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Effect of Ethyl Acetate Extract and Purified Compounds of *Alpinia galanga* (L.) on Immune Response of a Polyphagous Lepidopteran Pest, *Spodoptera litura* (Fabricius)

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ABSTRACT: Plant derived products provide defense to plants against herbivore insects by acting as antifeedant, growth regulators, toxins and modulate the activity of various enzyme. Additionally botanicals can also effect the immune system of insects. The present work reveals the impact of secondary metabolites of *Alpinia galanga,* a medicinal plant on humoral immune response of *Spodoptera litura* (Fab.). Addition of ethyl acetate extract and purified compounds viz. galangin and 1'-acetoxychavicol acetate induced the activity of phenoloxidase. Fluctuation in lysozyme activity was observed due to ethyl acetate extract. Although higher concentration of galangin induced the lysozyme level, but 1'-acetoxychavicol acetate did not significantly influence in enzyme level. Understanding the effect of biopesticides in immune response would help to design effective strategies for the control of insect pests.

Keywords: *Spodoptera litura*; *Alpinia galanga*; 1'-acetoxychavicol acetate; Galangin; Phenoloxidase; Lysozyme.

INTRODUCTION: Insects lack acquired immune system, but possess the capability to cope with a variety of parasites and pathogens through innate immune system. Insect immune response is further subdivided into cellular and humoral defenses. Cellular defense is hemocyte mediated response like phagocytosis, encapsulation and nodulation (Strand and Petch, 1995, Schmidt et al., 2001). Humoral immune response includes the production of antimicrobial peptides, PO cascade, lysozyme as well as production of reactive intermediates of oxygen and nitrogen (Ashida and Brey, 1998, Bulet et al., 1999, Lavine and strand, 2002, Cerenius and Soderhall, 2004). Humoral factors affect the function of hemocytes and hemocytes are important part of humoral molecules, so there is considerable overlap between cellular and humoral defenses. Both enzymes are important part of insect immune system. PO plays an important role in humoral immune response through melanization, wound healing, sclerotization of the cuticle and encapsulation (Ashida and Yamazaki, 1990, Sugumaran, 1998). It is present in an inactive form i.e. prophenoloxidase (Pro PO) and is released by hemocytes when activated by biological activators. Upon activation it catalyses the oxygenation of monophenols to o-diphenols and then oxidation to o-quinones which subsequently polymerize to melanin. Increased level of PO has been correlated with increased resistance to pathogens (Sugumaran 2002, Shelby and Popham 2006) and decline in activity is attributed to weakening of immune system (Hirmori and Nishigaki 2001). Lysozyme is another important enzyme of the humoral defense response and its activity increases during an immune challenge. Lysozyme is synthesised in hemocytes and fat body of lepidopteran insects and have antibacterial as well as antifungal activitiy (da Silva et al., 2000, Yu et al., 2002, Araujo et al., 2006, Wang et al., 2005).

Spodoptera litura (Fabricius), a polyphagous pest is cosmopolitan in distribution with wide host range of economically important crops such as groundnut, cruciferous vegetables, cotton, sunflower, tomato and many other crops (Rao et al., 1994, Qin et al., 2004). Excessive use of synthetic insecticides for its control has resulted in development of resistant populations of *S.litura* due to which sporadic outbreaks of this insect and failure of crops occur in several countries (Ahmad et al., 2007). Plant derived products have been intensively investigated for the past many years as an alternative to synthetic insecticides. Secondary metabolites are known to act as toxins, insect growth regulators, antifeedants and affect insect behaviour (Senthil-Nathan, 2013). Many phytochemicals from medicinal plants have been known to control a number of insect species in India (Saxena et al 2013). *Alpinia galanga* (L.) *a* medicinal plant belonging to family Zingiberaceae, has been widely cultivated in South-east Asia and its rhizomes have medicinal properties

like antibacterial (Khattak *et al.,* 2005), antiplasmid (Latha *et al.,* 2009), antioxidant and anti-microbial (Wong *et al.,* 2009). In addition to these, recent studies also demonstrated its insecticidal activity (Riyanto and Ohsawa, 1998, Sukhirun et al., 2011). A number of biologically active compounds viz. 1'-acetoxychavicol acetate, *p*-hydroxycinnamaldehyde, p-acetoxycinnamyl alcohol, p-coumaryl alcohol ethyl isolated from the rhizomes have been reported to be responsible for such activities (Latha et al., 2009, Barik et al., 1987, Sukhirun et al., 2011).

The effect of secondary plant metabolites on insect immune system has not been studied much. In light of this, the present study has been undertaken with the aim to evaluate the effect of ethyl acetate extract and purified compounds of *A.galanga* on immune response of *S.litura* larvae.

MATERIALS AND METHODS:

Plant extract and purification of compounds: The rhizomes of *A.galanga* purchased from the local market of Chandigarh, India, were identified and a sample (NIP-NPM-CD-134) was submitted at Natural Product Field laboratory (NPFL), NIPER, S.A.S. Nagar. The extraction and purification of the compounds from dried powdered rhizomes of *A.galanga* were done as described earlier (Kaur et al., 2010).

Insect rearing: The larvae of *S. litura* were collected from the fields surrounding Amritsar (Punjab), India. The culture was established in the laboratory at constant temperature and humidity conditions of 25 ± 2ºC and 65 ± 5 % respectively along with the photoperiod of 12:12 (D: L). The rearing was carried out in glass jars (15 × 10 cm) on castor leaves till pupation. The freshly emerged adults were kept in oviposition jars and fed on a mixture of water and honey solution (4:1v/v) soaked on a cotton swab which was replenished daily (Thakur et al., 2013). For experimental study the larvae from this culture were reared on artificial diet as recommended by Koul et al. (1997) with slight modifications.

Humoral response: The effect of ethyl acetate extract of *A.galanga* and purified compounds viz. galangin and 1'-acetoxychavicol acetate was investigated on the immune response of *S. litura*.

Ethyl acetate extract: Stock solution of 5000 ppm of ethyl acetate extract was prepared in DMSO (0.5%). The stock solution was appropriately diluted to get final concentrations of 500, 1000, 1500, 2000, 2500 ppm. Artificial diet was supplemented with these concentrations and placed in plastic container (4×6 cm). Diet without ethyl acetate extract and with DMSO (0.5%) served as control. The 3nd instar larvae were kept individually in plastic containers (4×6 cm) and fed on treated and control diets for different time intervals i.e. 24, 48, 72 and 96 hours. For each treatment group 30 larvae were used. The diet was refreshed regularly on alternate days. Throughout the experiment the temperature and humidity conditions were maintained at 25 ± 2ºC and 65 ± 5 % respectively. The hemolymph was collected by piercing the prothoracic legs with a sterile needle. The hemolymph was pooled and activity of phenoloxidase (PO) and lysozyme was estimated.

Estimation:

Phenoloxidase (PO) activity: The activity of PO was estimated according to the method of Cotter and Wilson, (2002) with certain modifications. From pooled hemolymph 4 µl was added to 200 µl of ice-cold PBS and vortexed on vortex mixer (SPINIX). Then 50 µl sample i.e. hemolymph-buffer mixture was added to 50 µl of 20 mM L-DOPA. The increase in absorbance was measured on microplate reader (EonBio Tek) at 475 nm with 1 min interval for 30 min.

Lysozyme activity: The activity of enzyme was estimated by using protocol given by Azambuja et al., (1991) with slight modification. 1 µl of hemolymph was added to 50 µl of ice cold phosphate buffer solution. Then 12.5 µl of hemolymph-PBS was added to 150 µl of *Micrococcus luteus* cell wall suspension and incubated at room temperature. The change in absorbance was measured on microplate reader (BioTek Eon) at 450 nm with 1 min interval for 30 min.

Purified compounds: Galangin and 1'-acetoxychavicol acetate were used to study the immune response of *S.litura.* Stock solutions (3125 ppm) of both the purified compounds were prepared in ethanol (0.5%) and appropriately diluted to get final concentrations of 125 and 625 ppm. Artificial diet was amended with these concentrations of the purified compounds and fed to 3rd instar larvae of *S. litura* for 48 and 96 hours. Larvae fed on diet amended with ethanol (0.5%) only, served as control. The activity of PO and lysozyme was estimated using the same experimental procedure as mentioned above for crude extract of *A.galanga*.

Statistical analysis: Experiment on immune response was replicated thrice. All the values were represented as their mean ± SE. To compare difference in means one way analysis of variance (ANOVA) with Tukey's test at $p \leq 0.05$ was performed. SPSS software for windows version 16.0 (SPSS Inc, Chicago) and Microsoft office Excel 2007 (Microsoft Corp., USA) were used to perform the statistical analysis.

RESULTS AND DISCUSSION: Secondary metabolites of *A.galanga* activated the PO system of *S.litura.* The larvae feeding on diet having 1500-2500 ppm of ethyl acetate extract for 24 hours showed significantly higher level of PO than control larvae (F=927.47, p≤0.05) (Table 1). Similarly all the concentrations differed significantly from control after 48 hours except for the lowest (F=477.83, p≤0.05). In comparison to 2.11 µM/mg/min of PO in control larvae, the activity level was 6.83 and 6.63 µM/mg/min in larvae feeding on 2000 and 2500 ppm of the extract. A slight decrease in PO activity was recorded after 72 and 96 hours of larval feeding on amended diet but in comparison to control it remained significantly higher showing a dose dependent response. Addition of 2500 ppm of ethyl acetate to larval diet induced the PO activity by 4.32 and 0.89 µM/mg/min in comparison to control after 72 and 96 hours respectively (Table 1).

Table 1: Influence of ethyl acetate extract of *A.galanga* on phenoloxidase activity of *S. litura* larvae.

Concentration (ppm)	Phenoloxidase activity (µM/mg/min)			
	24 (hrs)	48(hrs)	72(hrs)	96(hrs)
Control	1.33±0.03^{a}	2.11±0.08^{a}	1.02±0.16^{a}	1.84±0.04^{a}
500	1.06±0.04^{a}	2.63±0.16^{a}	1.08±0.16^{a}	2.06±0.04^{b}
1000	1.15±0.05^{a}	3.46±0.24^{b}	1.30±0.08^{a}	2.26±0.02^{c}
1500	2.39±0.04^{b}	5.04±0.05^{c}	2.34±0.06^{b}	2.12±0.02^{bc}
2000	3.73±0.10^{c}	6.83±0.11^{d}	3.18±0.13^{c}	2.57±0.18^{d}
2500	6.32±0.08^{d}	6.63±0.16^{d}	5.34±0.14^{d}	2.73±0.05^{d}

Figures are mean±S.E.Means followed by different superscript letters within a column are significant ly different. Tukey's test, p≤0.05

Table 2: Influence of ethyl acetate extract of *A.galanga* on lysozyme activity of *S. litura* larvae.

Concentration (ppm)	Lysozyme activity (µM/mg/min)			
	24(hrs)	48(hrs)	72(hrs)	96(hrs)
Control	10.08±.13^{a}	7.06±0.08^{a}	8.13±0.03^{a}	8.14±0.05^{abc}
500	7.79±0.02^{b}	8.41±0.06^{c}	8.16±0.13^{a}	9.49±0.03^{d}
1000	8.23±0.22^{b}	8.12±0.08^{bc}	6.84±0.03^{c}	8.93±0.17^{bcd}
1500	7.89±0.05^{b}	7.83±0.08^{b}	8.59±0.07^{b}	7.81±0.42^{a}
2000	8.11±0.15^{b}	7.82±0.08^{b}	8.56±0.07^{b}	8.98±0.02^{cd}
2500	6.75±0.30^{c}	8.32±0.20^{bc}	6.60±0.05^{c}	8.05±0.04^{ab}

Figures are mean±S.E.Means followed by different superscript letters within a column are significantly different. Tukey's test, p≤0.05

Table 3: Influence of galangin and 1'-acetoxychavicol acetate on phenoloxidase activity of *S. litura.*

Concentration (ppm)	Phenoloxidase activity (µM/mg/min)			
	Galangin		1'-Acetoxychavicol acetate	
	48 (hrs)	96 (hrs)	48 (hrs)	96 (hrs)
Control	1.86±0.02^{a}	1.68±0.04^{a}	1.86±0.03^{a}	2.66±0.04^{a}
125	1.74±0.08^{a}	1.59±0.07^{a}	2.36±0.05^{b}	2.39±0.04^{b}
625	2.09±0.02^{b}	1.50±0.05^{a}	2.24±0.02^{b}	1.83±0.04^{c}

Figures are mean±S.E.Means followed by different superscript letters within a column are significantly different. Tukey's test, p≤0.05

Table 4: Influence of galangin and 1'-acetoxychavicol acetate on lysozyme activity of *S. litura.*

Concentration (ppm)	Lysozyme activity (µM/mg/min)			
	Galangin		1'-Acetoxychavicol acetate	
	48 (hrs)	96 (hrs)	48 (hrs)	96 (hrs)
Control	7.89±0.14^{a}	8.14±0.10^{a}	7.74±0.15^{a}	8.07±0.09^{a}
125	8.07±0.20^{a}	8.00±0.10^{a}	7.92±0.15^{a}	8.00±0.08^{a}
625	8.24±0.11^{a}	9.05±0.17^{b}	8.02±0.08^{a}	7.92±0.02^{a}

Figures are mean±S.E.Means followed by different superscript letters within a column are significantly different. Tukey's test, p≤0.05

The larvae feeding on diet supplemented with ethyl acetate extract of *A.galanga* showed a significant reduction in lysozyme activity after 24 hours ($F=36.79$, $p\leq0.05$) (Table 2). Although the effect was not in a dose dependent manner, but the highest concentration decreased the activity level maximally (33.03%). However, after 48 hours, there was rise in enzyme level with 10.76- 19.12% over control at different concentrations. Although lysozyme activity of treated and control larvae differed significantly after 72 and 96 hours but no particular trend was recorded.

The purified compound of *A. galanga* was found to have significant influence on the PO and lysozyme activity of *S.litura* larvae. Consumption of 625 ppm of galangin induced the PO activity of *S.litura* larvae by 12.36% over control after 48 hours, ($F=11.77$, $p\leq0.05$) (Table 3) However, no significant differences were recorded between control and treated larvae after 96 hours. Addition of 1'-acetoxychavicol acetate to larval diet also significantly induced the PO activity. In comparison to control there was 26.88 and 20.43% rise due to 125 and 625 ppm of the compound after 48 hours. However, when the enzyme activity was estimated after 96 hours, a significant decrease was observed at both the concentrations ($F=77.20$, $p\leq0.05$) (Table 3). Supplementation of larval diet with 125 and 625 ppm of galangin seems to induce the level of lysozyme in *S.litura* after 48 hours, but the differences were statistically non significant. A significant increase of 11.18% was recorded after 96 hours of larval feeding on diet amended with 625 ppm of galangin with respect to control ($F=18.98$, $p\leq0.05$). However, 1'-acetoxychavicol acetate did not significant influence the level of lysozyme in *S.litura* larvae (Table 4).

Plant derived products are given importance in recent years for their use against several insect species. Their importance lies in the safety of compounds of plant origin to human and the environment. *A.galanga* a member of family Zingiberaceae has been known to exhibit a wide range of biologically activities (Chudiwal et al., 2010, Verma et al., 2011). Recently Sukhirun et al., (2011) demonstrated its insecticidal activity in *Bactrocera dorsalis* (Hendel).The present study demonstrated the effect of ethyl acetate extract and purified compounds of *A.galanga* on humoral immune response of *S.litura*. The plant metabolites significantly influenced the activity of PO and lysozyme. Incorporation of ethyl acetate extract to larval diet of *S.litura* increased the PO activity at all the time intervals as compared to control. Similarly purified compounds viz. 1'-acetoxychavicol acetate and galangin were found to induce the level of PO when the larvae were fed for 48 hours. The induction in PO activity has been attributed to activation of immune response as well as the activation of enzymes upstream from ProPO such as prophenoloxidase activating proteinase (Hartzer et al., 2005). There are reports documenting the effect of pathogens on humoral system of insect, but only few demonstrated the effect of secondary plant metabolites. Khanikor and Bora, (2012) reported a dose dependent increase in PO level on treatment of *Antheraea assama* (Helfer) by essential oils of *Ocimum sanctum, Ocimum gratissimum* and *Ageratum conyzoides.* Various studies reported that increase in PO activity is directly correlated with increased resistance to pathogen (Sugumaran, 2002; Shelby and Popham, 2006). Contrary to this Zibaee and Bandani, (2010) reported a decrease in PO activity with increasing concentration of *Artemisia annua* extract when fed to *Eurygaster integriceps* (Puton). Quercetin, a flavonoid has also been known to inhibit the activity of PO related enzymes such as monophenolase and o-diphenolase in *Spodoptera exigua* (Hubner) larvae (Luo et al., 2005). Similarly Zhiqing et al., (2008) documented suppression of PO level in 5th instar of the armyworm *Mythimna separata* (Walker) due to consumption of Terpinen-4-ol.

Ethyl acetate extract of *A.galanga* suppressed the level of lysozyme activity after 24 hours followed by a significant elevation after 48 hours as compared to control. The *S.litura* larvae fed on higher concentration of galangin also showed induced level of lysozyme activity while 1'-acetoxychavicol acetate did not significantly influence the lysozyme activity. The results are agreement with Gillespie et al., (2000) which reported that the lysozyme activity declined in *Schistocera gregaria* (Forskkal) with *Metarhizium anisopliae* infection. Admao, (2004) reported that lysozyme activity increased in *Gryllus texensis* (Cade and otte) after immune challenge and increase in level is related to survival of *G. texensis*. Kaur et al., (2015) reported that lysozyme activity in *S.litura* larvae significantly increased till 72 hours when fed in diet supplemented with ethyl acetate extract of *Alternaria alternata,* however, reduction in activity was observed thereafter.

CONCLUSION: In conclusion, this study revealed that ethyl acetate extract and purified compounds of *A.galanga* elicit immune response of *S.litura* by induction in PO and lysozyme activity. However suppression in enzyme activity at higher intervals indicates that enzyme induction does not completely overcome the toxicity of crude and purified compounds. A better understanding of insect immune defense mechanism would pave the way for success of pest management strategies like botanical, biocontrol agents etc.

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