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Oxoproline induced acetylcholinesterase activity on subterranean termite *Odontotermes obesus*

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Abstract
In this paper, impact of termites as serious structural insect-pests is highlighted with relevant information in Indian context. *Epipremnum aureum* (Linden and Andre) G.S. Bunting is an ornamental foliage rich in phytoconstituents and serves as a potential source of bioactive compounds possessing beneficial biological activities. Present study was carried out to isolate oxoproline along with other pyrrolidine alkaloids (PAs) from leaf explants and checked for its neurotransmitter effect on subterranean termite *Odontotermes obesus*. Spectrometric analysis via Gas Chromatography Mass Spectrometry (GC-MS) helped in the characterization of pyrrolidine alkaloids. Acetylcholinesterase activity affirmed the neurotransmitter effect of pyrrolidine alkaloids on termites. It is assumed that presence of higher contents of oxoproline in comparison to other alkaloids showed effective antitermite activity. Percentage mortality of termites by pyrrolidine alkaloids was similar in results when compared with the standard alkaloid Nicotine and chemical termiticide Monocrotophos. The neurotransmitter effect of pyrrolidine alkaloids on *O. obesus* via acetylcholinesterase induction were also supported by SEM studies. Presence of oxoproline has exposed high *in-vitro* antitermite activity against subterranean termites *Odontotermes obesus* and strongly supports the use of *E. aureum* as eco-friendly, environmentally sustainable approach for termite control.

Keywords: *Epipremnum aureum*; gc-ms; oxoproline; acetylcholinesterase; antitermite

Introduction

Termites are the most damaging pests found in a wide range of terrestrial environments distributed throughout the world and cause considerable problems in housing, agriculture and forestry (Ahmed *et al*., 2016) [1]. In order to cope with a huge diversity of unfavourable biotic conditions, plants have developed defense strategies against feeding arthropods are highly diverse, including constitutive and inducible, direct and indirect defense mechanism. Many of the bioactive compounds are toxic; others act as repellents or are attractive cues for organisms belonging to other trophic levels. Within the plants' reservoir of chemical defensive compounds, alkaloids, terpenoids, phenolic compounds, and many polypeptides can be found (Mithöfer and Maffei, 2017) [11].

Alkaloids play a significant role for the plants survival against micro-organisms, insects and herbivores (Molyneux etal., 1996). Currently, prevention of wood damage via termites is controlled by synthetic pesticides like chlorpyrifos, chromated copper arsenate and alkaline copper quaternary. Natural pesticides with their wide range of effectiveness have been used to overcome the drawbacks of synthetic pescticides (Hsu *et al*., 2016) [6].

The enzyme acetylcholinesterase (AChE) catalyses the hydrolysis of the ester bound of acetylcholine (ACh) to terminate the impulse transmitted action of Ach through cholinergic synapses (Stryer, 1995) [20]. Many alkaloids exhibit a pronounced toxicity in animal models and humans. A major target in animals is the nervous system and many alkaloids structurally resemble neurotransmitters (Wink, 2016) [22].

Pyrrolidine alkaloids from *Epipremnum aureum* had shown antitermite effect against *Odontotermes obesus*, with SEM studies supporting the neurological effect of botanicals over synthetic pesticides (Meshram *et al*., 2017) [18]. Purification of methanolic extract of *Epipremnum aureum* by GC-MS reveals presence of antioxidant, anti-inflammatory, diuretic, antiulcer, antimicrobial, antifungal and pesticide activities (Meshram *et al*., 2016) [18]. The plant *E. aureum* is reported as rich source of alkaloids as many alkaloids were isolated from the leaves of the plant using GC-MS (Meshram *et al*., 2015) [9]. *In vitro* antitermite activity of alkaloids isolated from leaf, root and stem of *E. aureum* against *Odontotermes obesus* have been reported (Srivastava and Meshram, 2015) [9].

The present study is a pioneer approach to study the effect of alkaloid identified from *Epipremnum aureum* on enzyme acetylcholinesterase to formulate eco-friendly, environmentally sustainable and economic module of termite control.

Materials and Methods

Alkaloid isolation and Characterization

The isolation of alkaloid oxoproline and other pyrrolidine alkaloids (fraction a37) from *E. aureum* leaf explant and its spectral data were described in previous study (Meshram *et al*., 2017) [18]. In brief, dry powder plant parts were dampened with a weak base, extracted with chloroform, acidified with sulfuric acid, defatted with chloroform, alkalized with ammonium hydroxide and further extracted with chloroform. The extracted alkaloids were purified via column chromatography silica gel using mixtures of chloroform-methanol. Ten alkaloid fractions were selected for the termite assay namely a4, a8, a14, a19, a23, a27, a31, a37, a41 and a45. Characterization of highest active

alkaloid fraction was done by GC-MS with GCMS-2010 Shimadzu instrument operating in EI mode at 70ev. A Restek-5MS column (30m x 0.25mm x 0.25µm) was used and a series of n-alkanes were run under the same conditions along with the sample involving a range of C9-C34 alkanes. Identification of components was conducted using the database of National Institute Standard and Technology (NIST) library. The name, molecular weight and structure of the components of the test materials were ascertained.

Antitermite Assay (No-choice test)

Antitermite assay was carried out as described in previous study (Meshram *et al*., 2017) [18]. In brief, Alkaloid in each petriplate was in 0.4 mg/ml concentration with methanol as control, 0.01mg/ml Nicotine (Sigma-Aldrich Chemie GmbH Germany) as standard alkaloid and Monocrotophos (0.1%) from Dhanuka Aritech Limited as chemical termiticide. Fifty active worker termites were placed on each petriplate. The petriplates were closed and kept at 25ºC in an incubator. The numbers of living termites were counted each day. Each treatment was performed in triplicate and percentage mortality of termites was calculated using the below mentioned formula:

$$\text{Termite Mortality Rate (\%)} = \frac{\text{Number of termites dead}}{\text{Total number of termites}} \times 100$$

Scanning Electron Microscopy (SEM)

Termite (*Odontotermes obesus*) workers treated with alkaloid extract of *E. aureum* were collected were observed under Scanning Electron Microscope (Oxford Instruments, X-Man^{N}, Tescan) to get the detailed ultrastructure of the surface. From the petriplates of antitermite assay. A thin conductive coat of palladium was applied to the sample using Sputter Coater (Quorum) for 1 min maintaining a low vaccum (10^{-2} mbar/P_3).

Lethal Concentration Determination

Lethal concentration of alkaloid fractions were determined with the mortality of termites *O. obesus* (Ravikumar *et al*., 2011; Wachira *et al*., 2014) [15, 21]. For a37 fraction, six petriplates were prepared with 0 mg/g, 100 mg/g, 200 mg/g, 300 mg/g, 400 mg/g and 500 mg/g of alkaloid fraction with respect to the body weight of termites respectively and mortality data was recorded after 24 hours. The experiment was repeated thrice and median lethal concentration (LC_{50}) and LC_{90} was determined.

Achetyl cholinesterase Activity (AChE)

The effect of the alkaloids on the head AChE activity of LC_{50}-exposed termites was monitored at 24 h. Twenty termites were taken from each control and treatment replicate and their heads chopped for the estimation of AChE activity. They were washed twice with ice-cold phosphate buffer (0.1M, pH 7.5) and homogenized in 1 ml of ice-cold phosphate buffer (0.1M, pH 7.5) containing 10ml litre^{-1} Triton X- 100. The homogenate was centrifuged at 20,000 *g* for 15min at 4ºC in a table top Ultra Centrifuge (Thermo Scientific WX Ultra 100). The resultant supernatant was re-centrifuged at 20,000 *g* for 15min at 4ºC and used as an enzyme source. AChE was assayed as described by Ellman *et al*. (1961). Briefly, 100 µl of cocktail solution [0.2mM substrate (acetylthiocholine iodide) + 0.4 mM DTNB in phosphate buffer] was added to react with 50 µl of crude enzyme with three replicates each. The rate of reaction was monitored by a spectrophotometer (Systronics 2201) at 412 nm. The enzyme activity was expressed as µmoles of thiocholine hydrolysed min^{-1} mg^{-1} protein.

Statistical Analysis

The experiments were conducted in triplicates. The data were represented as Mean ± S.E. and one-way analysis of variance (ANOVA) was performed by Tukey's Multiple Range test using software SPSS (version 16.0, SPSS Inc.). Significant differences between means were determined and $p<0.001$ was regarded as a significant value.

Result and Discussion

Pyrrolidine alkaloids were isolated from the leaves of *Epipremnum aureum* using solvent extraction and separation techniques. Ten alkaloid fractions were selected for the termite assay namely a4, a8, a14, a19, a23, a27, a31, a37, a41 and a45. Similarly pyrrolidine alkaloid was isolated from the leaves of Piper amalago (Carrara *et al*., 2017) [2].

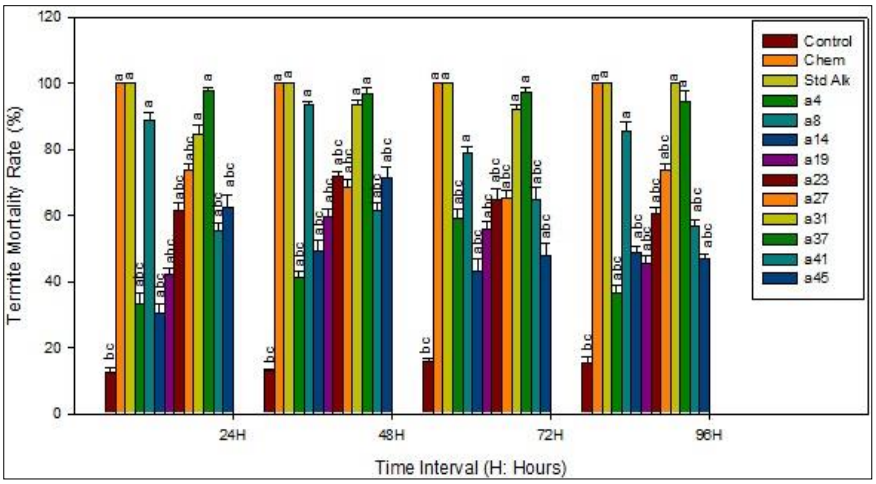


Fig 1: Termiticidal activity of alkaloid fractions isolated from *Epipremnum aureum* against *Odontotermes obesus* on paper *in vitro* conditions. Data are expressed as mean ± SE (n = 3). One way analysis of variance is done and columns with the same letter at specific time interval are not significantly different at p<0.001 by Tukey's test

Quantification of fractions a37 was carried out via GC-MS. The absolute contents of 5-Oxoproline and 1H-Tetrazole-1, 5-diamine in Fraction a37 were 104.0 µg/ml and 144.0 µg/ml, respectively. The lethal dose of highest active fraction a37 was found to be 500 µg/g body weight of termites being associated with high levels of 5-Oxoproline. 1H-Tetrazole-1, 5-diamine is the major component of Fraction a37 also shows activity against mortality of termites.

The acute toxicity of monocrotophos to subterranean termites, *Odontotermes obesus* (Rambur), has been studied by a paper contact method having affinity towards the neurotransmitter enzyme, acetylcholine estrase (Rao *et al*., 2005) [13]. Alkaloids are insecticidal at low concentrations, their mode of action varies, but many affect acetylcholine receptors in the nervous system (e.g. nicotine) or membrane sodium channels of nerves (e.g. veratrin) (Rattan, 2010) [14].

The effect of pyrrolidine alkaloids on LC_{50}-exposed *O. obesus* head was monitored for the AChE activity. From the current study, it is clear that *E. aureum* possess compounds that promote acetylcholinesterase activity, leading to decrease in acetylcholine level, thus affecting neuron degeneration and causing mortality (Fig.2).

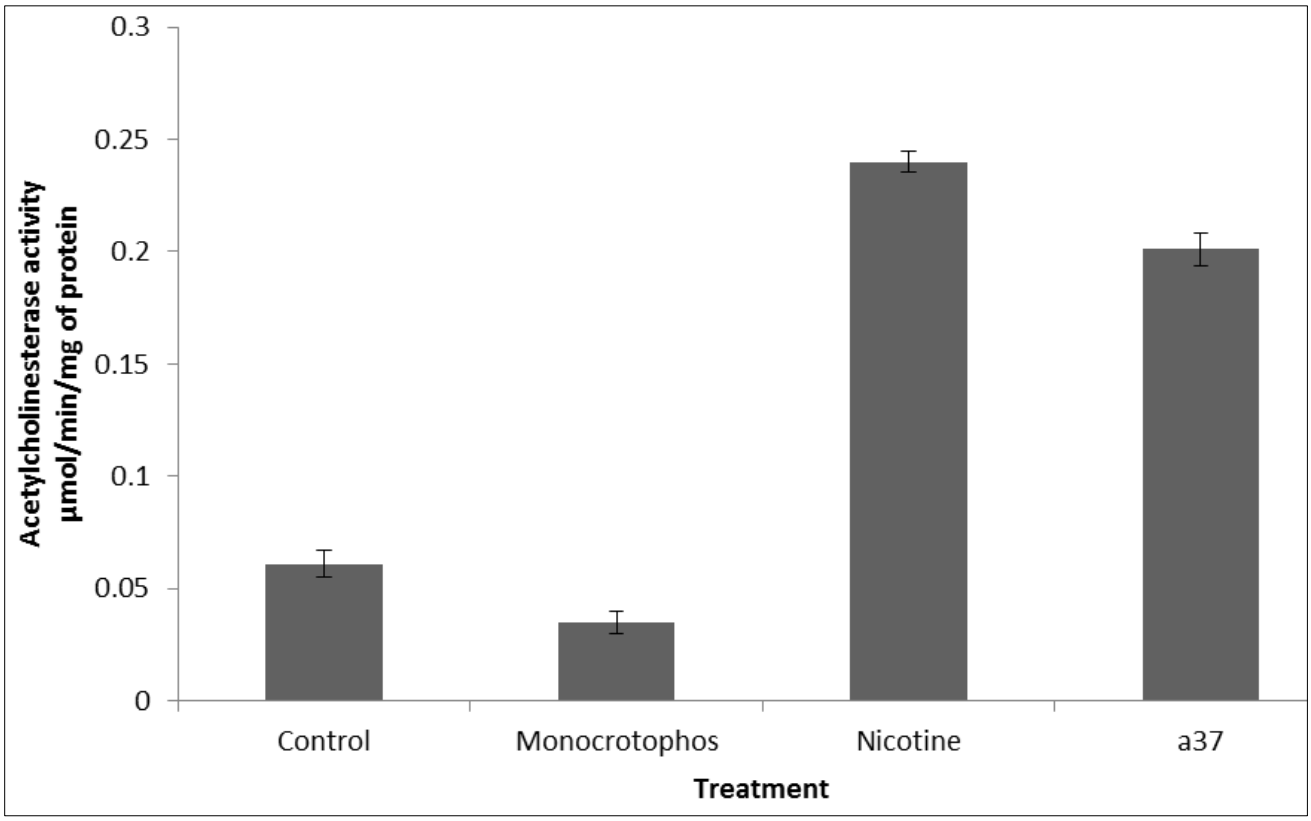
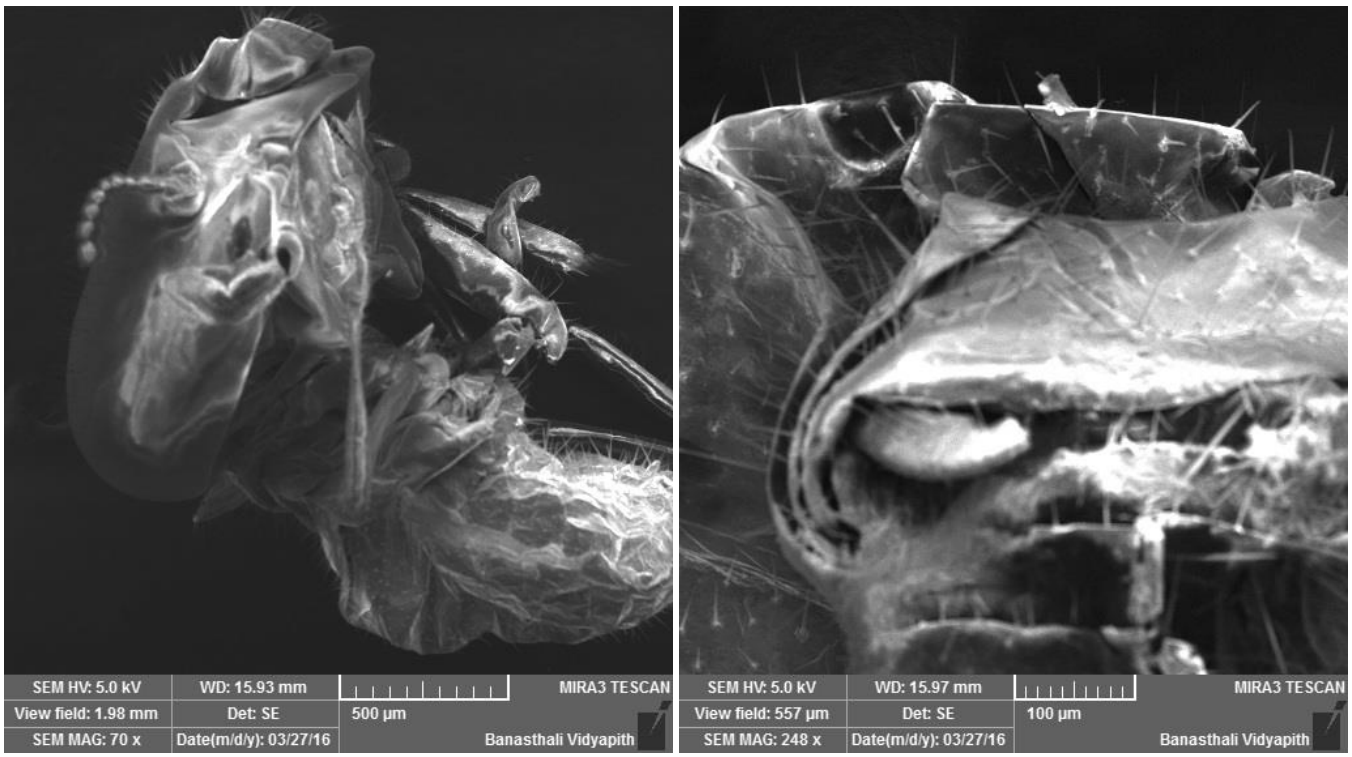


Fig 2: Acetylcholinesterase activity of alkaloid fraction a37

New metabolites were characterised in extracts prepared from transformed root cultures of the plant *Bethencourtia hermosae* (Asteraceae) from La Gomera (Canary Islands) (Fraga *et al*., 2014) [4]. In addition to the known pyrrolizidines senecionine, seneciphylline, and senkirkine, a new pyrrolizidine hermosine was also isolated and characterised through NMR analysis; the stereochemistry around the g-lactone remained undetermined. Along with many of the other metabolites isolated from this plant, hermosine exhibited some antifeedant activity against aphids (Yoon *et al*., 2015) [23].

The structure of the novel pyrrolizidine, (-) madhumidine was assigned by NMR experiments that showed weak cytotoxicity (IC50 > 100 mM) against cancer cell lines (Mandi´c *et al*., 2013) [7].

The scanning electron microscopic observation also revealed changes in the morphology of *E. aureum* alkaloids treated termites. The morphological changes visualized through SEM images revealed deformation of cuticular sensilla with no proper orientation present in various locations of the body of (Fig. 3). Deformation of these sensory structures may deform the sensing ability of the termites in its microhabitat leading to abnormal behaviour. The abnormal ultra-structural features of the cuticle, sensilla and hairs of the *O. obesus* suggest that chemical/ mechanical sensory system of this pest might be affected due to presence of pyrrolidine (alkaloid) in *E. aureum* that facilitates the abnormalities in the cuticle and sensory organs of the termite leading to unusual behaviour like sluggishness in movement, shrinkage of the body surface, brittleness of the appendage and ultimately death. Similar results were obtained with termites *Microtermes obesi* exposed to entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Singha *et al*., 2010) [17].



A B

Fig 3: SEM of a37 alkaloid treated termite: (a) anterior view at magnification 70X (b) posterior view at magnification 240X

Conclusion

The effect of pyrrolidine alkaloids on LC_{50}-exposed *O. obesus* head was monitored for the AChE activity. Present study has revealed the neurological effect of Oxoproline (PA) in the mortality of Indian white termites, *O. obesus*. From the current study, it is clear that *E. aureum* possess oxoproline that promote acetylcholinesterase activity, leading to the mortality of termites. At commercial level, production of oxoproline will promote ecologically friendly removal of termites that is cost-effect, safe and also favour environment protection.

List of Abbreviation

PA: Pyrrolidine Alkaloids; Ach: acetylcholine; GC-MS: Gas Chromatography Mass Spectrometry; AChE: Achetyl cholinesterase activity; LC: Lethal Concentration

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