Phytochemical Screening, Chromatographic Profiling and Biological Activities of Ethanol Extract of *Raphia australis*

ABSTRACT

Aim: This study evaluated the phytochemical constituents and the biological activities of ethanol extract of the fruit of *R. australis.*

Methodology: Phytochemical screening was carried out on the dried ethanol extract of *R. australis* pulp and seed. Column chromatography and Liquid chromatography-mass spectrometer (LCMS) were utilized to identify the phytochemicals present in the ethanol extract of the pulp. The extract was further subjected to Novelty Induced behaviour test as well as anxiolytic tests using 4 groups of rats in each case. Group one was administered normal saline (0.09 % w/v NaCl) while 2 and 3 were administered 500 $mgKg^{-1}$, 1000 $mgKg^{-1}$ of ethanol extract of R. australis respectively. Group four was administered 2 $mgKg^{-1}$ of diazepam (control).

Results: The phytochemical screening result was positive for all the secondary metabolites tested for. The LCMS results revealed the presence of proanthocyanidin (m/z 577.1354), catechin (m/z 289.0704), epicatechin (m/z 289.0700), protocatechuic acid (m/z 153.0200), umbelliferon (m/z 353.0868) and quinic acid (m/z 191.0571). At the doses of 500 mg Kg^{-1} and 1000 $mgKg^{-1}$, statistically significant results were obtained for grooming ($p>0.01$), head dipping ($p>0.01$) and anxiolytic ($p>0.05$) tests.

Conclusion: *Raphia australis* contains phenolic compounds which could be utilized for relief of stress and anxiety.

Key words: *Raphia australis*, chromatography, phytochemical screening, umbelliferon, anxiety, sedate.

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1.LCMS used for pulp , what about seed?
2. Animal grouping should make it more clear , one disease cntrol group??
3.In title can mention the focuseed disease

1. INTRODUCTION

Palm trees are evergreen perennial plants with characteristic long stems. Palm tree is a general word for a set of perennial plants comprising trees from various genera and species including *Elaeis guineensis*, *Phoenix reclinatazDypsis canaliculata*, *Raphia farinifera*, *Raphia hookeeri*, *Raphia vinifera* and *Raphia australis* (*R. australis*) [1,2]. Palm leaves are larger than those of all other trees and *Raphia* palm leaf is the longest among palm leaves. The *Raphia* palm belongs to the branch of spermaphytes, sub-branch angiosperms, class monocotyledons, super-order Spadiciflores, order Palmae, family Palmaceae, subfamily Lepidocaryoids, genus *Raphia* [3].

Raphia Palm can grow as tall as 16 metres. They are unique for their compound leaves. Some species have leaves that grow as long as 25 metre with a width of three metres. The plants are either monocarpic flowering once and dry after the maturation of the seeds or with individual stem drying after fruiting while the root system remains alive and continue to grow new stems. The genus *Raphia* has various species distributed throughout Africa, Central America and South America and comprises about 20 species distributed all over the world, with *Raphia australis* being the only species indigenous to South Africa [4].

R. australis is called unVuma by the Zulu speaking tribes in South Africa while it is called 'Kosi palm' by the Afrikaans. Unlike West African palm trees, *R. australis* is not cultivated for palm wine making. *R. australis* fruits grow in bunches and each fruit varies in size depending on the species and maturity stage. The fruit has a hard pulp covered with hard interlocking scales [1].

The different parts of various species in the Raphia genus have been used in fish harvesting [5] and traditionally to manage a wide variety of metabolic disorders such as sickle cell anaemia [6], alcoholic intoxication [7], filariasis, antihyperglycemia [8], benign prostatic hyperplasia and diabetes [9]. This is due to the presence of bioactive compounds which are produced either as a by-product of primary metabolism or for the defense of plants. Therefore, this study was aimed at screening the ethanol extract of the pulp of *R. australis* for biologically active secondary metabolites.

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2. MATERIALS AND METHODS

2.1 Collection and Identification

The fresh fruits were collected from Mr Bruce Hooper of KwaZulu-Natal and transferred in jute bags to Organic Chemistry Research Laboratory, Walter Sisulu University Mthatha, within 48 hours. Authentication of *R. australis* was done by Dr. K. L. Immelman of Department Biological and Environmental Sciences, Walter Sisulu University, Mthatha. The seed of *R. australis* was manually separated from the pulp and both were sun-dried.

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2.2 Preparation of Plant Extract

600g of the dried sample of *R. australis* pulp was steeped sequentially using hexane, dichloromethane, ethyl acetate and ethanol solvent. For each solvent protocol, the mixture was filtered after every 24 hours, and the filtrate was concentrated using a rotary evaporator at 40ºC. The concentrated filtrate was then transferred into a beaker to dry until a constant weight was obtained. Ethanolic extract gave the highest percentage yield.
The extract from 500g of the seed was also obtained by following this procedure but using only ethanol as the extracting solvent.

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2.3 Animal Handling

Mice (20-30g) and rats (150-260g) were obtained from the South African Vaccine Initiative, Johannesburg and kept at Animal Holding Facility at Walter Sisulu University, NMD campus, Mthatha. They were acclimatized to the laboratory environment for 1 week, maintained under 12 h light/dark circle at temperature of 22 ± 2 °C and housed (5 animals per cage) in a Plexiglas cage with wood shavings as beddings. The animals were fed with standard laboratory food for rodents and water was provided freely except during the experiment. This study was approved by the Department of Higher Education, WSU and Ethical Clearance Approval obtained from Walter Sisulu University Ethics Committee with Reference No. DVC (AA&R) DRD/SREC: FNS 01/02/2017.

2.3 Experimental Setup

2. 3.1 Phytochemical screening and gravity chromatographic separation of R. australis pulp extract

Qualitative phytochemical screening, using the method of Trease and Evans, 1987 [10], was carried out on the dried plant samples to determine the presence of phytochemicals in the ethanolic extract of *R. australis*
After several solvent systems available in literature proved inefficient for the TLC analysis of *Raphia australis* ethanol extract, ethyl acetate-ethanol-ammonia (5:3:2 respectively) was found to be the most suitable for TLC separation. Thus, this solvent system was used for column chromatography separation of *R. australis ethanol* crude

extract using a glass column (780 mm by 2.50 mm). The various test tube fractions obtained from the column were pooled together into four groups [B (21-25), H (66-75), K (86-95) and U (121-125)] based on similarity in their R_f values.

2.3.2 Liquid chromatography-mass spectrometry (LC-MS) of R. australis pulp extract

The four combined fraction samples (B, H, K and U) from the column were subjected to Liquid chromatography/ Mass Spectrometry analysis. Waters Synapt G2 quadrupole time-of-flight mass spectrometer was used for LC-MS analysis. It was fitted with a Waters Ultra pressure liquid chromatograph and photo diode array detection. Separation was achieved on a Waters BEH C18, 2.1x100 mm column with 1.7 µm particles. A gradient was applied using 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient started at 100% solvent A for 1 minute and changed to 28 % B over 22 minutes in a linear way. It then went to 40% B over 50 seconds and a wash step of 1.5 minutes at 100% B, followed by re-equilibration to initial conditions for 4 minutes. The flow rate was 0.3 ml/min, and the column was kept at 55 ºC. The injection volume was 2 µL. Data was acquired in MS^E mode which consisted of a low collision energy scan (6V) from *m/z* 150 to 1500 and a high collision energy scan from *m/z* 40 to 1500. The high collision energy scan was done using a collision energy ramp of 30-60V. The photo diode array detector was set to scan from 220-600 nm. The mass spectrometer was optimized for best sensitivity, a cone voltage of 15 V, desolvation gas was nitrogen at 650 L/hr and desolvation temperature 275 ºC. The instrument was operated with an electrospray ionization probe in the negative mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determinations.

2.4 Evaluation of biological activities of R. australis pulp extract

2.4.1 Acute toxicity test

Acute toxicity of the ethanol extracts of the pulp and seed of *Raphia australis* was assessed in mice using oral route (p.o) according to Lorke's method [11]. Each extract was tested for acute toxicity (LD_{50}) effect orally using 13 animals each. The procedure was divided into two phases, phase I used 3 animals per dose of 10, 100 and 1000 mg/kg. Phase II used one animal per dose levels of 1000, 1600, 2900 and 5000 mg/kg. Each animal after treatment was observed for a period of one h initially to check for immediate effect and then for up to 24 h after mortality. Animals that survived for more than 24 h were scored no mortality. The LD_{50} of the infusion extract was estimated as the geometric mean of the lowest dose causing death and the highest dose causing no death according to the following formula: $LD_{50} = \sqrt{(A \times B)}$. A is the maximum dose producing 0% death and B is the minimum dose that produces 100% death (Lorke,1983). From the result of LD_{50}, the working doses was chosen such that the highest working dose is below half of the LD_{50} according to the following relationship: Working dose $\leq$ ½ (LD_{50}).

2.4.2 Novelty-induced behavior

Novelty-induced behaviour was assessed by methods described by Akanmu *et al.*, [12] with modification. Five groups (n=5) of rats were randomly selected. Group 1 was administered the vehicle (5% Tween 80, 10mg/kg. p.o.). Groups 2 to 4 were injected with the extract (500mg/kg, 1000mg/kg, p.o.) respectively. Group 5 was injected diazepam (1 mg/ kg) to serve as positive control. Mice in all the groups were pre-treated for 30 minutes prior to the test. Each animal was placed inside an observation cage and assessed for rearing (when the animal stands on its hind limbs and raises its fore limbs in the air or places them against the wall of the cage), grooming (when the animal licks or washes its body with its mouth) for a period of 20 minutes, and locomotion was scored by the number of lines crossed with all limbs for the first 10 minutes).

2.4.3 Anxiolytic test

The anxiolytic test was carried out on an elevated plus maze (EPM) and hole-board models.

2.4.4 Elevated Plus-Maze (EPM)

Mice were randomly distributed and treated as in the previous section. After 30 min pre-treatment, each mouse was placed in the central section of the EPM. The time spent in the open arms and closed arms as well as the number of times the animal entered each arm was recorded for 5 min [13]. The results obtained were analysed and compared among the groups.

2.4.5 Head dipping test (hole board)

Mice were randomly distributed as described above. After 30min of pre-treatment, each mouse was placed in the centre of the hole board. The number of head-poking demonstrated by each mouse in 5 minutes was recorded. The results were analysed and compared among the groups [14,15].

3. RESULTS

3.1 Sequential extraction results

The result of the sequential extraction showed that *R. australis* has a high concentration of polar secondary metabolites due to the highest yield of ethanol as shown in table 1 below.

Table 1: Percentage yields of sequential extraction of the pulp of *R. australis*

Extracts	Mass yield (g)	Percentage yield
Hexane extract	1.9	0.38
Dichloromethane extract	1.5	0.30
Ethyl acetate extract	1.7	0.34
Ethanol extract	3.8	0.76
Total	8.9	1.78

Sequential extraction was not performed on the seed. Instead, ethanolic solvent extraction was done (Table 2).
Table 2: Percentage yields of ethanol extract of the seed of *R. australis*

Plant part	Mass of sample (g)	Mass of extract obtained (g)	Percentage yield
R. australis dried fruit seed	500	52	10.4

The yield of the ethanol extract of the seed of *R. australis* was found to be almost 6 times more than the yield of the pulp. This shows that the seed has even more polar secondary metabolites than the pulp as shown in Table 2.

3.2 Phytochemical screening results

Both the seed and the pulp of *R. australis* were found to contain all the secondary metabolites tested for.
Nine groups of secondary metabolites were identified in both the pulp and seed ethanolic extract (Table 3).

Table3: Qualitative phytochemical screening of ethanol extract of the fruit pulp and seed of *R. australis*

Phytochemicals	Results	
	Pulp	Seed
Saponins	+	+
Tannins	+	+
Flavonoids	+	+
Steroids	+	+
Terpenes	+	+
Phenolic Compounds	+	+
Phytosterols	+	+
Glycosides	+	+
Alkaloids	+	+

+ = present - = absent

3.3 LC-MS of the column fractions of the pulp of R. australis ethanol extract

Six compounds were identified from the LCMS result, and these results are listed in Table 4 below along with their corresponding literature references. The LC-MS results have shown that fractions contain mostly tannins and flavonoids. From the results of LC-MS analysis, six compounds were identified which are catechin (m/z= 289.0712), protocatechuic acid (m/z= 153.0182), proanthocyanidin (m/z= 577.1354), Epicatechin (m/z= 289.0704), umbelliferon also known as 7-hydroxycoumarin (m/z= 353.0873), quinic acid (m/z =191.0556).

Table 4: LC-MS of the column fractions of *R. australis* ethanol extract.

Compound name	m/z experimental $M-H^-$	m/z literature	Retention time (min)
Epicatechin	289.0700	289.0712 [16]	13.20
Proanthocyanidin	577.1354	577.1340 [16]	12.57
Protocatechuic acid	153.0200	153.0188 [16]	7.79
Quinic acid	191.0571	191.0556 [17]	1.76

Catechin	289.0704	289.0712 [16]	11.12
7-hydroxycoumarin (umbelliferon)	353.0868	353.0873 [17]	11.81

m/z = mass/ charge ratio

3.4 Biological results

3.4.1 Acute toxicity result

The results showed that oral administration (5000 mg/kg) of ethanolic extract of the pulp did not result in mortality after 24 h (Table 5).

Table (5): Acute toxicity profile of the ethanol extracts of the fruit pulp of *R. australis* in mice

Treatment **mg/kg, (p.o.)**	*R. australis* pulp ethanolic extract Death pattern after 24 h
Phase 1	
10	0/3
100	0/3
1000	0/3
Phase 2	
1000	0/1
1600	0/1
2900	0/1
5000	0/1
LD_{50}	**≥5000 mg/kg**

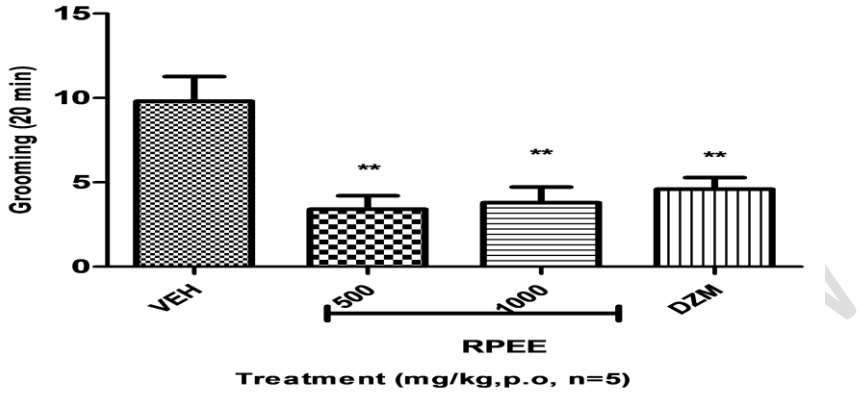

Figure 1: Effect of *R. australis* pulp ethanol extract on novelty-induced grooming behaviour in mice
VEH, RPEE and DZP represent vehicle (normal saline), *R. australis* ethanol extract and diazepam respectively.
**p > .01 statistically significant compared to the vehicle (ANOVA, Dunnett's test)

The extract (500 and 1000 mg/kg) and the standard drug (diazepam, 2 mg/kg) caused significant ($p < .01$; $F_{(6, 13)} = 8.64$) decrease in grooming activity compared to the vehicle (Fig. 1).

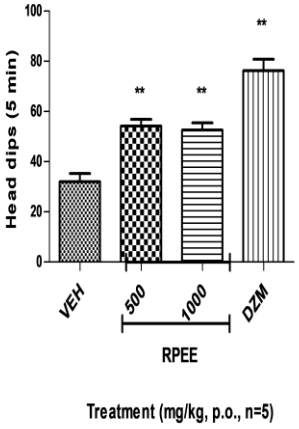

Figure 2: Effect of *R. australis* pulp ethanolic extract head dip behaviour in mice.
VEH, RPEE and DZP 1mg/kg represent vehicle (normal saline), *R. australis* ethanol extract and diazepam (1 mg/kg) respectively.
**p < .01 statistically significant compared to the vehicle (ANOVA, Dunnett's test)

3.4.2 Anxiolytic test

The effect of the extract on the elevated plus maze (EPM) is presented in Figure 3. The extract caused significant ($p < .01$; $F_{(3,16)} = 15.63$) increase in the time spent on the open arms of the EPM compared to the vehicle.

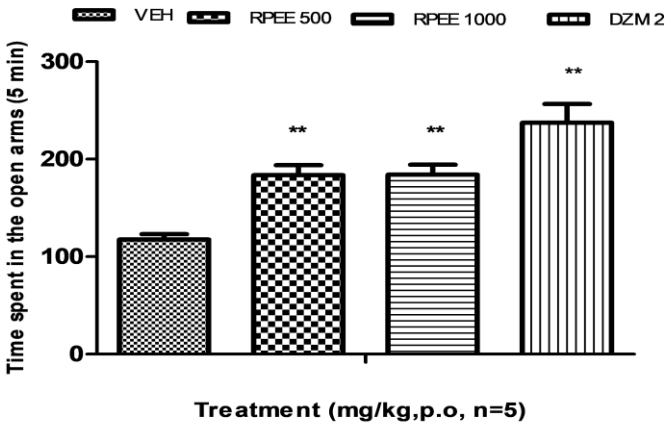

Figure 3: Effect of *R. australis* pulp ethanolic extract on anxiolytic elevated plus maze (EPM) behaviour in mice. VEH, RPEE represent vehicle (normal saline), *R. australis* ethanol extract and diazepam (1 mg/kg) respectively. $^{**}p > .05$ statistically significant compared to the vehicle (ANOVA, Dunnett's test)

4. DISCUSSION

Ethanol extract of the pulp of *R. australis* gave positive results for all secondary metabolites tested for, showing that it is a rich source of secondary metabolites. Phenolic compounds were found to be present in high concentration in the crude extract. This was confirmed by the fact that most compounds identified *via* LCMS analysis in this work were phenolic compounds except for quinic acid which is also flanked by 4 hydroxyl groups on a single homocyclic ring. The solvent system (ethyl acetate: ethanol: aqueous ammonia in the ratio 5:3:2) discovered during this study is therefore recommended for chromatography separation in future work relating to selective isolation of flavonoids, phenolic or poly-hydroxyl secondary metabolites from this plant.

The implication of the acute toxicity results shown in table 5 above suggest that consumption of the fruit of this plant is safe and may not constitute severe health hazard. According to Lorke [11, 22], LD50 values above 5000 mg/kg indicate that the extract is non-toxic and safe. Further research is vital to evaluate the long-term toxicological profile of oral administration of this extract in preclinical studies using 2 different species of animal models.

As reported by Moreira *et al* [21], proanthocyanidin B1 exhibited sedative and anxiolytic activity. The presence of proanthocyanidin B1 in the ethanol extract may be responsible for the sedative and the anxiolytic properties of the ethanol extract of the pulp *R. australis*. The anxiolytic result obtained via elevated plus maze test could probably be suggested as a scientific backing for its use for relieving anxiety in some regions of Cameroon.

Quinic acid, umbelliferone (7-hydroxycoumarin) and protochatechuic acid have all been reported to exhibit anti-inflammatory activity [23,24. It is hence imperative to conduct anti-inflammatory study on the ethanol extract in future studies. Quinic acid, although not synthesised in human body, supports the synthesis of nicotinamide and tryptophan which are essential for DNA repair [25].

Grooming reflects the state of arousal or stimulation of the animal. Drugs that have depressant effect are known to suppress grooming in experimental animals, while those that have stimulatory effects increase grooming behaviour and vice versa [18]. The effect of the extract on head dipping exploratory activity on the hole board, as presented in Figure 2, caused a significant ($p<0.01$; $F_{(3, 16)}=28.6$) increase in the number of head dipping when compared to the vehicle thereby signifying anxiolytic activity [19, 20]. Hence, the effect of this extract on head dipping behaviour is hereby suggested to be anxiolytic.

It is also noteworthy that the fruits and seeds of *R. hookeri* is a popular substance being consumed by Cameroonians to relieve tension or anxiety. This probably explains why the plant is being voraciously consumed. Further studies

can be undertaken to evaluate long term effect of this plant and a potential product to be developed further as an effective remedy for treating anxiety and related disorders in humans.

5. CONCLUSION

From all the results above, it can be inferred that *R. australis* is rich in phytochemicals which could be of good pharmacological relevance. The phenolic compounds some of which act as either CNS stimulants or as sedatives. These significant biological results obtained from grooming, head dipping and anxiolytic tests were suggested to be due to the presence of some constituent phytochemicals including proanthocyanidin.

The impetus behind this study was an undocumented report that *R. hookeri* from West Africa, which was often consumed by Cameroonians, effected a state of calm on the mind. This shows that *R. australis* palm from Kwazulu Natal, much more than its use as a decorative tree, the source of brooms or strong ropes, could be exploited for its far-reaching medicinal potential.

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