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Involvement of cholinergic-muscarinic receptor in *Anaphe venata*-induced stretching-ataxia behavioral effects in rats

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ABSTRACT

Anaphe venata entomophagy has previously been implicated in the aetiopathogenesis of seasonal ataxia in humans and altered motor function in rodents. Thus, we investigated the effect of *A. venata* Phosphate Buffer Saline (PBS) extract on stretching, ataxia and the possible mechanism(s) of action. Animals were divided into four groups (n = 6-12 per group) and graded doses of extract (100, 200 or 400 mg/kg) were administered intraperitoneally (i.p.) while the control group received saline. Behavioral scores were recorded for a period of 30 min immediately after the administration of saline or extract. The role of various receptors in the extract induced stretching and ataxia was evaluated using known receptor antagonists in other groups of rats. The *in-vitro* cholinesterase inhibition assay of the extract was also performed. The protein profile of the extract was evaluated using the Sodium Dodecyl Sulphate (SDS)-Polyacrylamide gel electrophoresis. Results showed that the extract induced significant ($p<0.01$) stretching and ataxia behavioural effects dose-dependently when compared to vehicle-treated rats. Pretreatment with the non-selective muscarinic antagonist, scopolamine, significantly ($p<0.05$) reversed both stretching and ataxia-induced behaviour of PBS extract at all dose levels however both flumazenil and naloxone did not show any significant effects. Anticholinesterase assay also provided evidence that the extract has inhibitory effect on acetylcholinesterase enzyme. Electrophoresis assay suggested that the major proteins in the extract are probably of small molecular weight. In conclusion, the *A. venata* PBS extract induced-behaviours are probably mediated via the activation of cholinergic-muscarinic receptor systems.

Keywords: *Anaphe venata*, stretching-ataxia syndrome, behavior, rat.

INTRODUCTION

The roasted larvae of African silk worm, *Anaphe venata* Butler (Lepidoptera: Notodontidae) known as 'Kanni' or 'Monimoni' among the people of South-western part of Nigeria has been implicated in the epidemic acute seasonal ataxic syndrome (Adamolekun, 1992). This syndrome was initially called encephalitis tremens because it was thought to be an encephalitic process with a viral etiology (Adamolekun et al., 1997). The larvae have been reported to contain

several amino acids but lack cysteine and methionine, sulfur–containing amino acids (Ashiru, 1989). Further studies showed that those individuals that consumed the larvae developed acute seasonal ataxic syndrome (Adamolekun et al., 1997; Ogunniyi, 2010). The disease symptoms include acute postprandial effects with a triad of cerebella ataxia**,** intention tremors, and varying levels of impaired consciousness with associated nystagmus and cogwheel rigidity. Previous study by Onayade et al. (2004) reported that the LD_{50} values of non-polar and polar extracts were 2300 mg/kg and 1500 mg/kg, respectively. Furthermore, Iwalewa et al. (2005) reported that the sub-acute administration of anaphe extract induced both ataxia and stretching behaviours. However, previous studies showed that peptides (Gessa et al., 1967) and a variety of neurotransmitters such as acetylcholine (Ferrari et al., 1963), dopamine (Ferrari et al., 1993; Argiolas and Melis, 1998), neuropeptides such as morphine (Bertolili and Gessa, 1981), serotonin (Bentley et al., 1999) and inorganic ions such as calcium (Argiolas et al., 1980) and nitric oxide (Poggioli et al., 1995) are involved in the stimulation of stretching behaviour. Ashiru (1989) reported that the anaphe larvae are made up of crude protein and some natural mineral elements. Current management of seasonal ataxia involves oral thiamine replacement therapy (Adamolekun et al., 1994; Hossler, 2009) and since other neurotransmitters have been implicated in stretch and ataxia-induced behaviours, it is therefore pertinent to investigate the possible involvement of some of these neurotransmitters or receptors in anaphe-induced behaviour in order to determine the holistic approach in the management of the syndrome. Therefore, we decided to investigate the stretch and ataxia-induced behavioral effects of phosphate buffer saline (PBS) extract of *Anaphe venata* (non-polar extract) and possible mechanism(s) of action in rats.

MATERIALS AND METHODS

Preparation of *Anaphe venata* extracts

Dried *Anaphe venata* larvae were purchased from the market at Ile-Ife, Osun State and authentication was done by Professor W. A. Muse in the Department of Zoology, Obafemi Awolowo University, Ile-Ife, Nigeria. The powdered larvae (225 g) were extracted with 1.5 liters of Phosphate Buffer Saline (PBS), pH 7.4 and kept in the refrigerator for 12 hours. The extract was filtered and centrifuged the filtrate at 15,000 x g for 10 min at 4 ºC (IEC B-20A Centrifuge, Damon/IEC Division U.S.A). The resulting supernatant was freeze-dried to obtain the crude PBS extract (SB-4 Freeze-dryer, Benhay, SB4, UK). The total amount of crude extract obtained was 16.128 g (7.1% yields w/w).

Animals

One hundred and ten adult male Wistar rats weighing 150-200 g were purchased from College of Health Sciences Animal House, Obafemi Awolowo University, Ile-Ife, Nigeria and used in this study. The animals were housed (four to six per cage) in standard plastic cages with stainless steel coverlids and wood shavings as bedding. All rats had free access to water and rodent feeds (Bendel Feeds, Ewu, Nigeria) *ad libitum*. The experimental protocols were approved by the University Research Committee of Obafemi Awolowo University, Ile-Ife in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

Drugs and chemicals

The drugs scopolamine hydrochloride, naloxone hydrochloride, acetylthiocholine iodide, flumazenil, 5, 5´-di-thio-bis (2-nitrob-enzoic acid) [DTNB], acetylcholine iodide [ATChI], and MW-SDS-70 [Molecular weight marker kit] were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, USA). Acetylcholinesterase and all other reagents were of analytical grades.

General behavioural research procedure

Animals were allowed to acclimatize to the laboratory condition for at least one week and then divided into sixteen groups. All animals were observed and scored individually in an opaque Plexiglas (60 x 60 x 60 cm) after the administration of the test substance(s). Each animal was used only once with wood shavings bedding changed after each assessment to remove olfactory cue from one animal to the other. The time of experiment was kept constant at 10.00 a.m. - 2.00 p.m. daily. The laboratory was brightly lit, with an ambient temperature of 27 ± 2 ºC. The scoring for each parameter was done over a period of 30 min for each animal.

Assessment of the effect of PBS *Anaphe* extract on stretching and ataxia behavior

The animals were divided into four groups based on the three dose levels (100, 200 or 400 mg/kg, i.p., n=6/group) of the extract and control (PBS vehicle: 1 ml/kg, i.p., n=12). The number of stretches and ataxia were counted for 30 min in each animal immediately after the administration of PBS extracts of *Anaphe venata* (100, 200 or 400 mg/kg, i.p.) or vehicle in the opaque plexiglas. Stretching phenomenon simply involved stretching of the forelimb and hind limbs by the animal (extension or dorsal flexion of the trunk, causing lengthening of the body). Ataxia was assessed as the number of repeated episode of falling or falling tendency upon movement (Andine et al., 1999).

Assessment of receptor antagonists effects on *Anaphe venata* PBS extract induced-stretching or ataxia behavior.

The animals were divided into twelve groups and graded doses of *Anaphe* PBS extract (100, 200, 400 mg/kg) were administered intraperitoneally (i.p.) to each animal in the experimental groups respectively and vehicle (1 ml/kg, i.p.) was administered to the control group 15 min after pretreatment with scopolamine (3 mg/kg, i.p., n=6-12.) (Chapman et al., 1997; Eghashira et al., 2008), flumazenil (2 mg/kg, i.p., n=6-8) (Ayoka et al., 2005) and naloxone (2.5 mg/kg, i.p., n=6) (Gallate et al., 1999). The numbers of repeated episodes of stretching or ataxia in 30 min were scored.

Biochemical studies of the crude extract:
Polyacrylamide gel electrophoresis – SDS (Sodium Dodecyl Sulphate)

Polyacrylamide gel electrophoresis of the *Anaphe* extract was performed to ascertain its proteins profile and also for the determination of subunit molecular weight in accordance with the standard method described by Weber and Osborn (1975) using rod gels. The SDS-Phosphate system at 10% gel concentration was used. An aliquot of the *Anaphe* extract (3 mg/ml) was diluted with the sample buffers (Phosphate buffer system) and then incubated for 2 min in boiling water bath. Twenty micro liters (20 µl) of this protein was mixed with 5 µl of tracking dye solution (0.05 M bromophenol blue dissolved in 0.01 M phosphate buffer pH= 7), 5 µl of 2-mercaptoethanol and a drop of glycerol. This was layered on the 10% Polyacrylamide gels.

A lyophilized mixture of low molecular weight calibration kit protein standard (Sigma MW-SDS-70 kit) was reconstituted in 1.5 ml sample buffer, and heated for 2 min at 100 ºC. This reconstituted protein solution was then mixed with 50 µl of tracking dye solution, 40 µl of glycerol and 50 µl of 2-mercaptoethanol. About 10 µl of this sample, protein concentration 2 mg/ml, was used per gel. For the SDS-Phosphate system, the current was 8 mA/gel. After electrophoresis, the length of the gels and distances migrated by the tracking dye were measured before the gels were stained. The gels were stained with Coomassie brilliant blue R-250 solution [Brilliant Blue R-250 (1.25 g) was dissolved in 227 ml methanol, 46 ml glacial acetic acid and distilled water was added to 500 ml level]. The gels were de-stained manually in the de-staining solution [50 ml of methanol, 75 ml glacial acetic acid and distilled water added to 1 liter level]. After the de-staining procedure, the length of the gels and the distances migrated by various protein bands were measured. The relative mobility (Rf) of each of the protein bands was determined in

accordance with standard method (Weber and Osborn, 1975).

Anticholinesterase (AChE) assay

An African giant land snail (*Archachatina marginata)* was purchased from a commercial outlet at the Farm gate, Obafemi Awolowo University Ile-Ife. The snail was washed clean of slime and then sacrificed in order to obtain the haemolymph. The bluish colored fresh haemolymph was then collected into a clean baker placed in an ice bath. The haemolymph was sieved with a muslin cloth to remove impurities. The filtrate was diluted with phosphate buffer in the ratio 1:100 to give a working enzyme source for Acetylcholinesterase (Jaiswal et al., 2010). The resulting working enzyme source was then dispensed into aliquots in 1.5 ml eppendorf centrifuge tubes and kept in the freezer(-20 ºC) until required for assay.

In-vitro anticholinesterase activity of the PBS extract of Anaphe venata

The anticholinesterase activity of the PBS *Anaphe venata* extract was carried out following the standard method of Ellman et al. (1961) as modified by Jaiswal et al. (2010) using acetythiocholine iodide (ATChI) as substrate. In this assay, acetylthiocholine iodide (ATChI) was hydrolyzed to acetate and thiocholine. The thiocholine subsequently reacted with dithiobis-nitrobenzate (DTNB) to produce a yellow color that was monitored colorimetrically at 412 nm. Generally, a kinetic profile of the enzyme activity was measured at the interval of 30 s at 412 nm (Jaiswal et al., 2010). To 1.5 ml of phosphate buffer (100 nM, pH = 8.0) in each assay tube, the appropriate volume (equivalence of varying concentrations) of the extract was added, followed by the addition of appropriate volume of distilled water, in order to obtain a total reaction mixture of 2.0 ml. Then 100 µl of enzyme was added at 5 min intervals. The resulting mixture was then vortexed and incubated for 15 min at 37 ºC in a water bath after which 100 µl of DTNB was added. The mixture was transferred into a 2.5 ml cuvette and placed inside the spectrophotometer (zeroed with distilled water at 412 nm). The appropriate volume equivalence of the varying concentration of ATCHI (initiates the reaction) was added immediately and absorbance at 412 nm was taken at 30 s interval for 4 min. The change in absorbance per min (ΔA / min) was then obtained by subjecting the data recorded from the spectrophotometer to linear regression analysis using Sigma plot graphical software, version 1.02 (Jendel Scientific, 1994).

Statistical analysis

Each value was expressed as mean ± SEM. Significance changes in stretching and ataxia following various treatment was analyzed using one-way analysis of variance (ANOVA) followed by Student-Neuman-Keuls test. P-value equal to or less than 0.05 was taken as significant. Data management was carried out using "The Primer of Biostatistics programme" version 3.0 (by Glantz, McGraw-Hill Inc., U.S.A, 1992).

RESULTS

Effect of PBS *Anaphe* extract and antagonists on the stretching-induced behaviour.

PBS extract (100-400 mg/kg, i.p.) induced significant [$F(7, 59) = 13.47$; $p<0.01$] dose-dependent stretching behaviour when compared to vehicle treated rats. It also showed that pretreatment with the non-selective muscarinic antagonist, scopolamine (3 mg/kg, i.p.) significantly reversed PBS extract induced-stretching behaviour in rats (Table 1) but a specific antagonist for $GABA_A$ receptor, flumazenil (2 mg/kg, i.p.) and opioid receptor antagonist naloxone (2.5 mg/kg, i.p.) have no effects (Table 1).

Effect of PBS *Anaphe* extract and antagonists on ataxia-induced behaviour.

PBS extract (100-400 mg/kg, i.p.) induced significant [$F(7, 59) = 12.13$, $p<0.01$] ataxia behaviour in rats (Table 2). Pretreatment with the non-selective muscarinic antagonist, scopolamine (3 mg/kg, i.p.) significantly ($p<0.01$) reversed PBS extract induced-ataxia behaviour in rats (Figure 1) but a specific antagonist for $GABA_A$ receptor, flumazenil (2 mg/kg, i.p.) and opioid receptor antagonist- naloxone (2.5 mg/kg, i.p.) had no effects (Table 2).

Biochemical studies of the PBS extract

SDS-Polyacrylamide gel electrophoresis of the PBS extract was done to ascertain the protein profile of the larva extract. The sample major proteins separated in the electrophoretic gel are represented by letters a, b, c. (Figure 1). The molecular weight ranges of the proteins identified in the extract are observed to fall within the molecular weight range of the marker proteins (14,000-70,000 daltons) (Table 3 and Figure 2).

The results on anticholiestrase activity are presented in Figures 3, and 5. Anticholinesterase assay showed the activity profile of the extract. From the Line weaver-Burk and Dixon plots, there is evidence to indicate that the nature of inhibition of the constituents in the crude extracts of *Anaphe venata* was a linear mixed type of inhibition for AChE in that there were decreases in the V_{max} values with increase of K_m values. However, the dissociation constant (K_i) and the IC_{50} values could not be determined accurately from the Dixon plot due to the inhibition profile of the extract. Lineweaver-Burk plot showed that at high concentration (1000 µg/ml) of the extract the inhibition was non-competitive while at 1, 10 and 100 µg/ml the inhibition was mixed with respect to the enzyme (Figure 4).

Table 1: Effects of PBS extract of *Anaphe venata* on stretching behavior in rats and the involvement of cholinergic, GABAergic, opioidergic systems.

	PBS Extract of *Anaphe venata* (mg/kg, i.p.)			
	Vehicle	**100**	**200**	**400**
Vehicle	0.00 ± 0.00 (n=12)	16.33 ± 2.47* (n=6)	21.00 ± 5.14* (n=6)	23.50 ± 5.93* (n=6)
SCOP	0.00 ± 0.00 (n=12)	2.50 ± 1.33** (n=6)	4.17 ± 1.67** (n=6)	6.67 ± 3.68** (n=6)
FMZ	0.00 ± 0.00 (n=8)	16.33 ± 1.85 (n=6)	18.83 ± 2.48 (n=6)	14.00 ± 2.83 (n=6)
NAL	0.00 ± 0.00 (n=6)	16.50 ± 3.50 (n=6)	25.50 ± 5.46 (n=6)	19.33 ± 7.35 (n=6)

Administration of PBS extract induced stretching behaviour dose-dependently. All the antagonists used had no effect on their own. Pretreatment with scopolamine (SCOP: 3 mg/kg, i.p.), flumazenil (FMZ: 2 mg/kg, i.p.) and Naloxone (NAL: 2.5 mg/kg, i.p.) inhibited the anaphe-induced stretching behaviour that was only siginificant ($P<0.05$) with scopolamine (a non-selective muscarinic-cholinergic antagonist). *$p<0.05$ vs vehicle; **$p<0.05$ vs corresponding extract dose level.

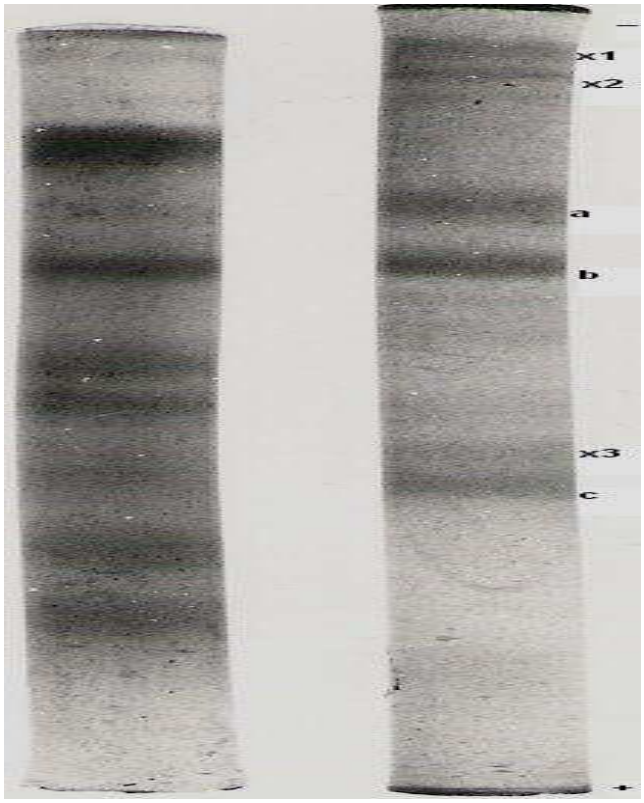
Table 2: Effects of PBS extract of *Anaphe venata* on ataxia behaviour in rats and the involvement of cholinergic, GABAergic, opioidergic systems.

	PBS Extract of *Anaphe venata* (mg/kg, i.p.)			
	Vehicle	**100**	**200**	**400**
Vehicle	0.00 ± 0.00 (n=12)	11.00 ± 2.36* (n=6)	26.33 ± 7.01* (n=6)	40.33 ± 11.41* (n=6)
SCOP	0.00 ± 0.00 (n=12)	0.00 ± 0.00** (n=6)	3.17 ± 1.83** (n=6)	6.50 ± 4.87** (n=6)
FMZ	0.00 ± 0.00 (n=8)	19.83 ± 4.34 (n=6)	21.00 ± 5.10 (n=6)	36.00 ± 8.15 (n=6)
NAL	0.00 ± 0.00 (n=6)	8.00 ± 2.37 (n=6)	18.67 ± 2.17 (n=6)	23.17 ± 6.62 (n=6)

Administration of PBS extract induced ataxia behaviour dose-dependently. All the antagonists used had no effect on their own. Pretreatment with scopolamine (SCOP: 3 mg/kg, i.p.), flumazenil (FMZ: 2 mg/kg, i.p.) and Naloxone (NAL: 2.5 mg/kg, i.p.) inhibited the anaphe-induced ataxia that was only siginificant ($P<0.05$) with scopolamine (a non-selective muscarinic-cholinergic antagonist). *$p<0.05$ vs vehicle; **$p<0.05$ vs corresponding extract dose level.

Table 3: Molecular weight range of proteins present in PBS *Anaphe venata* extract.

Protein band	R_f Values	Molecular weight (Daltons)
x_1	0.07	80,000
x_2	0.12	72,000
a	0.31	49,000
b	0.42	38,000
c	0.77	18,000
x_3	0.69	23,000



CONTROL **SAMPLE**

Figure 1: Polyacrylamide gel electrophoresis of the PBS extract of *Anaphe venata.*
Electrophoresis was done in the presence of SDS. The sample major proteins separated in the electrophoretic gel all fall within the range of molecular weight marker proteins (represented as a, b, c) (14,000-70,000 daltons) as shown above. Experimental details are as described in the text.

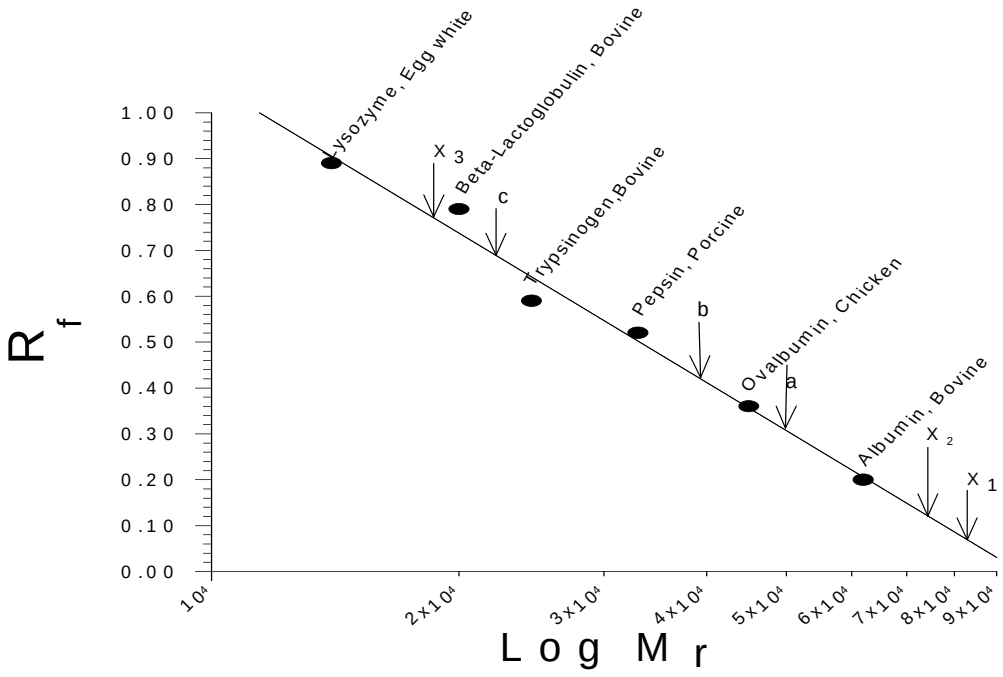


Figure 2: Plot of molecular weight range of proteins present in the PBS extract of *Anaphe venata*. The distinct individual protein bands are represented by the letters a, b, c, x_1, x_2, x_3 compared to standard molecular range of proteins bands.

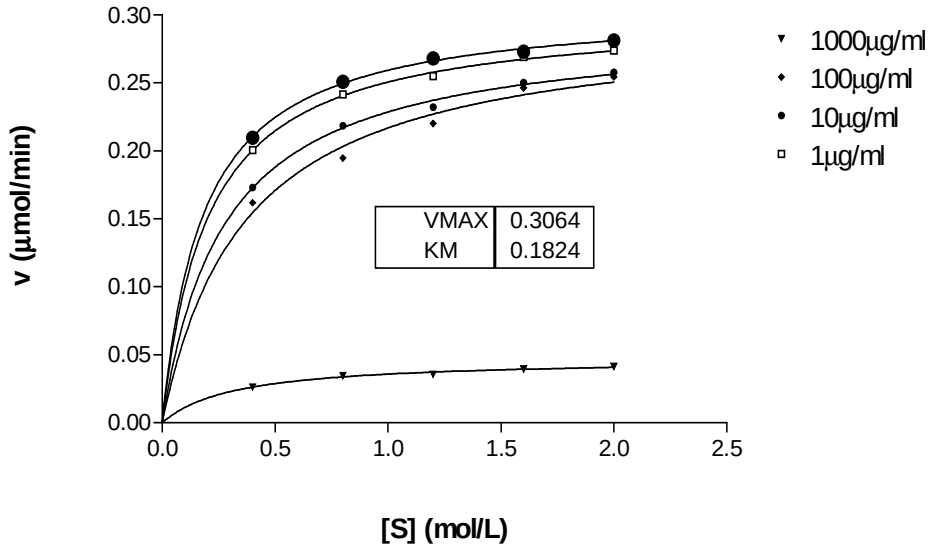


Figure 3: Michaelis-Menten plot of cholinesterase inhibition by the PBS extract of *Anaphe venata*. The figure shows the activity of the extract at various concentrations of 1 µg/ml, 10 µg/ml, 100 µg/ml, 1000 µg/ml respectively. The dark circles: cholinesterase activity in assays without anaphe PBS-extract.

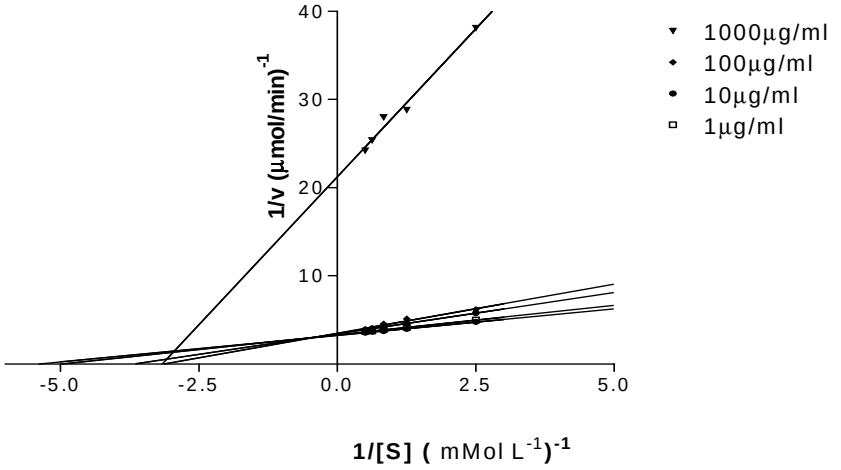


Figure 4: Lineweaver-Burk plot of cholinesterase inhibition by the PBS extract of *Anaphe venata* 1 µg/ml, 10 µg/ml, 100 µg/ml, 1000 µg/ml are the various concentrations of the extract.
The dark circles: cholinesterase activity in assays without anaphe PBS-extract

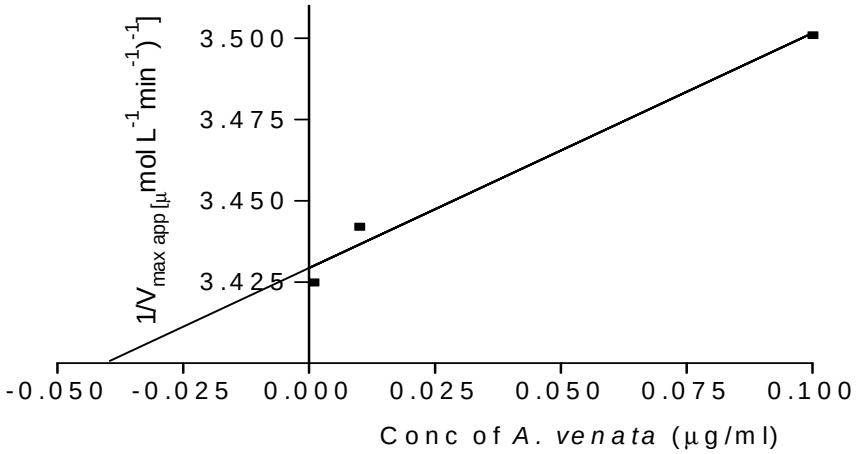


Figure 5: Dixon plot of cholinesterase inhibition by the PBS extract of *Anaphe venata.*

DISCUSSION

Anaphe venata has been implicated in the epidemic acute seasonal ataxic syndrome. Therefore, in the present study, we have investigated the effect of the crude PBS extract of *Anaphe venata* on stretching and ataxia behaviours and also determined the possible mechanism(s) of action in rats. The results obtained showed that PBS *Anaphe* extract caused significant induction of both stretching and ataxia behaviours in rats. Further investigation showed that non-selective muscarinic antagonist, scopolamine significantly reversed the induced-stretching/ataxia behaviors. Thus, the present data suggest the possible involvement of muscarinic-cholinergic system in the induction of these behaviours. This

observation is consistent with previous findings that cholinergic neurotransmission was involved in stretching behavior in rats that was abolished by pretreatment with scopolamine, a muscarinic receptor antagonist (Bentley et al., 1999). However, naloxone, an opioid receptor antagonist has no effect on these behavioral effects even though previous reports showed that opioidergic system has been involved in stretching behavior (Szechtman, 1986), motor coordination and ataxia (Castellani et al., 1982) therefore suggesting that the opioid system is not involved in anaphe PBS extract induced-behavioural effects. Similarly we observed that pretreatment with flumazenil, a benzodiazepine-$GABA_A$ receptor antagonist has no significant effect. Anticholinesterase assay showed that the activity profile of the extract is inhibitory in nature and the results revealed that the nature of inhibition of the constituents in the crude extracts of *A. venata* was a linear mixed-type of inhibition for AChE as it was observed that the V_{max} values decreased with increase in K_m values (Figure 3) (Choulhary et al., 2005). It could therefore be suggested that the anticholinesterase constituents of crude extract of *Anaphe venata* bind randomly and independently at different sites of the enzyme. The cholinesterase inhibition studies showed that this extract has inhibitory effects on acetylcholinesterase enzyme *in-vitro* and therefore may have cholinomimetic effect *in-vivo* as suggested by the *in-vivo* behavioural studies.

The profile of protein identified in the extract as observed in the electrophoresis revealed that they are within the molecular weight range of the marker proteins (14,000-70,000 daltons) and therefore, the major proteins of interest in the extract are probably of small molecular weight.

In summary, it is hereby concluded from this study that PBS extract induced-stretching-ataxia syndrome is probably mediated via the activation of muscarinic-cholinergic receptors in the central nervous system. Therefore, the muscarinic-cholinergic antagonist may be useful in the management of this syndrome.

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