in the mitochondria whereas ALT is a purely cytosolic enzyme. Therefore, AST appears in higher concentrations in a number of tissues (liver, kidneys, heart and pancreas) and is released slowly in comparison to ALT. But since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Al-Mamary et al., 2002). Therefore, the significant elevation in ALT level at 400 mg/kg could be an indication of hepatocellular changes induced by NCB. Surprisingly, the liver histology did not reveal any evidence of centrilobular degenerative changes, steatosis or necrosis at this dose level. Indeed microscopic examination of the selected organs did not reveal any treatment-related effects showing that the observed elevations in some biochemical parameters might actually be artefactual in nature (Salazar et al., 1998). This view is also strengthened by the fact that the relative weights of the liver, heart and testes did not show any evidence of toxicity.

The significant reduction in ALP levels by NCB shows that no possible cholestasis occurred at the dose levels tested since a rise in plasma alkaline phosphatase (ALP) level is usually a characteristic finding in cholestatic liver disease (Kaneko, 1989). This was further confirmed by the fact that at 200 and 400 mg/kg, significant hypobilirubinaemic effects were observed, testifying possibly that the hepatic capacity to excrete bilirubin was not impaired by NCB (Mayne, 1996).

The significant increase in serum glucose levels in treated rats shows that NCB could produce some hyperglycaemic effects. Hence caution and close monitoring of glucose levels might be necessary when patients are placed on this herbal remedy. The increased albumin levels (hyperalbuminaemic effect) were probably secondary to hypoproteinaemia indicating that the synthetic function of the liver might not have been tampered with (Mayne, 1996).

On serum lipid profile, the dose-dependent reductions in serum triglyceride and total cholesterol concentrations is a positive attribute of NCB and might just be a revelation of the potentials of this herbal formula as a lipid-lowering drug in mixed hyperlipidaemic states. The observed dose-dependent hypocholesterolaemic effect may be important because of the salient relationship that exists between serum cholesterol levels and the incidence of Ischaemic and coronary heart diseases such as atherosclerosis (Stamler, 1986; Dixit et al., 2000).

The reduced levels of urea, uric acid and BUN probably indicate that NCB did not interfere with the renal capacity to excrete these metabolites. It may also be a reflection of the preserved renal integrity of treated rats

(Kaneko, 1989).

In addition, the diets were well accepted by the treated rats suggesting that NCB did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. It also shows that NCB did not adversely interfere with the nutritional benefits (e.g. weight gain, stability of appetite) expected of animals that are continually supplied with food and water *ad libitum*.

100 and 200 mg/kg of NCB produced a significant increase in the relative weight of the lungs, thus revealing the possible toxic effects of NCB to the lungs. In an analogous manner, the spleen and kidneys were adversely affected judging by the significant increase in their relative weights at 400 mg/kg. Predictably, the PCV and Hb levels were reduced in treated rats indicating that NCB could produce some anaemic effects. How applicable this observation could be to humans remains an issue yet to be investigated. But if such results are extrapolable to man, then in the case of sickle cell anaemia patients, long-term exposure to this drug might precipitate a crisis situation (Sofowora, 1989).

The dose-dependent elevations in total leucocyte counts (TLC) could be an attestation of the fact that NCB may contain biologically active principles that have the ability to boost the immune system through increasing the population of defensive white blood cells. The reduction in lymphocyte count was compensated by an increased neutrophil count suggesting that NCB may possess some anti-lymphocytic activity (Garg et al., 1997).

The present investigations could be regarded as preliminary probes, necessitating further studies to establish the mechanisms of toxicity of NCB. Prospective studies should include amongst other things a battery of reproductive toxicity, genetic toxicity, mutagenicity and carcinogenicity tests in addition to effects on drug metabolising enzymes (especially cytochrome P-450s) and toxicokinetic profiling. When such data are available, a conclusive remark can then be made on the safety profile of NCB.

Nevertheless, the present findings have shown that NCB is not likely to produce severe toxicological effects on organ weights, haematological and biochemical indices when given at normal therapeutic doses.

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