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Toxicological effects of the essential oil of *Premna angolensis* leaves used as a rice protector against *Sitotroga cerealella* (Lepidoptera: Gelechiideae)

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Abstract

Cereals are increasingly attacked by *Sitotroga cerealella*, a highly destructive and economically important insect pest. The need to control this micro Lepidoptera to reduce its pressure on rice stocks is therefore acute. Today, the use of plant extracts, particularly essential oils, is a promising natural alternative to synthetic insecticides in the fight against crop pests. The objective of this study was to evaluate the acute oral toxicity of *Premna angolensis* in rats in order to propose an effective means of controlling *S. cerealella* in rice stocks without adversely affecting human health. The toxicity of this essential oil was assessed in male Wistar rats at a single dose of 2000mg/kg. The animals' behavior was observed for 14 days. The results showed that the lethal dose 50% (LD50) is strictly higher than 2000mg/Kg bw and that this extract does not influence the normal weight growth in the treated rats. Also, the evaluation of biochemical parameters of rats proved that *P. angolensis* essential oil is not toxic to the liver, kidneys and did not disturb lipid metabolism in rats at 2000mg/kg bw. Haematological analyses showed no significant difference from the control ($p>0.05$), however, the blood platelet count was significantly increased in treated rats compared to the control at the 5% threshold. Thus, essential oil treatments that could be applied at much lower doses to rice stocks could not be harmful to humans. It could therefore be used without risk by the rice industry to control *S. cerealella*.

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Introduction

In Benin, as in many West African countries, rice is increasingly stored for consumption, marketing and seed (Adegbola and Sodjinou, 2003; Dossouhoui *et al.,* 2017; Fiamohe, 2018). To secure the quantity and quality of stored grain, it is necessary to control pests that are economically important for this cereal. The cereal leaf beetle *Sitotroga cerealella* is among the most devastating primary pests of this cereal. Their populations and damage have been increasingly observed in many rice producing areas of the country in recent years. This situation requires the rice industry to look for ways to control post-harvest insects, mainly *S. cerealella*, without harming human health. Several research studies have demonstrated the antifungal, antibacterial, antimicrobial, insecticidal and insect repellent properties of essential oils. *Premna angolensis* essential oil is known to have strong insect repellent, insecticidal, ovicidal and larvicidal activity against *S. cerealella* (Adjalian *et al.,* 2015). Similarly, restrictions imposed by international bodies on the use of chemically synthesised insecticides due to health and environmental risks (Degnon *et al.,* 2016) are increasingly driving the use of essential oils in the control of insect pests (Soumanou and Adjou, 2016). The essential oil of *P. angolensis* can therefore be recommended for the post-harvest conservation of rice stocks if its safety for humans is proven. In order to ensure that it does not present any health risk related to its use in the protection of stored foodstuffs, the acute toxicity of this oil was studied in male Wistar rats.

Materials and methods

Materials

Plant biological material and distillation of volatile constituents

Premna angolensis leaves were collected in Comé, in the Mono department, Benin. It was identified at the National Herbarium of the University of Abomey-Calavi and stored in the laboratory at 18-20°C in the shade during the whole extraction period. The essential oil was extracted by hydrodistillation of the leaves (450g) for 4 hours using a Clevenger type extractor according to the British Pharmacopoeia method (British Pharmacopoeia, 1980). It was then dried over anhydrous sodium sulphate and analyzed by GC/MS.

Animal biological material

The animal material used consists of male rats of the Wistar strain. The rats are bred at the Laboratory of Physiology and Experimental Pharmacology of the Faculty of Technical Sciences (FAST) at the University of Abomey-Calavi. They are approximately 3 to 4 months old with a body weight of between 110g and 190g. These animals were placed in cages and had free access to a standard diet (pellets rich in carbohydrate, protide and lipid) and water to which they had free access except in case of fasting.

Technical equipment

The equipment consists essentially of a Compact Scal SF- 400C electronic precision balance for weighing the animals, a cannula for gavage, haematocrit tubes for capillary blood sampling, tubes without and with anticoagulant for blood sampling, automatons and spectrophotometers, respectively, for the determination of haematological and biochemical parameters, a centrifuge, an experimental rodent jar

Methods

Acute oral toxicity

Choice of dose

Acute toxicity studies were conducted in accordance with the OECD Guideline (Organisation for Economic Co-operation and Development, Guideline-423, adopted 17 December 2001) (OECD, 2001). Referring to traditional use and literature (Degnon *et al.,* 2016; Kpadonou *et al.,* 2019), the limit dose of 2000mg/kg bw was chosen as the initial dose.

Treatment of the animals

Preparation of the rats

The experiment was performed on eight (08) male Wistar rats. The choice of this animal model was made for the following reasons: rats are docile and easy to handle animals; they are not very aggressive; they are the best adapted and most used animal models in similar studies cited in the literature: The animals were randomly selected, then individually

marked for identification and underwent an adaptation period of seven (07) days prior to the administration of the treatment, in order to acclimatize them to the laboratory conditions.

The rats were divided into two experimental batches of four (04) rats each, the batch of normal control rats that received no extract and the batch of normal rats that received *Premna angolensis* essential oil by gavage at a dose of 2000mg/kg body weight. The animals were housed in cages that were cleaned daily. They were fed a standard complete feed in pellet form, 'mother rabbit' feed supplied by Groupe Véto Service S.A. (G.V.S.) and maintained at room temperature (23°C) and supplied with borehole water. Artificial lighting was provided, alternating 12 hours of light and 12 hours of darkness.

Administration of the essential oil extract of P. angolensis

For 12 hours, the animals were deprived of food but had access to drinking water. After fasting, the animals were weighed and then the essential oil of *P. angolensis* was administered. The dose was calculated according to the fasting body weight of each animal. The essential oil was administered by gavage (per os) using a suitable intubation cannula to the rats in batch T. The essential oil of *P. angolensis* was administered as a single dose at 2000mg/kg body weight. The animals were observed regularly, for the first 30 minutes, after 6 hours and daily for 14 days. Any clinical signs observed were noted.

Blood sampling

Blood sampling was performed as described by (Costa-Silva *et al.,* 2008) using haematocrit tubes through the retro-orbital sinus at eye level, 14 days after the rats were gavaged with essential oil. Rats were anaesthetised with ether by inhalation for 2-3 minutes under a hermetically sealed laboratory rodent jar. Blood was collected in two different tubes, a dry tube for the determination of haematological parameters (CBC) and a second EDTA tube for the determination of some biochemical parameters. Each tube was numbered to facilitate identification throughout the analysis process.

Effects of P. angolensis essential oil on behavioral parameters and weight growth of experimental rats

The weight growth and behavioral parameters of the rats were monitored before and for 14 days after administration of the essential oil. The skin, hair, eyes, mucous membranes, respiration, motor activity, morbidity, mortality and behaviour of the experimental rats were carefully observed for the first six hours (06h) after administration of the extract and then daily until day fourteen (14). The weight was measured with a scale in grams.

Effects of P. angolensis essential oil on biochemical parameters

By means of a HACH LANGUE DR 3900 spectrophotometer the biochemical parameters were performed. The cholesterol content was determined according to the kinetic method described by Gabriela *et al.* (2005). Transaminases (Asat and Alat), urea and creatinine were determined following the method described by Sangare *et al.* (2012).

Effects of P. angolensis essential oil on haematological parameters

Using the SYSMEX KX 21N haematology machine, haematological parameters such as the average blood levels of white blood cells (WBC), lymphocytes (Lymph), granulocytes (Gran), haemoglobin (HGB), red blood cells (RBC), haematocrit (HCT), mean corpuscular volume (MCV), mean haemoglobin content (MHC), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT) were assessed in these rats following the method described by Sangare *et al.* (2012).

These analyses of biochemical and haematological parameters were performed at the Laboratoire de Recherche en Biologie Appliquée (LARBA).

Statistical analysis

The biochemical and haematological data obtained were processed using R software version 4.1 with a significance level of 5%. The variation of the mean of haematological parameters, biochemical parameters and weight of the subjects according to the experimental batches was compared using the non-parametric

Kruskal wallis test, taking into account the very small size of the experimental numbers (n= 4). When the test was significant for a parameter, the comparison of the two means was carried out using the non-parametric Wilcoxon test.

Results

Effects of Premna angolensis essential oil on behaviour and mortality of animals at a single dose of 2000mg/kg bw

Table 1 presents the results of the monitoring of behavioural parameters in the investigated rats. Analysis of the table reveals that after oral administration of *P. angolensis* essential oil at 2000mg/kg to the experimental rats, no mortality and no changes in behaviour and changes in skin, hair, eyes, mucous membranes and respiratory tract, no physical or behavioural signs of toxicity such as sleep, hyperactivity, restlessness, respiratory distress or convulsions were observed in the rats of the treated batch. These results indicate that the essential oil does not have a proven toxic effect after administration at 2000mg/kg body weight, implying that the LD50 lethal dose is greater than 2000mg/kg body weight.

Table 1. Clinical signs of toxicity observed in male rats treated with *Premna angolensis* essential oil at 2000mg/Kg body weight.

	30 mn		6 hours		1 week		2 weeks	
Parameters	C	T	C	T	C	T	C	T
Skin	N	N	N	N	N	N	N	N
Hair	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N
Breathing	N	N	N	N	N	N	N	N
Motor activity	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N
Drowsiness	—	—	—	—	—	—	—	—
Diarrhea	—	—	—	—	—	—	—	—
Trembling	—	—	—	—	—	—	—	—
Morbidity	—	—	—	—	—	—	—	—
Mortality	—	—	—	—	—	—	—	—

Legend: C: Control; T: Treated; N = Normal; —: Absent

Effects of Premna angolensis essential oil at a single dose of 2000mg/Kg bw on the weight growth of male rats

All treated animals showed a positive and consistent weight development during the 14 days of observation (Table 2). Gavage of the essential oil at 2000mg/kg to the animals did not cause any alteration in the weight status of the rats in the experimental batch. There was a gradual change in the weight of the rats from 164.5g±19.12 to 179.25g±21.07 in the rats gavaged with *P. angolensis* essential oil; then 176.5g±19.09 to 185.5g±23.33 in the control. Statistical analysis of the mean weight values reveals that there is no significant difference ($p>0.05$) between the weights of the animals of the two batches on days 0 and 14.

Table 2. Effects of *Premna angolensis* essential oil at 2000mg/Kg body weight orally on weight growth in adult male rats.

	Body weight (g) ± standard deviation	
Lots	Day 0	Day 14
Control	176,5±19,09[a]	185,5±23,33[a]
P. angolensis	164,5±19,12[a]	179,25±21,07[a]

Values in the same row followed by the same letters are not significantly different at the 5% level.

Effects of P. angolensis at a single dose of 2000mg/Kg bw on biochemical parameters in male rats

Several biochemical parameters such as transaminases (Asat and Alat), uraemia, creatinine and cholesterol were measured in order to assess the effect of *P. angolensis* essential oil on the functioning of vital organs in animals (Table 3). The table shows the average levels of the supra-indicated parameters in the experimental batches as a function of time. The analysis of Table 3 shows that no significant differences ($p>0.05$) were observed between the levels of these parameters at day 14. However, the levels of Asat, Urea, Creat and Chol in the treated batch were slightly lower than in the control but without any significant change ($p > 0.05$).

Effects of P. angolensis at a single dose of 2000mg/Kg bw on haematological parameters in male rats

Several haematological parameters were evaluated in order to assess the effect of *P. angolensis* essential oil on the functioning of vital organs in animals (Table 4). On analysis of the data, there was no significant difference ($p>0.05$) in the levels of white blood cells (WBC), lymphocytes (Lymph), granulocytes (Gran), haemoglobin (HGB), red blood cells (RBC), haematocrit (HCT), mean corpuscular volume (MCV), haemoglobin (TMH), mean

corpuscular haemoglobin concentration (MCHC). As for neutrophils, eosinophils and monocytes, despite the few variations noted in the treated batch compared to the control batch, statistical analyses revealed that there were no significant differences ($p>0.05$) between the haematological parameters of the rats given *P. angolensis* essential oil and the control rats. For platelets (PLT), there was a significant difference between the values obtained in the two batches.

Table 3. Effects of *Premna angolensis* essential oil on biochemical biochemical parameters of rats.

Treatment	Alat (UI/L)	Asat (UI/L)	Urée (g/L)	Créat (mg/L)	Chol (g/L)
Control	221±5,89[a]	277,65±21[a]	0,64±0,36[a]	3,155±0,28[a]	0,64±0,04[a]
P. angolensis	234,6±13,9[a]	224,85±48,15[a]	0,375±0,02[a]	2,925±0,45[a]	0,605±0,02[a]
P-value	0.5611	0.06646	0.1133	0.3932	0.05817
significance level	NS	NS	NS	NS	NS

Alat: Alanine aminotransferase; Asat: Aspartate aminotransferase; Chol: Total cholesterol; Créat: Creatinine; NS: Not significant; Within the same column, values assigned the same different letter are not significantly different at the 5% threshold

Table 4. Effects of *Premna angolensis* on haematological parameters of rats.

Variables	Control	*P. angolensis*	P-value	significance level
Red blood cells (/mm3)	701000±98994,9[a]	681500±9192,39[a]	0.1133	NS
White blood cells (/mm3)	7850±636,4[a]	8750±919,2[a]	0,1842	NS
Haemoglobin (g/dl)	14,6±0,77[a]	14,25±0,49[a]	0,05091	NS
Haematocrit (%)	39±4,66[a]	36,25±0,63[a]	0,4911	NS
CCMH (%)	37,8±2,54[a]	39,3±0,56[a]	0,1842	NS
MCHT (Pg)	21±1,9[a]	21,3±1,55[a]	0,528	NS
GMV (µm3)	56±1,41[a]	53±1,41[a]	0,05492	NS
Platelets (/mm3)	548500±28991,37[a]	597000±9899,49[b]	0,02732	S
Neutrophils (/mm3)	560,75±322,08[a]	348,8±64,2[a]	0,3932	NS
Eosinophils (/mm3)	235,5±19,09[a]	158±90,5[a]	0,3932	NS
Basophils (/mm3)	0±0	0±0	0,3527	NS
Lymph (/mm3)	6226,35±150,68[a]	7200,4±1329,36[a]	0,3292	NS
Monocytes (/mm3)	827,4±144,53[a]	697,2±117,37[a]	0,1133	NS

NS: Not significant; WBC: White blood cells; Lymph: Lymphocytes; HGB: Haemoglobin; RBC: Red blood cells; HCT: Haematocrit;mgV: Mean blood volume; MCH: Mean haemoglobin content; MCHC: Mean corpuscular haemoglobin concentration; PLT: Platelet; Values in the same row followed by the same letters are not significantly different at the 5% threshold.

Discussion

The toxicological study of *Premna angolensis* essential oil, extracted by hydrodistillation, in wistar rats showed that the 50% lethal dose (LD50) of this volatile extract is greater than 2000mg/kg body weight. The estimation of the LD50 at a value above 2000mg/Kg shows that these biopesticides would be relatively harmless (Hodge *et al.,* 1943). This result could be explained by the fact that the leaves and stems of *Premna angolensis* are traditionally used by farmers for the protection of grain stocks against post-harvest pests. They put the leaves in grain bags or use the stems in making their granaries or they use them as a fumigant or repellent under the granaries or in the enclosure of grain storage shops.

P. angolensis essential oil administered orally at a dose of 2000mg/kg bw in male rats did not result in any deaths throughout the study. No changes were observed after 14 days of observation. This result suggests that this biopesticide presents very low health risks at a single dose.

To assess the toxic effect of a substance, body weight, food intake and general behaviour, which are the first signs of toxicity, must be checked (Mbaka *et al.,* 2010; Almança *et al.,* 2011). During the 14 days of the study, male rats showed a positive and regular weight evolution. This could explain the non-toxicity of *P. angolensis* essential oil. The evaluation of the effect of the oils on the weight growth of the rats showed that

the extracts did not influence the normal weight growth in the treated rats.

The results of the evaluation of the effects of *P. angolensis* on the biochemical parameters of the rats showed that there were no significant variations ($p>0.05$) between the levels of Alat, Asat, cholesterol, urea and creatinine of the rats subjected to the extract compared to the controls. Asat and Alat are two hepatic enzymes whose role is to transfer an amine group during the many chemical processes that take place in the liver (Rye, 2009). Their activity is proportional to the degree of liver damage (Sacoti, 2012). They are therefore two good indicators of hepatotoxicity (Pratt and Kaplan, 2000; Al-Habori *et al.,* 2002; Shen, 2009; Song *et al.,* 2007). An increase in serum Asat activity reflects an inflammatory, traumatic or degenerative state of Asat-rich tissues (Da Silva *et al.,* 2010; Sow *et al.,* 2012). The non-significant variation in Alat and Asat levels observed in both control and *Premna angolensis* treated rats indicates the absence of:

- Of liver cytolysis, inflammation and tissue degeneration in the investigated rats. Therefore, we can say that these oils had no toxic effect on the liver of rats at 2000mg/ kg live weight. The cholesterol level provides information on the mobilisation of body fat reserves by the animal. Cholesterol is present in the diet and can be synthesised by the liver in a mechanism that is subject to very fine metabolic regulation (Marshall and Bangert, 2005).

- The decrease in cholesterol may be related to environmental conditions, dietary intake deficits or pathologies (Faye *et al.,* 2004). The increase in cholesterol levels is thought to be dietary in origin. Thus, the non-significant increase noted in the three batches would not be linked to the essential oil, but rather to the food given to the experimental rats, since during the entire experimental period, the food was served to the animals ad libitum. Renal function was assessed by measuring serum creatinine and urea. Urea and creatinine are significant markers of renal function (Pauly, 2012). These metabolites are end products of protein metabolism and are generally constant in concentration under normal conditions (Whitby *et al.,* 1988). Their increase or decrease reflects renal dysfunction (Sirwal *et al.,* 2004). Pritchard *et al.*, (2009) showed that a decrease in serum creatinine concentration can be a sign of cachexia. With regard to serum urea concentration, its increase can be a sign of nephropathy, dehydration, electrolyte imbalance, hypo-albuminemia, tissue catabolism (fever, muscle trauma, myositis) (Pitel *et al.,* 2006). These levels did not vary in the treated rats compared to the controls, indicating normal renal function. Overall, since there were no significant variations in the levels of Asat, Alat, urea, creatinine and cholesterol, we can say that the essential oil of Premna angolensis is not toxic to the liver or kidneys and did not disturb lipid metabolism in rats at 2000mg/ kg body weight.

P. angolensis essential oil administered to rats at 2000mg/ kg body weight did not cause significant changes in white blood cell or red blood cell counts. White blood cells, being a family of cells composed of granulocytes, lymphocytes and monocytes, play an important role in fighting infections and in the development of resistance to infection in response to natural exposure or vaccination (WHO, 2009). Indeed, their levels are increased by infections (viral or severe), inflammation, cancer or leukaemia and may be decreased by certain:

- Drugs, during certain autoimmune diseases, bone marrow failure, splenomegaly, liver disease (WHO, 2009). This level did not vary significantly in treated rats compared to controls, indicating that *P. angolensis* essential oil had no toxic effect on the white blood cells of rats at 2000mg/ kg body weight.

- Regarding the effect of *P. angolensis* essential oil on red blood cells, the red blood cell count did not change significantly. The red blood cell level decreases in case of anaemia and increases in case of exaggerated production or fluid losses (diarrhoea, dehydration, burns) (Olivier, 2011). An increase in VGM, TMH and CCMH indicates the presence of normochromic bmacrocytic red blood cells, while a decrease in their levels reflects the presence of hypochromic microcytic red blood cells (Agourram, 2013).

In our case, there were no significant variations in these constants, which show that the red blood cells of the rats are normocytic normochromic, and therefore, the essential oil of *Premna angolensis* had no toxic effect on the red blood cells of the rats at 2000mg/ kg body weight.

- The essential oil of *P. angolensis* significantly changed the platelet levels of rats. The platelet assay can be used to detect a risk of haemorrhage or infectious or inflammatory syndromes after a major haemorrhage (Olivier, 2011).

Overall, there was no significant difference between the calculated means for different parameters in the three batches. However, a significant increase in the number of blood platelets was observed for the treated batch compared to the controls at the 5% threshold. Therefore, the non-significant fluctuations recorded in this study could be related to variation factors related to the individual concerned and his environment (e.g. stress induces leucocyte margination), and to all the factors related to cell variability, notably their life span (Lecomte, 1998). All in all, the essential oil of *P. angolensis* can be applied in trials in food or rice stocks in particular for the control of *S. cerealella* without any health risk for humans.

Conclusion

Premna angolensis essential oil administered orally has a lethal dose 50 (LD50) greater than 2000mg/kg; this allows it to be classified as non-toxic. No signs of behavioural toxicity were observed in treated rats compared to controls. In addition, *Premna angolensis* oil induced a positive and regular weight development in the rats. No significant changes in biochemical and haematological parameters were observed. The essential oil of *P. angolensis* is not toxic to rats by the oral route at a dose of 2000mg/kg; therefore, it would not be harmful to humans. It could therefore be safely used in the protection of rice stocks against *Sitotroga cerealella*.

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