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Toxicological Evaluation of the Anti-Malarial Herb *Cryptolepis sanguinolenta* in Rodents

[1]C. Ansah, [1]E.A. Mfoafo, [1]E. Woode, [2]C. Opoku-Okrah,
[3]W.K.B.A. Owiredu and [1]M. Duwiejua
[1]Department of Pharmacology,
[2]Department of Laboratory Technology,
[3]Department of Molecular Medicine, College of Health Sciences,
Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

Abstract: In this study, we evaluated the aqueous extract of the roots of *Cryptolepis sanguinolenta* (Periplocaceae), an anti-malarial herb in the West African sub-region for possible toxicity in rodents. Administration of cryptolepis (10-1000 mg kg^{-1}) daily for two weeks did not cause significant changes in most of the haematological parameters assessed. However, the MCV reduced from a vehicle-treated value of 63.1±0.6 to 58.1±0.9 g dL^{-1} at a dose of 10 mg kg^{-1}, which reflected in an increased MCHC (27.8±0.3 to 30.5±0.3 g dL^{-1}), since the Hb concentration remained unchanged. Serum transaminase levels did not change significantly suggesting a limited effect on the liver. Administration of the extract (50-1000 mg kg^{-1}, p.o.) 30 min before pentobarbitone (50 mg kg^{-1}, i.p.) caused a dose-dependent prolongation of the rat sleeping time from 66.6±8.1 min (vehicle-treated control) to 266.5±7.0 min (1000 mg kg^{-1}). Similarly, daily treatment with the extract (50-1000 mg kg^{-1}) for 2 weeks prolonged the sleeping time from 155±28.4 to 292.8±28.7 min. This effect appeared to be CNS-related rather than an enzymatic as reflected in a decreased locomotor activity (19.4±1.5 to 1.8±0.8 min^{-1}) at a dose of 500 mg kg^{-1} body weight. All together, our results suggest that *Cryptolepis* could synergize with hypno-sedatives or other CNS depressants and therefore caution needs to be taken in the concomitant administration of *Cryptolepis* and other CNS depressants.

Key words: *Cryptolepis sanguinolenta*, antimalarial, rodents, haematological parameters, sleeping time, CNS depressant

INTRODUCTION

Malaria kills more than one million people every year, mostly young children under 5 years of age (WHO, 2003). In developing countries, most people in rural communities are low income earners who are unable to afford conventional anti-malarial drugs. The reported resistance to chloroquine (Mockenhaupt *et al*., 2005) and the resultant change in malaria treatment policy from chloroquine as the first choice medication to artemisinin-based combination therapy imposes a financial burden of approximately USD 4.0 for an episode of malaria in comparison to the previous cost of under 50 cents per episode when choloroquine was the drug of choice (Mutabingwa, 2005). The alternative for many people is to resort to herbal products with doubtful claims of efficacy and safety.

Extract of *Cryptolepis sanguinolenta* (Periplocaceae) root sold under various trade names as herbaquine, malacure and phytolaria for uncomplicated malaria have been used in Ghanaian traditional medicine for many years (Boye and Ampofo, 1983). *Cryptolepis sanguinolenta* is a thin-stemmed plant

Corresponding Author: Dr. Charles Ansah, Department of Pharmacology, College of Health Sciences, KNUST, Kumasi, Ghana Tel: +233 (0) 243 382661/+233 (0) 51 60372

occurring on high ground and in the wild and may be twinning or scrambling. The leaves are generally elliptic, appear lateral on branched shoots and are about 7 cm long and 3 cm wide. The roots are yellowish brown on the outer surface with a faint odour and a bitter taste (Mshana *et al*., 2000).

In addition to the established anti-malarial action (Noamesi *et al*., 1991; Kirby *et al*., 1995; Wright *et al*., 2001), the major alkaloid of the roots, cryptolepine is reported to exhibit several biological effects including anti-bacterial (Cimanga *et al*., 1991; Paulo *et al*., 1994; Sawer *et al*., 1995), anti-muscarinic (Rauwald *et al*., 1992), anti-thrombotic (Oyekan and Okafor, 1989); noradrenergic (Noamesi and Bamgbose, 1980); vasodilator (Oyekan, 1994); hypoglycaemic (Bierer *et al*., 1998) and anti-inflammatory (Bamgbose and Noamesi, 1981). Recent reports from *in vitro* studies also indicate that cryptolepine interacts with DNA (Lisgarten *et al*., 2002) provoking marked toxicity in mammalian cells (Bonjean *et al*., 1998; Dassonneville *et al*., 2000; Ansah and Gooderham, 2002). To date however, there is little knowledge on the possible systemic toxicity of this popular anti-malarial remedy. We had reasoned from the reports on the potent toxicity to some mammalian cells including hepatoma cells *in vitro* (Ansah and Gooderham, 2005), that the agent may affect the cellular elements of blood and possibly cause damage to the liver. The objective of the present study was to investigate the possible toxicity of *Cryptolepis in vivo* using rodent models.

MATERIALS AND METHODS

Animals

The animals used in the study, Sprague-Dawley rats (180-220 g) and ICR mice (20-30 g), were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana in February 2006 and housed in the animal unit of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. They were housed in groups of 5 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding and fed with normal commercial pellet diet (GAFCO, Tema, Ghana) and water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Preparation of the Aqueous Root Extract

Powdered roots of the whole plant *Cryptolepis sanguinolenta* were authenticated and obtained from the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong Akwapim, Ghana, where the plant is routinely used for the treatment of malaria. The powdered roots (600 g) were boiled for 30 min in 5 L of distilled water, decanted and filtered. The filtrate was transferred into a container and kept. The residue was further extracted successively with (3×2500 mL) quantities of distilled water. The filtrates were pooled together and freeze-dried to obtain a crude extract (yield = 9.2% w/w), referred to in this study as extract or cryptolepis. Appropriate amounts of cryptolepis were reconstituted in distilled water immediately before use.

Effect of Extract on Haematological Parameters

Male Sprague-Dawley rats (180-220 g) were placed into 7 groups (n = 5). Animals in the groups received orally 10, 50, 100, 250, 500 and 1000 mg kg^{-1} body weight of cryptolepis daily or distilled water (10 mL kg^{-1} body weight; control group) respectively for two weeks. The animals were monitored for signs of toxicity. At the end of the two-week period, the rats were anaesthetized with ether and blood was collected through a cardiac puncture into polystyrene tubes coated with Ethylenediaminetetraacetic Acid (EDTA) as anticoagulant. Red Blood Cells (RBC), total White Blood

Cells (WBC), granulocytes, Haematocrit (HCT), Platelets (PLT), Mean Corpuscular Volume (MCV), Haemoglobin Concentration (HB), Mean Haemoglobin Concentration (MHC), Mean Corpuscular Haemoglobin Concentration (MCHC) were determined. using an automated analyzer, Cell Dyne: Model. 331430 (Abbott Laboratories, IL USA)

Serum Biochemical Analysis

Blood was collected from the heart (without anticoagulant) for biochemical determinations. Serum was separated by centrifugation (750 g for 10 min). Serum determination of total proteins, total bilirubin, direct bilirubin, indirect bilirubin, albumin, globulin and albumin/globulin (A/G) ratio were performed using an automated analyzer, ATAC 8000 (Elan Diagnostics, CA, USA). Levels of the liver enzymes Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST) and γ-Glutamyl Aminotransferase (GGT) were also determined.

Determination of Relative Organ Weights

All the animals were euthanized at the end of the two-week period. The spleen, liver, kidney and stomach were excised from each rat and weighed. The organ-to-body weight ratios were then calculated.

Effect of Cryptolepis on Pentobarbitone-Induced Sleeping Time

Male rats were placed into 6 groups (n = 5). Animals in the groups received orally 50, 100, 250, 500 and 1000 mg kg^{-1} body weight of cryptolepis, daily or distilled water (10 mL kg^{-1} body weight; control group) respectively for 2 weeks. Thirty minutes after administration of cryptolepis, the animals were challenged with 50 mg kg^{-1} body weight of pentobarbitone (i.p). Sleeping times in the different groups were recorded as the time between the loss and gain of the righting reflex. In another study, the rats were treated daily with cryptolepis for two weeks before the sleeping times were determined.

CYP_{450} Carbon Monoxide Binding Assay

Male rats were placed into 4 groups (n = 5) and treated with cryptolepis (500 mg kg^{-1} body weight), phenobarbitone (100 mg kg^{-1} body weight), ketoconazole (80 mg kg^{-1} body weight) or distilled water (control group).

Hepatic microsomes were prepared by calcium precipitation (Gibson and Skett, 1994) and the cytochrome P_{450} content determined by the method of Omura and Sato (1964). Briefly, samples of microsomes to be assayed were suspended in phosphate buffer (pH 7.4) with 20% glycerol to a final concentration of 2 mg of protein mL^{-1}. Protein concentration was determined by the method of Lowry *et al*. (1951). Equal volumes of the suspension were added to both matched and reference cuvettes and then a baseline spectrum was recorded between the two cuvettes in a dual beam UV-visible spectrophotometer (Cecil Instrument, Cambridge, England) between 400 and 500 nm. Samples were measured in the presence and absence of carbon monoxide and the P_{450} content determined by the change in absorption between 450 and 490 nm using the extinction coefficient, ε 450-490 = 91 mM^{-1} cm^{-1}.

Effect of Extract on Locomotor Activity in Mice

Twenty five mice were placed into 5 groups (n = 5). The mice were orally treated with 50, 100, 250 and 500 mg kg^{-1} body weight of cryptolepis or distilled water (10 mL kg^{-1} body weight). The group that received distilled water served as the control. Thirty minutes after cryptolepis administration, the activities of mice in the different groups were determined over a period of thirty minutes using the activity cage (Ugo Basile, Comerio, VA, Italy). The mean activity per minute for each of the groups was then calculated.

Statistical Analysis

Group means were compared using one-way ANOVA followed by Tukey's multiple comparison test. $p \leq 0.05$ was considered significant.

RESULTS

Haematological Assessment

The extract did not cause significant changes to the levels of RBC, WBC, Hb, HCT, PLT or the MCH at the dose levels tested (Table 1). However, for MCHC, there were increases at all levels of treatment compared with vehicle-treated control. From the vehicle-treated control of 27.82±0.26, the MCHC increased to a significant value of 30.52±0.34 g dL^{-1} at a dose of 10 mg kg^{-1} body weight. In contrast, MCV levels were reduced significantly ($p<0.05$) at dose levels of 10 and 50 mg kg^{-1} body weight from the control value of 63.12±0.56 to 58.12±0.90 g dL^{-1} and 59.04±0.97, respectively. (Table 1). Lymphocytes increased (but only significantly ($p<0.05$) at 100 mg kg^{-1}) up to 500 mg but dropped to 6.64±0.97 at a dose of 1000 mg kg^{-1} body weight.

Serum Biochemical Evaluation

The extract did not cause significant changes to the serum levels of bilirubin, albumin, globulin, A/G ratio and total protein (Table 2). Similarly, mean serum levels of AST, ALT, GGT and ALP in the test groups were not significantly different from those from the vehicle-treated control group (Table 3).

Table 1: Effect of *Cryptolepis* on haematological indices in rats treated for 2 weeks

Dose (mg kg^{-1})	RBC (M uL^{-1})	Hb (g dL^{-1})	HCT (%)	MCV (fl)	MCHC (g dL^{-1})
Control	7.57±0.39	13.28±0.58	47.74±2.12	63.12±0.56	27.82±0.26
10	7.88±0.15	13.96±0.18	45.76±0.78	58.12±0.90**	30.52±0.34***
50	8.13±0.29	14.36±0.50	48.00±1.38	59.04±0.97*	29.94±0.42***
100	8.02±0.29	14.60±0.36	48.30±1.26	60.32±1.31	30.24±0.31***
250	7.71±0.13	14.04±0.25	46.96±0.87	61.36±0.49	29.72±0.15**
500	7.83±0.28	13.82±0.17	47.52±1.05	60.84±1.03	29.12±0.39
1000	7.98±0.11	14.46±0.15	50.24±0.93	62.94±0.66	28.78±0.26
Dose (mg kg^{-1})		MCH (pg)	PLT (K μL^{-1})	WBC (K μL^{-1})	LYMP (%)
Control		17.58±0.20	660.80±176.2	7.00±1.02	5.64±0.85
10		17.76±0.37	848.40±48.24	11.44±1.68	7.34±0.64
50		17.68±0.46	844.00±108.60	8.56±1.38	7.04±0.95
100		18.08±0.12	663.80±82.41	14.80±3.14	11.64±1.61*
250		17.74±0.48	580.00±75.36	11.44±1.71	10.90±1.97
500		17.74±0.48	613.00±94.24	13.32±2.20	11.16±1.81
1000		18.12±0.16	470.80±52.75	9.26±1.53	6.64±0.97

Values are expressed as means±SEM (n = 5), *: Indicates significance ($p<0.05$), **: ($p<0.01$), ***: ($p<0.005$) compared to the control by Tukey's test

Table 2: Effect of *Cryptolepis* on plasma proteins and bilirubin in rats treated for 2 weeks

Dose (mg kg^{-1})	Globulin (g dL^{-1})	Albumin (g dL^{-1})	Total proteins (g dL^{-1})	Total bilirubin (mg dL^{-1})	Conjugated bilirubin (mg dL^{-1})	Unconjugated bilirubin (mg dL^{-1})	A/G
Control	4.34±0.26	3.84±0.17	8.18±0.30	2.52±0.41	2.04±0.36	0.48±0.07	0.90±0.07
10	3.84±0.40	3.76±0.14	7.60±0.31	2.40±0.74	1.62±0.56	0.78±0.21	1.04±0.16
50	3.48±0.41	3.76±0.07	7.24±0.44	2.00±0.16	1.88±0.53	0.90±0.24	1.12±0.11
100	3.18±0.15	3.78±0.10	6.96±0.22	1.00±0.33	0.48±0.24	0.52±0.11	1.20±0.04
250	3.24±0.19	3.58±0.12	6.70±0.25*	1.15±0.41	0.80±0.33	0.30±0.03	1.10±0.08
500	3.32±0.24	3.76±0.13	7.08±0.30	1.34±0.24	0.62±0.32	0.72±0.11	1.16±0.08
1000	4.74±0.20	3.84±0.17	8.74±0.27	3.48±1.14	2.52±0.89	0.96±0.28	0.86±0.05

Values are expressed as means±SEM (n = 5), *: Indicates significance ($p<0.05$) compared to the control by Tukey's test

Table 3: Effect of *cryptolepis* on liver enzymes in rats treated for 2 weeks

Dose (mg kg^{-1})	AST	ALT	ALP	GGT
	----------	----------(U L^{-1})----------	----------	----------
Control	244.00±11.56	198.40±25.41	458.40±26.64	13.75±1.78
10	208.80±11.15	180.20±8.350	515.60±52.86	18.60±3.89
50	145.50±37.51	142.40±10.00	448.00±31.95	13.54±2.17
100	223.80±27.89	150.20±4.710	401.60±24.42	11.50±1.19
250	214.50±22.25	148.20±4.940	403.00±12.81	7.75±1.49
500	254.00±44.57	163.20±14.74	410.00±46.80	11.40±2.44
1000	257.20±15.92	140.00±30.70	349.00±64.78	11.00±2.52

Values are expressed as means±SEM (n = 5), Values not statistically significant compared to control by Tukey's test

Table 4: Effect of *cryptolepis* on relative organ weights in rats treated for 2 weeks

Dose (mg kg^{-1})	Relative weights (%) Spleen	Liver	Kidney	Stomach
Control	0.27±0.06	3.03±0.17	0.59±0.04	1.01±0.08
10	0.45±0.08	3.80±0.20	0.66±0.07	1.14±0.11
50	0.46±0.09	3.35±0.26	0.85±0.17	1.17±0.12
100	0.39±0.04	3.64±0.23	0.48±0.11	1.11±0.12
250	0.47±0.06	4.18±0.36*	0.75±0.05	1.18±0.09
500	0.35±0.02	3.41±0.19	0.56±0.03	1.11±0.07
1000	0.36±0.05	2.61±0.14	0.56±0.02	0.99±0.07

Values are expressed as means±SEM (n = 5), *: Indicates significance ($p<0.05$) compared to control by Tukey's test

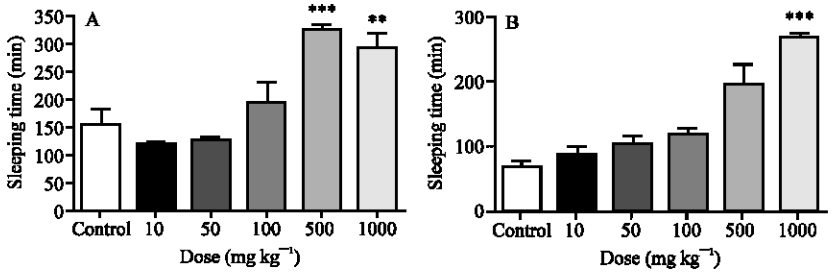

Fig. 1: Effect of *Cryptolepis* on pentobarbitone-induced sleeping time in rats. Animals either received the extract 30 min (A) or 2 week (B) before the pentobarbitone (50 mg kg^{-1}) challenge. The sleeping times in the different groups were recorded as the time between the loss and gain of the righting reflex. Values are presented as the mean±SEM (n = 5), **: Indicates significance ($p<0.01$) and ***: Indicates ($p<0.0001$) compared to the control group by Tukey's test

Relative Weight of Organs

Relative weights of the liver increased in cryptolepis-treated animals over a dose range of 50-500 mg kg^{-1} body weight. However, the increase was only significant ($p<0.05$) at a dose of 250 mg kg^{-1} body weight, which was 4.18±0.11 compared to the control value of 3.03±0.17. There were no significant changes in the weights of the kidney, spleen and the stomach (Table 4).

Effect of *Cryptolepis* on Pentobarbitone-Induced Sleeping Time

The extract caused a profound increase in pentobarbitone-induced sleeping time in both the acute (30 min before pentobarbitone administration) and sub-acute (administration of cryptolepis daily for two weeks before the pentobarbitone challenge) studies. Cryptolepis (50-1000 mg kg^{-1}) caused a dose-dependent prolongation of the sleeping time from the vehicle-treated control group of 66.6±8.1 to 266.5±7.0 min at a dose of 1000 mg kg^{-1} (Fig. 1A). Similarly, two-week treatment with cryptolepis caused a dose-dependent increase in the sleeping time of the vehicle-treated control group from 155.0±28.4 to 292.8±28.7 min (Fig. 1B).

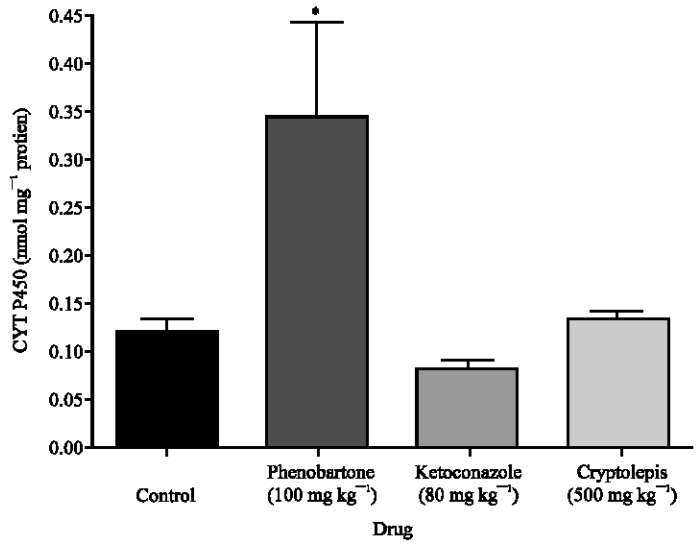

Fig. 2: Effect of *Cryptolepis* on CYP450 in rats. Male rats received cryptolepis (500 mg kg^{-1}), phenobarbitone (100 mg kg^{-1}), ketoconazole (80 mg kg^{-1}) or distilled water daily for one week and the CYP450 content determined as described in the methods. Values are presented as the mean±SEM (n = 5),*: Indicates significance ($p<0.05$) by Tukey's test

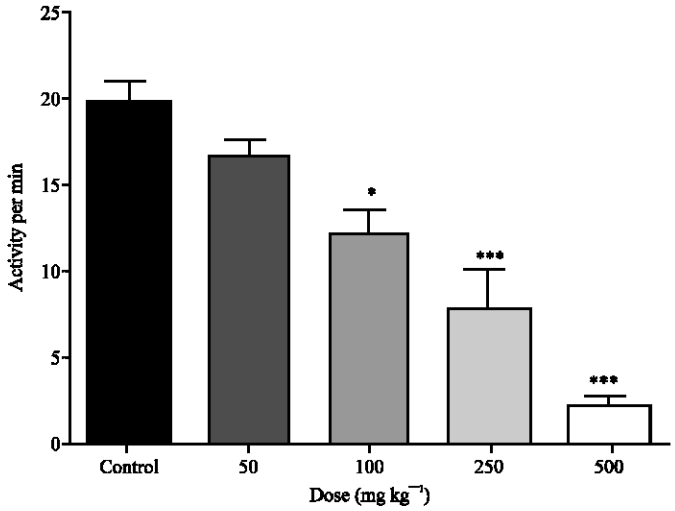

Fig. 3: Effect of *Cryptolepis* on spontaneous activity of mice. Thirty minutes after treatment with the extract, the activities of the mice were determined using the activity cage at 5 min intervals over a period of 30 min. Values are presented as the mean±SEM (n = 5), *: Indicates significance ($p<0.05$) and ***: ($p<0.0001$) compared to the control group by Tukey's test

CYP_{450} Carbon Monoxide Binding Assay

CYP_{450} level (0.132±0.009 nmol mg^{-1} protein) after treatment with cryptolepis (500 mg kg^{-1}) was comparable to the vehicle-treated control (0.118±0.015 nmol mg^{-1} protein; Fig. 2). Treatment with phenobarbitone (100 mg kg^{-1}), increased CYP_{450} levels to 0.345±0.098 nmol mg^{-1} protein (Fig. 2), typical of an inducer.

Effect of Cryptolepis on Locomotor Activity of Mice

A dose-dependent decrease in spontaneous activity was observed. From a vehicle-treated basal activity of 19.4±1.5 min^{-1}, the spontaneous activity reduced to 1.8±0.8 min^{-1} at a dose of 500 mg kg^{-1} body weight (Fig. 3).

DISCUSSION

Safety is the most important consideration for the assessment of any agent which is administered for the treatment of disease in humans. This is especially important for herbal products since evidence from several reports indicate that contrary to popular perception that natural remedies are safe, profound toxicity can result from their use (Perharic *et al*., 1995; De Smet, 1997; Bateman *et al*., 1998; Moss, 1998). In addition, current regulatory requirements for registration of herbal medicines in many countries do not require extensive safety assessment before they are released on to the market.

Aqueous extracts of *Cryptolepis sanguinolenta* have been used for years in the West African sub-region for the treatment of malaria. Though there are no official reports of toxicity or adverse reactions to *cryptolepis* in humans, absolute safety cannot be assumed, since there is no effective adverse reaction monitoring on herbal medications. We were prompted by recent reports on both the aqueous extract and cryptolepine, the major alkaloid of the roots as *in vitro* mammalian cytotoxins (Bonjean *et al*., 1998; Dassonneville *et al*., 2000; Ansah and Gooderham, 2002) and the interaction of cryptolepine with DNA (Lisgarten *et al*., 2002) to investigate the possible systemic toxicity of cryptolepis.

The extract had no effect on the haematological indices assessed except a decrease in the MCV. The MCV measures the average volume of the cell and a decrease as observed in the present study would suggest the presence of microcytic cells. It is presently unknown if this observation is due to iron deficiency or a haemoglobinopathy. Consistent with the reduced MCV and the unchanged haemoglobin concentration, the MCHC, a measure of the average concentration of haemoglobin in a red cell increased.

Treatment with the extract caused an increase in lymphocyte number. Lymphocytes are the immunologically competent cells which assist the phagocytes in the defense of the body against infections and other foreign invasion. The current findings suggest that cryptolepis may augment the immune system through an increase in the number of lymphocytes. The reported antimicrobial actions of cryptolepis (Boakye-Yiadom and Herman-Ackah, 1979; Cimanga *et al*., 1991; Paulo *et al*., 1994; Sawer *et al*., 1995) could in part be due to this immunostimulant activity.

Though, we found marginal increases in the relative liver weights of treated animals, serum bilirubin and globulin levels were not affected, suggesting that the capacity of the liver to transport bilirubin and synthesize globulins was not compromised by cryptolepis. Similarly, protein synthesis was not affected. Additionally, serum transaminase levels which are elevated several fold following hepatic damage was normal. These findings indicate that the extract had only a minor effect on the liver, which is in sharp contrast to *in vitro* observations where the extract showed profound toxicity to hepatoma cells (Ansah and Gooderham, 2005). The contrast may be ascribed to species differences in response to cryptolepis by the rat liver used in this experiment and the human hepatoma cells used for the *in vitro* study (Ansah and Gooderham, 2005). Secondly, pharmacokinetic and pharmacodynamic variations *in vivo* and *in vitro* could contribute to the present observations.

Further to the observed increase in the relative weight of the liver, we sought to determine if the metabolic capacity of the liver could be affected by cryptolepis. For this purpose, we investigated the effect of the extract on pentobarbitone-induced sleeping in rats. Pentobarbitone, a hypno-sedative is metabolized by the microsomal enzymes in the liver. The duration of sleep is therefore a marker of the ability of the liver to convert pentobarbitone into inactive metabolites (Gibson and Skett, 1994). Interestingly, our treatment prolonged the sleeping time under both acute and sub-acute conditions,

suggesting a possible enzyme inhibition or a CNS depressant action by the extract. However, we did not find the extract to be an enzyme inhibitor in the carbon monoxide binding assay. The prolongation of the sleeping time is therefore unlikely to be due to an enzymatic effect but possibly a CNS effect. We therefore determined the effect of the extract on the spontaneous activity of mice using the activity cage. Here, we observed a clear dose-dependent reduction in spontaneous activity by the extract. We suggest that the prolongation of the pentobarbitone sleeping time by the extract could in part be due to a possible CNS depressant action of cryptolepis. This effect is in agreement with the use of cryptolepis in the management of insomnia (Mshana *et al*., 2000).

All together, the study has shown that cryptolepis increases the number of lymphocytes, an effect which could contribute to the reported antimicrobial activity of cryptolepis. Cryptolepis has little effect on the cellular elements of blood but profoundly prolongs the pentobarbitone-induced sleeping time in rats. This effect together with the reduced CNS activity suggests that caution should be taken in the concomitant use of cryptolepis and other medications that depress the CNS.

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