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PHYTOCHEMISTRY, ISOLATIONSAND SOME PHARMACOLOGICAL STUDIES OF ETHANOL LEAF EXTRACT OF *PILIOSTIGMA THONNINGII*

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ABSTRACT

This study was aimed at evaluation of phytochemical constituents and the effect of ethanol leaf extract of *Piliostigma thonningii* on the central and peripheral nervous systems in laboratory animals. Fresh leaves of *Piliostigma thonningii* were air-dried, pulverized extracted using soxhlet extraction technique with ethanol 148.24% w/w after being concentrated. The extract was screened for phytochemicals using standard methods. 20 g of the ethanol extract was subjected to column chromatographic (CC) analysis using ethylacetate and n-butanol as mobile phase at different ratios and silica gel of 60-120 mesh as the stationary phase. Fractions obtained with similar retention factor (R_f) using thin layer chromatography (TLC) were combined, coded and subsequently screened for phytochemicals. Subsequent purification of fraction PTE3 was carried out using CC (ethylacetate and methanol were used as mobile phase at different ratios) and TLC until a sub-fraction PTE34 amongst other fractions gave a single spot on TLC and had a melting point of 102-103 °C. The phytochemical studies of the ethanol leaf extract of *Piliostigma thonningii* revealed the presence of some useful chemical compounds such as flavonoids, cardiac glycosides, tannins, saponins, and terpenoids. The pharmacological effects of *Piliostigma thonningii* was

determined by examining the effects of the leaf extract on phenobarbitone sleeping time, analgesic and muscle relaxant activities using experimental animals. The analgesic effect of the leaf extract was evaluated with acetic acid induced writhing and thermally induced Nociception for pain. It was observed that the extract conferred 48.00 and 57.20% protection from writhes induced by acetic acid on mice when extract doses of 200 and 400 mg/Kg were administered. Similarly, there was a significant ($p<0.5$) dose dependent effect conferred on mice when pain was induced by heat. The extract also had a muscle relaxant effect as 20%, 60% and 80% were observed to slide down an inclined board in a dose dependent manner. The extract also significantly potentiated sleeping time of phenobarbitone dose dependently in rats of which the mean time duration of (72.0±04.64) min, (83.40±02.11) min, and (123.60±11.57) min were observed when rats were administered extract doses of 200, 400 and 600 mg/Kg b wt. Thus the ethanol leaf extract of *Piliostigmathonningii* was able to providedepressant effects which were shown in its ability to potentiate barbiturate sleeping, analgesia and muscle relaxant effect.

Keywords: *Piliostigma thonningii*, Phytochemicals, Ethanol Leaf Extract, Chromatography, Pharmacology

INTRODUCTION

Medicinal plants have been the source of traditional herbal medicine amongst rural dwellers since antiquity. The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premises [1]. In Africa, knowledge of traditional medicine as part of a holistic

system was passed through generations by oral communication and indigenous practices [2]. In more recent years, with considerable research, it has been discovered that many plants possess medicinal values [3].Approximately 70,000 plant species have been used for medicinal purposes[4]. These plants are cheaper and more accessible to most of the population in the world whose medicinal value lies in some chemical substances that produce a definite physiological action on the human body [5].The global demand for herbal medicinal products has increased significantly in recent years. It is estimated that the world's population will be more than 7.5 billion in the next 10 to 15 years. This increase in population will occur mostly in the southern hemisphere, where approximately 80 % of the population still relies on a traditional system of medicine based on herbal drugs for primary healthcare [6].A complete understanding of medicinal plants involves a number of disciplines including commerce, botany, horticulture, chemistry, enzymology, genetics, quality control and pharmacology [1]. As a result of recent interest in the plant kingdom as a potential source of new drugs, strategies for the fractionation of plant extracts based on biological activity rather than a particular class of compound have been developed and the chemical examinations follow after isolation of the active fractions [7].*Piliostigma thonningii* (Schumach) Milne-Redhead [Leguminosae-Caesalpinioideae] is a plant used for medicinal purposes in many African countries. Different parts of the plant have been used traditionally for the treatment of various diseases in humans and animals [8]. For example, the roots and twigs have been used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections [9, 10]. The leaf is useful in the treatment of malaria fever [11, 12]. The leaves of *Piliostigma*

thonningii are used to treat wounds, chronic ulcers, diarrhoea, toothache and gingivitis, cough, and bronchitis [13].The dry leaf powder has been reported to contain alkaloids, saponins, flavonoids and tannins [14]. Besides carbohydrates, glycosides, flavonoids, tannins, saponins, balsams, volatile oil and terpenes have also been isolated from the leaves of *Piliostigma thonningii* [15].

MATERIALS AND METHODS

Sample Collection, Identification and Preparation

Fresh leaves of *Piliostigma thonningii* (Schumach) Milne-Redheadwere collected in the month of May, 2013 from Jumu'a village in Potiskum, Yobe State, Nigeria. Jumu'a village is about 7 km east of Potiskum. Potiskum is a Local Government Area in Yobe State in Nigeria lying at 11°42'36" North of the equator and 11°4'12" East of Greenwich Prime Meridian, which lies approximately 1297 km north of equator (NIPOST, 2009). It has an area of 559 km^2 (216 sq mi) and a population of 205,876 at the 2006 census(NIPOST, 2009).

The plant material was identified and authenticated to be *Piliostigma thonningii*by a plant Taxonomist in the Department of Biological Sciences, University of Maiduguri, Borno State, Nigeria. It was given a voucher specimen number 548A and deposited at the Postgraduate Research Laboratory of the Department of Chemistry, Faculty of Science, University of Maiduguri.

The leaves were cleaned and air-dried under shade at room temperature for several days (Rates, 2001) and pulverized using mortar and pestle and then subjected to the following analysis.

Plant Extraction

Chemicals and Reagents

All the chemicals and reagents used were of analar grade and were used as such without further purification. They are: ethylacetate (Sigma-Aldrich), n-butanol (Keshi, Japan), methanol and chloroform (JHD-China, and Merck Chemical).

Plant Extraction and Preliminary Phytochemical Screening

The chemical used for the extraction is of analar grade and was used as such without further purification. Eight hundred grammes (800 g) of the powdered leaves was exhaustively and sequentially soxhlet extracted using ethanol. The extract was concentrated to dryness by slow evaporation process at room temperature. They were weighed, labelled and kept asceptically in a desiccator until required.

The extract fractions of the leaf of *Piliostigma thonningii* were screened qualitatively for phytochemical constituents using standard procedures[1, 3, 16, 17, 18, 19].

Chromatographic Separationsof Bioactive Components from Ethanol Leaf Extract of *Piliostigma thonningii*

Column Chromatography (CC)

A glass tube with a diameter 2.8 cm and a height of 90 cm with a tap at the bottom was used for the column chromatographic technique. A plug of cotton wool was well placed at the bottom of the column very close to the tap so as to prevent the stationary phase from blocking the column. About 250 g of silica gel 60-120 mesh (Quikelem, India) was used to prepare a slurry by wet method. The silica gel was mixed with chloroform and stirred with a clean glassrod until a uniform mixture was obtained then it was packed cautiously and manually to about two third the size of the column tube using a glass funnel. The gel was then allowed to settle and

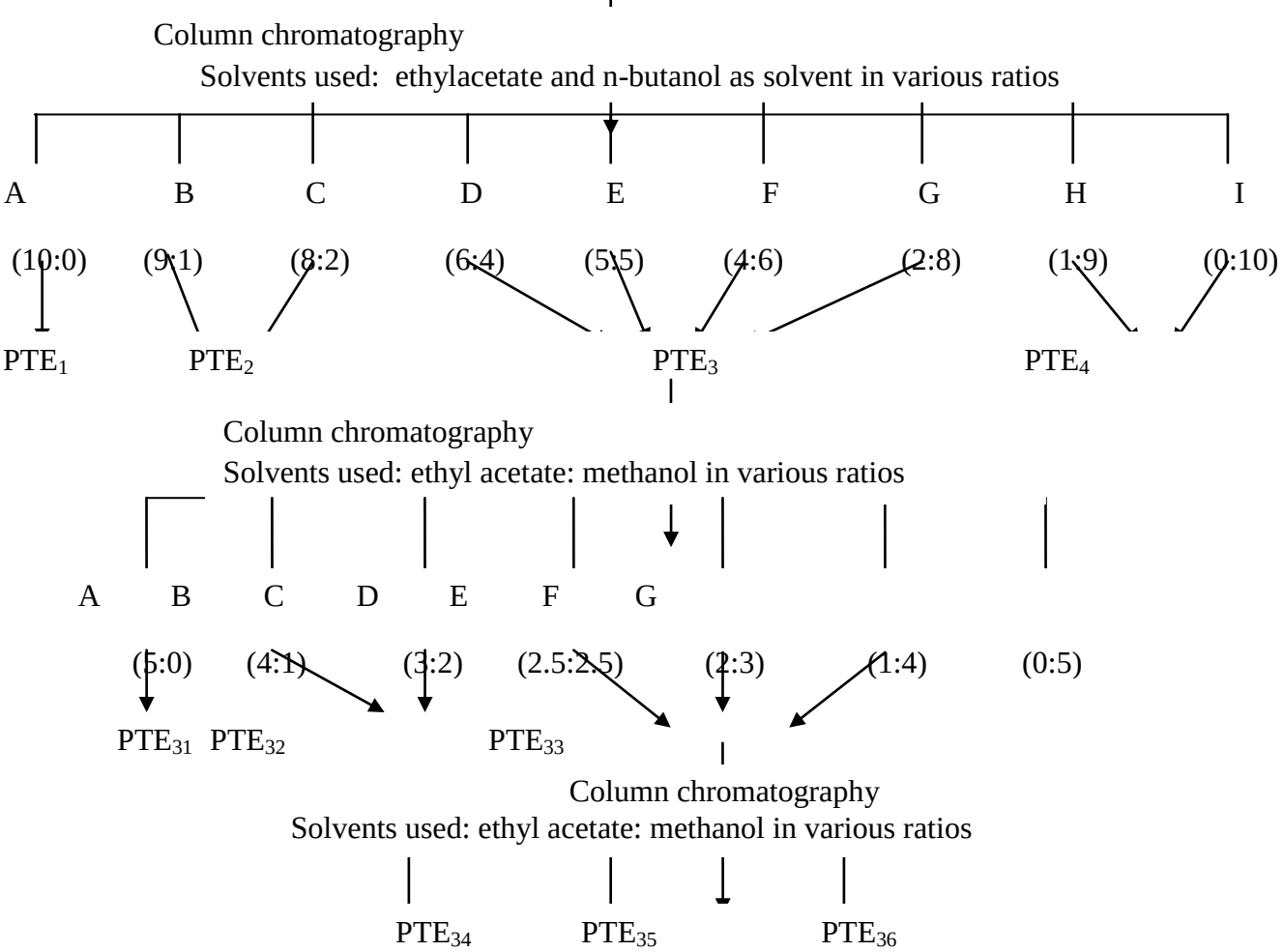
pack for 24 hrs. The air bubbles were avoided and care was taken not to dry the column by maintaining the level of the chloroform to that of the silica gel. About 20 g of the mixture of the ethanol extract and 5 g of silica gel in methanol was mounted on the already equilibrated silica-fixed column on top of the stationary phase. This was topped with a small layer of cotton, then sand to protect the shape of the organic layer from the velocity of newly added eluent (stationary phase). The eluting solvent initially was 100 % ethyl acetate and the polarity was gradually increased at 90:10, ethyl acetate: n-butanol ratio until 0:100 ethylacetate: n-butanol ratio was used. Forty (40) sub-fractions were collected. This method was used to further purify the column fractions using ethylacetate and methanol in increasing polarity as mentioned above until a compound of single spot on thin layer chromatography was achieved.

Thin Layer Chromatography (TLC)

Commercially prepared aluminium back TLC plates of 20 x 20 cm x 0.25 mm activated silica gel of 60 F_{254} (Merck, Germany) was cut to size of 5 x 5 cm. Ethanol extract of *Piliostigma thonningii* which contained the most phytochemicals and had the largest quantity was preferred for the chromatographic analysis. Methanol was used to dissolve the extract and allowed to stand for 30 minutes and then it was spotted at the bottom of the TLC plate (about 1.5 cm from the base). The extract which was dissolved in a few drops of methanol was dried in air for 30 minutes and was used for the spotting by the aid of a capillary tube. The spotted plate was kept in a previously saturated developing chamber containing the chosen solvent system as mobile phase and was covered with a

watch glass and allowed to run 3/4th of the height of the prepared plates [14].

Solvent combination of ethyl acetate and n-butanol was used in increasing polarity starting with 100 % ethyl acetate to 100 % n-butanol. The fractions collected were monitored by TLC and similar fractions were combined together. Solvent was then evaporated from the bulked fraction, allowed to dry and weighed. TLC analysis was carried out on the semi dry bulked fraction using various polar solvents to ensure the purity of the fraction. Fractions which had a better resolution and more phytochemicals were further purified using chromatographic techniques as earlier mentioned and used. Phytochemical screening of the sub-fractions and subsequent TLC to analyse the fractions were carried out. Further purification of the column fractions was carried out using chromatographic technique. Ethylacetate and methanol were used as solvent for the purification with gradual increase in polarity as stated earlier at different solvent combinations. 20 column fractions were obtained which were recombined and TLC analysis was again carried out on the column fractions. Fraction which gave a single spot when developed using chromatographic tank and then sprayed with 10 % sulphuric acid in ethanol was allowed to dry. A crystalline solid was obtained which was soluble in methanol and ethyl acetate.



PTE = Ethanol leaf extract of *Piliostigma thonningii*

Scheme: Fractionation profile diagram of column chromatography of ethanolic extract of *Piliostigma thonningii*

Statistical Analysis

Results of pharmacological studies were analysed using GraphPad InStat 2000 Model, Version 3.10 for windows (Graphpad Software, 2000). One way Analyses of Variance (ANOVA) test followed by Tukey-Kramer's Multiple Comparison test was used to analyse and compare the results at 95 % confidence level. Values of $p<0.05$ were considered significant. Results were expressed as mean ± standard error of mean.

RESULTS AND DISCUSSIONS

Gradient Extraction and Phytochemical Screening of the Various Fractions of the
Leaf of *Piliostigma thonningii*

Soxhlet Extraction

The extraction of the leaf of *Piliostigma thonningii* using ethanol produced extract with brown colour. It had a % yield of 21.04 %. The result of the extraction profile is shown on Table 1.

Phytochemical Screening of Gradient Extract of the Leaf of *Piliostigma thonningii*

The preliminary phytochemical screening of the leaf extract of *Piliostigma thonningii* using ethanol solvent revealed the presence of some phytochemicals such as flavonoids, terpenoids, cardiac glycosides, saponins and tannins. The result of the phytochemical screening of the extraction is shown in Table 2.

Chromatographic Separation of Ethanol Leaf Extract of *Piliostigma thonningii* and Phytochemical Screening of Fractions

Column Chromatographic Analysis ofEthanol Leaf Extract of *Piliostigma thonningii*

Components present in the ethanol leaf extract of *Piliostigma thonningii* were separated through . Similar fractions were combined together and were coded: PTE (1-5) as PTE_1, PTE (6-15) as PTE_2, PTE (16-35) as PTE_3 and PTE (36-45) as PTE_4. Weights of the combined fractions determined ranged from 13.5% withfraction PTE_1 as the highest, to 2.0 % of PTE_4 as the combined fraction with the lowest weight. The colours of the column fractions ranged from light green of PTE_1 to pale yellow of PTE_4 as shown on Table 3.

Thin Layer Chromatographic Analysis of the Column Fractions

The fractions collected from column chromatographic analysis were monitored by thin layer chromatographic technique. The combined column fractions encoded PTE_1 and PTE_2 did not resolve while PTE_3 and PTE_4 fractions resolved when solvent system of ethyl acetate and methanol was used. The retardation factors of the spots observed were calculated using the equation below while values obtained are shown on Table 4

Phytochemical Screening of 1stColumn Chromatographic Fractions

Phytochemical screening of the column fractions showed a significant separation of the bioactive components present in the ethanol leaf extract of *Piliostigma thonningii.* Column fractions PTE_3 had the highest number of phytochemicals

which are carbohydrates, tannins, cardiac glycosides, terpenes saponins and flavonoids, while column fractions PTE_1 have the least number of phytochemicals (tannins, cardiac glycosides terpenoids and flavonoids) Results of the phytochemical screening of the combined column fractions are shown in Table 5.

Column Chromatographic Analysis of Fraction PTE_3

The combined fraction obtained from column chromatography encoded PTE_3 was subjected to further column chromatographic analysis. Similar column fractions collected were recombined together and encoded: PTE (1-3) as PTE_{31}, PTE (4-9) as PTE_{32}, PTE (10-18) as PTE_{33}. Weights of the combined fractions were determined and are shown in Table 6.

Thin Layer Chromatographic Analysis of Fraction PTE_3

The fractions collected from column chromatographic analysis of fraction PTE_3were monitored by thin layer chromatographic technique. Fractions PTE_{31}, PTE_{32} and PTE_{33} were obtained as combined sub-fraction of PTE_3. The compounds of combined column fractions encoded PTE_{33}resolved in a solvent system of ethyl acetate, acetic acid methanol and water while PTE_{31} and PTE_{32} did not resolve. The retardation factor of all visible spots observed were calculated and presented in Table 7.

Phytochemical Screening of Recombined Column Chromatographic Sub- Fractions of PTE_{33}

Phytochemical screening of the recombined column fractions obtained from PTE_{33} fraction shows the presence of tannins,

cardiac glycosides, terpenoids saponins and flavonoids. The results of the screening are shown in table 8.

Column Chromatographic Analysis of Fraction PTE_{33}

The combined fraction obtained from column chromatography encoded PTE_{33} was subjected to further column chromatographic analysis. Similar column fractions collected were recombined together and coded: PTE (1) as PTE_{34}, PTE (2-3) as PTE_{35}, PTE (4-8) as PTE_{36}. Weights of the combined fractions were determined and are shown in Table 9

Thin Layer Chromatographic Analysis of column Fractions of PTE_{33}

The fractions collected from column chromatographic analysis of fraction PTE_{33} were monitored by TLC technique. Fractions PTE_{34}, PTE_{35} and PTE_{36} were obtained as combined sub-fraction of PTE_{33}. The compounds of the combined column sub-fractions resolved in a solvent system of ethyl acetate, methanol and water while. The R_f values of all visible spots were calculated as shown in table 4.10. The melting point of sub-fraction PTE_{34} with single spot was determined.

Table 1: The extraction profile of air dried powdered leaves of *Piliostigma thonningii*

S/N	Fraction	Mass (g)	%Yield ($^{w}/_{w}$)	Colour
	Texture			
	Ethanol	148.29	21.04	brown
	gummy mass			

Table 2: Phytochemical Ethanol *Piliostigma thonningii* leaf

S/N	Active component/Test	PTET
1	**Carbohydrates**	
i	Molisch's test	+
ii	Barfoed's test	-
iii	Fehling's test	+
iv	Combined reducing sugar	+
v	Ketoses (seivanoff's test)	+
vi	Pentoses	+
vii	Soluble starch	-
2	**Tannins**	
i	Ferric chloride test	+
ii	Lead acetate test	+
3	**Phlobatannins**	-
4		
i	Free anthraquinones	-
ii	Combined anthraquinones	-
5		
i	Salkowski's test	+
ii	Liebermann-burchard's test	+
6	**Terpenes**	+
7	**Saponins**	

i	Frothing test	+
8		
i.	Shinoida,s test	+
ii	Ferric chlorides test	+
iii	Lead acetate test	+
iv	Sodium hydroxide test	-
9	**Alkaloids**	
i	Dragendroff's test	-
ii	Meyer's test	-

Key: (+) = Present, **(-)** = Absent, **PTEA PTET**= *Piliostigma thonningii*ethanol extract,

Table 3: Combined fractions from column chromatographic analysis

Recombined fraction	weight of dried recombined fraction (g)	% $^{w}/_{w}$ of Component fraction	Colour of fraction
PTE_1	2.7	13.5	PTE (1-5) dark green
PTE_2	1.6	8.0	PTE (6-15) green
PTE_3	1.9	9.5	PTE (16-35) yellow
PTE_4	0.4	2.0	PTE (36-45) pale yellow

Initial weight of crude aqueous extract = 20 g
Amount recovered = 6.6 g
Percentage recovered = 33% (w/w)
PTE= *Piliostigma thonningii* ethanol extract
Mobile phase= Ethyl acetate and methanol

Table 4: Thin layer chromatography (TLC) examination of column fractions

Fraction	**No. of spots**	**Distance (cm)**	**Retardation factor (R_f)**
PTE_1	NR		
PTE_2	NR		
PTE_3	5	5.10	0.83
		4.60	0.75
		4.50	0.73
		2.10	0.34
		1.70	0.27
PTE_4	5	5.20	0.85
		4.80	0.78
		4.40	0.72
		3.50	0.57
		2.90	0.47

PTE = *Piliostigma thonningii* ethanol extract; NR= Not Resolved

Solvent front (cm) = 6.10

Solvent system = Ethylacetate: Methanol: Water; 7:4:3.4

Table 5: Phytochemical screening of combined column fractions of ethanol leaf extract of *Piliostigma thonningii*

Active component/ Test	PTE_1	PTE_2	PTE_3
1. Carbohydrate			
i. Molisch's test	-	+ +	
ii. Barfoed's test	-	- -	
iii. Fehling's test	-	-	+
iv. Combined reducing sugar	-	+ +	
v. Ketoses (Selivanoff's test)	-	-	
vi. Pentoses	-	- -	
2. Tannins			
i. Ferric chloride test	+	+	+
ii. Lead acetate test	+	+ +	
3. Cardiac glycosides			
i. Salkowiski's test	+	+ +	
ii. Liebermann- Burchard's test	+	+ +	
4. Terpenes	+	+	+
5. Saponins			
i. Frothing test	-	-	+
6. Flavonoids			
i. Shinoida's test	+	+ +	
ii. Ferric chloride test	+	+ +	

iii.	Lead acetate test	+	+ +
iv.	Sodium hydroxide test	+	+ +

Key: (+) = Present, **(-)** = Absent, **PTE** = *Piliostigma thonningii* column fractions of ethanol leaf extract

Table 6: Recombined fractions from 2nd column chromatographic analysis

Recombined fraction	Weight of dried recombined fraction (g)	%(w/w) of Component fraction	
PTE_{31}	0.35	19.40	PTE (1-3)
PTE_{32}	0.28	15.50	PTE (4-9)
PTE_{33}	0.92	51.10	PTE (10-18)

Initial weight of crude aqueous extract = 1.80 g
Amount recovered = 1.55 g
Percentage recovered = 86.11 ($^{w}/_{w}$)
PTE= *Piliostigma thonningii* ethanol extract
Mobile phase = Ethyl acetate and methanol

Table 7: Thin layer chromatography (TLC) examination of column fractions of the ethanol
leafextract of *Piliostigma thonningii*

Fraction	**Colour of fractions**	**No. of spot**	**Distance (cm)**	**Retardation factor (R_f)**
PTE_{31}	greenish yellow	NR		
PTE_{32}	pale yellow	NR		
PTE_{33}	yellow	4	5.20	0.78
			4.00	0.60
			3.80	0.58
			0.70	0.11

NR = Not Resolve
PTE = Ethanol leaf extract of *Piliostigma thonningii*
Solvent front = 6.60 cm
Solvent system = Ethyl acetate, Acetic acid : Water : Methanol; 13:3:3:4

Table 8: Phytochemical screening of combined fractions of 2nd column chromatography analysis of ethanol leaf extractof *Piliostigma thonningii*

Active component/ Test	PTE_{31}	PTE_{32}	PTE_{33}
1. Carbohydrate			
i. Molisch's test	-	-	-
ii. Barfoed's test	-	-	-
iii. Fehling's test	-	-	-
iv. Combined reducing sugar	-	-	-
v. Ketoses (Selivanoff's test)	-	-	+
2. Tannins			
i. Ferric chloride test	+	+	-
ii. Lead acetate test	-	-	-
3. Cardiac glycosides			
i. Salkowski' test	+	+	+
ii. Liebermann- Burchard's test	-	+	+
4. Terpenes	-	-	+
5. Saponins			
i. Frothing test	-	+	-
6. Flavonoids			
i. Shinoida's test	-	-	-

ii.	Ferric chloride test	+	--
iii.	Lead acetate test	-	--
iv.	Sodium hydroxide test	-	--

Key: (+) = Present, **(-)** = Absent, **PTE** = *Piliostigma thonningii* column fractions of ethanol leaf extract

Table 9: Recombined fractions from 3rd column chromatographic analysis of fraction PTE_{33}

Recombined fraction	**Weight of dried recombined Component fraction (g)**	**%(w/w) of fraction**
PTE_{34} PTE (1)	0.19	20.60
PTE_{35} PTE (2-3)	0.26	28.30
PTE_{36} PTE (4-8)	0.21	22.80

Initial weight of crude aqueous extract = 0.80 g
Amount recovered = 0.66 g
Percentage recovered = 82.50 ($^{w}/_{w}$)
PTE= *Piliostigma thonningii* ethanol extract
Mobile phase = Ethyl acetate and methanol

Table 10: Thin layer chromatographic (TLC) examination of column fraction of PTE_{33}

Fraction point (°C)	Colour of fractions	No. of spot	Distance (cm)	R_f	Melting
PTE_{34}	pale yellow	1	11.300.73	102-103	
PTE_{35}	pale green	4	11.200.72		
			9.60	0.62	
			7.10	0.46	
PTE_{36}	greenish yellow	2	7.00	0.45	
			2.00	0.19	

PTE = Ethanol leaf extract of *Piliostigma thonningii*
Solvent front = 15.40 cm
Solvent system = ethyl acetate : methanol : water; 7:4:3.4
$\mathbf{R_f}$ = Retention factor

Analgesic Effect of Ethanol Leaf Extract of *Piliostigma thonningii*

Acetic Acid-Induced Writhing

The ethanol leaf extract of *P. thonningii* caused an inhibition on the writhing response induced by acetic acid in a dose dependent manner at ($P< 0.05$) [fig. 4.3]. 26.40±0.51 and 31.60 ±0.51 mean number of writhing for doses of 400 and 200 mg/Kg bd. wt.(*i.p*) was observed as compared to the control (60.80±0.37) as shown in figure (4.3) and Appendix (vii). The effect was more pronounced at a high dose of 400mg/kg bd. wt. which gave a high percentage of inhibition (57.20 %) of the abdominal constriction induced by acetic acid. This was found to be significantly lower than the effect

of the synthetic drug (pentazocine, 20 mg/kg bd. wt) as shown in the figure with mean number of writhes at 14.80±0.37 in the extent to which the writhing or stretching induced by acetic acid was reduced.

Thermally-Induced Nociception

Figure 2 represent the mean time of pad licking at increasing doses of ethanol leaf extract of *P. thonningii* in the evaluation of thermally induced nociception of ethanol extract on rats. The extract doses of 200 and 400 mg/kg bd. wt. significantly ($p<0.05$) increased the time of pad licking (4.20±0.20 and 5.40±0.25) respectively. However pentazocine significantly increased the time of pad licking with a superior effect when compared to the extract.

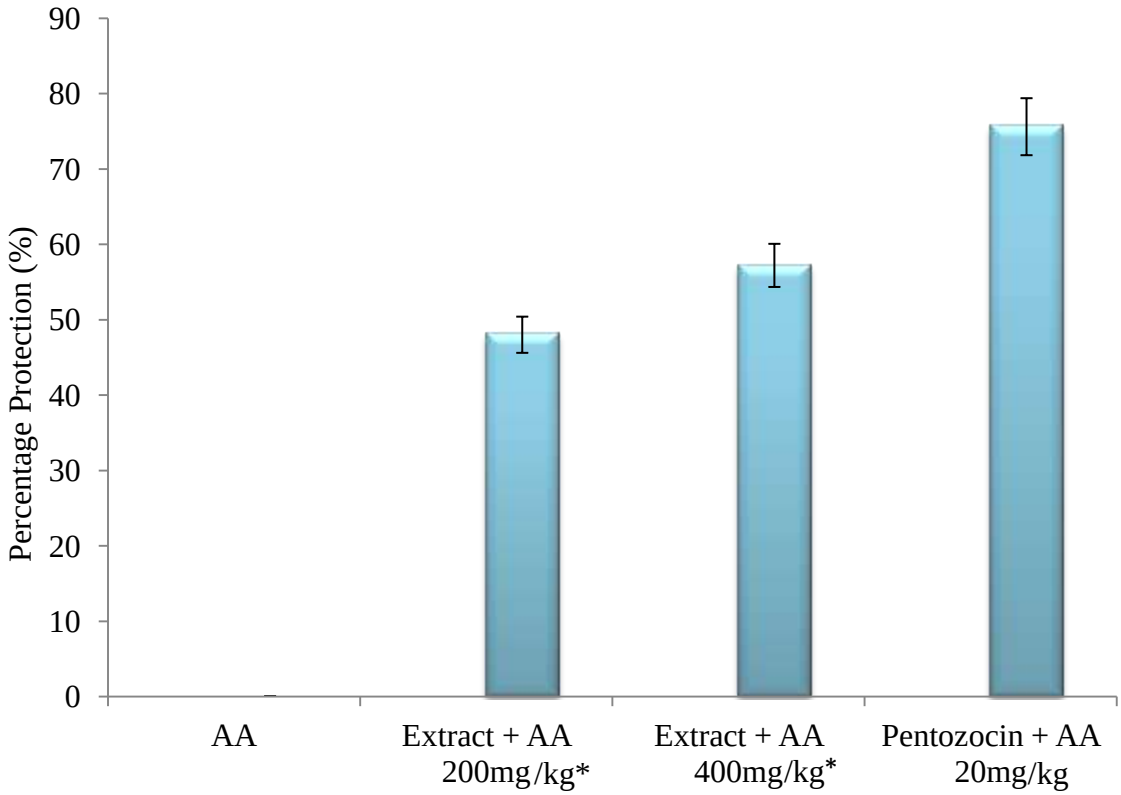
Muscle Relaxant Effect ofEthanol Leaf Extract of *Piliostigma thonningii*

The effect of the ethanol leaf extract of *P. thonningii* on muscle relaxation shows that20, 60 and 80% of the rats treated with extract doses of 100, 200 and 400 mg/kgslid down the board. The extract's muscle relaxant activity was dose dependent. Figure 3 show meannumber of rats that slid down an inclined plane at increasing doses of the extracton muscle relaxant effect on rats. The result is statistically significant at $P<0.05$ at all doses administered when compared to the control.

Phenobarbitone Sleeping Time Effect

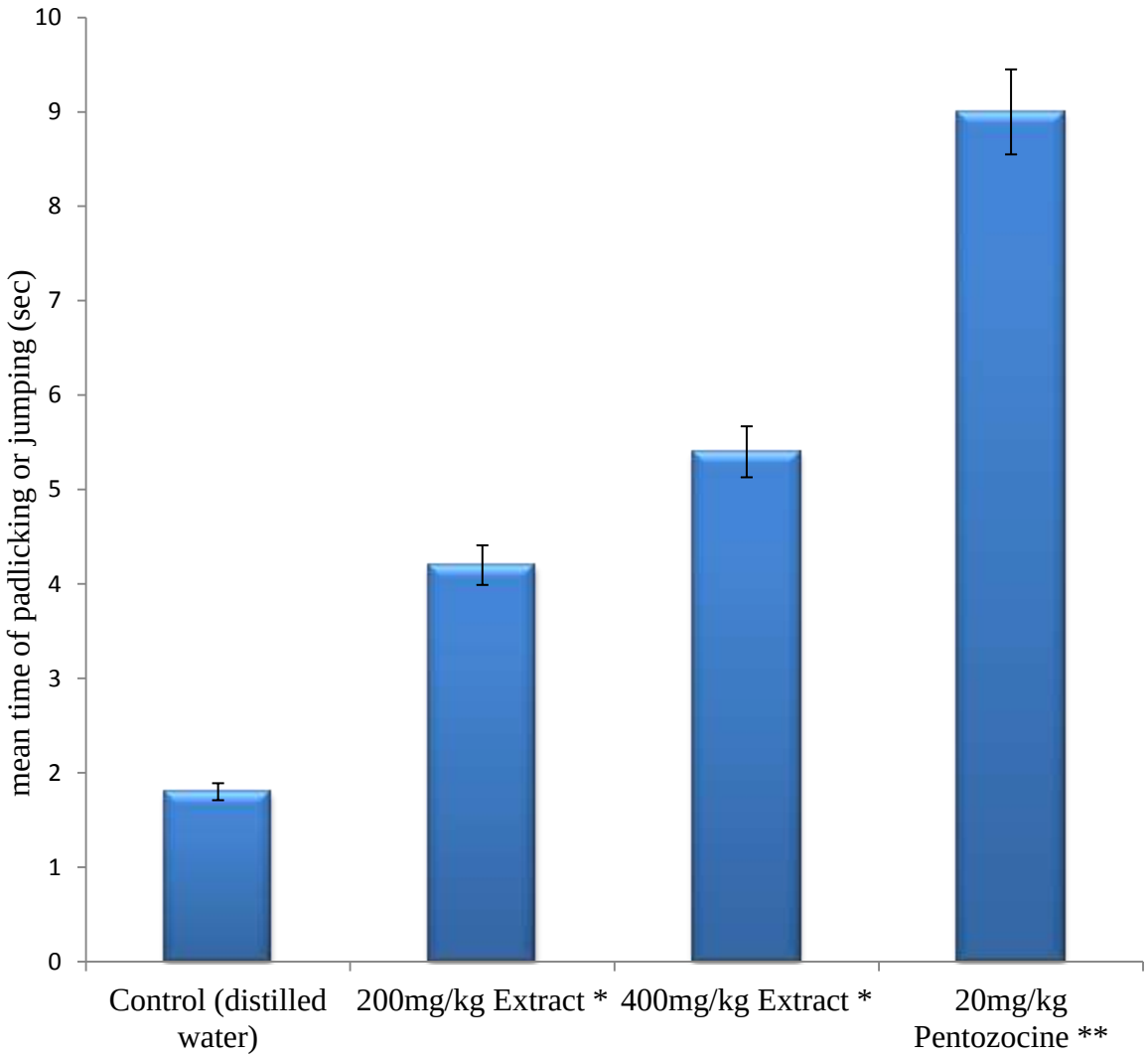
The ethanol extract of *P. thonningii* significantly and dose dependently increased ($p<0.05$) the sleeping time of phenobarbitonein rats (Table 11). The mean time duration of (72.0±04.64) min, (83.40±02.11) min, and (123.60±11.57) min

were observed when rats were administered extract doses of 200, 400 and 600 mg/kg bd. wt. after phenobarbitone was administered (*i.p*) 30 minutes before extract administration when compared to the control (phenobarbitone alone)[36.00±03.67] min. An increase in the dose of extract resulted in the increase in duration of sleep which were significantly different from the control group at $p<0.05$



* = diff controls

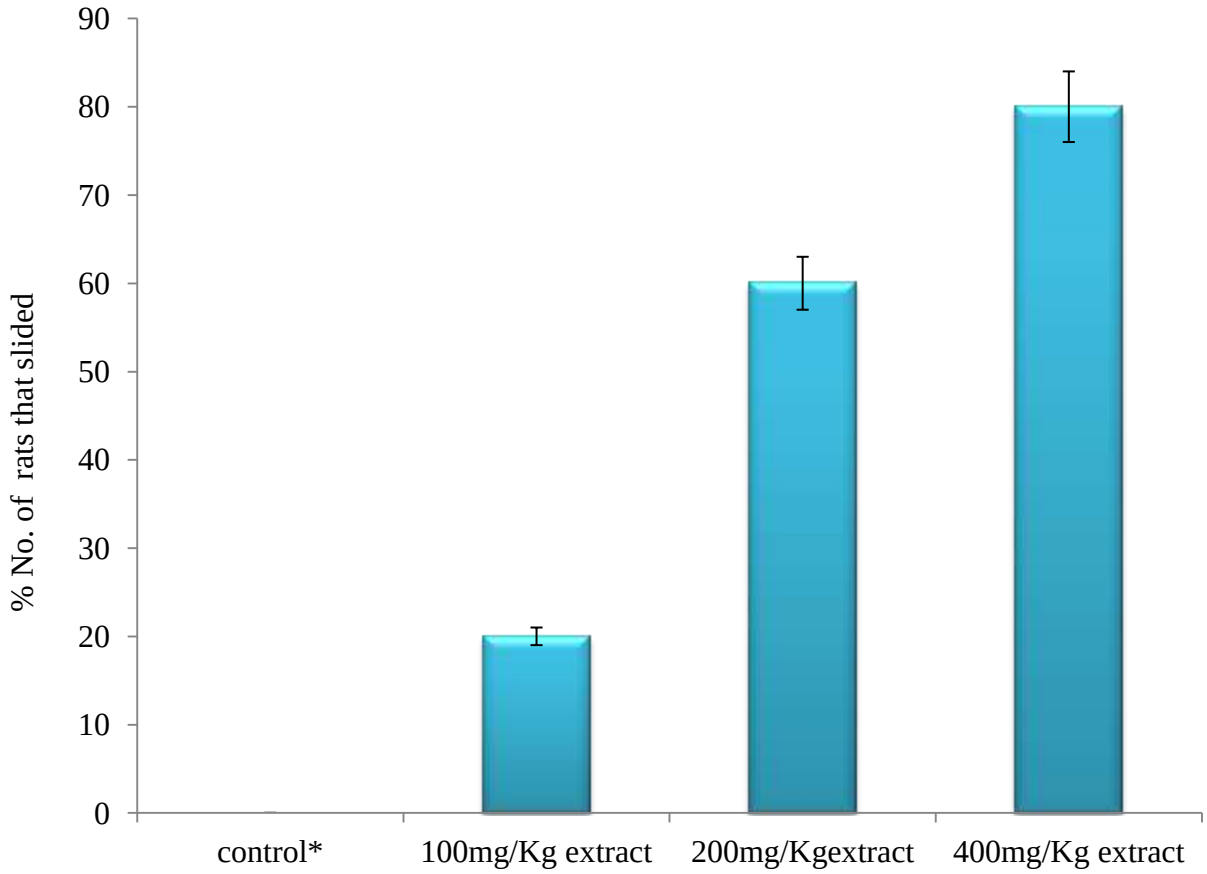
Figure 1:Acetic Acid Induced Writhing Effect of ethanol leaf extract of *Piliostigma thonningii* on mice.



* = significantly different at *p<0.05*

**= significantly different compared to extract doses administered at p<0.05

Figure 2: Thermally induced nociception effect of ethanol leaf extract of *Piliostigma thonningii* on rat



* =distilled water (10 ml)

Figure 3: Muscle relaxant effect of the ethanolic leaf extract of *Piliostigma thonningii*

Table 4.15: Effect of ethanol leaf extract of *Piliostigma thonningii* on phenobarbitone sleeping time

Group	Dosage of extract (mg/kg) Mean±SEM	Onset of sleep (min) Mean±SEM	Sleeping time (min)
A	Control (35 mg/kg) Phenobarbitone	7.00±0.84	36.00±03.67
B	Extract (200 mg/kg) + Phenobarbitone (35 mg/kg)	13.00±0.32*	72.0±04.64*
C	Extract (400 mg/kg) + Phenobarbitone (35 mg/kg)	14.00±0.32**	83.40±02.11*
D	Extract (600 mg/kg) + Phenobarbitone (35 mg/kg)	14.80±0.20**	123.60±11.57*

Significantly different at *$p<0.05$, compared to control
**$p<0.05$ no significant difference
n= 5= number of rats

DISCUSSION

The phytochemical studies of the ethanol leaf extract of *P. thonningii* revealed some useful chemical compounds such as flavonoids, cardiac glycosides, tannins, saponins, and terpenoids. These compounds have been known to exert pharmacological and antagonistic effects and still some are capable of protecting the active ingredient in herbs from decomposing either chemically or physiologically [21]. Flavonoids exhibit several biological effects such as antihepatotoxic, anti-inflammatory and antiulceractivity [22, 23]. They also inhibit enzymes such as aldose reductase, cycloxygenase, Ca^{2+}-ATPase, xanthine oxidase, phosphodiesterase and lipoxygenase. They are

potentantioxidants and have free radical scavenging abilities[24]. Many have antiallergic, antiviral actions and some of them provide protection against cardiovascular mortality [25, 26]. They have shown to inhibit the growth of various cancer cell lines in vitro and reduce tumor development in experimental animals [27].Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [28].
Saponins have been reported to posses insecticidal activity [29], antitumorigenic effect [30], molluscicidal effect [31], spermicidal activity [32], anxiolytic activity [33] and Anti-bacterial activity [34].

Tannins are polyphenols that are obtained from various parts of different plants [35]. In addition to its use in leather processing industries, tannins have shown potential antiviral [36], antibacterial [37,38], and antiparasitic effects[39, 40]. In the past few years tannins have also been studied for their effects against cancer through different mechanisms.

Terpenes have been reported to posses important biological activities, such as analgesic [41, 42]anticonvulsant [43], cardiovascular [44] antimalarial and antibacterial [1],
The successful separation of biomolecules by the chromatographic technique depended upon suitable solvent system which needs an ideal range of partition coefficient (K) for each target compound[45]. Chromatographic techniques (CC and TLC) showed significant effect in the purification process for the isolation of possible bioactive compounds. Thin Layer Chromatography is the simplest and cheapest method for detecting plant constituents because the method is easy to run, reproducible and requires little equipment. Of the various

methods of separating plant constituents, thin layer chromatography the most powerful technique used for the separation, identification and estimation of single or mixture of components present in various extracts. The TLC profiling of the extracts gave an impressive result that directing towards the presence of number of phytochemicals. The various phytochemicals gave different R_f values in different solvent system. This variation in R_f values of the phytochemicals provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by column chromatography. The isolated compound of sub-fraction PTE_{34} showed high R_f value in less polar solvent system which indicated the compound has a low polarity. The mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extracts was achieved by analyzing the R_f values of compounds in different solvent system. In the present state of affairs, TLC profile of all the plant extracts of the leaf of *P. thonningii*in different solvent system indicated the presence of diverse type of phytochemicals in this plant. Different R_f values of the compound also reflects an idea about their polarity. This information will help in selection of appropriate solvent system for further separation of compound from this plant extracts. However, for efficient separation of metabolites, good selectivity and sensitivity of detection, together with the capability of providing on-line structural information was needed. This was achieved through the use of the hyphenated system of Gas Chromatography-Mass spectroscopic Technique. The ethanol leaf extract of *Piliostigma thonningii* from this study

produced profound pharmacological effects on both the peripheral and the central nervous systems. The central action of the leaf extract was shown by its ability to induce sleep, in the phenobarbitone sleeping time experiment, analgesics and muscle relaxant activities.

The abdominal constriction method used in evaluation of the effect of the plant extract is a very sensitive one and can detect antinociceptive effect of a substance at a dose that cannot be detected by other methods such as tail-flick test [46, 47]. Inhibition of acetic acid-induced writhing in mice by extract (200 and 400 mg/kg) suggested that the analgesic affect of the extract may beperipherally mediated via the inhibition of the synthesis and release of prostaglandins [46].The acetic acid induced mouse writhing test has been used extensively to qualify analgesic agents that have peripheral analgesic activity [48]. Writhing induced by chemical substances injected intraperitoneally, are due to sensitization of nociceptors by prostaglandins. The ethanol leaf extract of *P. thonningii* caused an inhibition on the writhing response induced by acetic acid in a dose dependent manner. The effect was more pronounced at a higher dose which gave high percentage of inhibition of the abdominal constriction induced by acetic acid. This was found to be significantly lower than the effect of the synthetic drug (pentazocine) in the extent to which the writhing or stretching induced by acetic acid was reduced.

The leaf extract produced analgesia (antinociception) when pain was induced on the laboratory rats using heat (Eddy's hot plate). The superiority of the analgesic effect of pentazocine was expected, since pentazocine is a narcotic analgesic used

to alleviate deep-seated pain [49,50]. This effect may be due to the presence of bioactive compounds such as flavonoids and tannins that are present in the extract. They possess analgesic and anti-inflammatory activities [51, 52].According to previous reports, tannins such as ellagic acid and gallic acid exhibit analgesic potential through peripheral and central mechanisms [53, 54, 55, 56].Therefore it is possible that the analgesic effects observed in the extract might be attributed to these phytochemical constituents present in the leaf of *P. thonningii*.

The ethanol leaf extract of *P. thonningii* also possessed muscle relaxant activity evident by its effect on the inclined board test. Certain centrally acting agents are available which have the effect of reducing the background tone of the muscle without seriously affecting their ability to contract transiently under voluntary control [57].

CONCLUSION

The phytochemical study revealed the presence of saponins, cardiac glycosides, tannins, flavonoids, terpenoids and carbohydrates. Isolation of bioactive compound was achieved using thin layer and column chromatographic techniques. It is therefore recommended that, Further purification and characterization using Nuclear Magnetic Resonance (NMR), (^{13}Carbon Nuclear Magnetic Resonance and ^{1}H Nuclear Magnetic Resonance) should be carried out on ethanol extract of *Piliostigma thonningii* in order to confirm the active principles responsible for wide use of the plant traditionally.

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