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SUB-CHRONIC ORAL TOXICITY PROFILE OF *CARPOLOBIA LUTEA* LEAF FRACTIONS IN RATS

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Abstract: *Carpolobia lutea* (CL) (Polygalaceae) leaf is widely reported to be effective for the treatment of diarrheal diseases. The subchronic oral toxicological investigation is yet to be executed with both the crude ethyl acetate extract (CEAE) and ethyl acetate fraction (EAF) to verify suitability of plant for the management of diarrhea diseases. The pathological alteration following oral administration of CEAE and EAF of *C. lutea* leaf were explored in rodents over a period of 60 days adopting Good Laboratory Practice (GLP) conditions. Thirty adult male and female wistar rats were randomized to four treatment groups of 6 rats /sex/group and were administered orally with three doses of CEAE, 192.5, 385.0, 770 mg / kg and one dose of EAF 770.0 mg/kg; while 10ml/kg of 20% Tween 80 was used for the control test. Weekly relative body weight and vital signs were assessed. Blood samples were collected weekly for hematological assessment. At autopsy, the major organs were cautiously excised and weighed. The study showed that both CEAE and EAF do not have significant impact ($P > 0.05$) on the hematopoietic system rather it impact more on the biochemical parameters which increased significantly ($P < 0.05 - 0.001$) and dose dependently. These results indicate that oral administration of CEAE and EAF in rats mediates biochemical but not haematopoietic sub-chronic toxicity. Its pharmacological and therapeutic effectiveness is not without toxicity implication and should be used cautiously sub-chronically.

Keywords: *Carpolobia Lutea*, Polygalaceae, Subchronic Toxicity, Haematological, Biochemical Toxicity

INTRODUCTION

Carpolobia *lutea* (CL) G. Don (Polygalaceae) is a small tree which grows up to 15ft high [1]. It is widely dispersed in West and Central areas of Tropical Africa [2]. The stem is used as chewing stick [3]; the root is chewed at bed time because of its passion power. Its shrubby and smallish stem enhances its ornamental use as sweeping material or broom (indiyan) in rural areas of Ibibio tribe of Akwa Ibom State, Nigeria [4]. The toughness of the woody stem promoted its use by cattle herders as cane to control their cattle heads. The decoction of the root is used in locally-made alcohol as aphrodisiac. It is used in the treatment of genitourinary infections, gingivitis and waist pains [5]. The root decoction is also useful in the treatment of internal heat. The hot water extract of the root was reported to have antimicrobial activity [6]. CL has anti-inflammatory, anti-arthritic properties [7], gastro-protective effects [8]; antinociceptive effects [9]; antimicrobial activities [10, 11]; antidiarrhoeal and anti-ulcerogenic properties [12]; antimalarial activity and moderate toxicity [13]. The antimicrobial effects of leaf fraction have been published [14] and the antidiarrheal mechanisms of the leaf extract established [15]. The root is used to facilitate childbirth, headache, expel worm infestation and as aphrodisiac and stimulant [2].

It has analgesic, androgenic properties and it is reputed to cure rheumatism, fever and to combat sterility. The leaf is reported to be effective in the management of fever associated with diarrheal, headache, leprosy, snakebite, venereal disease, wounds [16, 17]. Three new triterpene saponins were isolated from the root [2]. Polyphenols have been isolated from the ethyl acetate fraction (EAF) of *C. lutea* leaf [9]. The acute and sub acute oral administration of both CEAE and EAF reveal that the EAF but not CEAE to have high acute toxicity, significant biochemical but not hematopoietic sub-acute toxicity [18].

This study is designed to investigate the subchronic oral toxicity profile of the crude ethyl acetate extract and ethyl acetate fraction (EAF) reported to have antinociceptive [9] and antidiarrheal [15] effects to elucidate suitability for approval for chronic human use. This is the first report of subchronic oral toxicity profile of the leaf extract in rodent.

MATERIALS AND METHODS

The leaf of *C. lutea was* collected by Mr. Etefia Okon from Ikot Itak Town in Ibeno Local Government area, Akwa Ibom State, Nigeria and authenticated by Dr. (Mrs.) Margaret Bassey of Department of Botany of the same University. Voucher specimen (UUH 998) was deposited at the University Herbarium, University of Uyo, Nigeria.

Preparation of plant extract and fraction: The preparation of crude ethyl acetate extract (CEAE) was performed by macerating air-dried powdered samples (0.57 kg) of *C. lutea* in 2.5 L of ethyl acetate solvent at room temperature for 3 days. After suction filtration through a Buchner funnel, the ethyl acetate filtrates were evaporated by a rotary evaporator (BUCHI, USA) at 40–60°C. The ethyl acetate recovered after rotary evaporation was further used to extract the air-dried plant residue (marc). This procedure was repeated three times. The filtrate was pooled together and evaporated to dryness in the rotary evaporator (BUCHI, USA). The yield was estimated by weighing to constant dryness to yield CEAE.

The ethyl acetate fraction (EAF) was prepared as reported earlier by Nwidu et al. [9]. Both extract and fraction were kept in desiccators until utilized for pharmacological assay. For pharmacological studies EAF and CEAE were suspended in 20% aqueous solution of Tween 80. The doses employed for various pharmacological studies were expressed as milligram of the dried extract or fraction per kilogram body weight. The highest dose of EAF (the most effective dose) used for anti-inflammatory, antiulcer, antidiarrheal, antimicrobial, antinociceptive studies was the same dose used for the subchronic toxicological investigation. The quantification of total phenolic content (TPC) using Folin Ciocalteu's reagent revealed the TPC content of EAF and CEAE as 78.67 and 90.78μg/ml respectively [19]. Three doses of CEAE (192.5; 385.0 and 770.0 mg/kg) were chosen to see if progressive increased in TPC may account for dose-dependent toxicity profile.

Animals: Adult wistar rats (n=30) of either sex, aged 6–10 weeks weighing between 160–230g were purchased from the University of Jos and Laboratory Animal Center, Vom, Plateau State Nigeria. The animals were kept in an animal room where the temperature was maintained at 22 ± 3 C under a 12h light–dark cycle. They were provided with food and water *ad libitum* for one week to acclimatize them before starting the experiment. The protocols were approved by the University of Uyo Institutional animal Care and Use Committee which follows the guidelines of Committee for the purpose of control and supervision of experimental on animals (CPSCEA; UUAEC No. 2008/014).

Animal pre-treatment and blood sampling: Rats were divided into five groups of six animals/equal sex/group. For each of the treatment groups, 192.5, 385, 770 mg/kg crude ethyl acetate extract (CEAE) and 770 mg/kg ethyl acetate fraction (EAF) were orally administered by gavages' using orogastric tube on alternate days. The control group of animals received 10 ml/kg 20% Tween 80 orally only on alternate days. All animals received treatment for 60 days. One set of six animals in each group were weighed on day 0 and then weekly until termination at day 60. For blood parameter studies, blood samples (1ml each) of these six animals in each treatment group, were taken by caudal vein puncture following chloroform anesthesia, on days 0, 7, 14, 21, 28, 35, 42 and 60 into separate Eppendorf tubes containing EDTA (1.5mg) for haematological analyses, respectively. For the biochemical studies, the same animals were euthanised on termination of experiment and their principal organs excised, weighed and prepared for homogenization.

Weekly body weight: The body weight of each rat was assessed during the acclimatization period once before commencement of dosing. Weight of each animal was taken once a week during the dosing period and once at the day of euthanasia. The relative body weight (RBW) of each animal was calculated as follows:

$$RBW = \frac{\text{Absolute weight of one time interval (g)}}{\text{Body weight of rat on commencement of dosing day (g)}} \times 100$$

Haematology : For haematological studies, blood samples (1 ml each) of these six animals in each treatment group, were taken from the caudal vein puncture on days 0, 7, 14, 21, 28, 36, 42 and 49; but on day 60 by cardiac puncture after autopsy. The blood samples were collected into separate Eppendorf tubes containing heparin (0.125 mg) for hematological analyses using standard procedure [20] (Jain, 1986). Red blood cell (RBC), white blood cell (WBC), and platelet counts were done electronically using Coulter Counter. The haemoglobin estimated concentration by cyanomethaemoglobin method. The packed cell volume (PCV) was done using capillary method and clotting time by needle streaking of a drop of blood sample on transparent surface. The differential leucocytes count for neutrophils (N), eosinophils (E), basophils (B), lymphocytes (L) and monocytes were estimated by examination of Giemsa stained blood samples. The mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) were calculated from the data obtained.

Serum biochemistry: Blood samples for biochemical analyses were centrifuged at 5000 × g for 5

min and the plasma collected and stored in Eppendorf tubes at –20 °C and used for the analysis of the following parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine and albumin. The Enzymatic activities of AST and ALT were analysed colorimetrically at 546nm according to standard methods [21]; while ALP was analysed calorimetrically at 405nm using standard procedure [22]. Serum samples were analysed for the determination of the following: total protein, urea and creatinine respectively according to the methods described by Gornall [23]; Hartmann [24] and Cheesbrough, [25]1991. Serum concentration of glucose and total cholesterol were determined using Accu-Chek Active test strips and kits respectively. The effects of extract on triglycerides levels were also examined.

Relative organ weight and macroscopic examination: After taking the blood, organs such as the heart, liver, lungs, spleen, stomach, brain, ovary, testes and kidneys were quickly removed, cleaned with ice-cold saline and weighed. Each organ was examined macroscopically using hand lens for any visible lesions. The relative organ weight (ROW) of each animal was then calculated as follows:

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on day of eutanasia (g)}} \times 100$$

Histopathological evaluation was executed adopting the methodology described by Lamb [26]. Briefly, after euthanising the animals, small organ pieces (3–5mm thick) small pieces of liver, kidney, lungs, spleen, heart, testes, stomach, ovary and brain were fixed in 10% formal-saline for 24 h, and washed in flowing water for 24 h. Samples were dehydrated by passing through 50, 70, 90 and 100% alcohol above a 2-day period, and then cleared in benzene to eliminate alcohol pending when the tissues became more or less transparent. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50 C and then in a cubical block of paraffin made by the 'L' moulds. Besides, this was followed by sectioning and staining with haematoxylin and eosin. The effects of extract treated group and fraction were estimated as mild, moderate to severe in all treated groups.

Determination of LD_{50}: The determination of LD_{50} and subacute toxicity of the extract and fractions have already being established [18].

Statistical methods: Data obtained were analyzed by Student's *t*-test and multiple comparisons were done by ANOVA followed by Turkey Kramer's Multiple Comparison Test. A probability level of less than 5% was considered significant ($p < 0.05$).

RESULTS

The yield of CEAE was 13.72% and that of EAF is as stated in previous article [8, 12]. Polyphenols: Cinnamoyl 1-deoxyglucopyranosides, p-coumaroyl 1-deoxyglucopyranosides and cinnamic acid were isolated from EAF [9] but TPC of EAF and CEAE is 78.67 and 90.78µg/ml respectively as reported in Nwidu [19].

Table.1 shows changes in body weight following treatment of rats with extract and fraction. The body weight in the EAF group significantly increases ($p < 0.05$) progressively from day 7 to day 28 when compared to the control group. The initial and final dose of the CEAE did produce significant (p<0.05-0.01) increase in weight when compared to control. However, the percentage increases in mean body weights of EAF were greater than the extract treated group for same period. The percentages change in mean body weights from commencement to termination of experiment reveals that control (± 4.9%), 192.5, 385.0, 770.0 mg/kg CEAE (±13.4%, ±29.4%, ±6.2%) and 770 mg/kg of EAF (±17.1%). There were however no significant differences in liver, kidneys, lungs, heart, testes, ovaries, brain, stomach and spleen weights, expressed as percent of body weight, between control and the extract and fraction treatment groups at termination of experiment (Table.2).

Table 1: Effects of *C. lutea* extracts and fraction on Weekly and Relative body weight for subchronic oral intake for 60 days

Dose(mg/kg)	Days of weighing								
	0	7	14	21	28	35	42	49	60
Control	212.7±28.5	209.5±28.7	203.7±26.0	214.5±26.4	204.2±20.2	218.6±26.8	189.0±28.6	210.6±26.3	202.3±31.1
192.5 CEAE	192.8±26.0	190.3±26.5^b	193.7±27.0^b	190.0±24.6^b	191.7±18.6^b	180.8±23.3	164.3±18.9	178.0±22.8	167.0±20.6
385.0 CEAE	189.3±25.9	195.8±25.4	198.7±26.6	199.0±27.2	197.0±26.8	228.8±21.6	206.5±15.9	222.0±21.6	244.3±46.9
770.0 CEAE	176.5±23.6	178.8±21.7^b	182.0±22.0^b	186.7±19.5^b	184.3±19.7^b	182.4±18.3	189.0±27.6	182.40±22.9	187.2±21.8
770.0 EAF	210.2±16.8	215.7±18.0^a	218.3±19.1^a	221.3±18.2^a	231.0±18.2^a	218.3±34.3	242.0±12.1	218.8±54.3	246.0±12.1

Significance relative to control: *p<0.05; **p<0.01; values represent mean ± S.E.M (n=6)

Table 2: Effects of EA extracts and fractions of *C. lutea* on relative organ weights after 60 days oral dosing.

Dose /mg/kg	AV. Body wght	Organ weights/ Relative organs weights in bracket								
		Stomach	Heart	Kidneys	Lungs	Spleen	Liver	Brain	Ovaries	Testes
Control	202.3±31.1	1.84±0.23	0.76±0.10	1.46±0.24	1.62±0.27	0.79±0.17	7.60±1.07	1.83±0.14	0.09±0.07	2.64±1.50
192.5 CEAE	167.3±20.7	1.35±0.10	0.65±0.08	1.19±0.12	1.40±0.22	0.57±0.07	5.55±0.53	1.68±0.04	0.09±0.01	3.36±0.74
385.0 CEAE	219.3±21.0	1.69±0.21	0.65±0.08	1.19±0.12	1.40±0.22	0.93±0.09	7.07±0.66	1.76±0.58	0.12±0.01	1.76±0.58
770.0 CEAE	187.20±21.8	1.38±0.13	0.63±0.5	1.20±0.10	1.21±0.11	0.80±0.05	5.78±0.57	1.70±0.04	0.07±0.03	4.48±0.16
770.0 EAF	246.0±121.0	1.91±0.49	0.81±0.36	1.60±0.62	1.74±0.61	0.84±0.30	7.99±3.18	1.57±0.14	0.05±0.00	5.64±0.00

Significance relative to control: $p>0.05$; values represent mean ±S.E.M (n=6)

Results of the hematological studies are presented in Table.3. The data shows that Hb, PCV, RBC, platelets, lymphocytes and granulocytes levels for control rats were not significantly different from those treated with CL extracts and fraction during the period of study (Table.3).

Baseline WBC levels were similar in all treatment groups. However, WBC counts, increased generally, it was not significant in all treatment groups when compared to control from 7th to 49th days of treatment.

Table 3.0: Effects of sub-chronic oral intake of *C. lutea* on Haematological parameter for 60 days

Parameters/ Doses	Control	192.5 CEAE	385.0 CEAE	770.0 CEAE	770.0 EAF
0 Day					
Hb(g/dl)	11.83±0.70	11.78±1.27	12.08±0.77	11.55±1.01	13.52±0.39
Hb(%)	80.67±4.99	80.00±8.73	83.00±5.71	79.00±7.04	92.00±3.57
PCV(%)	54.50±2.66	55.00±1.72	53.33±3.16	51.17±2.64	46.83±1.59
MCV(μm^3)	49.00±9.94	85.07±16.53	76.31±12.43	75.84±21.59	29.67±3.58
MCHC(%)	21.73±0.93	21.65±2.79	23.29±2.8	29.03±1.41	22.96±2.59
MCH(ρg)	10.53 ±1.96	16.63±1.90	17.43±3.17	17.30±4.78	8.53±0.95
Total WBC×10^9(N/L)	6.33±0.81	5.32±1.25	6.27±1.09	9.73±1.86	7.07±1.61
Neutrophils (%)	19.50±1.97	15.00±1.67	21.67±2.15	22.33±3.22	21.00±5.27
Lymphocytes (%)	77.00±1.15	80.00±1.26	77.00±2.45	75.67±9.74	76.00±4.45
Eosinophils (%)	3.00±1.41	1.50±0.68	0.33±0.37	1.33±0.73	2.00±0.99
Monocytes (%)	4.00±0.10	2.00±0.80	0.00	1.00±0.75	1.33±0.73
RBC × 10^6(N/L)	12.00±1.66	7.93±2.24	7.87±1.46	10.97±2.82	16.67±1.80
Platelets(× 10^3/mm^3)	68.00±25.57	85.33±35.18	94.67±34.35	94.67±50.72	161.33±40.4
Clothing time	1.46±0.15	1.30±0.08	1.38±0.15	1.41±0.09	1.35±0.09
Day 7					
Hb (g/dl)	12.42±0.33	11.33±0.56	12.00±0.57	10.58±0.84	12.00±0.32
Hb (%)	84.00±2.88	76.17±3.28	82.17±4.20	71.00±5.48	80.83±2.27
PCV(%)	51.83±1.28	53.50±1.38	54.83±1.31	51.83±2.37	50.17±0.96
MCV(μm^3)	38.93±6.26	38.83±7.28	58.68±19.9	41.11±8.60	45.10±15.20
MCHC(%)	23.97±	21.29±1.34	21.94±1.20	8.57±2.22	10.44±3.09
MCH(ρg)	9.38±1.67	8.01±1.35	12.48±5.16	20.74±2.20	23.98±0.91
Total WBC ×10^9(N/L)	8.92±1.68	10.20±0.87	10.87±1.52	9.95±2.13	9.13±1.09
Neutrophils (%)	12.00±3.20	17.67±7.07	17.33±5.08	14.67±2.87	15.67±2.07
Lymphocytes (%)	87.33±0.35	82.00±7.31	72.50±14.89	84.67±3.23	83.00±2.58
Eosinophils (%)	0.67±7.31	0.00	2.00±1.79	0.00	1.00±0.75
Monocytes (%)	0.00	0.33±0.37	0.67±0.45	0.67±0.45	0.33±0.35
RBC × 10^6(N/L)	14.80±2.25	16.93±4.42	13.87±2.24	6.00±4.05	14.67±0.85
Platelets (× 10^3/mm^3)	53.33±14.25	37.33±7.73	51.20±9.21	44.00±10.31	73.60±25.47
Clothing time	0.87±0.14	1.19±0.05	1.16±0.06	1.17±0.20	1.34±0.45
14 Days					
Hb (g/dl)	11.20±0.52	12.08±0.26	11.42±0.26	10.75±0.88	11.50±0.20
Hb (%)	75.40±3.35	81.33±2.01	76.83±1.71	72.50±5.92	77.67±1.01
PCV(%)	51.33±1.08	54.17±1.56	56.67±1.51	51.17±1.43	53.12±1.68
MCV(μm^3)	18.64±2.51	21.87±2.26	28.94±5.51	21.94±5.56	23.82±2.21
MCHC(%)	21.82±1.12	22.37±0.65	20.20±0.65	21.14±1.96	21.75±0.91
MCH(ρg)	3.96±0.34	4.86±0.45	5.77±0.96	6.61±1.93	5.15±0.46
Total WBC ×10^9(N/L)	15.66±4.03	8.68±1.67	14.38±1.82	12.55±2.76	7.47±1.31
Neutrophils (%)	17.60±5.40	20.67±4.65	18.67±5.17	16.00±188	32.00±3.30
Lymphocytes (%)	82.0±2.92	78.67±4.62	80.00±5.15	80.67±3.82	66.67±3.43
Eosinophils (%)	0.33±0.37	0.33±0.37	0.00	2.00±1.79	1.33±1.46
Monocytes (%)	1.67±1.19	0.33±0.37	3.00±1.57	1.33±0.92	0.00
RBC(× 10^6 N/L)	29.08±3.00	25.83±2.63	21.88±1.40	20.37±3.61	23.20±2.42

Platelets (× 10^3/mm^3)	193.00±45.4	266.67±53.86	156.00±34.59	234.17±42.62	120.00±32.0
Clothing time	0.39±0.03	0.45±0.17	0.35±0.04	0.52±0.12	0.47±0.04
21Days					
Hb (g/dl)	11.50±0.45	10.56±0.96	11.00±0.57	11.50±1.09	11.17±0.77
Hb (%)	78.00±3.27	71.67±6.36	74.17±3.40	78.83±7.70	76.50±5.66
PCV(%)	55.3±2.6	58.2±1.5	60.51.8	53.5±1.1	55.5±1.7
MCV(μm^3)	30.78±4.20	40.82±7.31	44.69±4.38	34.58±4.75	48.09±9.30
MCHC(%)	20.90±1.00	18.32±1.93	18.25±1.11	21.45±1.90	20.22±1.64
MCH(ρg)	7.44±0.54	7.41±1.20	8.02±0.61	7.13±0.54	9.27±1.45
Total WBC ×10^9(N/L)	9.8±0.56	10.22±2.85	12.78±2.73	16.07±5.93	14.40±1.31
Neutrophils (%)	19.67±1.19	15.67±2.62	18.67±3.70	14.67±2.17	15.67±1.91
Lymphocytes (%)	79.33±1.76	78.67±1.08	80.00±3.84	80.33±2.96	82.00±1.50
Eosinophils (%)	0.67±0.73	0.33±0.37	0.00	0.33±0.37	0.00
Monocytes (%)	1.67±1.19	3.33±2.38	1.33±0.73	5.60±1.92	1.00±1.10
RBC × 10^6 (N/L)	15.73±1.30	15.87±2.58	13.93±1.00	16.43±1.74	13.22±2.134
Platelets (× 10^3/mm^3)	213.33±26.93	206.67±20.91	160.00±27.71	240.00±51.85	186.67±39.87
Clothing Time	1.05±0.16	1.19±0.11	1.53±0.19	1.09±0.13	1.19±0.10
28 Days					
Hb (g/dl)	11.50±0.79	12.58±0.22	12.38±0.28	11.50±0.53	11.70±0.65
Hb (%)	77.80±5.18	86.17±2.83	83.00±2.75	77.50±3.66	80.40±5.01
PCV(%)	47.60±1.04	44.67±3.76	49.50±0.58	48.67±2.93	48.60±1.89
MCV(μm^3)	25.91±3.92	19.84±2.01	25.97±2.22	24.61±2.62	29.12±2.41
MCHC(%)	23.05±2.08	29.03±2.45	25.02±0.75	23.86±1.32	24.10±1.16
MCH(ρg)	6.26±1.11	5.24±0.49	6.54±0.75	5.82±0.57	6.94±0.34
Total WBC (×10^9 N/L)	12.96±5.02	9.03±2.46	8.90±0.67	12.97±2.00	12.10±1.53
Neutrophils (%)	37.20±4.16	35.33±4.18	26.50±1.45	29.67±1.19	33.20±5.41
Lymphocytes (%)	62.00±4.18	63.00±3.61	71.00±2.40	68.00±0.80	65.20±5.03
Eosinophils (%)	0.00	0.33±0.37	0.50±0.57	0.00	1.20±0.89
Monocytes (%)	0.80±0.89	1.33±0.73	0.50±0.57	2.33±0.88	0.40±0.45
RBC × 10^6 (N/L)	19.70±2.85	25.08±2.74	19.43±1.93	20.43±1.93	16.96±1.14
Platelets (× 10^3/mm^3)	139.20±51.07	128.00±29.68	108.00±41.05	97.33±25.34	58.40±20.96
Clothing time	0.98±0.20	1.18±0.50	1.25±0.58	0.80±0.15	1.01±0.50
35 Day					
Hb(g/dl)	13.30±0.42	13.00±0.47	13.55±0.74	12.80±0.45	12.60±0.50
Hb(%)	88.80±3.80	87.40±3.67	92.50±4.79	88.80±2.86	85.40±3.38
PCV(%)	49.60±2.08	50.80±3.19	51.00±1.56	49.40±1.79	49.80±1.14
MCV(μm^3)	24.77±3.20	21.69±2.95	20.54±2.70	19.40±1.91	21.46±1.89
MCHC (%)	26.92±1.18	25.78±0.50	26.56±1.13	26.02±1.33	25.36±1.03
MCH(ρg)	6.15±0.69	5.59±0.77	5.39±0.54	4.99±0.45	5.41±0.41
Total WBC (×10^9(N/L)	14.74±5.34	9.42±2.68	13.43±2.66	9.78±1.40	9.10±0.51
Neutrophils (%)	44.40±6.69	30.80±5.94	27.00±2.21	34.00±5.70	36.40±9.68
Lymphocytes (%)	54.00±6.40	65.60±5.45	73.00±2.21	65.20±5.59	62.80±9.48
Eosinophils (%)	0.00	0.40±0.45	0.00	0.00	0.00
Monocytes (%)	1.60±1.30	0.40±0.45	0.00	0.00	0.00
RBC × 10^6 (N/L)	22.40±2.28	25.14±4.57	26.00±4.00	26.40±3.03	23.80±2.13
Platelets (× 10^3/mm^3)	68.80±18.89	36.80±14.24	66.00±44.68	34.40±13.08	29.20±1.32
Clothing time	1.10±0.10	0.97±0.10	1.10±0.00	0.92±0.20	1.10±0.50
42 Days					
Hb (g/dl)	13.50±0.35	13.40±0.41	13.00±0.47	14.04±0.33	12.80±0.38
Hb (%)	92.60±3.33	90.00±3.06	89.25±4.04	96.80±2.04	88.00±3.79
PCV(%)	51.00±1.6	51.60±0.91	53.00±1.41	52.00±0.87	52.00±1.12
MCV(μm^3)	16.94±1.98	16.61±071	28.83±3.69	18.65±1.99	26.61±1.84
MCHC(%)	26.51±0.67	26.01±1.07	24.62±1.55	27.02±0.74	24.67±1.06
MCH(ρg)	4.50±0.59	4.31±0.16	7.14±1.40	5.01±0.45	6.59±0.63
Total WBC ×10^9(N/L)	9.88±1.08	8.32±1.23	9.30±2.27	5.24±1.10	9.70±0.93
Neutrophils (%)	40.40±6.14	28.00±3.81	20.50±1.97	29.20±4.77	28.80±4.34
Lymphocytes (%)	58.40±5.63	70.00±3.08	77.50±1.11	68.40±4.21	70.40±4.09
Eosinophils (%)	0.00	0.40±0.45	0.50±0.58	0.00	0.00
Monocytes (%)	1.20±1.34	0.80±0.56	1.50±1.11	1.20±1.34	0.80±0.56
RBC × 10^6 (N/L)	31.20±2.95	31.12±0.98	19.13±2.65	28.80±2.73	19.84±1.43
Platelets (× 10^3/mm^3)	29.60±5.59	25.60±6.57	16.00±3.27	41.60±22.16	35.20±13.45
Clothing Time	1.11±0.10	0.97±0.11	1.12±0.00	0.92±0.10	1.12±0.49
60Days					
Hb (g/dl)	9.50±0.24	9.50±0.20	9.50±0.33	11.10±0.33	11.25±0.33
Hb (%)	64.50±1.45	64.67±1.22	65.00±2.00	74.60±2.28	76.50±2.12
PCV(%)	47.00±2.35	45.83±1.71	44.50±2.89	48.00±3.22	48.00±1.41
MCV(μm^3)	21.69±4.97	22.39±2.70	34.28±7.83	30.91±2.41	36.74±5.79
MCHC(%)	20.31±1.03	20.81±0.68	21.55±1.52	23.43±1.54	23.43±0.04

MCH(ρg)	4.33±0.83	4.64±0.52	7.13±1.05	7.17±0.46	8.61±1.37
Total WBC ×10^9(N/L)	5.15±1.77	3.15±0.55	3.80±1.40	5.90±1.85	7.85±0.49
Neutrophils (%)	33.00±10.77	24.00±2.12	31.00±3.59	32.40±1.64	61.00±1.41
Lymphocytes (%)	73.50±2.73	74.67±1.67	68.00±3.13	65.60±2.28	34.00±5.66
Eosinophils (%)	0.00	0.00	0.00	0.80±0.89	0.00
Monocytes (%)	1.00±0.67	1.33±0.92	1.00±1.15	1.20±0.80	0.00
RBC × 10^6 (N/L)	24.00±4.99	21.67±2.76	14.00±2.05	11.10±0.33	11.25±0.35
Platelets (× 10/mm^3)	42.00±15.26	38.67±9.17	33.00±18.89	52.00±11.49	40.00±33.94
Clothing time	1.55±0.17	1.17±0.15	1.52±0.01	1.43±0.11	1.11±0.11

Significance relative to control: p.>0.05; values represent mean ±S.E.M (n=6)

Plasma biochemical analysis data at termination of the study are presented in Table 4. Significant changes were observed in the clinical chemistry parameters (creatinine, albumin, globulin, BUN, AST, ALT and ALP) measured in the CL treatment groups compared to control. Baseline levels of these parameters for the groups were significantly different. There were also no significance differences observed between treatment groups and controls in respect of lipid profile examined (Table 5).

Table 4: Effect of Sub-chronic oral intake of CL leaf extract and fraction on renal functions

Parameters	**Na^+ (mmol/L)**	**K^+ (mmol/L)**	**Cl^- (mmol/L)**	**HCO_3^- (mmol/L)**	**BUN (mmol/L)**	**Creatinine (μmol/L)**	**UA (mmol/dL)**	**Glucose (mmol/L)**
Control	130.33±0.71	13.03±0.93	106.0±0.43	27.0±0.0.6	36.0±6.76	79.33±0.36	0.27±0.03	7.00±0.12
192.5 CEAE	122.83±0.85	13.82±0.36	106.62±0.42	29.0±0.70	40.33±0.75	74.12±0.53	0.28±0.01	5.26± 0.5
385.0 CEAE	132.0±0.69	9.14±0.44	103.0± 0.07	25.4±0.15	29.8±0.68	44.20±0.19	0.38±0.13	6.15±0.35
770.0 CEAE	127.25±0.46	15.82±0.45	111.0±0.08	30.75±0.14	27.00±0.50	40.25±0.17	0.22±0.02	5.91± 0.11
770.0 EAF	137.0±0.60	21.5±0.30	110.5±0.65	27.0±0.35	21.0±0.30	24.0±0.12	0.27±0.03	3.47±0.18

Significance relative to control: p>0.05; values represent mean ±S.E.M (n=6)
BUN: Blood urea nitrogen; UA: uric acid

Table 5: Effects of Sub-chronic oral intake of leaf extracts of *C. lutea* on Liver functions

Parameters	**Total proteins (mmol/dL)**	**Albumin (mmol/dL)**	**Globulin (mmol/dL)**	**ALT (mmol/dL)**	**AST (mmol/dL)**	**ALP (mmol/dL)**
Control	58.60±0.91	38.20±0.65	19.4±0.45	18.20±0.50	15.20±0.22	18.40±0.15
192.5 CEAE	54.0±0.47	37.0±0.47	15.75±0.55	14.75±0.55	15.5±0.33	14.5±0.33
385.0 CEAE	59.12±0.66	39.0±0.23	20.0±0.27	39.67±0.24	24.50±0.10	19.67±0.73
770.0 CEAE	65.4±0.35	37.20±0.96	28.6±0.83	22.0±0.23	19.40±0.17	23.40±0.22
770.0 EAF	60.50±0.33	40.75±0.55	30.50±0.33	25.0±0.17	21.0±0.40b	28.5±0.18

Significance relative to control: p.>0.05; values represent mean ±S.E.M (n=6 ALP, Alanine phosphates; AST, Aspartate aminotransferase;ALT, Alanine transferase

Table 6: Effects of Sub-chronic oral intake of CL leaf extract and fraction on lipid profile

Parameters	**TC (mmol/dL)**	**TG (mmol/dL)**	**HDL (mmol/dL)**	**LDL (mmol/dL)**
Control	1.87±0.11	1.2±0.14	1.00±0.25	0.35±0.22
192.5 CEAE	2.22±0.19	1.30±0.08	1.03±0.14	0.53±0.17
385.0 CEAE	2.04±0.40	1.22±0.17	1.14±0.26	0.70±0.23
770.0 CEAE	1.80±0.14	1.28±0.05	0.83±0.25	0.76±0.21
770.0 EAF	2.20±0.20	1.13±0.08	0.85±0.55	0.70±0.05

Significance relative to control: Not significant, values represent mean ±S.E.M (n=6)
Tc, Total cholesterol; TG, triglycerides; HDL, High density lipoproteins; LDL, Low density lipoproteins; UA, uric acid.

Ponderal development of the treated groups was increased when compared to control. All organs in the

treated group exhibit dose dependent toxicity ranging from mild to severe cellular alterations as summarized in table 6.

Table 6. Histopathopathological effects *C. lutea* extracts and fraction on organs

Treatment organs	Control (Tween 80 20%)	192.5 mg/kg CEAE	385.0 mg/kg CEAE	770 mg/kg CEAE	770 mg/kg EAF
Liver	No cirrhotic lesions	Severe liver infarct	Severe liver infarct	Severe liver infarct	Moderate liver infarct
kidney	No nephritic lesions	Acute tubular necrosis	Acute tubular necrosis	Acute tubular necrosis + hydronephrosis	Acute tubular necrosis + hydronephrosis
Lungs	No pulmonary oedema	Severe pulmonary oedema	Severe pulmonary oedema	Severe pulmonary oedema	Severe pulmonary oedema
Heart	No cardiac lesions	Heart infarct + mild thrombus formation	Heart infarct + severe thrombus formation	Heart infarct + severe thrombus formation	Heart infarct + severe thrombus formation
Brain	No cerebral lesion	Mild cerebral lesions	Mild cerebral lesions	Severe cerebral lesions	Severe cerebral oedema
Stomach	No gastric pathology	Gastric atrophy	Mild gastric atrophy	Severe gastric atrophy	Severe gastric atrophy
Spleen	No splenic infarct	Splenic infarct	Severe splenic infarct	Severe splenic infarct	Splenic infarct
Testes	No testicular atrophy	Severe oligospermia	Testicular atrophy with severe oligospermia	Severe testicular atrophy	Severe testicular atrophy
Ovary	No ovarian pathology	Mild thicker endometrium & and dilated uterine glands	Moderate thicker endometrium & and dilated uterine glands	Severe thicker endometrium & and dilated uterine glands	Severe thicker endometrium & and dilated uterine glands

DISCUSSION

Toxicological studies are important for the approval of pharmaceutical products for human use [27, 28] Animals in the study groups gained significant ($p<0.05$- 0.01) weight from 7th to 28th days of treatment; beyond these days the gain in body weight was not significant. Although the mean increase in body weights double (13.4 to 29.4%) when the extract dose was increased from 192.5 to 385 mg/kg but however diminishes to 6.2% with the highest dose of extract (770 mg/kg) but it was considerably higher, 17.1%, in EAF (770 mg/kg). Unlike the body weight, no treatment-related changes produce significant alteration in organs weights, suggesting that the fraction and extract may not promote cell proliferation or cause cellular damage and hence oedema. Though TPC in extracts were higher than fractions, this appears not to influence the results.

Certain medicinal herbal preparations or conventional drugs/chemicals adversely affect various blood components [29, 30] For example, some flavonoids and including those isolated from herbs cause haemolytic anaemia and thrombocytopaenia [31]. CL extract and fraction contains polyphenols but it did not affect Hb and other haematological indices that would suggest adverse effects on bone marrow, which is the source of reticulocytes.

It is likely that the polyphenol content of the CL administered did not impinge on the haematological parameters. CL leaf treatment also did affect increase in blood platelet counts which account for insipient thrombocytosis observed though not significant.

CL treatment increased WBC counts and caused slight depression in lymphocyte and granulocyte counts. This observation suggests that the elevation of WBCs caused by CL was compensated for by decreased bone marrow production of granulocytes, the precursors of WBCs. Thus, the profile of WBC counts could be a reflection of the balance between the rate of granulocyte production and that of WBC destruction which may be as a result of direct actions of CL.

Some herbal medicines exhibit nephrotoxic and hepatotoxic effects [32, 33]. Damage to these organs often results in elevation in clinical chemistry parameters [34,35] such as serum enzymes like AST and ALT and analytes like total and conjugated bilirubin, urea and creatinine [33]. CL leaves contain polyphenols and other compounds [19]. Although certain flavonoid-containing herbal medicines impair liver and kidneys functions [36, 37], we did observe some abnormalities in clinical chemistry parameters and urinalysis that would suggest that CL treatment had adverse effects on either the liver or kidney. For example, the extract did alter plasma albumin levels, an indication that it did inhibit protein biosynthesis and thus would have some adverse effect on oncotic pressure [38]. The elevation of plasma ALP levels

indicate that CL either caused damage to cardiac or skeletal muscle or affected hepatic excretory dysfunction / cholestasis or the bone marrow [35, 39] Mild elevations of AST have been associated with liver injury or myocardial infarctions [40]. The higher the activity of AST, the larger the infarction size [41, 42]. In chronic liver diseases such as hepatitis C and cirrhosis, the serum ALT level correlates only moderately well with liver inflammation [43]. A typical myocardial infarction gives an AST/ALT ratio greater than 1; however, AST/ALT ratios of less than 1 are found due to the release of ALT from the affected liver [42]. Since the results gave an AST/ALT ratio to be less than 1, the extract is less likely to lead to myocardial infarction if taken over a long period of time.

Since the standard range for plasma ALT levels for rats is 21–52 UI/L [43], our results provide evidence of no hepatic overload. Fluctuations in ALT levels are usually accompanied by an alteration of AST levels, however, AST is essentially a mitochondrial enzyme and it is not released as fast as ALT, which is cytosolic [44]. This could explain why ALT levels are higher than AST level.

The histopathologic alterations observed in various organs evaluated were attributed degenerative and necrotic changes, and inflammatory reactions and not circulatory disturbances. The seemingly increase in titer of liver enzymes observed with higher dose of extract and fraction could be related to the antioxidant (lower doses) and prooxidant (higher doses) properties of flavonoids [45]. Indeed, the hepatotoxic effect observed in our work could be due polyphenol prooxidant properties.

CONCLUSION

We have demonstrated for the first time that oral sub-chronic administration of ethyl acetate extract and fraction of CL to rats did showed some changes on the biochemical but not the hematological parameters. Similar effects were noted in the acute and subacute studies with the same extract and fraction [18] (Nwidu et al., 2012). Therefore, precaution should be taken with high doses. However further research is required to clearly elucidate the mechanism.

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