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Phytochemical Screening, Chromatographic Profiling and Anxiolytic Effect of *Raphia australis* Ethanol Extract in Adult Male Mice

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study seeks to evaluate the anxiolytic effect of the ethanol extract of the pulp of *Raphia australis* when tested in adult male mice, and to identify some of the anxiolytic secondary metabolites present in the plant's pulp extract.
Methodology: Phytochemical screening was carried out to determine the various classes of secondary metabolites present in the ethanol extract of *R. australis*. Both column chromatography

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and Liquid chromatography-mass spectrometer (LC-MS) were utilized to identify the bioactive compounds present in the ethanol extract of the pulp of *Raphia australis*. The extract was further subjected to Novelty Induced behaviour and anxiolytic tests using 4 groups of mice for each experiment comprising 5 mice in each group (n=5). Group one was administered normal saline (0.09% w/v NaCl. p.o) while 2 and 3 were administered 500 $mgKg^{-1}$ and 1000 $mgKg^{-1}$ (p.o) of ethanol extract of *Raphia australis* respectively. Group four was administered 2 $mgKg^{-1}$, (p.o.) of diazepam (control).

Results: The phytochemical screening result was positive for all the classes of secondary metabolites tested for. The LC-MS 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). When mice were injected with *Raphia australis* extract 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 bioactive compounds which could be utilized for relief of stress and anxiety.

Keywords: Raphia australis; chromatography; phytochemical screening; umbelliferon; anxiety; sedate.

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 jacq., Phoenix reclinate jacq., Dypsis canaliculate (jum.)beentje & j.dransf, Raphia farinifera (gaertn.)Hyl., Raphia hookeeri G.Mann & H.Wendl, Raphia vinifera P.Beauv., and Raphia australis Oberm. & Strey. (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 *R. 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] (Fig. 1).

Fig. 1. Images of *R. australis* fruit used for this work

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, hyperglycemia [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 bioactive secondary metabolites.

Anxiety is generally described as a feeling of uneasiness, tension, depression or apprehension. The physical symptoms of anxiety include sweating, palpitation, (tachycardia) and, sometimes, trembling. Anxiety disorders are the major causes of mental disability. Majority of the medicinal approach to the treatment of anxiety utilizes orthodox drugs known as anxiolytics. These include classes of drugs and their examples: Barbiturates (pentobarbital and phenobarbital), benzodiazepines (clonazepam, lorazepam, triazolam and diazepam) and nonbenzodiazepines (zaleplon and zolpidem) [10].

Natural products have gained ethno-cultural popularity around the world, perhaps because they are known to exhibit mild to no side effects compared to orthodox drugs [11]. The impetus behind this study was an undocumented report that *R. hookeri* from West Africa (Cameroon), which was often consumed by Cameroonians, effected a state of calm on the mind. This report necessitated the need to embark on scientific inquiries tailored towards the anxiolytic relevance of the fruit pulp of *R. australis*. To date and to the best of our knowledge, there has been no documented report on the use of *R. australis* for the treatment of anxiety, hence the rationale for this work.

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 pulp of *R. australis* was manually separated from the pulp and both were sun-dried.

2.2 Preparation of Plant Extract

The scale of *R. australis* fruit was peeled off, and the pulp was separated from the fruit. The pulp was sun dried, and size reduction was done to enhance easy extraction. 600 g of the dried grinded 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 40^{o}C. The concentrated filtrate was then transferred into a beaker to dry until a constant weight was obtained. Ethanolic extract gave the highest percentage yield.

2.3 Animal Handling

Mice (20-30 g) 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 cycle at temperature of 22 ± 2 ℃ 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.

2.4 Experimental Setup

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

Qualitative phytochemical screening, using the method of Trease and Evans, 1987 [12], was carried out on the dried plant samples to determine the presence of phytochemicals in the ethanol 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 two groups [B (21-25), K (86-95) based on similarity in their R_f values.

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

The two fractions (B and K) which were further analysed using Liquid chromatography/ Mass Spectrometry. 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.

2.5 Evaluation of Biological Activities of *R. australis* Pulp Extract

2.5.1 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 [13]. 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}).

2.5.2 Novelty-induced behavior

Novelty-induced behaviour was assessed by methods described by Akanmu et al. [14] with modification. Four groups of mice comprising 5 mice in each group (n=5), were randomly selected. Group 1 was administered the vehicle (5% Tween 80, 10 mg/kg. p.o.). Groups 2 and 3 were injected with the extract (500 mg/kg, 1000 mg/kg, p.o.) respectively. Group 4 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).

2.5.3 Anxiolytic test

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

2.5.4 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 [15]. The results obtained were analysed and compared among the groups.

2.5.5 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" [16,17].

2.5.6 Analysis of results

All results were presented as Mean ± SEM and further analysed with one way analysis of variance (ANOVA), followed by Dunnet's post hoc test, and values were considered significant at $p<0.05$. GraphPad Version 3.0 and GraphPad Prism Version 5 copyright © 2013 by GraphPad Software Inc. USA were used for analyzing the results.

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.

3.2 Phytochemical Screening Results

The pulp of *R. australis* was found to contain all the secondary metabolites tested for.

Nine groups of secondary metabolites were identified in the ethanol extract of the pulp (Table 2).

The results of the phytochemical screening in Table 2 shows that *R. australis* enriched with various classes of secondary metabolites.

3.3 LC-MS of the Column Fractions of the pulp of *R. australis* Ethanol Extract

Six compounds were identified from the LC-MS. The chromatograms of the compounds are shown below [Figs. 2-7] below. Each compound was identified by correlating the experimental mass to charge ratio and retention time with a corresponding literature reference. As shown summarized in Table 3, the compounds, mostly tannins and flavonoids, are epicatechin (m/z= 289.0704), proanthocyanidin (m/z= 577.1354), protocatechuic acid (m/z= 153.0182), quinic acid (m/z =191.0556), catechin (m/z= 289.0712), 7-hydroxycoumarin (m/z= 353.0873) also called umbelliferon.

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

Table 2. Qualitative phytochemical screening of ethanol extract of the fruit pulp of *R. australis*

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

+ = present; - = absent

Table 3. LC-MS of the column fractions of *R. australis* pulp ethanol extract.

Compound name	Experimental m/z Ionization mode $[M-H]^-$	m/z [Literature references]	Retention time (min)
Epicatechin	289.0700	289.0712 [18]	13.20
Proanthocyanidin	577.1354	577.1340 [18]	12.57
Protocatechuic acid	153.0200	153.0188 [18]	7.79
Quinic acid	191.0571	191.0556 [19]	1.76
Catechin	289.0704	289.0712 [18]	11.12
7-hydroxycoumarin (umbelliferon)	353.0868	353.0873 [19]	11.81

m/z = mass/ charge ratio

The Chromatograms of the secondary metabolites identified by LCMS analysis:

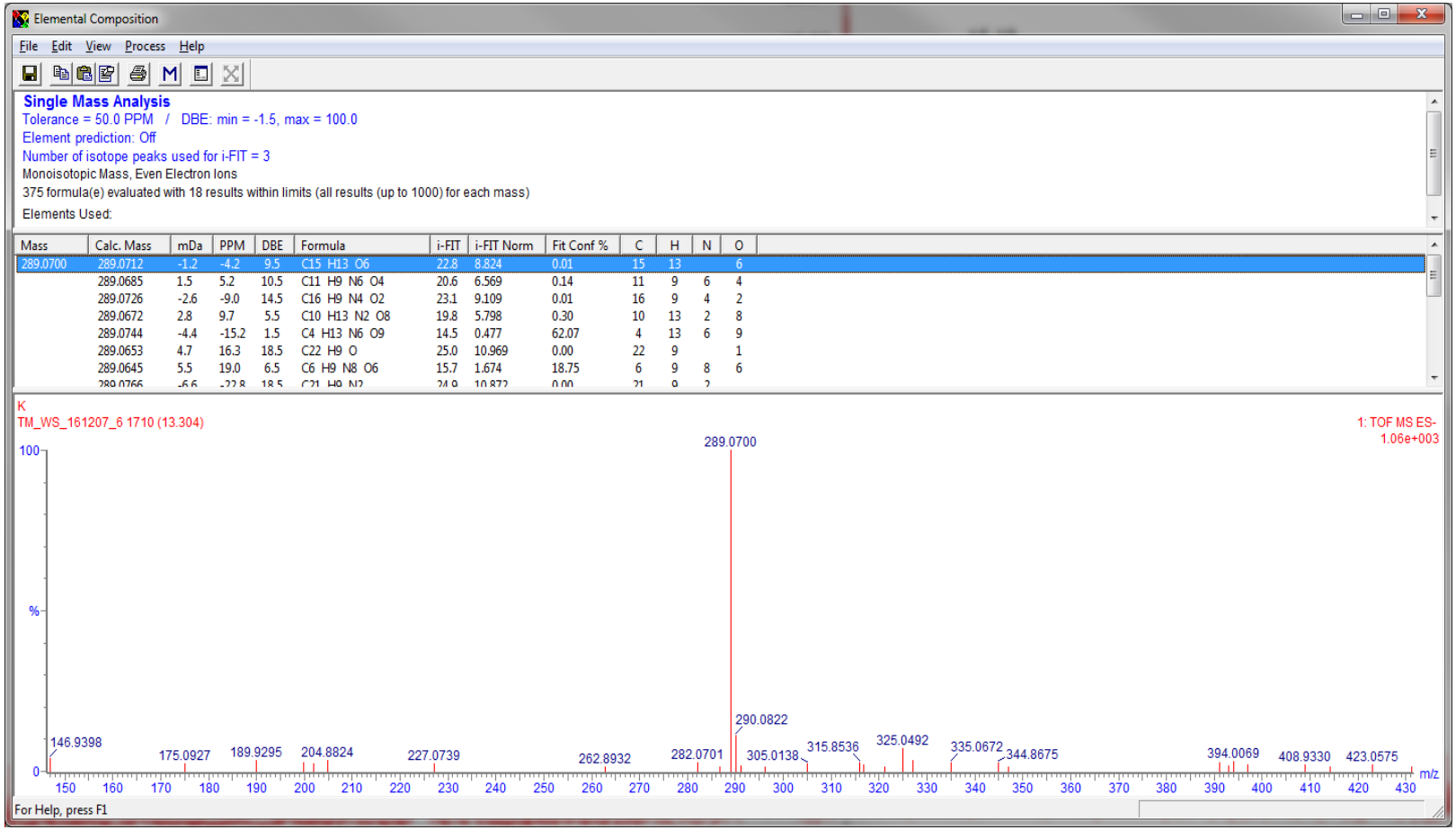

Fig. 2. LC-MS chromatogram of epicatechin

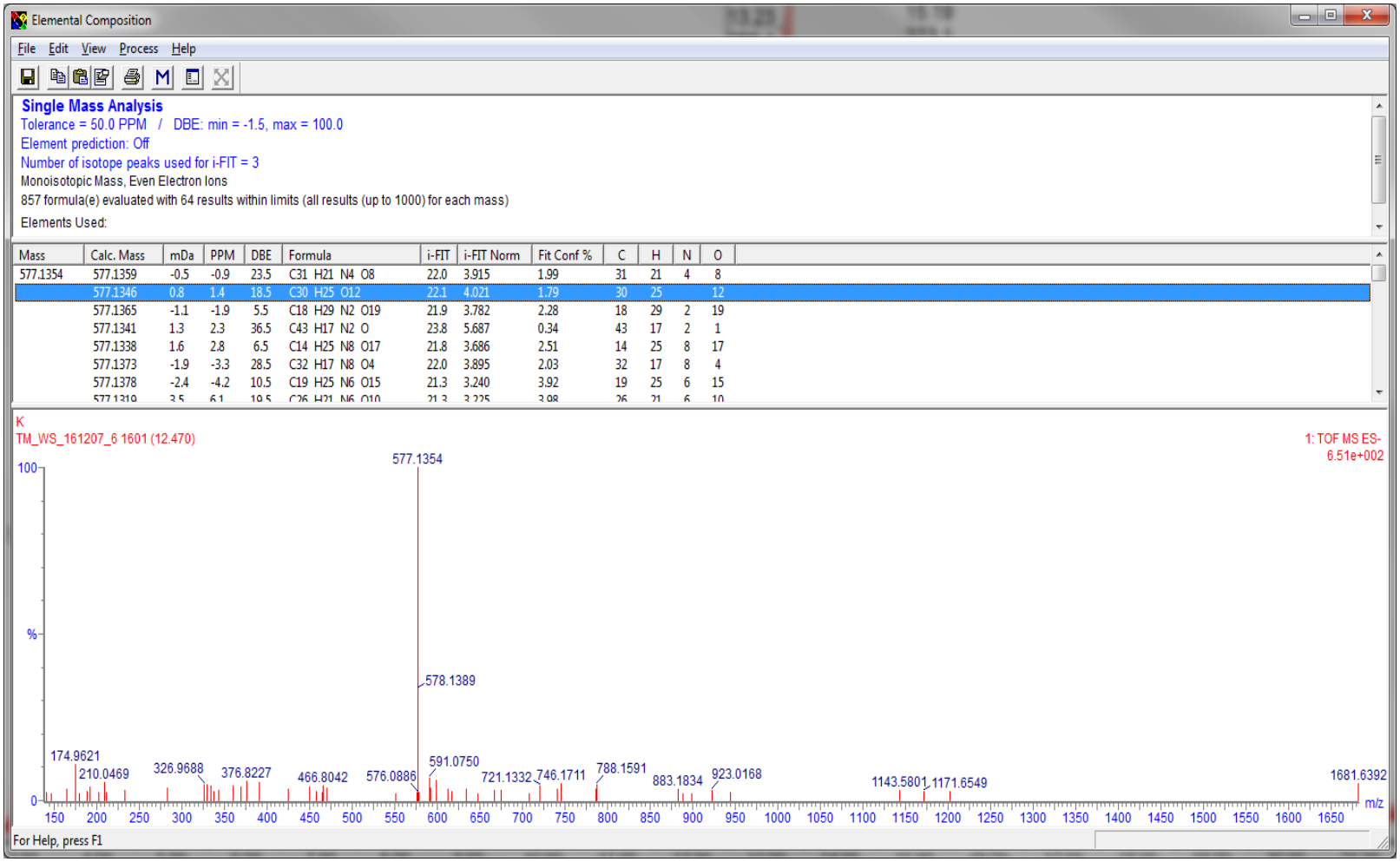

Fig. 3. LC-MS chromatogram of proanthocyanidin

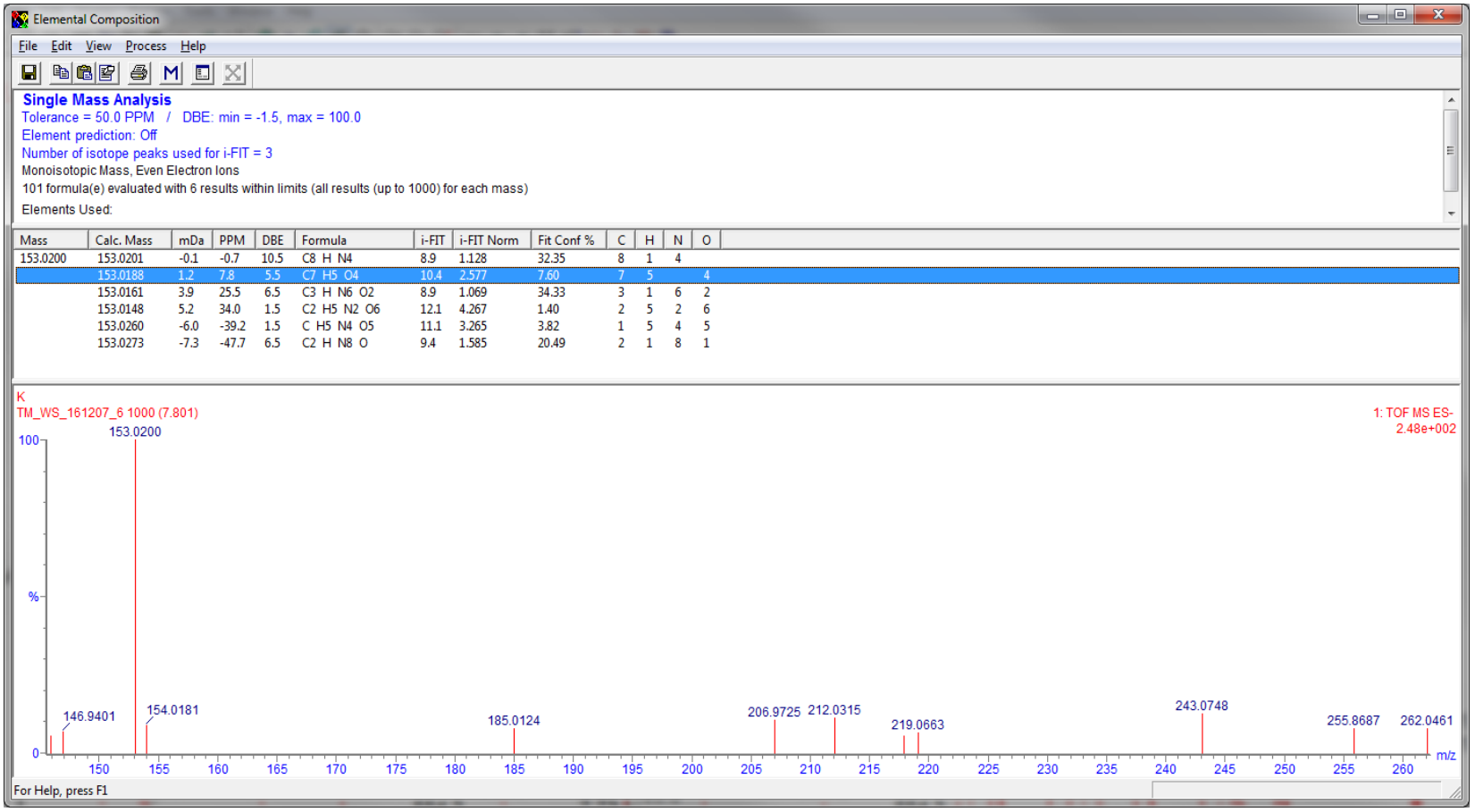

Fig. 4. LC-MS chromatogram of protocatechuic acid

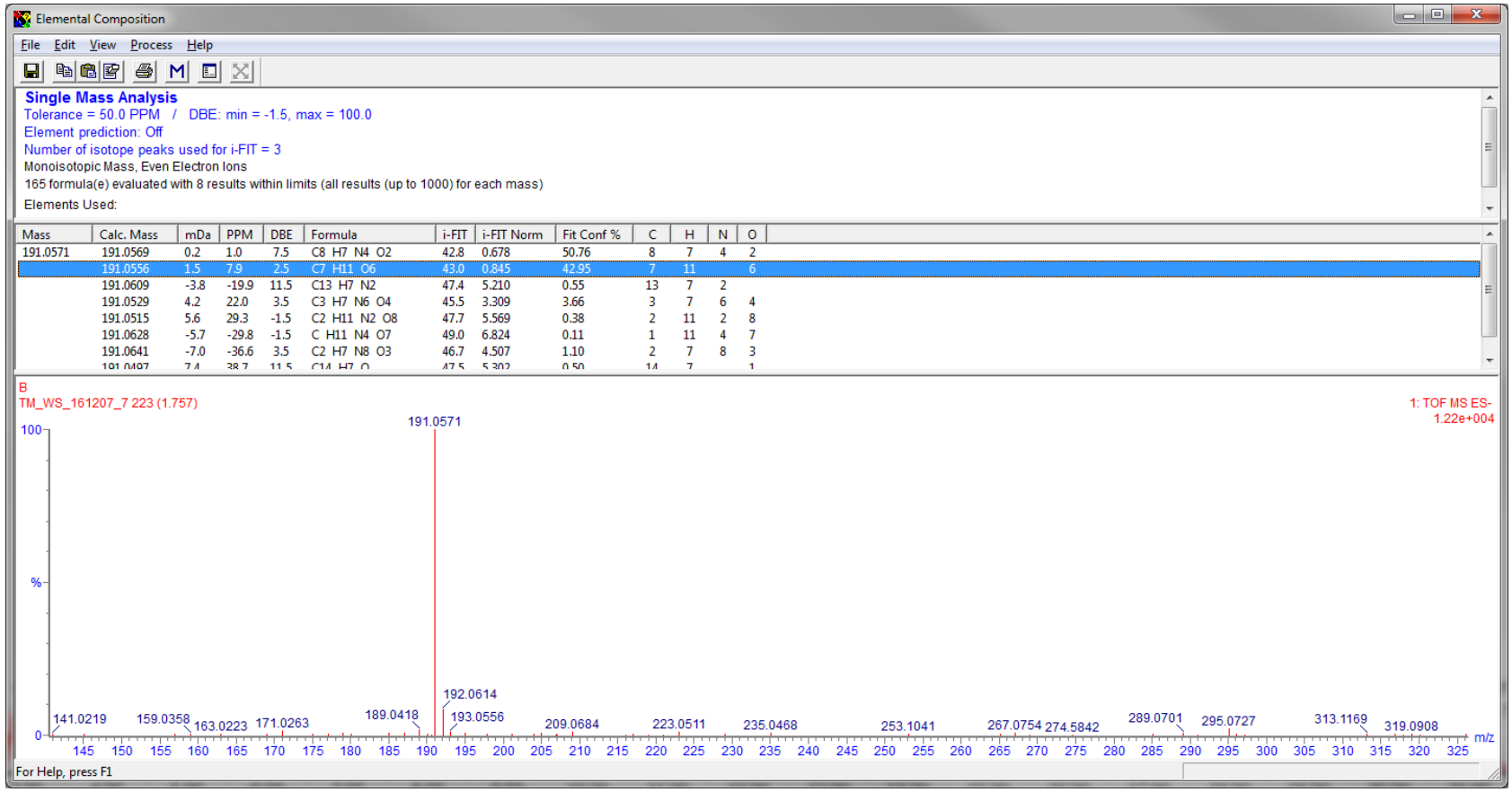

Fig. 5. LC-MS chromatogram of quinic acid

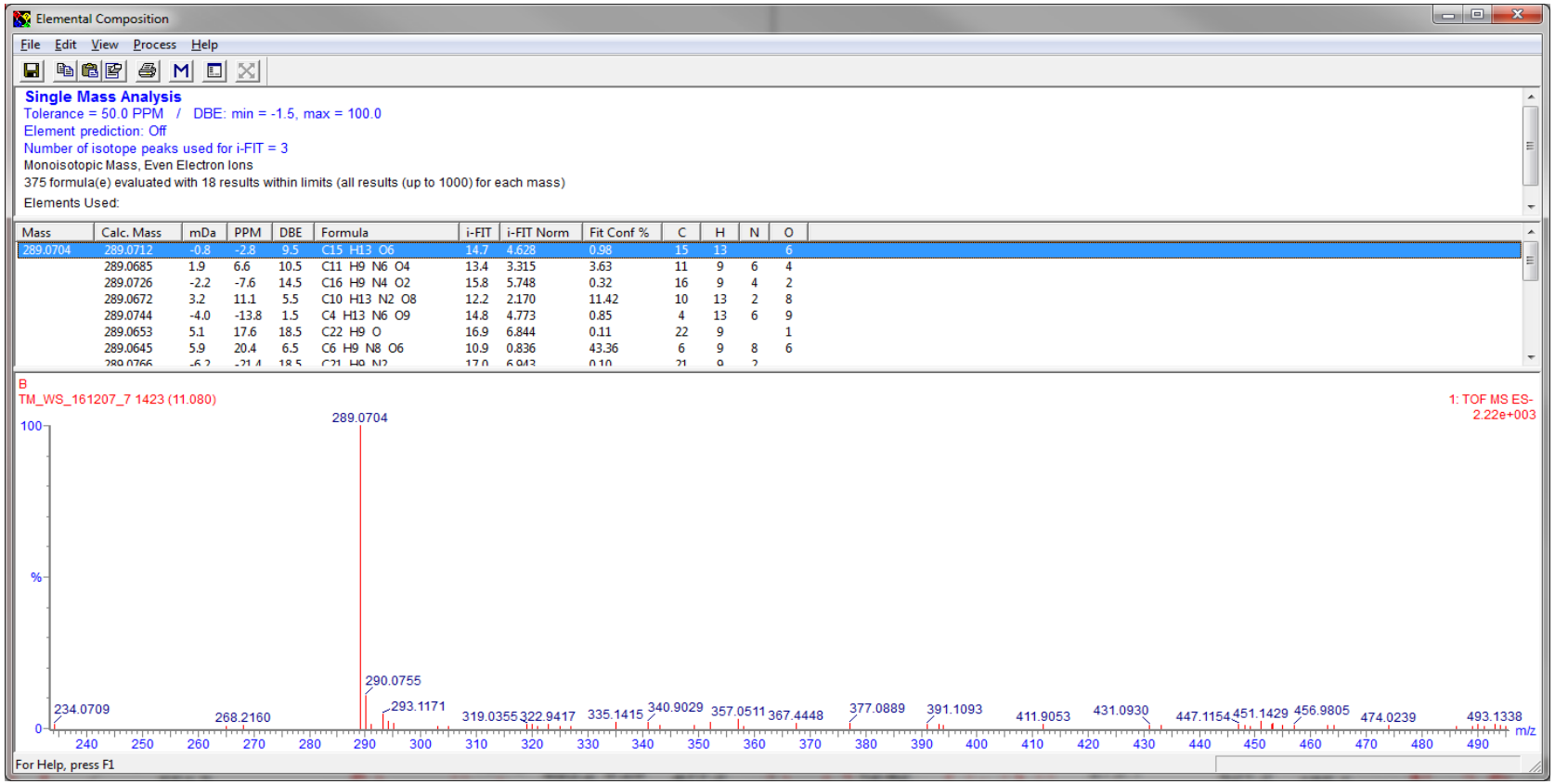

Fig. 6. LC-MS chromatogram of epicatechin

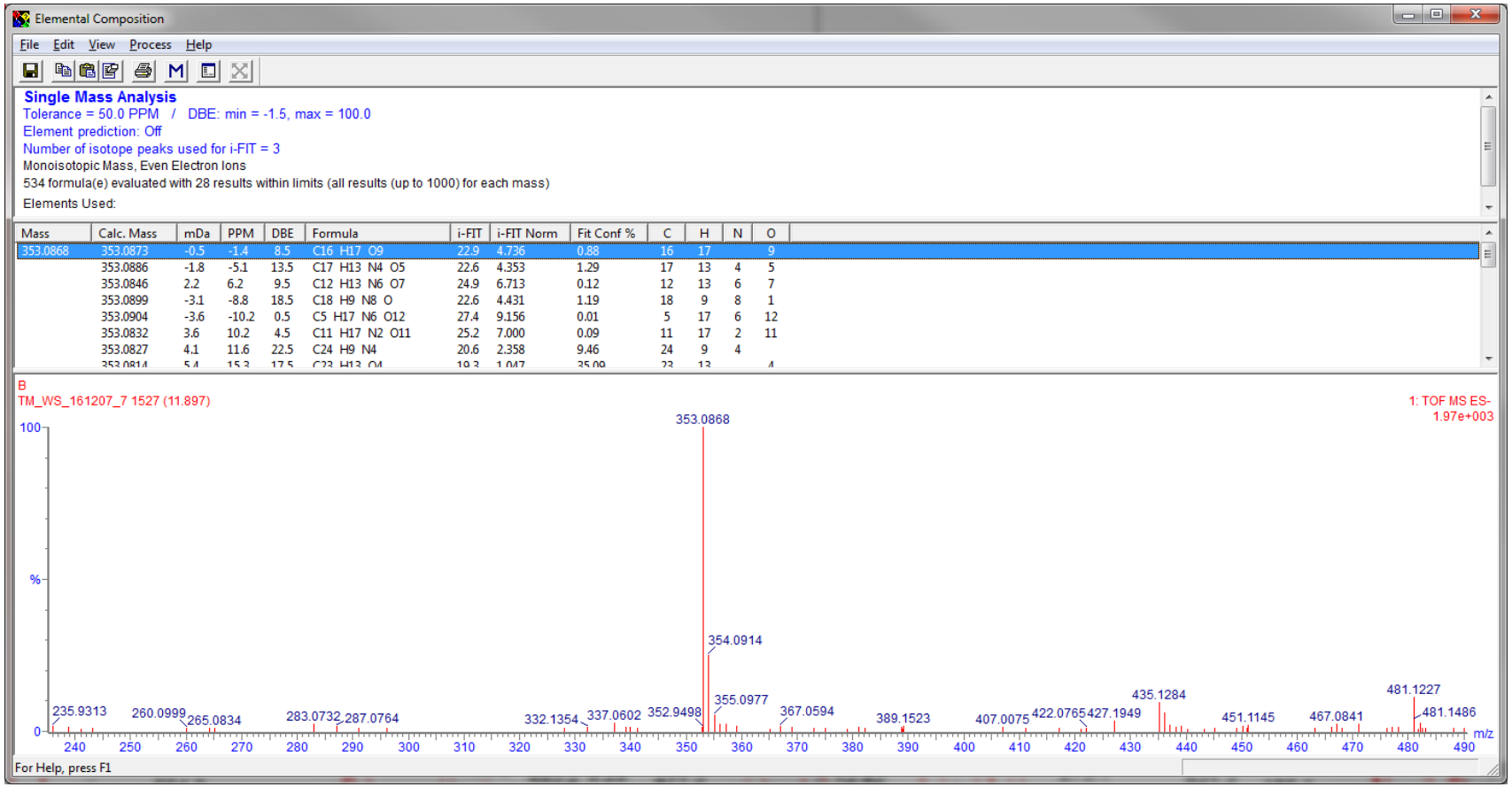

Fig. 7. LC-MS chromatogram of 7-hydroxycoumarin

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 4).

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. 8).

The extract (500 and 1000 mg/kg) and the standard drug (diazepam, 1 mg/kg) caused a significant decrease (**$p < .01$) in the number of head dips in mice compared to the vehicle (Fig. 9).

3.4.2 Anxiolytic test

The effect of the extract on the elevated plus maze (EPM) is presented in Fig. 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.

The extract (500 and 1000 mg/kg) and the standard drug (diazepam, 1 mg/kg) caused a significant increase in time spent in the open arm compared to the vehicle.

Table 4. 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**

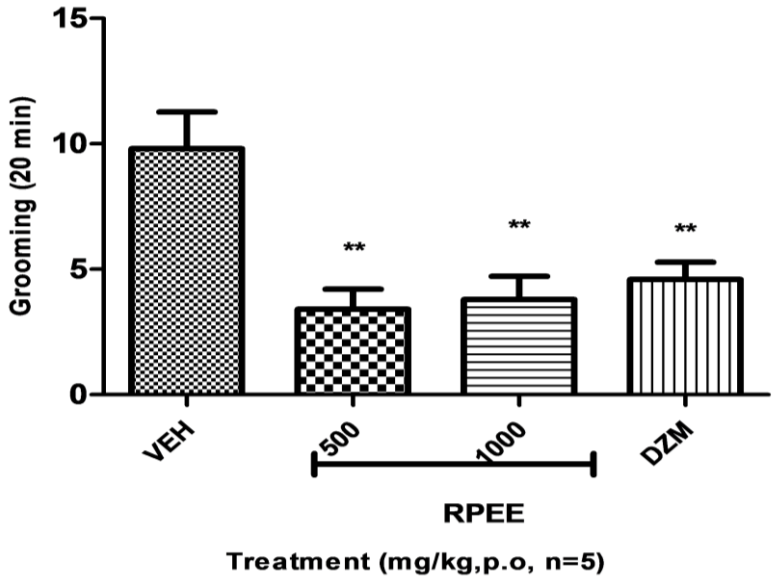

Fig. 8. Effect of *R. australis* pulp ethanol extract on novelty-induced grooming behaviour in mice

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)*

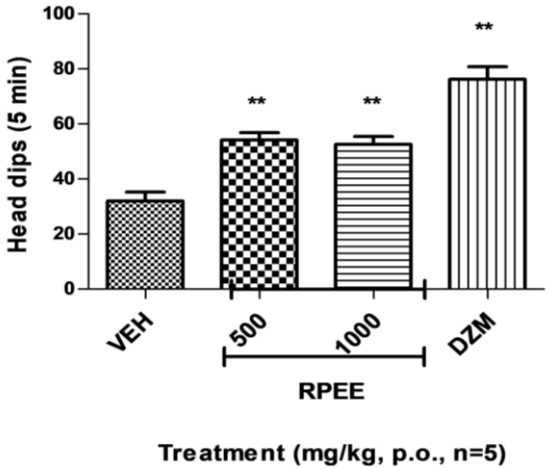

Fig. 9. Effect of *R. australis* pulp ethanolic extract head dip behaviour in mice

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)*

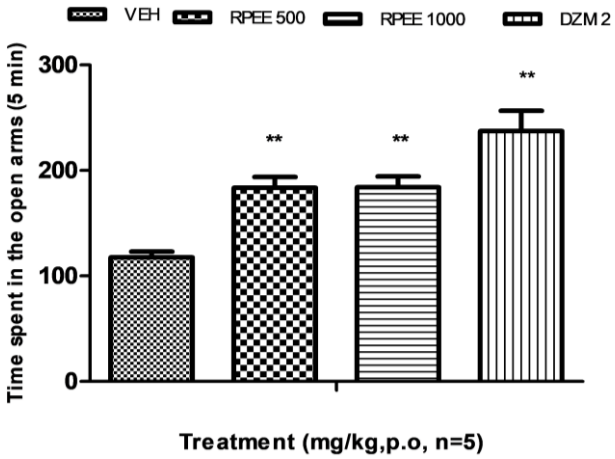

Fig. 10. Effect of *R. australis* pulp ethanolic extract on anxiolytic elevated plus maze (EPM) behaviour in mice

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 an alicyclic ring. The solvent system (ethyl acetate: ethanol: aqueous ammonia in the ratio 5:3:2) discovered during this study is therefore recommended for chromatograph 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 4 suggest that consumption of the fruit of this plant is safe and may not constitute severe health hazard. According to Lorke [13,20], 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.

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 [22]. The effect of the extract on head dipping exploratory activity on the hole board, as presented in Fig. 9, 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 [23,24]. Hence, the effect of this extract on head dipping behaviour is hereby suggested to be anxiolytic.

It is also noteworthy that the fruit 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 preclinical studies can be undertaken to evaluate a potential chronic effect of this plant on humans. Isolation of bioactive compounds from the various parts of this plants need to be explored to obtain isolated leads or scaffolds from which analogues with greater efficacy and potency can be synthesized for the treatment of anxiety and related disorders in humans.

5. CONCLUSION

From all the results above, it can be inferred that *R. australis* is rich in phytochemicals, especially the phenolic compounds, which could exhibit therapeutic effects by acting 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. 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.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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