

Research Article

Characterization and Toxicity Studies of Caffeine Isolated from Root Bark Extract of *Piliostigma thonningii* (Schum.)

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ABSTRACT

Caffeine is the most frequently ingested pharmacologically active substance in the World. It is found in common coffee, tea, soft drinks, in products containing cocoa or chocolate, and in medications among others. Because of its wide consumption at different levels by most of the people, the scientific community as well as the general public have expressed interest in the potential for caffeine to produce adverse effects on human health. This study was carried out to isolate and characterize caffeine from root bark extract of *Piliostigma thonningii* and to study the toxicity profile of the isolated isolate. The ethyl acetate fraction was gradiently eluted in a silica gel column to afford an isolate coded EA, in which its purity was confirmed by preparative thin layer chromatography (TLC). Structure of the isolate was elucidated using spectroscopic techniques such as ¹H NMR, ¹³C {¹H}NMR and Infrared spectroscopy (IR). and literature comparison suggested the isolated compound EA as an alkaloid namely 1,3,7-Trimethylpurine-2,6-dione. *Drosophila melanogaster* oral toxicity of the EA against 30 adults fruit flies (15 males and 15 females) was carried out using standard procedure. results showed mortality rate of 0 % at 14 mg/10g diet for 7 days. The Locomotor assay was done using negative geo-axis assay, results showed 70 % of flies reached the top of the glass bottle (10 cm height) at concentration of 5 mg/10g diet. The isolate EA from *P. thonningii* root bark is safe for oral consumption up to a concentration of 14 mg/10gdiet.

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INTRODUCTION

Plant substances have continued to serve as a viable source of drugs for the World population and several plant-based drugs are in extensive clinical use (Roja and Rao, 2000). In the past few decades, a number of plants have been widely used for the treatment of various diseases due to their antioxidant properties (Mshelia *et al.*, 2008). Approximately 80% of the World's population relies on traditional medicine for their primary health care, and most of these practices include the use of plant extracts or their active components (WHO, 2000). With the increase in people's demand and preference for natural products and medicines, herbal product industries are increasing day by day (Shinwari *et al.*, 2006). Many medicinal plants have been investigated worldwide for their chemical components and some of the isolated compounds have been shown to possess biological activity. Among these plants are *Melia azedarach* and *Xylopi aethiopica* used for the treatment of cough, stomach upset, skin infections, and dysentery among others. About 40% of recent drugs in practice have been developed from natural products mostly plants (Jacob, 2009). Research indicates that caffeine may help protect human brain cells, which lowers the risk of developing some diseases, such as Parkinson's. It also relieves asthma attacks and increases the potency of analgesics (Aniket *et al.*, 2019). Death due to caffeine intake is not common, and only a few cases have been reported in the literature. The survival of a patient who allegedly ingested 24 g of caffeine was reported. There is ample evidence indicating that for the general population of healthy adults, moderate caffeine intake at a dose level of 400 mg/day is not associated with adverse effects such as general toxicity, cardiovascular effects, changes in adult behavior, and increased incidence of cancer and effects on

male fertility. Nor are moderate intakes of caffeine associated with adverse effects on bone status and/or calcium balance if adequate intakes of calcium are being consumed (Nawrot *et al.*, 2003).

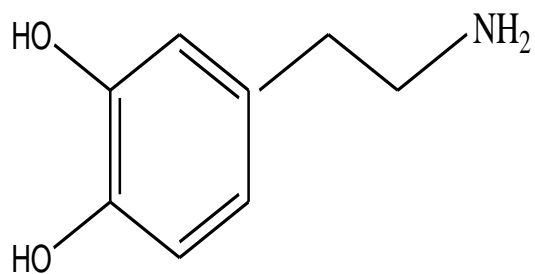
Figure 1 (a-d) showed some plant-derived alkaloids

Toxicity testing is important in evaluating the safety profile of drugs and herbs intended to be used by humankind for the treatment of diverse ailments (Rajalakshmi *et al.*, 2014). *Drosophila* has been used to gain insight into molecular, cellular, developmental, and disease processes that are conserved in mammals, including humans, as most of these fundamental biological mechanisms are shared throughout the animal kingdom. Although mammals have two to three times as many genes as flies, they have approximately the same number of gene families (Holland, 2003).

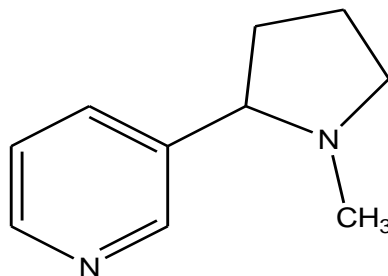
Flies and mosquitoes transmit various diseases by acting as a vector for pathogens. Flies can rapidly distribute, reproduce and have good ecological adaptability. Flies are the carriers of more than 100 different types of pathogens and develop diseases in both humans and animals (Bonney *et al.*, 2008).

The therapeutic effectiveness of medicinal plants lies in their phytochemical components especially the secondary metabolites like alkaloids, tannins, anthocyanins, anthraquinone derivatives, flavonoids, and other phenolic compounds present in these plants (Anyasor *et al.*, 2011). People consume medicinal plants especially caffeinated products without knowing the toxicity profile, in this paper, *Drosophila melanogaster* is used as a model for testing the toxicity profile of the isolated compound which is considered one of the most important genetic models' organisms for biomedical science (Nicholas, 2017). It is very much used for genetic research because the relationship between humans and the fruit fly

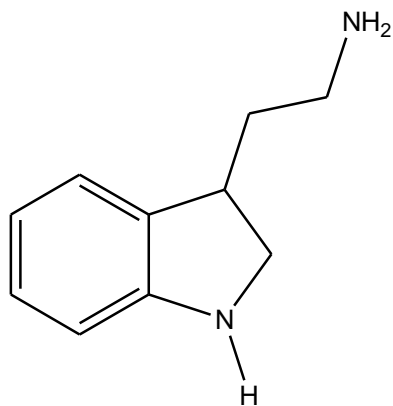
genes is very close. Plate 1 showed the life cycle of *Drosophila melanogaster*.



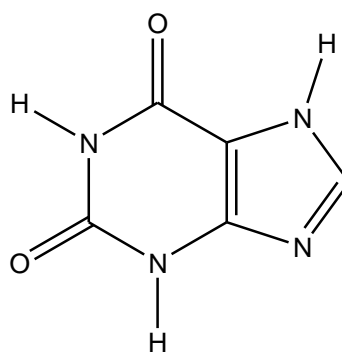
(a) Dopamine



(b) Nicotine



(c) Tryptamine



(d) Xanthine

Figure 1: Some Plants Derived alkaloids (Tadeusz, 2007; Junior, *et al.*, 2013).



Plate 1: *Drosophila melanogaster* (Mathew and Krishnamurthy, 2018)

MATERIALS AND METHODS

Chemicals and Reagents

All the solvents and reagents were purchased from Sigma Aldrich (USA) and JHD, China and were all of analytical grade. The deuterated CDCl_3 was purchased from Merck.

Instruments

The ^1H NMR (600 MHz) and ^{13}C {H}NMR (150 MHz) spectra were recorded on an NMR spectrometer (Jeol). All NMR analyses were conducted at room temperature and chemical shifts are reported as parts per million (ppm) relative to tetramethylsilane (TMS) which is used as internal standards for ^1H NMR and ^{13}C {H}NMR for CDCl_3 . Infrared spectra were recorded on an FTIR (Thermo Nicolet). For preparative TLC (Kieselgel 60 F₂₅₄, Merck), the spots were visualized by UV light (254 and 365 nm) and spraying with a 10 % (v/v) H_2SO_4 . Column chromatography was performed with silica gel (Kieselgel 60, 70– 230 mesh; Merck).

Sample Collection

Fresh *P. thonningii* root barks were collected in October, 2018 from pharmacognosy garden, Faculty of Pharmaceutical Science, Sokoto State North Western-Nigeria. They were washed with water to remove earthy impurities, identified and authenticated at the Herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Usmanu Danfodiyo University Sokoto where a herbarium specimen was deposited and a voucher number PCG/UDUS/Legus/0001 issued. They were then air dried for three weeks, powdered with the aid of a clean mechanical grinder and stored in an air tight glass container until use.

Extraction

About 300 g of the powdered sample was packed into a thimble and subjected to gradient extraction with n-hexane, ethyl acetate and methanol. The extracts were freed from the solvent using a rotary evaporator. The yield was determined according to equation 1 (Sofowora, 2008).

$$\% \text{ Recovery} = (\text{weight of extract}) / (\text{weight of sample}) \times 100 \dots \dots \dots 1$$

Characterization and Isolation

i. Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was carried out using TLC pre-coated plates (silica gel 60F254) by ascending order. Spotting was carried out manually using capillary tubes and developed in an air tight chromatographic tank at room temperature. A preliminary TLC separation of all the extracts was carried out using different solvent system. The developed

chromatographs were air dried and visualized under normal day light, ultraviolet light (254 nm and 365 nm) and by spraying with 10 % sulphuric acid followed by heating at 105 ° C for 5 min in an oven.

Different solvent system was used to develop the plate for the different extracts. The solvent system used for ethyl acetate extract is ethyl acetate: n-hexane (8:2; 7:3) and the R_f values of distinct components were calculated using the formula in the equation 2.

$$R_f = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by solvent}} \dots \dots \dots (2)$$

ii. Column

The Column Chromatography was carried out according to the method used by Kwazo *et al.* (2015). A piece of glass wool was inserted at

the base of the glass column, and then 120 g silica gel power (120 μm mesh) was made into a slurry using n-hexane and then packed into the column and a glass wool was inserted just

above the silica gel surface. The ethyl acetate extract was pre-adsorbed with 3g of silica gel. The pre-adsorbed sample on silica gel was transferred onto the column and sequentially eluted with n-hexane: ethyl acetate; 100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100. 85 (85) fractions were collected and regrouped to seven (7) based on their TLC profiles. Similar procedure was carried out for the ethyl acetate and methanol extracts. These fractions consist of major spots with some impurities. The impurities were removed using preparative TLC.

Toxicity Study

Drosophila melanogaster Flies

Seven (7) day old adult flies were obtained and identified from the Department of Pharmacology, Faculty of Pharmacy University of Maiduguri, Borno state. They were reared under controlled temperature of 25-35 °C by use of air conditioner (Syed *et al.*, 2017). The flies were kept in a bottle of 10 × 7 cm³ with the lid closed using cotton wool. The bottles were kept away from sunlight, heat source, chemicals, predator and parasitic organisms.

Preparation of 1000 g Diet Feed

Clean tap water (1.7 L) was used for preparation of the fruit flies feed. One-liter portion of the water was heated to boiling in a 3L container, agar slurry 10,000 mg in 200 cm³ of water was added to the boiling water and stirred for 10 min. Yeast slurry (20 g) and

yellow corn flour slurry (100 g) was also added and stirred for another 10 min. The feed was removed and allowed to cool to 55 °C followed by addition of 0.059 M propionic acid (5 cm³) into the mixture and stirred. The feed was quickly transferred into the bottles and further allowed to cool before introducing the flies (Bloomington *Drosophila* Stock Center, 2018).

Preparation of Ethyl Acetate Isolate for Toxicity Assay

Flies were anesthetized by introducing 1 cm³ chloroform vapour soaked with cotton wool for 2 min. Thirty adult flies (15 males and 15 females) each were introduced with the aid of a magnifying lens and the lid of the bottles covered with cotton wool. After 10 min the flies woke up from the sleep and started feeding on the isolate mixed with the food. Percentage death was calculated after one day and recorded for seven days, the concentrations of the isolates used are; 0 mg/g, 1 mg/g, 2 mg/g, 3 mg/g, 4 mg/g and 5 mg/g diet (Syed *et al.*, 2017).

Locomotor Assay

Locomotor activity was determined using the modified negative geotaxis assay.

After treatment for 72 h (3 days) three groups of 10 flies per treatment were immobilized on ice (anesthetized) for 1–2 min and were gently tapped to the bottom of a glass column and after 1 min, the number of flies that reach 10 cm of the bottle (bottom to top) were counted as described by (Jimenez-Del-Rio *et al.*, 2009).

RESULTS AND DISCUSSION

The percentage yield of the extract is presented in Table 1

Table 1: Percentage Recovery of *P. thonningii* Root Bark extracts

Extract	% Yield
n-Hexane	5.60
Ethyl acetate	11.22
Methanol	21.50

Physicochemical Properties

The physicochemical properties of the isolated compound (EA) are presented in Table 2

Table 2: Physicochemical properties of Isolate EA

Mass (g)	R _f value	Melting Point (°C)	Murexide Test
0.120	7.1	234 ± 0.2	+

¹H NMR signal observed from the isolate EA compared with literature is presented in Table 3.

Table 3: ¹H NMR for Isolate EA Compared with Reported Literature Data

Position	¹ H (ppm)#	¹ H (ppm)*
1	3.35	3.37(s)
3	3.53	3.55(s)
7	3.95	4.01(d)
8	7.49	7.58(d)

*Sitwoski *et al.*, 1995; #this work

The ¹³C {H} NMR chemical shifts observed for compound EA compared with literature values for caffeine are presented in Table 4.

Table 4: ¹³C {H} NMR for Isolate EA Compared with Reported Literature Data

Position	¹³ C {H} NMR (ppm)#	¹³ C {H} NMR (ppm)*
C-1	27.90	27.50
C-2	151.70	151.30
C-3	29.80	29.30
C-4	148.60	148.30
C-5	107.60	107.10
C-6	155.40	154.90
C-7	33.60	33.20
C-8	141.40	141.20

*Sitwoski *et al.*, 1995; #this work

Spectroscopic Analysis of EA

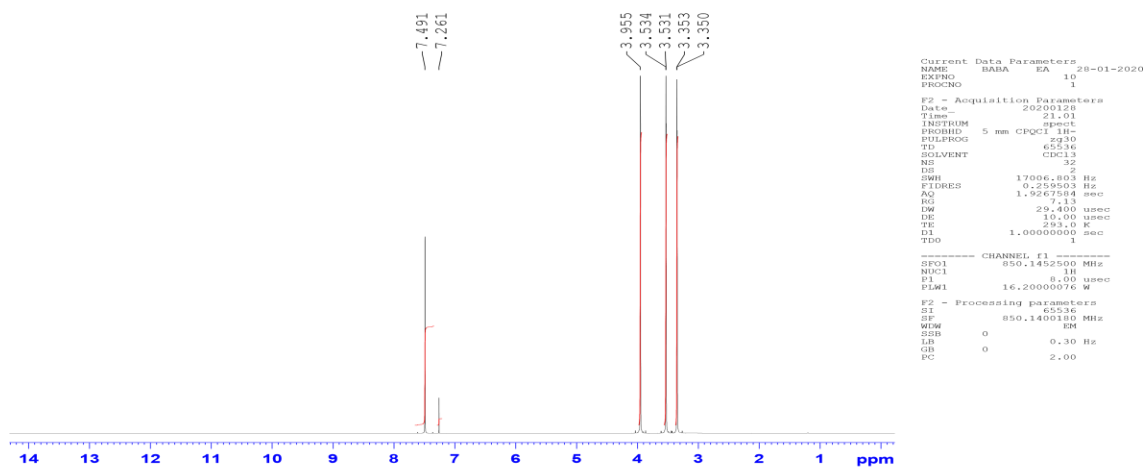


Figure 2: ^1H NMR spectrum of the isolate EA (600 MHz, CDCl_3)

The Infrared signal observed from the isolate EA compared with literature is presented in Table 5.

Table 5: Comparison of the Infrared Peak of the Isolate (EA) with Reported Literature Data

Bands	ν (cm^{-1})#	ν^* (cm^{-1})
C-H	3138	3130
N-CH ₃	3800	3855
C=O	1690	1692
C-N	1261	1238
C=C	1578	1577

*(Al-Hitti and Ibrahim, 2009; Yunus and Nulamuga, 2020); #this work

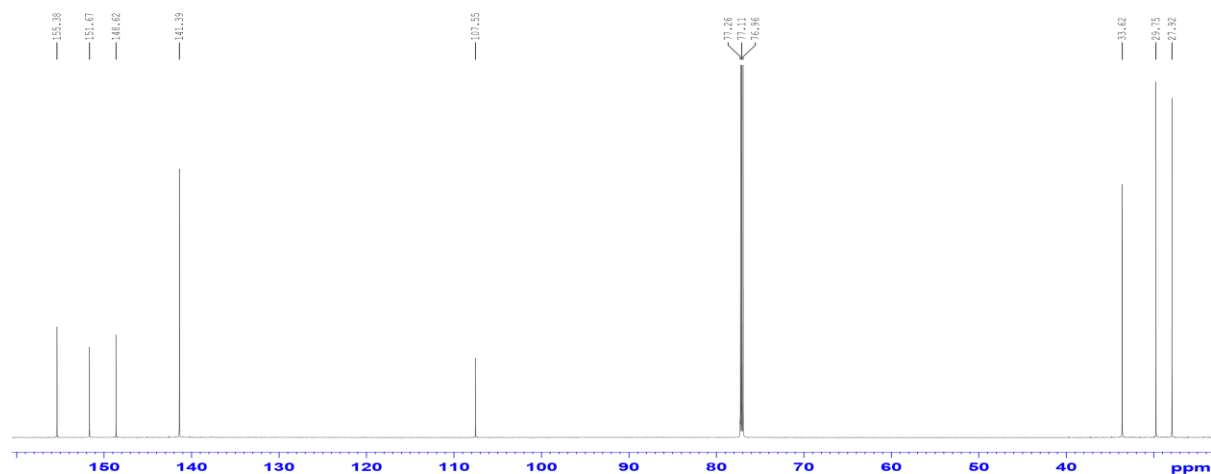


Figure 3: $^{13}\text{C}\{\text{H}\}$ NMR spectrum of the isolate EA (150 MHz, CDCl_3)

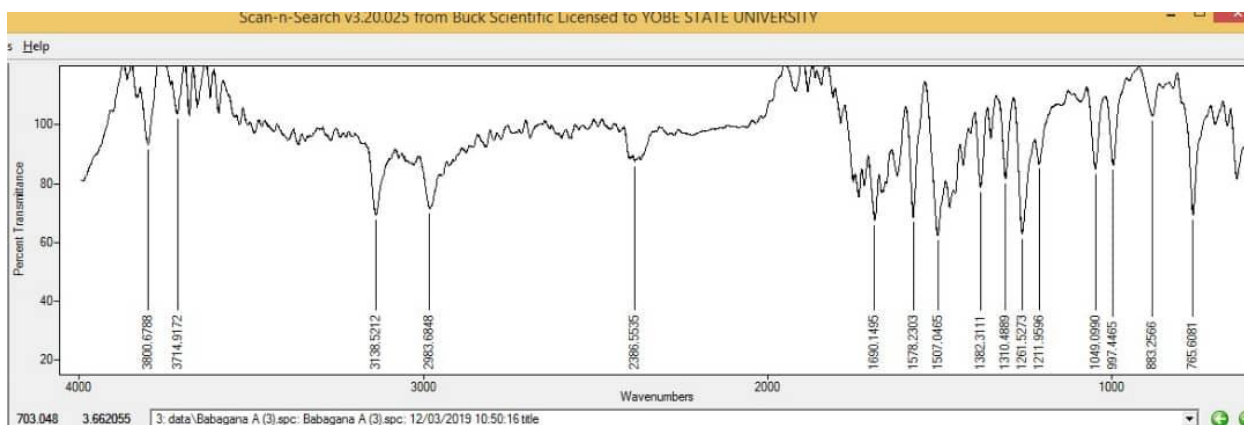


Figure 4: IR Spectrum of isolate EA

Based on the spectral data (^1H NMR, $^{13}\text{C}\{^1\text{H}\}$ NMR and IR) and literature comparison, the structure of isolate EA is presented in Figure 5.

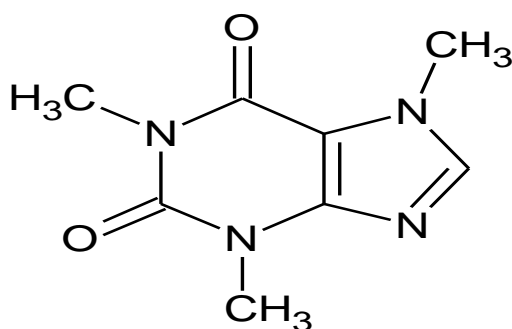


Figure 5: Caffeine ($\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2$).

Toxicity of EA

The detailed profile of toxicity of the isolate EA against the fruit flies (*D. melanogaster*) is presented in Table 6.

Table 6: Toxicity Profile of EA against *D. melanogaster*

Conc. (mg/10g diet)	Number of Flies reaching the bottle top (%)
1	0
2	0
3	0
4	0
5	0

Locomotion Assay

The locomotion assay of flies after 3 days of exposure to the isolate EA are presented in Table 7

Table 7: locomotion assay of flies after 3 days of exposure to the EA

Conc. (mg/10g diet)	Number of Flies reaching the bottle top (%)
2	90
4	88
6	85
8	84
10	80
12	79
14	70

DISCUSSION

The percentage yield of the extracts used for the analysis showed that methanol had the highest percentage recovery, followed by ethyl acetate and then n-hexane extracts (Table 1), this variation could be due to the polarity of methanol which is greater than the other solvents. This is in line with the results obtained from (Halilu *et al.*, 2017).

The isolate EA is a white and amorphous solid at room temperature, it is soluble in ethyl acetate and chloroform. It has a melting point of 234 ± 0.2 °C. This value is in close agreement with the value 234-239 °C as reported (Pradeep, 2015). The isolate is positive when tested for caffeine using Murexide test. its mass was recorded as 120 mg and R_f value of 7.1 (Table 2).

$^1\text{H-NMR}$ of EA (Table 3 and Figure 2) showed the isolate contains 4 protons. One aromatic proton and three protons due to methyl groups. A chemical shift of 7.49 ppm (Figure 3) is characteristic of the aromatic signal which is assigned to the proton at position C_8 , this might be due to the deshielding effect of the neighboring electronegative nitrogen atom and the proton experience a higher external magnetic field while the three methyl protons

(CH_3) at 1, 3 and 7 positions are highly shielded and are at the furthest upfield region of the spectrum (Sitwoski *et al.*, 1995).

The result of the $^{13}\text{C} \{^1\text{H}\}$ NMR of the isolate (Table 4 and Figure 3) showed that the isolate EA consists of 8 carbon atoms and has an aromatic character (Mohamed *et al.*, 2015). The chemical shift of 107.55 ppm and 148.6 ppm are characteristic of the aromatic signal which was assigned to C_4 and C_5 which are downfield due to sp^2 hybridization of the aromatic ring. The chemical shift of δ 27.92, δ 29.75, and δ 33.62 ppm which are attributed to C_1 , C_3 , and C_7 respectively are carbons of methyl groups that are all upfield due to the shielding effects of the protons. The overall $^{13}\text{C} \{^1\text{H}\}$ NMR data were in full agreement with data on caffeine (Sitwoski *et al.*, 1995).

The frequencies of the functional groups in the isolate compared with the literature (Table 5 and Figure 4) showed a Chemical shift of 3138 cm^{-1} was also attributed to the hydrogen region (C-H). Signals of 1261 and 1690 cm^{-1} are attributed to C-N and C=O respectively. A signal of 1578 cm^{-1} is a result of the alkene C=C double bond. The overall IR data are in agreement with data reported for caffeine

(Sitwoski *et al.*, 1995; Al-Hitti and Ibrahim, 2009; Yunus and Nulamuga, 2020).

To the best of our knowledge, this is the first report of the presence of caffeine isolated from the root bark of *Piliostigma thonningii*, the caffeine might have arisen from Mycorrhizal infection in the roots or rhizobial bacteria as nearly all *Fabaceae* plants have N-fixing root nodules (Vivienne and Felix, 2004; Wink, 2013). The toxicity profile of the plant was studied for seven days using adult *Drosophila melanogaster*. The result of the toxicity assay of EA isolate from ethyl acetate extract was presented in Table 6, from the results the mortality rate of this isolate tested against the 30 adults (15 males and 15 females) fruit flies was found to be 0 % at all concentrations of the isolate for 7 days. Ventrella *et al.* (2016) reported that glycoalkaloids α -solanine extracted from the potato when exposed to *D. melanogaster* at different concentrations reduced the number of fruit flies reaching the pupal and imaginal stages, which has no effect on the adult fruit flies.

Table 7 showed the locomotion of flies exposed to isolate EA. Negative geotaxis consists of the ability of flies to move vertically when startled and is a common measure of locomotor behaviour. From the Figure, the number of flies at the top of the bottle (10 cm long) was 90 %, 88 %, 85 %, 84 %, 80 %, 79 % and 70 % at concentrations of 1, 2, 3, 4 and 5 mg/10g diet respectively. Age-related defects in physiological processes and behaviors depend on the normal function of the nervous system (Malenie and Mike, 2011).

CONCLUSION

Caffeine (1,3,7-Trimethylpurine-2,6-dione) was isolated from the ethyl acetate fraction of *Piliostigma thonningii* root bark using low-pressure column chromatography and characterised. The structure of the compound was unveiled using ^1H NMR, $^{13}\text{C}\{\text{H}\}$ NMR, and FTIR spectroscopic studies. *Drosophila*

melanogaster oral toxicity of the isolate against 30 adults fruit flies (15 males and 15 females) showed a mortality rate of 0 % at a 14 mg/10g diet for 7 days. The locomotor assay showed that 70 % of flies reached the top of a glass bottle (10 cm height) at a concentration of 5 mg/10g diet.

Declaration of Competing Interest

The authors listed on this paper, were fully involved in the authentication, data generation, examination, and evaluation of manuscript among others.

Declaration of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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