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Anaphe venata larva extract-induced purposeless chewing in rats: the role of cholinergic, GABAergic and opioid systems

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Summary: Seasonal ataxia was reported in humans following the consumption of *Anaphe venata* larva as protein supplement in diet and altered motor function in rodents when the extract was administered intraperitoneally. In this study we investigated the effect of the crude aqueous and Phosphate Buffer Saline (PBS) extracts of *this larva* on altered spontaneous rat behavior in a novel environment particularly chewing behaviour, with a view to determine the mechanism(s) involved in these behavioural alteration. Animals were randomly assigned into four groups (n = 6-12 per group) and graded doses of aqueous and PBS extracts (100-400 mg/kg) were administered dissolved in saline intraperitoneally (i.p.) to each animal in the experimental groups. The control group received an equivalent volume of saline. Behavioral scores were recorded for a period of 30 minutes after the administration of saline or extract. The role of various receptors in the extract induced chewing was evaluated using known receptor agonist/antagonists. Results revealed a significant increase in purposeless chewing ($F_{(7, 95)} = 7.85$; $p<0.05$) by the aqueous extract compared to saline control at all dose levels, which was significantly attenuated by scopolamine (3 mg/kg, i.p) and thiamine (1 mg/kg, i.p) respectively ($p<0.05$); while flumazenil (2 mg/kg, i.p) and naloxone (2.5 mg/kg, i.p) did not alter the induced purposeless chewing behaviour. Also, administration of PBS induced a significant ($F_{(7, 95)} = 6.11$; $p<0.05$) increase in chewing behaviour but only at 400 mg/kg dose level which was attenuated by scopolamine (3 mg/kg, i.p); while flumazenil (2 mg/kg, i.p), naloxone (2.5 mg/kg, i.p), and thiamine (1 mg/kg, i.p) potentiated purposeless chewing behaviour respectively. It may therefore be concluded from this study that *Anaphe* extract-induced purposeless chewing behaviour in rat is mediated via the activation of cholinergic neurotransmission which is modulated by GABAergic and opioid receptor systems.
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Key: *Anaphe*, chewing, cholinergic, opioid, GABA, receptor, behaviour, rat

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INTRODUCTION

Seasonal ataxia was reported in humans following the ingestion of roasted larvae of *Anaphe venata*. The disease, onset is acute postprandial and presents with a triad of cerebellar ataxia, intention tremors, and other motor abnormalities (Adamolekun, et al., 1994).

Anaphe venata (Lepidoptera, Notodontidae), a moth reported to occur mainly in Africa. Its larva is a defoliator of *Triplochiton scleroxylon* K. Schum (Obeche); an economically important timber species in Nigeria. The *Anaphe venata* larva referred to as African Silkworm, is eaten in some part of Western Nigeria particularly among Ijesa people as a culturally acceptable means of dietary protein supplementation (Golding, 1942; Umoh et al., 1980; Ashiru, 1988) which is widely available in the raining season (July-September) (Adamolekun, 1993; Adamolekun *et al.*, 1994).

The larva is reported to be of high protein content (Umoh et al., 1980; Ashiru, 1988). Available data on the chemical constituents of *A. venata* larvae revealed the presence of amino acids, fats, proteins and sterols (Onayade and Adamolekun, 1996); existence of thiaminase type I enzyme has also been confirmed by some Japanese researchers (Nishimune et al., 2000) supporting the earlier proposal of Adamolekun (1993). In view of the above findings, the phenomenon of acute seasonal ataxic syndrome raises an issue of public health importance. This serves as impetus to investigate the behavioural effects of the extract of this larva in animals. Initial investigations in rodents using the extract of this larva showed that extract of this larva induced some behavioral alterations characterized by motor disturbances among which is alteration in novelty-induced behaviours like purposeless chewing, stretching and ataxia in animals (Onayade *et al.*, 2004; Iwalewa *et al.*, 2005). But the neuropharmacological mechanism(s) of these behaviours has not been explored. Our study therefore examined the neuropharmacological mechanisms involved in the *Anaphe venata* extract induced purposeless chewing behaviour in the 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 (150 g) were extracted cold in 2 litres of water with continuous shaking for 24 hours in a mechanical shaker. The mixture was filtered and then freeze-dried to obtain the crude aqueous extract. The total aqueous extract obtained was 6.5 g (4.3% yield w/w). Phosphate Buffer Saline (PBS) extract of the larvae was obtained by soaking 225 g of powdered larvae in 1.5 litres of freshly prepared Phosphate Buffer Saline (PBS) at pH 7.4 and kept in the refrigerator for 12 hours. The extract was filtered and the filtrate centrifuged at 15,000 x g for 10 minutes at 4 ºC (IEC B-20A Centrifuge, Damon/IEC Division U.S.A). The resulting supernatant, freeze-dried to obtain the crude PBS extract (SB-4 Freeze-dryer, Benhay, SB4, UK). The total amount of crude PBS extract obtained was 16.128 g (7.1% yields w/w).

Animals

Male Wistar rats (Vom strains), obtained from College of Health Sciences Animal House, Obafemi Awolowo University Ile-Ife, Nigeria were used for all investigations and were fed with Guinea Feed Growers (Bendel Feeds and Flour Mills, Ewu Edo State, Nigeria), water available *ad libitium*.

Drugs: Scopolamine Hydrochloride, Naloxone Hydrochloride, Flumazenil, Thiamine hydrochloride (Sigma-Aldrich, St Louis, USA).

Observation cage: Plexiglas observation cage (45 x 25 x 25 cm) with only one side transparent for observation.

The general protocol for the behavioural assays

Rats were placed directly from their home cage into an opaque Plexiglas observation cage (45 x 25 x 25 cm) with only one side transparent for observation. All animals were observed and scored singly in the cage after the administration of the test substance or saline. Each animal was used only once with wood shavings bedding changed after each assessment to remove olfactory cue from one animal to the other (Brown *et al.,* 1999). All experiments were carried out between 10.00 a.m-2.00 p.m. daily to avoid behavioural variations due to circadian rhythm (Siquera *et al.,* 1998). The laboratory was brightly lit, with an ambient temperature of 27 ± 2ºC. All animals were randomly assigned into groups consisting of 6-12 rats per group. The scoring for each parameter was done over a period of 30 minutes for each animal. All Investigations were conducted in accordance with the internationally accepted principles for laboratory animal use (EEC Directive of 1986; 86/609/EEC).

Assessment of effect of *Anaphe* extracts and antagonists on purposeless chewing

The animals were randomly assigned into 4 groups and graded doses (100, 200, 400 mg/kg, i.p.) of aqueous and PBS *Anaphe* extracts were administered in normal saline to each animal respectively while equal volume (1 ml/kg, i.p.) of normal saline were administered to the control group. Effect of pretreatment with scopolamine (3 mg/kg), flumazenil (2 mg/kg), naloxone (2.5 mg/kg) and thiamine (1 mg/kg) 15 min prior to extract administration respectively on chewing were also evaluated. Purposeless chewing was assessed by direct observation and counting the number of chews following drugs/extracts administration. Purposeless chewing was defined as rapid, repetitive vertical deflections of the lower jaw that resembles chewing but not directed at any object; each individual deflections of the jaw was counted as a single jaw movement (Mayorga et al., 1999)

Statistical Analysis

Each value was expressed as mean ± SEM. Significance changes in purposeless chewing 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 Primer of Biostatistics programme version 3.01(Glantz, 1992).

RESULTS:

ANOVA revealed a significant increase in purposeless chewing ($F_{(7, 95)} = 7.85$; $p<0.05$) by the aqueous extract compared to saline control at all dose levels. Similarly ANOVA revealed a significant ($F_{(7, 95)} = 6.11$; $p<0.05$) increase in chewing behaviour by the PBS extract but only at 400 mg/kg dose level. Effect of the various individual antagonists on the extract induced chewing behaviour is as follows:

a.) Aqueous *Anaphe* extract + antagonist:

i. Scopolamine (3 mg/kg, i.p) attenuated the dose-dependent increase in purposeless chewing significantly ($p< 0.05$) at all doses used (fig. 1).

ii. Thiamine (1 mg/kg, i.p) attenuated the dose-dependent purposeless chewing significantly ($p< 0.05$) at the highest dose of 400 mg/kg of the extract (fig. 4).

iii. Flumazenil (2 mg/kg, i.p) did not have any effect on the extract induced purposeless chewing behaviour (fig. 2).

iv. Naloxone (2.5 mg/kg, i.p) did not have any effect on the extract induced purposeless chewing behaviour (fig. 3).

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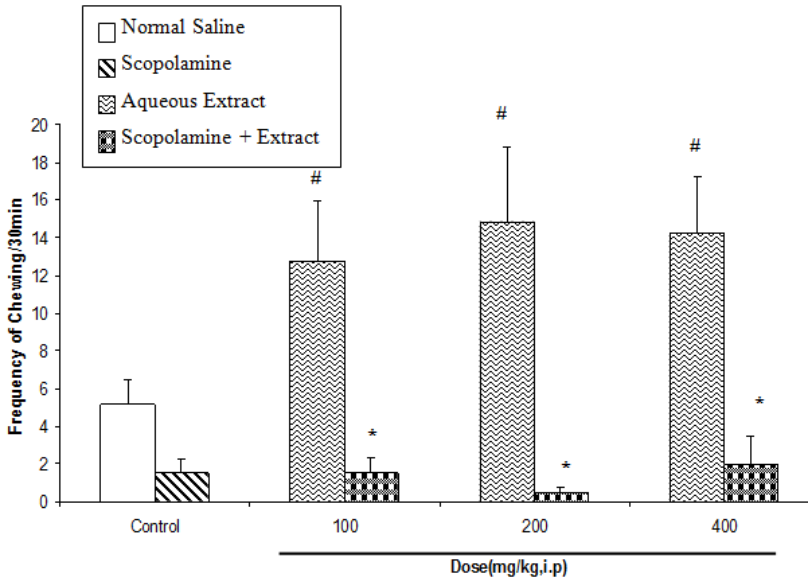

Fig. 1
Effect of aqueous *Anaphe* extract and Scopolamine on chewing behaviour. Each bar is expressed as Mean ± SEM.; (n =12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 95)} = 7.85$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced significant chewing behaviour in rat compared to saline control. Scopolamine (3 mg/kg, i.p), attenuated extract- induced chewing behaviour. # $p<0.05$: *Anaphe* extract vs Saline control; * $p<0.05$: *Anaphe* extract vs. *Anaphe* + Scopolamine.

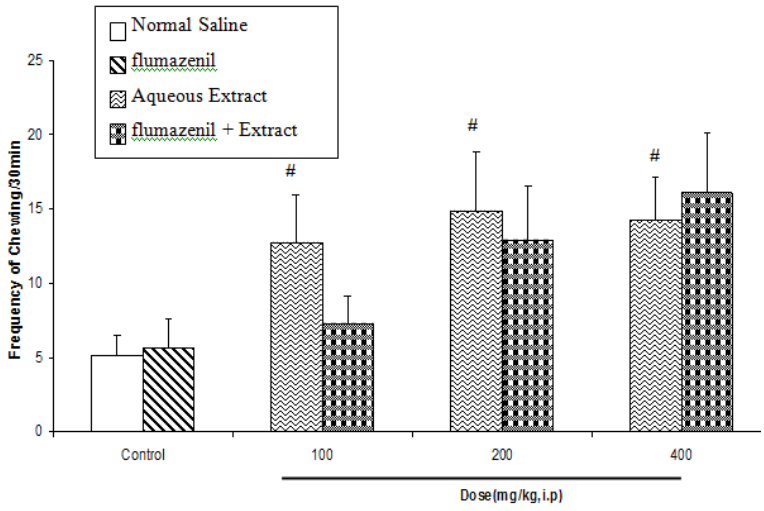

Fig. 2
Effect of aqueous *Anaphe* extract and flumazenil on chewing behaviour Each bar is expressed as Mean ± SEM.; (n =8-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 79)} = 1.97$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced significant chewing behaviour in rat compared to saline which was not altered by flumazenil (2 mg/kg, i.p). # $p<0.05$: *Anaphe* extract vs Saline control

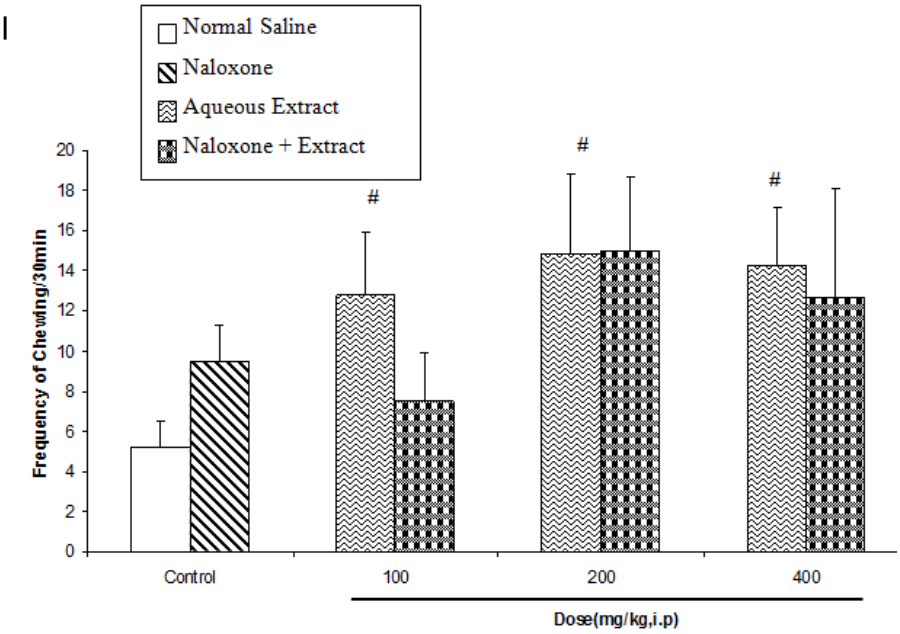

Fig.3
Effect of aqueous *Anaphe* extract and naloxone on chewing behaviour.. Each bar is expressed as Mean ± SEM.; (n =8-12) per treatment group. One-way ANOVA revealed no significant differences between the control and the treatment groups $F_{(7, 79)} = 1.31$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced significant chewing behaviour in rat compared to saline which was not altered by naloxone (2.5mg/kg, i.p). # $p<0.05$: *Anaphe* extract vs Saline control

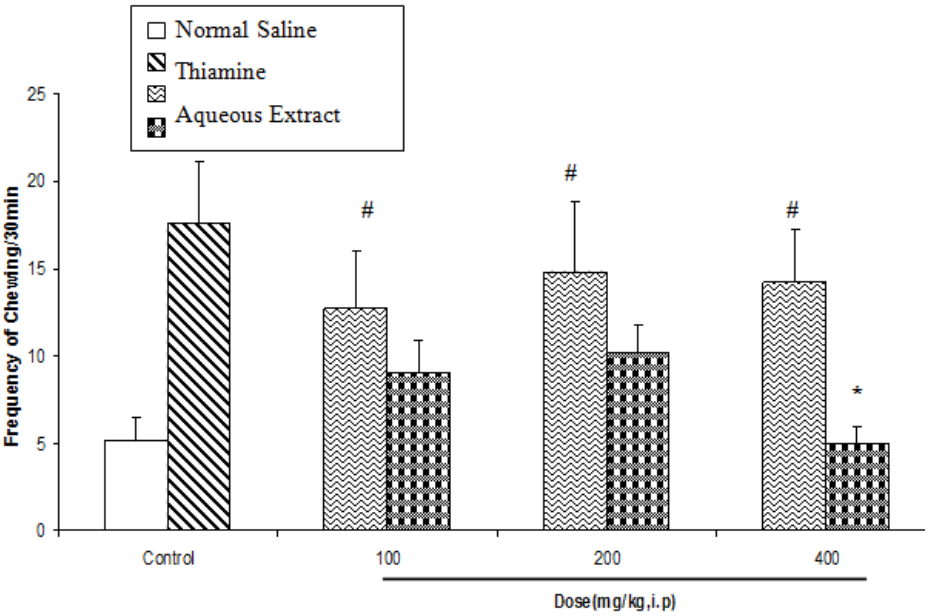

Fig.4
Effect of aqueous *Anaphe* extract and thiamine on chewing behaviour.. Each bar is expressed as Mean ± SEM.; (n = 6-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 71)} = 2.11$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced significant chewing behaviour in rat compared to saline $p<0.05$, which was attenuated by thiamine (1 mg/kg, i.p). But also thiamine alone increased chewing behaviour in rat compared to saline. # $p<0.05$: *Anaphe* extract vs Saline control; * $p<0.05$: *Anaphe* extract vs. *Anaphe* + thiamine.

b.) PBS *Anaphe* extract + antagonist

i. Scopolamine (3 mg/kg, i.p) attenuated the dose-dependent increase in purposeless chewing significantly ($p< 0.05$) at the dose of 200 mg/kg and 400 mg/kg respectively (fig. 5).

ii. Flumazenil (2 mg/kg, i.p) significantly ($p<0.05$) potentiated the extract induced purposeless chewing behaviour at the dose 200 mg/kg and 400 mg/kg respectively (fig. 6).
iii. Naloxone (2.5 mg/kg, i.p) significantly ($p<0.05$) potentiated the extract induced purposeless chewing behaviour at the dose 200 mg/kg and 400 mg/kg respectively (fig. 7).
iv. Thiamine (1 mg/kg, i.p) significantly ($p<0.05$) potentiated the extract induced purposeless chewing behaviour at the dose 200 mg/kg and 400 mg/kg respectively (fig. 8).

DISCUSSION

Anaphe extracts induced significant purposeless chewing in the animals compared to saline control. In this study it was observed that aqueous extract was more effective in inducing purposeless chewing as all doses used produced significant increase in chewing while chewing was only significant at the maximum dose used with PBS extract.

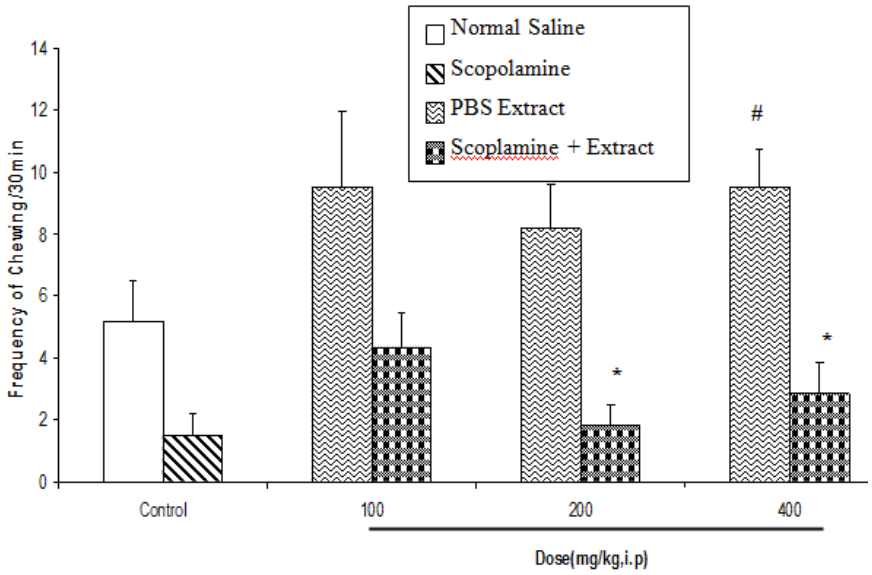

Fig. 5
Effect of PBS *Anaphe* extract and scopolamine on chewing behaviour.. Each bar is expressed as Mean ± SEM.; (n =6-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 59)} = 6.11$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced chewing behaviour in rat compared to saline which was blocked by scopolamine (2.5 mg/kg, i.p). # $p<0.05$: *Anaphe* extract vs saline control; * $p<0.05$: *Anaphe* extract vs. *Anaphe* + scopolamine.

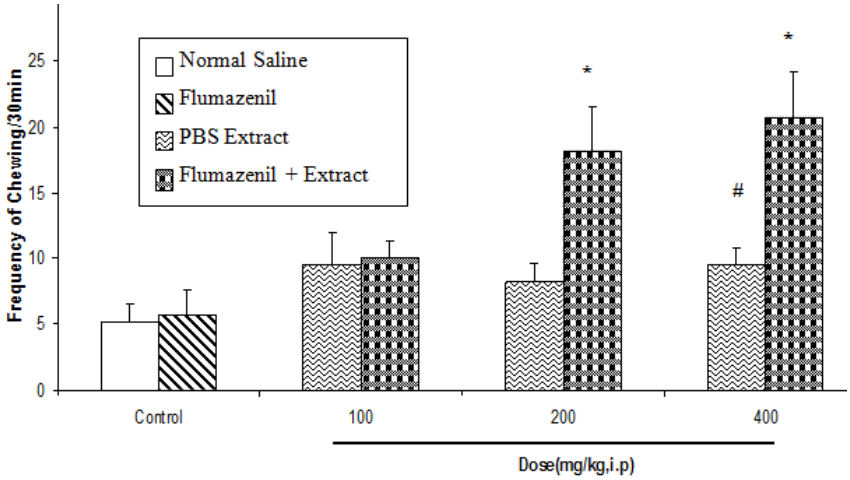

Fig.6
Effect of PBS *Anaphe* extract and flumazenil on chewing behavior. Each bar is expressed as Mean ± SEM.; (n =6-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 55)} = 7.16$; $p<0.05$. The extract at 100, 200, and 400 mg/kg induced chewing behaviour in rat compared to saline control which

was potentiated by flumazenil (2 mg/kg, i.p). # p<0.05: *Anaphe* extract vs saline control; * p<0.05: *Anaphe* extract vs. *Anaphe* + flumazenil.

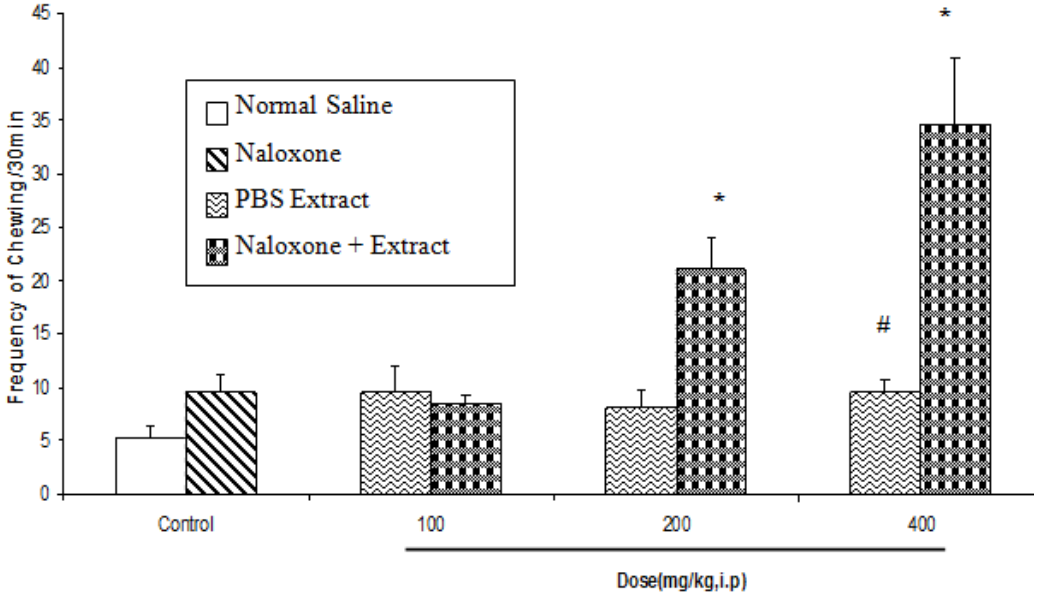

Fig.7
Effect of PBS *Anaphe* extract and naloxone on chewing behaviour. Each bar is expressed as Mean ± SEM.; (n =6-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 53)} = 13.51$; p<0.05. The extract at 100, 200, and 400 mg/kg induced chewing behaviour in rat compared to saline control which was potentiated by naloxone (2.5 mg/kg, i.p). # p<0.05: *Anaphe* extract vs saline control; * p<0.05: *Anaphe* extract vs. *Anaphe* + naloxone.

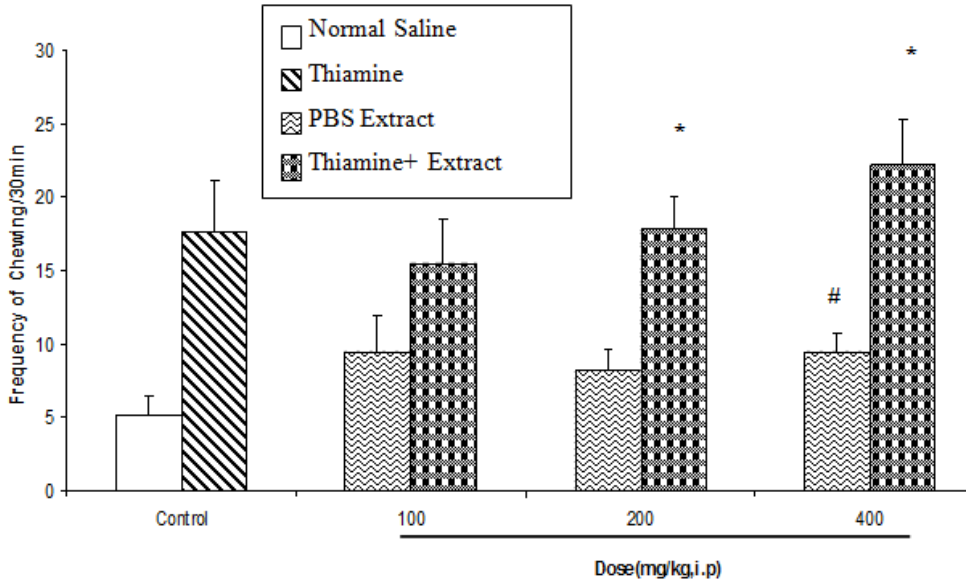

Fig.8
Effect of PBS *Anaphe* extract and thiamine on chewing behaviour. Each bar is expressed as Mean ± SEM.; (n =6-12) per treatment group. One-way ANOVA revealed significant differences between the control and the treatment groups $F_{(7, 53)} = 7.45$; p<0.05. The extract at 100, 200, and 400 mg/kg induced chewing behaviour in rat compared to saline control which was potentiated by thiamine (1 mg/kg, i.p). # p<0.05: *Anaphe* extract vs saline control; * p<0.05: *Anaphe* extract vs. *Anaphe* + thiamine.

This action was attenuated by scopolamine (a muscarinic antagonist) in the presence of both extracts, suggesting a central cholinergic (muscarinic) receptor involvement. Chewing mouth movement had

previously been attributed to direct activation of central cholinergic neurons (Rupniak et al., 1984; Stoessl *et al.,* 1988) and therefore a useful index of agonist action at the central muscarinic receptors (Salamone et. al., 1986).

However, it is interesting to note that scopolamine blocked chewing induced by both extracts which suggests that this extract acts via cholinergic muscarinic receptors to induce chewing similar to the mechanism involved in chewing behaviour as previously reported in the rat (Mayorga et al., 1999).

Thiamine behaved differently in both extracts; it exhibited blockade with aqueous extract which may be its direct blockade of the cholinergic neurotransmission (Harley and Flesher, 1946). On the other hand, it facilitated chewing with PBS extract which may probably be attributable to its central cholinomimetic effect (Meador et al., 1993) and its anticholinesterase activity (Rodgriguez-Martin et al., 2001).

Flumazenil (a GABA antagonist) and naloxone (an opioid antagonist) did not alter the extract-induced chewing with aqueous extract administration, which suggests that GABA and opioid systems were not involved in the aqueous extract induced chewing. However, flumazenil and naloxone potentiated chewing respectively with PBS extract, which may be a modulatory action of GABA and opioid receptors respectively on the cholinergic neurons (Cousins et al., 1999; Tien et al., 2004). The presence of cholinergic cells which serves as interneurons in the striatal brain area has been reported (Woolf, 1991) which are said to play a role in motor function which is involved in chewing behaviour and that they interact with other neurotransmitter systems such as GABAergic (Sivam et al., 1983; Harsing and Zigmond, 1998) and opioid systems (Tien et al., 2004).

In conclusion, our study therefore suggested that *Anaphe* extract-induced purposeless chewing behaviour in rat is mediated via the activation of cholinergic neurotransmission which is modulated by GABAergic and opioid receptor systems.

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