# DETECTION OF NATURAL AGGLUTININS IN A FEW COLEOPTERAN INSECTS WITH SPECIFIC REFERENCE TO *POPILLIA JAPONICA*

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**ABSTRACT** *Natural hemagglutinins with specific affinity for the glycocalyx of rabbit erythrocytes was identified in the extracts of the whole body of few Coleopteran insects Popillia japonica, Phyllophaga drakei, Phyllophaga tristis, Copris minutus of the family Scarabaeidae and Luprops tristis of the family Tenebrionidae. The agglutinins in the extracts of the whole body of all the five species also agglutinated rat, pig, human A and human B erythrocytes with various specificities. Further, the physico-chemical characterization of the extracts of the whole body of the insect, Popillia japonica revealed that the agglutinin was calcium dependent and sensitive to EDTA. Maximum hemagglutination titer was observed from pH 6.5 to 8 at 35 $^{o}$C. The hemagglutinability was inhibited by fetuin and lactoferrin with the same potency and sugars GluNAc, α-lactose and mellibiose. Disappearance of agglutinability following cross adsorption revealed the presence of a single agglutinin. Presence of $Ca^{2+}$ dependent agglutinin with affinity for sialoglycoproteins and GluNAc in the whole body extracts of Popillia japonica, may contribute to its immune strategies, which could be further analysed upon purification using affinity chromatography.*

***Keywords:*** *Agglutinin, Cross adsorption, Erythrocytes, Hemagglutination, Hemagglutination inhibition*

## INTRODUCTION

Coleoptera (beetles and weevils), the largest order in the class Insecta consists of 360,000 known species. The name Coleoptera, derived from the Greek words *"koleos"* meaning sheath and *"ptera"* meaning wings, refers to the modified front wings which serve as protective covers for the membranous hind wings. To survive in a world full of microorganisms and parasites, insects have developed a potent defense mechanism that recognizes and removes microbial threats. Innate immunity is the first line of defense against infection which is the less specific or non-specific one and eliminates infection within hours after introduction into body (Debnath *et al*., 2017). The innate immune system in insects is composed of a large variety of specific and nonspecific responses that are activated in response to the presence of foreign agents (Santoyo and Aguilar, 2011). Invertebrates, including insects mainly depend on the innate immune system for their survival, due to the lack an adaptive immune system (Ab-based immunity). Several reports suggest the role of endogenous serum lectins with opsonic activity in non-self recognition of the invading pathogens (Wilson *et al*., 1999).

Lectins comprise a structurally vary diverse class of proteins characterized by their ability to selectively bind carbohydrate moieties of the glycoproteins of the cell surface (Kumar *et al*., 2012; Anitha and Basil-Rose, 2017). They are involved in biological processes such as recognition and binding of carbohydrates, interactions with pathogens, cell-cell interaction, cancer metastasis, apoptosis and differentiation (Chidhambaradhas *et al*., 2017). These suggest that lectins may have important functions in the renewal of cuticle, the degeneration of larval tissues and in the development of adult tissues (Gul and Ayvali, 2002). Among insecta, purification, sugar binding and physico-chemical properties and functions of lectins were determined in dipterans (Cao *et al*., 2010; Seufi *et al*., 2012; Ayaad *et al*., 2015), hemipterans (Kawauchi *et al*., 1993), orthopterans (Basil-Rose *et al*., 2005), lepidopterans (Yu and Kanost, 2000; Gul and Ayvali, 2002; Jayalakshmi *et al*., 2004) and coleopterans (Jayalakshmi, 2005; Anitha *et al*., 2018). However, most of the coleopteran representatives are untouched for agglutinin/lectin study. Hence an effort was taken in this research to analyze the presence of the hemagglutinins in five different species of coleopteran insects and to study the physico-chemical characterization of the insect with high HA titer.

## MATERIALS AND METHODS

### Collection of insects

Live specimen of five different insects species were collected from Elavuvilai near Marthandam, VilavancodeTaluk, Kanyakumari District, Tamil Nadu, India.

### Preparation of whole body extract

The healthy anaesthetized insects were cleaned with distilled water and then rinsed in cold Tris buffered saline (TBS) to remove the dust. The whole body extracts of each insect were prepared separately following the modified method of Volf *et al*. (2002). The extract was prepared at 1:10 ratio ie. 1 gm beetle was ground in 10 ml cold TBS and centrifuged at 4000 g for 10 min. at 4 $^{o}$C and the supernatant was assessed for hemagglutination activity.

**Preparation of erythrocyte suspension**

Blood samples were collected from different mammals (human A, B, O, pig, rabbit, rat, buffalo, cow, goat) directly in modified Alseivier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulfate (100 µg/ml) and chloramphenicol (330 µg/ml). Erythrocytes were suspended and washed thrice by centrifugation at 4000g with ten volumes of physiological saline and with Tris-Buffered Saline (TBS) pH 7.5 (Tris-HCl: 50 mM, NaCl: 100 mM; $CaCl_2$: 10 mM) and resuspended in TBS as 1.5% suspension (Mercy and Ravindranath, 1993).

**Hemagglutination assay**

The whole body extract of the insects were assayed for the presence of agglutinins using TARSON 96 well U-bottom microtitre plates as described by the method of Ravindranath and Paulson, 1987. The sample (25 µl) was serially diluted in TBS (25 µl) and incubated with 1.5% suspension of RBCs (25 µl) at room temperature (30±2 $^{o}$C) for an hour or until the negative control showed a red button formation. Agglutination activity was detected based on the RBCs appearance on the well: a positive result appears as a red-carpet layer, while negative results appear as a red button in the bottom of the well.

**Effect of pH and temperature on hemagglutinating activity**

To study the effect of pH and temperature on agglutinability, whole body extract of *Popillia japonica* was incubated at specific pH (5 - 9.5) and temperature (0 - 95 $^{o}$C) for an hour before adding erythrocyte suspension.

**Effect of calcium ions and chelators on hemagglutination activity**

To assess the effect of calcium ions and chelating agents on HA activity of the whole body extract, the extract was serially diluted with 25 µl of TBS with different concentration of $Ca^{2+}$ and chelators (EDTA and trisodium citrate) and was incubated at room temperature (30±2 $^{o}$C) for an hour prior to the addition of rabbit erythrocytes and the hemagglutination titer was determined.

**Hemagglutination Inhibition assay**

To a known concentration of serially diluted inhibitor (sugars/glycoproteins) solution (25 µl), 25 µl of the extract of the whole body diluted to sub agglutination concentration was added, mixed and the plate was incubated for 1 hour at room temperature. Finally 25 µl of 1.5% rabbit erythrocytes suspension was added and incubated for 1 hour at room temperature (30±2 $^{o}$C). The minimum concentration of the inhibitors required to completely block the agglutination after 1 hour of incubation at room temperature (30 ±2 $^{o}$C) was reported as the HAI titer.

**Cross adsorption assay**

The cross adsorption assay was carried out following the modified method of Mercy and Ravindranath (1992). 500 µl of whole body extracts were prepared from the healthy insect *Popillia japonica* were mixed with an equal volume of washed and packed native rabbit, rat buffalo, human A or B erythrocytes and incubated for 2 hours with frequent shaking. The RBC suspension was centrifuged (3000 rpm, 5 minutes), the supernatant was collected and used for hemagglutination assay. The supernatant that gave hemagglutination activity was readsorbed in equal volumes of their respective washed and packed erythrocytes for the second, third and subsequent time till it showed no HA activity with the tested erythrocytes.

**RESULTS**

**Diversity and distribution of insects**

Five different species of insects *Popillia japonica*, *Phyllophaga drakei*, *Phyllophaga tristis*, *Copris minutus* and *Luprops tristis* representing two different families Scarabaeidae and Tenebrionidae of the order Coleoptera of the class Insecta of the phylum Arthropoda were used for this investigation (Table 1).

**Table 1 Taxonomic position of the experimental animals**

| **Phylum : Arthropoda<br>Class : Insecta<br>Order : Coleoptera** | |
|---|---|
| **Family** | **Name** |
| Scarabaeidae | *Popillia japonica* |
| | *Phyllophaga drakei* |
| | *Phyllophaga tristis* |
| | *Copris minutus* |
| Tenebrionidae | *Luprops tristis* |

**Hemagglutinability of the whole body extract of insects**

The agglutinin in the extract of the whole body all the insects agglutinated rabbit (HA Titer = 128-1024), rat (HA Titer = 32-256), human A (HA Titer = 4-256), human B (HA Titer = 2-16) and pig (HA Titer = 4-512) erythrocytes with varying specificities. Among the various erythrocytes tested, maximum agglutinability was observed with rabbit erythrocytes (Table 2). Since *Popillia japonica* was easily available, it was selected for further study on physico-chemical characterization.

Table 2 Natural agglutinins in the whole body extract of the selected coleopteran insects

| **Erythrocytes** | **HA Titer** | | | | |
|---|---|---|---|---|---|
| | ***Popillia japonica*** | ***Phyllophaga drakei*** | ***Phyllophaga tristis*** | ***Copris minutus*** | ***Luprops tristis*** |
| Rabbit | 1024 | 1024 | 512 | 1024 | 128 |
| Rat | 128 | 256 | 32 | 64 | 64 |
| Buffalo | 16 | 0 | 0 | 4 | 4 |
| Human A | 16 | 128 | 256 | 4 | 16 |
| Human B | 16 | 8 | 8 | 2 | 4 |
| Human O | 2 | 2 | 2 | 0 | 0 |
| Cow | 8 | 0 | 0 | 0 | 0 |
| Goat | 4 | 0 | 0 | 2 | 0 |
| Pig | 4 | 512 | 128 | 8 | 16 |

**Influence of pH on HA**

The optimum pH for maximum activity of the agglutinin in the extract of the whole body of *Popillia japonica* was observed from pH 6.5 to 8. The agglutinability was low at acidic and alkaline range (Figure 1).

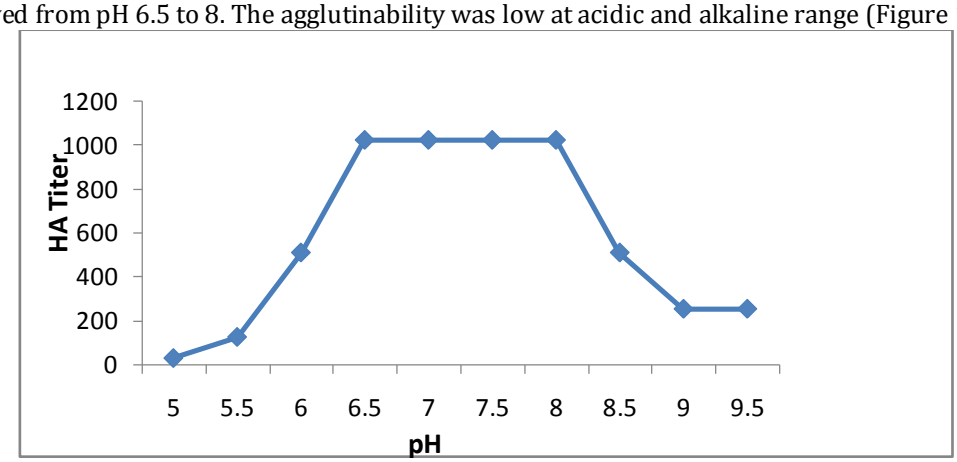


Figure 1 HA titer of the agglutinin in the whole body extract of *Popillia japonica* in relation to pH

**Impact of temperature on HA**

The maximum hemagglutination activity of the extract of the whole body of *Popillia japonica* was observed at 35 $^{o}$C, which got gradually reduced above and below 35 $^{o}$C (Figure 2).

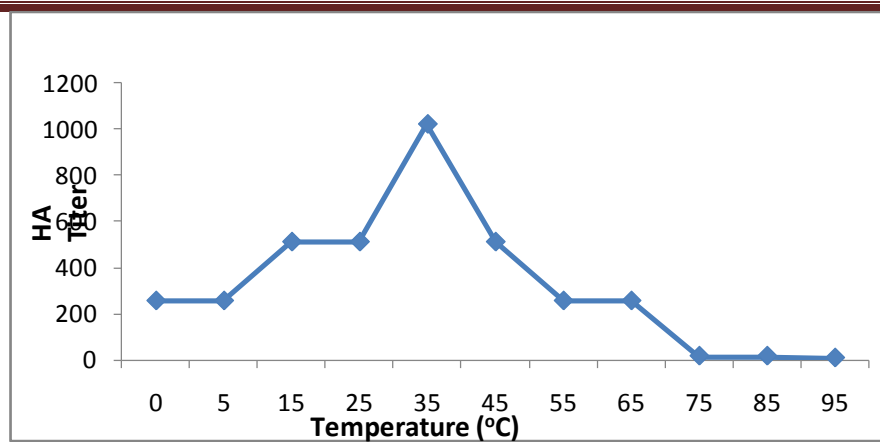


Figure 2 HA titer of the agglutinin in the whole body extract of *Popillia japonica* in relation to temperature

**Effect of cations and chelators**

Maximum hemagglutination was observed in the presence of 10 mM $Ca^{2+}$ (Figure 3). An increase in HA titer was observed with the addition of 0.01 to 0.1mM disodium EDTA which got reduced on further increase in concentration of disodium EDTA. However, the agglutinability was minimally affected by the addition of increasing concentrations of tetrasodium EDTA and trisodium citrate (Table 3).

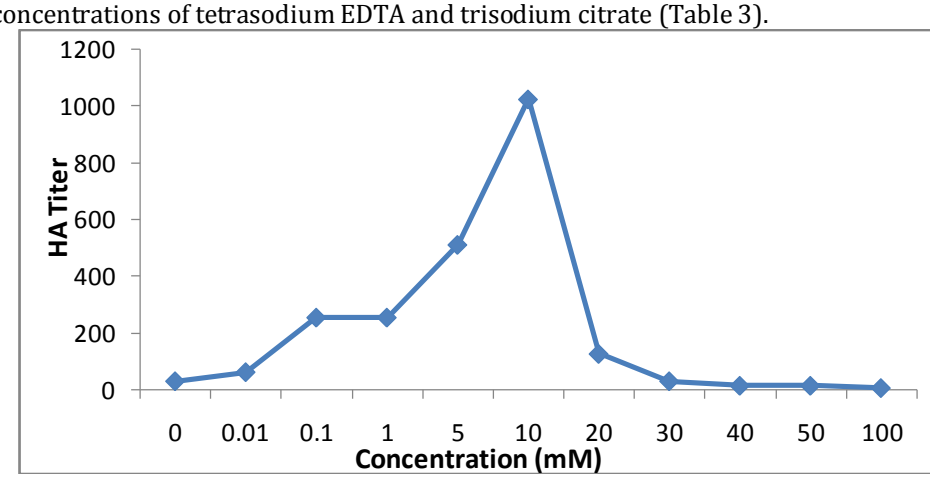


Figure 3 Effect of calcium ions on HA activity of the agglutinin in the whole body extract of *Poppilia japonica*

**Table 3 Effect of chelators on HA activity of the agglutinin in the whole body extract of *Popillia japonica***

| Concentration (mM) | HA Titer | | |
|---|---|---|---|
| | EDTA | | Trisodium citrate |
| | Disodium | Tetrasodium | |
| 0 | 32 | 32 | 32 |
| 0.01 | 512 | 32 | 64 |
| 0.1 | 512 | 32 | 64 |
| 1 | 256 | 32 | 64 |
| 5 | 256 | 32 | 64 |
| 10 | 4 | 32 | 64 |
| 20 | 0 | 32 | 64 |
| 30 | 0 | 32 | 16 |
| 40 | 0 | 32 | 16 |
| 50 | 0 | 16 | 8 |
| 100 | 0 | 16 | 4 |

**Cross adsorption test**

Agglutinating activity of the whole body extracts of the insect *Popillia japonica* was completely lost after adsorption with any one of the erythrocytes initially recognized by the agglutinin.

Table 4 HA titer of the whole body extracts of the insect *Popillia japonica* after adsorption with different erythrocytes

| Erythrocyte adsorbed | Rabbit | Rat | Buffalo | Human A | Human B |
|---|---|---|---|---|---|
| None | 1024 | 128 | 16 | 16 | 16 |
| Rabbit | 0 | 2 (0) | 0 | 0 | 0 |
| Rat | 8 (0) | 0 | 0 | 0 | 0 |
| Buffalo | 2 (0) | 0 | 0 | 0 | 0 |
| Human A | 0 | 0 | 0 | 0 | 0 |
| Human B | 0 | 0 | 0 | 0 | 0 |

**HAI assay**

Among the inhibitors tested, the agglutinability of the agglutinin was inhibited by glycoproteins fetuin, lactoferrin (HAI titre=32) followed by BSM, PSM (HAI titre=4), thyroglobulin (HAI titre=2) and sugars GluNAc (HAI titre = 16), α-lactose, mellibiose (HAI titre = 8), D-fructose (HAI titre = 4), galactose, raffinose (HAI titre = 2) (Table 4).

Table 4: HAI titer of the agglutinin in the whole body extract of *Papillia japonica* by various glycoproteins and sugars

| Inhibitors (Glycoproteins/Sugars) | | HAI titer | Minimum Conc. Required (mg/ml) / (mM) | Relative inhibitory potency (%) |
|---|---|---|---|---|
| **Glycoproteins** | Fetuin | 32 | 156.25 | 100 |
| | Lactoferrin | 32 | 156.25 | 100 |
| | BSM | 4 | 1250 | 12.5 |
| | PSM | 4 | 1250 | 12.5 |
| | Thyroglobulin | 2 | 2500 | 6.25 |
| **Sugars** | GluNAc | 16 | 6.25 | 100 |
| | α-lactose | 8 | 12.5 | 50 |
| | Mellibiose | 8 | 12.5 | 50 |
| | D-Fructose | 4 | 25 | 25 |
| | D-Galactose | 2 | 50 | 12.5 |
| | Raffinose | 2 | 50 | 12.5 |

**Discussion**

The present study, the occurrence of agglutinins is identified in the extracts of the whole body of five different coleopteran insects by way of hemagglutination assay. All the tested insects showed specific affinity to the tested mammalian erythrocytes and with varying capacities. The difference in agglutination activity among the insects reflects that lectins in the insect are diverse and so, the binding specificity to cell surface glycoconjugates of mammalian erythrocytes vary as expressed in hemagglutination activity. Agglutination is facilitated by two or more combining sites on agglutinating molecules enabling the agglutinin to adhere to more than one erythrocyte species (Goldstein *et al*., 1980). The erythrocyte specificity of the agglutinin of the whole body extract argues for the specific recognition of the sugars constituting the glycocalyx of these erythrocytes, which serve as receptors to ligands as in the eukaryotic cells (Hakomori, 1973). It is interesting to note that all the erythrocytes recognized by the agglutinin possess sialic acid as the terminal sugar in the glycocalyx. Probably the agglutinin may bind to sialic acid of the glycocalyx of these erythrocytes (Yasue *et al*., 1978). Among the various mammalian erythrocytes tested against the whole body extracts of the insects, rabbit erythrocyte was agglutinated with high HA titer as reported in *Atractomorpha crenulata* (Basil-Rose *et al*., 2005), *Oxya hyla hyla* (Anusha, 2012), *Danaus chrysippus* (Jayalakshmi *et al*., 2004), *Oryctes rhinocerous* (Jayalakshmi, 2005) and *Phyllophaga* sp (Anitha *et al*., 2018). Due to the easy availabity, the physico-chemical characterization was carried out in *Papillia japonica.*

Physico-chemical characterization revealed the proteinaceous nature of the lectin. Generally, primary and secondary structures of the proteins were altered when the agglutinins were exposed to external stress

such as pH, temperature, humidity etc. The acidic and alkaline range of pH and very low and high temperature which alter the disulphide bond in the binding site of the agglutinin resulted in either reduction or enhancement of the agglutinating activity. The ability of the lectin to agglutinate erythrocytes with maximum HA titer in a wide range of pH from 6.5 to 8 explain its ability to function in wide range of ionic conditions. This is in support of the pH sensitivity reported in sand fly *Phlebotomus* sp (Palanova and Volf, 1997), giant stick insect *Extatosoma tiaratum* (Richards *et al*., 1988) and May beetle *Phyllophaga* sp (Anitha *et al*., 2018). However, the agglutinin is thermo-labile with optimum HA titer specifically at 35 $^{o}$C reported in a butterfly *Danaus chrysippus* (Jayalakshmi *et al*., 2004) and a beetle *Phyllophaga* sp (Anitha *et al*., 2018). Increase in activity with increase in temperature up to 35 $^{o}$C falls in line with the VantKoff's law for a biological activity.

Calcium dependency of the agglutinin was confirmed based on increase in HA titer with the addition of increasing concentration of calcium up to 10 mM and decrease in HA titer with the addition of increasing concentration of disodium EDTA to 10 mM and its total disappearance on further addition. This calcium dependency for hemagglutination indicates a preferential calcium ion requirement for the binding of agglutinin to the sugar moieties. Calcium dependency for hemagglutination is also reported in *Danaus chrysippus* (Jayalakshmi *et al*., 2004), *Silana farinosa* (Priya, 2013), *Mylabris pustulata* (Pramila, 2012), *Geotrupes stercorarius* (Rama Devi *et al*., 2014) and *Phyllophaga* sp (Anitha *et al*., 2018). Metal chelating agents as the strongest inhibitor may cleave the excess divalent cations resulting in an enhanced the agglutinability.

Cross adsorption assay resulted in the complete disappearance of agglutinability with all the erythrocytes after the first or second adsorption revealing the presence of a single agglutinin. The rat and buffalo erythrocytes may not have sufficient number of receptors for the binding of agglutinins resulting in the need to proceed with the second adsorption which completely removed the agglutinins from the extract of the whole body of the insect *Papillia japonica*. Presence of single agglutinin previously reported in insects *Oryctes rhinoceros* (Jayalakshmi 2005), *Oxya hyla hyla* (Anusha, 2012) and *Mylabris pustulata* (Pramila, 2012).

Of the different inhibitors tested fetuin, lactoferrin, BSM, PSM, thyroglobulin, GluNAc, α-lactose, mellibiose, D-Fructose, D-Galactose and raffinose inhibited the agglutinability of the agglutinin at varying capacities. HA titer with NeuAc / NeuGc containing erythrocytes and HAI titer with NeuAc / NeuGc containing glycoproteins suggest its affinity to sialic acid or glycosidic linkages which can be confirmed on purification by affinity purification.

## Conclusion

The present study revealed the presence of a calcium dependent agglutinin in the whole body extract of the insect *Popillia japonica*. The agglutinin recognized rabbit erythrocytes with great avidity in the presence of $Ca^{2+}$ ions at 35 $^{o}$C and pH from 6.5 to 8. The agglutinability was specifically inhibited by the glycoproteins fetuin and lactoferrin with equal affinity. This study provides the physico-chemical characteristics necessary to purify the agglutinin by affinity chromatography.

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