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ARTICLE

A purified lectin with larvicidal activity from a woodland mushroom, *Agaricus semotus* Fr.

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ABSTRACT This study investigated the larvicidal activity on *Culex quinquefasciatus* of lectin purified from fresh fruiting bodies of woodland mushroom, *Agaricus semotus*. *A. semotus* lectin (ASL) was purified via ion-exchange chromatography on DEAE-cellulose A-25 and size exclusion chromatography on Sephadex G-100 matrix. Molecular weight (16.6 kDa) was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The effects of temperature, pH, metal chelation- and larvicidal activity of ASL were also investigated. The ASL indifferently agglutinated the erythrocytes of the human ABO blood system and was stable at acidic pH and below 50 °C whereas 66% of its activity was lost at 60 °C with complete inactivation at 70 °C. ASL is a metalloprotein requiring barium ion as chelation of metals by 50 mM EDTA rendered the lectin inactive, while the addition of $BaCl_2$, among other metal salts, restored the activity. ASL showed larvicidal activity against *C. quinquefasciatus* larvae after 24 h with a mortality of 5 and 95% at 5 and 25 mg/mL respectively, and LC_{50} of 13.80 mg/mL. This study concluded that purified *A. semotus* lectin showed impressive larvicidal activity, which could be exploited in its development as an insecticidal agent. **Acta Biol Szeged 65(1):65-73 (2021)**

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Introduction

Diseases transmitted by mosquitoes are one of the endemic health-related environmental menaces on the rise worldwide, which contribute to a high loss of life, especially in tropical countries with low income (Wilke et al. 2017; Fonseca et al. 2019). *Culex quinquefasciatus* Say, 1823 (*Culicidae*, southern house mosquito) is vastly distributed in subtropical regions of the world (Lopes et al. 2019). It is incursive, and belongs to the *Culex pipiens* species complex, which feed on mammalian and avian blood, *C. quinquefasciatus* is considered a medically significant species, because of their implicated roles in the transmission of zoonotic diseases including those caused by *Wuchereria bancrofti,* West Nile virus, and arbovirus (Fonseca et al. 2004; Lima-Camara et al. 2016).

Although majorly found in Africa, *C. quinquefasciatus* usually spread beyond their domiciled environment, posing a severe risk to public health (Farnesi et al. 2015; Cuthbert et al. 2020). As part of control measures to curb mosquito establishment and proliferation, the World Health Organization (WHO) introduced a manual on the management of these quintessential vectors (Takken and van den Berg 2019). Due to the advent of mosquito strains resistant to synthetic insecticides, together with potential environmental and health threats associated with these chemicals, alternative bio-control agents are being sought (Bellinato et al. 2016; Camaroti et al. 2017; Santos 2020).

The non-enzymatic lectins with unique sugar-binding characteristics are ubiquitous proteins capable of reversible discrete interaction with cell surface carbohydrate associated structures and involved in diverse biological and pathological functions (He et al. 2015; Muszyńska et al. 2018; Nascimento et al. 2020; Perduca et al. 2020). Aside from being a remarkable tool in blood group determination, lectins also perform defense-related functions (Silva et al. 2020). Although originally identified in plants and animals, exploration of fungal lectins is becoming popular because of their peculiar carbohydrate specificity coupled with potential application in biotechnology and biomedicine (Singh et al. 2010; Varrot et al. 2013; Hassan et al. 2015; Singh et al. 2015). Mushrooms have attracted numerous research activities due their diverse inherent bioactive constituents including lectins (Singh et al. 2010;

Largeteau et al. 2011).

Worldwide, *Agaricus* genus has over 300 members, which are distinguished by their unique spore coloration, among other structural features (Zhang et al. 2019). In this study a lectin was purified from the fresh fruiting bodies of woodland mushroom and its larvicidal activity was investigated towards *C. quinquefasciatus* to explore its potential in insect control.

Materials and Methods

Materials and chemicals

Glutaraldehyde, Folin reagent, ethylenediaminetetraacetic acid (EDTA), acrylamide, bovine serum albumin, sodium dodecyl sulfate (SDS), sugars, Coomassie Brilliant Blue R 250 and molecular weight protein markers (10-170 kDa) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Purchase of Sephadex G-100 and DEAE cellulose A-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). The quality of all reagents was research-quality.

A, B, O human blood groups were collected with informed consent from healthy subjects.

Collection of mushroom

Woodland mushroom (*A. semotus* Fries) was collected on farmland near the Department of Electrical and Electronics Engineering, Obafemi Awolowo University, Ile-Ife (South-West Nigeria). Identification was done at the Department of Microbiology (Mycology Laboratory), Obafemi Awolowo University, Ile-Ife, Nigeria.

A. semotus crude extract preparation

Approximately 16 g mushroom sample was homogenized and extracted using 0.025 M Phosphate Buffered Saline, pH 7.2 (1:5 w/v) and was stirred at 4 °C overnight. Resulting mixture was centrifuged at 4000 rpm for 15 min and filtered through cheesecloth to get the crude extract, which was stored at -20 °C until use.

Protein concentration assay

Assay method of Lowry et al. (1951) was employed in estimating protein concentration. Standard protein used was bovine serum albumin (1 mg/mL).

Hemagglutination and sugar specificity assays

Human A, B, and O blood groups were collected into lithium-heparin bottles. Erythrocytes were recovered from the centrifuged blood samples and fixed using glutaraldehyde (Bing et al. 1967). Hemagglutination assay and sugar specificity test was performed as described by Kuku and Eretan (2004) in a U-shaped 96-well microtiter plate. Tested sugars were D-mannitol, fructose, L-sorbose, galactose, D-mannose, D-glucosamine hydrochloride, N-acetyl-D-glucosamine, inulin, maltose, glycogen, D-lactose monohydrate, D-glucose monohydrate, and starch.

Purification of A. semotus lectin (ASL)

Ion exchange chromatography on DEAE cellulose A-25

Chromatography column (1.5 x 20 cm, Bio-Rad) packed with DEAE- cellulose A-25 matrix, and equilibrated with 10 mM Tris-HCl buffer, pH 7.3, was used to purify the crude protein extract (3 mL, ≈33 mg protein). Fractions (2 mL) were collected at 24 mL/h flow rate. Unadsorbed fractions were eluted with the equilibration buffer (10 mM Tris-HCl buffer, pH 7.3), while adsorbed fractions eluted with the same buffer containing 0.2 M NaCl. Fractions were monitored at 280 nm, and hemagglutinating activities were assayed. Active peak fractions (unadsorbed peak) were pooled, dialyzed (against 10 mM Tris-HCl buffer, pH 7.3, and distilled water) for 48 h, freeze-dried, and kept at -20 °C.

Size exclusion chromatography on Sephadex G-100

Five milliliters (≈ 30 mg protein) of the pooled DEAE active fractions (unadsorbed peak) was loaded on Sephadex G-100 column (2.5 x 20 cm, Bio-Rad) equilibrated with phosphate-buffered saline (0.02 M, pH 7.2), and fractions (4 mL) collected at 30 mL/h flow rate. Fractions were monitored at 280 nm, and hemagglutinating activity assayed. Active peak fractions were collected, dialyzed, freeze-dried, and kept frozen at -20 °C.

Physicochemical characterization of ASL

Denaturing gel electrophoresis (12.5% acrylamide gel, Tris-HCl system) was employed in the determination of ASL subunit molecular weight using standard marker proteins of 10-170 kDa range, as described by Laemmli

Table 1. Purification summary.

Fractions	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield (%)	Purification fold
Crude extract	5550	1024	0.184	100.0	1.00
Ion exchange chromatography (IEC-1)	585.90	512	0.873	50.0	4.74
Size exclusion chromatography (SEC-2)	101.8	128	1.257	12.5	6.83

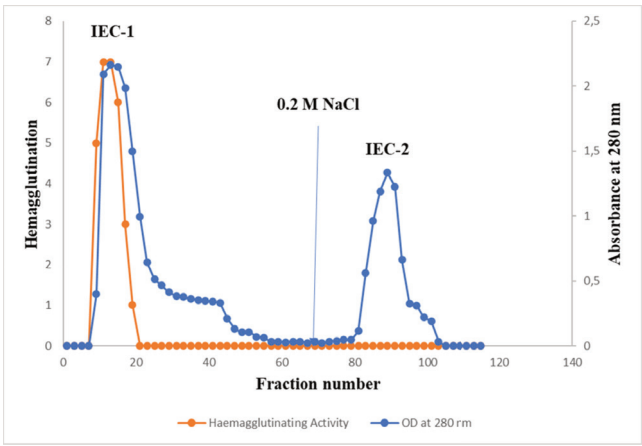

Figure 1. Ion exchange chromatography of *A. semotus* crude extract.

and Favre (1970).

Assay method of Sampaio et al. (1998) was employed in investigating temperature effect on ASL hemagglutinating activity. Aliquot of ASL (500 µL) was subjected to temperature variation (30-90 °C) for 60 min using a water bath (Model DK-420, Movel Scientific Instruments, Zhejiang, China). Incubated ASL was swiftly iced-cooled and activity was assayed. Activity of ASL was served as control at 25 °C.

The pH effect on ASL hemagglutinating activity was investigated by incubating lectin aliquots (500 µL) at 25 °C for 60 min with buffer solutions of pH 2-11 range (0.2 M citrate, pH 2-6; 0.2 M Tris-HCl, pH 7-8, and 0.2 M glycine-NaOH, pH 9-11). Control was ASL incubated in PBS, pH 7.2 and results presented as described by Nakagawa et al. (1996).

To investigate the divalent cation requirement of ASL, assay experiment of Wang et al. (1996) was employed. Aliquot of ASL was dialyzed for 24 h against 50 mM EDTA and hemagglutinating activity assayed. Thereafter, the demetallized lectin aliquots were incubated differently with $BaCl_2$, $HgCl_2$, $MgCl_2$, $SnCl_2$, and $CaCl_2$ (10 mM, 50 µL) for 2 h, and each time, assayed for hemagglutinating activity.

Larvicidal assay

C. quinquefasciatus larvae were cultured in an insectarium under regulated conditions of relative humidity 70 ± 10% and at 27 ± 2 °C with a 12-12 light-dark regime at the Drug Research and Production Unit (DRPU), Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. Free adult mosquitoes were allowed to lay eggs in the habitats provided for them in the laboratory and egg rafts collected for hatching in glass and plastic bowls. To promote larva development and female fecundity, hatched mosquito larvae were fed daily with fish food *ad libitum*.

Larvicidal activity of ASL was investigated as described in our previous study (Johnny et al. 2016). Laboratory–reared fourth instar larvae of *C. quinquefasciatus* were exposed to varying concentrations (5-25 mg/mL) of ASL according to standard procedure (WHO 1981). Comprehensively, 20 larvae were introduced into plastic beakers (50 mL) for each concentration of ASL. Experimentations were done in triplicates at 27 ± 2 °C, with one control (distilled H_2O) set for each. Motionless larvae or those which settled at the bottom of test beakers with no sensitivity to involuntary stimulus or light, or after mild probing failed to move were considered dead. Percentage mortalities were recorded for each concentration after 24 h of the exposure period and obtained data statistically analyzed.

Statistical analysis

Experimental data were analyzed using StatPlus® 2006 (AnalystSoft, Canada) to find the lethal concentrations (LC_{50}) of larvae in 24 h by probit analysis with a reliability interval of 95% (Finney 1971; Islam et al. 2013).

Results

Purification, hemagglutinating activity, and sugar specificity of Agaricus semotus lectin (ASL)

Ion exchange and size exclusion chromatography purified ASL from *A. semotus* to homogeneity with yield and purification fold of 12.5% and 6.83, respectively (Table 1).

Two distinct peaks were obtained from the elution profile of *A. semotus* crude extract on DEAE cellulose A-25 column (Fig. 1), of which only the unadsorbed peak (IEC-1) exhibited hemagglutinating activity. Size exclusion chromatography of the DEAE-active peak (IEC-1) on Sephadex G-100 matrix also resulted in two protein peaks (Fig. 2), but only second peak (SEC-2) showed hemagglutinating activity, which constituted the homogeneous ASL used for further studies.

Hemagglutination assay showed that ASL indifferently agglutinated the erythrocytes of the human ABO blood group, and this activity was strongly inhibited by inulin among tested sugars (Table 2).

Physicochemical characterization of ASL

SDS-PAGE analysis of ASL revealed a distinct band with a relative molecular weight of 16.6 kDa (Fig. 3). ASL lost its activity at 70 °C (Fig. 4) and stable at acidic pH (Fig. 5).

Larvicidal study

The ASL treatment resulted the concentration-dependent mortality of *C. quinquefasciatus* larvae with the lowest mortality of 5% at 5 mg/mL and the highest mortality of

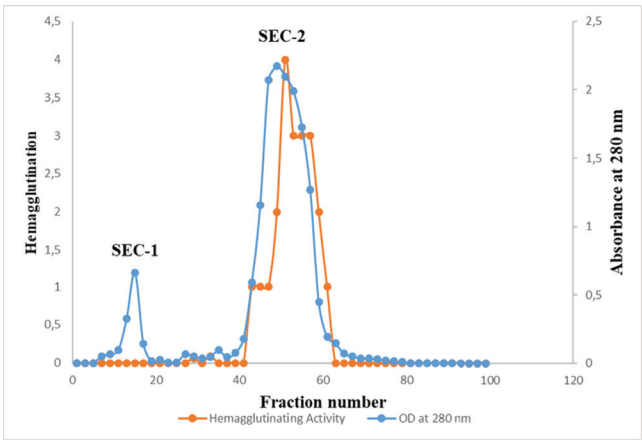

Figure 2. Size exclusion chromatography of the DEAE-Unadsorbed peak (IEC-1).

95% at 25 mg/mL concentration after 24 h (Table 3) with LC_{50} of 13.767 mg/mL (Fig. 6).

Table 2. Hapten inhibition test of ASL purified from *A. semotus.*

Sugar	Hemagglutination titre
Control	2^9
Fructose	2^7
L-Sorbose	2^7
Galactose	2^7
D- Mannose	2^7
D- Mannitol	2^9
D- Glucosamine-hydrochloride	2^9
D- Glucose monohydrate	2^8
N-acetyl-D-glucosamine	2^8
Inulin	2^2
Maltose	2^8
Glycogen	2^8
D-Lactose monohydrate	2^9
Starch	2^{12}

Experiments comprised ASL (100 µL) diluted serially in a 96-well microtitre plate. Equal volumes (50 µL) of respective sugar solutions (0.2 M) and 2% human blood group A erythrocytes suspension were introduced to the wells. Positive control contained no sugars, and negative control contained neither ASL nor sugars. Experiments were done in triplicates.

Discussion

Purification, hemagglutinating activity and sugar specificity of Agaricus semotus lectin (ASL)

Diverse chromatographic techniques are being used in the purification of mushroom lectins, however, recent studies have reported the effectiveness of ion-exchange and size exclusion chromatographic techniques as an alternative to highly preferred single-step affinity chromatography, which exploits sugar specificity of the lectin (Chandrasekaran et al. 2016; Wang et al. 2018; Panchak 2019). Elution profile of ASL on DEAE cellulose A-25 matrix reported in this study was similar to the chromatographic behavior of *A. arvensis* lectin (AAL) (Zhao et al. 2011).

A. semotus lectin (ASL) exhibited a non-specific agglutination towards the human ABO erythrocytes, characteristics mainly associated with an unique group of promiscuous lectins (panalectins), which have been identified mainly in plants, but rarely in mushrooms (Kuku et al. 2000; Akinyoola et al. 2016; Oladokun et al. 2019). However, lectins have been already identified and isolated within the genus *Agaricus*, there is not any previous report on the lectins of *A. semotus* (Kawagishi et al. 1988; Mikiashvili et al. 2006; Nakamura-Tsuruta et al. 2006; Zhang et al. 2019).

The hapten inhibition test to determine the sugar specificity of ASL showed that D-mannose, L-sorbose, galactose, and fructose slightly decreased the hemagglutinating activity, while inulin (a fructan) strongly inhibited the activity, although starch enhanced activity among the sugars tested (Table 2). Inhibition of the hemagglutinating activity of ASL by more than one simple and/or complex sugar reported in this study is not uncommon among mushroom lectins, especially within the genus *Agaricus*, as similar characteristics have been reported for lec-

Table 3. Larvicidal activity of ASL on *C. quinquefasciatus*.

S/N	Concentration (mg/mL)	Number of larvae	Recorded death	Mortality %
1	Control (distilled water)	20.00	0.00 ± 0.00	0.00 ± 0.00
2	5.00	20.00	1.00 ± 0.05	5.00 ± 0.12
3	10.00	20.00	5.00 ± 0.25	25.00 ± 0.31
4	15.00	20.00	8.00 ± 0.40	40.00 ± 0.11
5	20.00	20.00	15.00 ± 0.75	75.00 ± 0.25
6	25.00	20.00	19.00 ± 0.95	95.00 ± 0.11

Experiments were done in triplicates. Data were presented as mean ± SEM (standard error of mean).

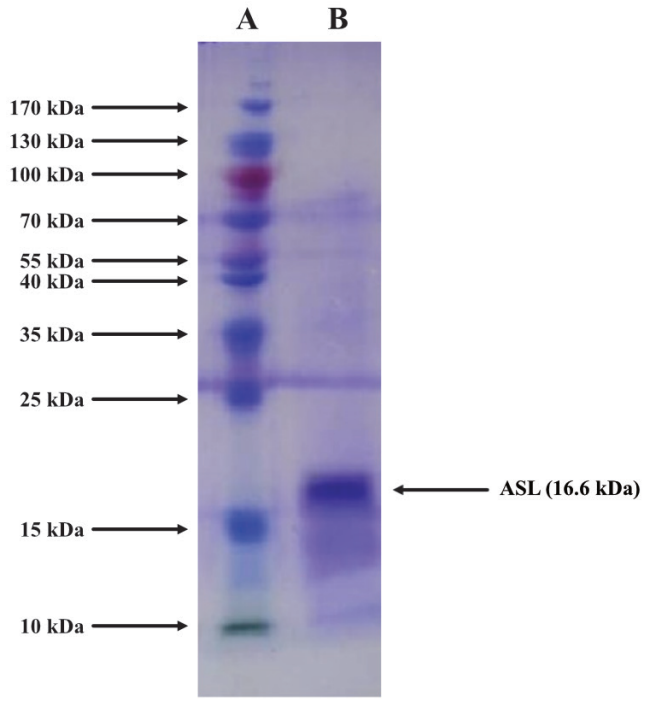

Figure 3. Electrophoretogram of SDS-PAGE of *A. semotus* lectin (ASL) and molecular weight markers (10-170 kDa). Lane A (Standard marker proteins), lane B (purified ASL).

tins isolated from *A. blazei*, *A. bisporus,* and *A. pilatianus* (Kawagishi et al. 1988; Nakamura-Tsuruta et al. 2006; Mikiashvili et al. 2006).

Inulin specificity obtained for ASL is similar to that of *A. arvensis* lectin (Zhao et al. 2011). Zhang et al. (2019) also reported inulin inhibition of *A. bitorquis* lectin suggesting a conserved inulin-binding specificity regarding isolated lectins from the genus *Agaricus*, which might be exploited in a structure-function relationship for practicable biotechnological application, especially through molecular docking. Other mushroom lectins reportedly inhibited by inulin include *Armillaria luteo-virens* lectin, *Pholiota adipose* lectin, and *Hericium erinaceum* lectin (Feng et al. 2006; Zhang et al. 2009; Li et al. 2010).

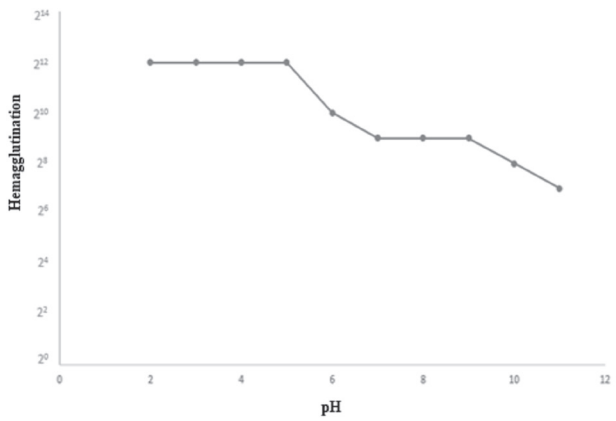

Figure 4. Effect of temperature on the hemagglutinating activity of ASL.

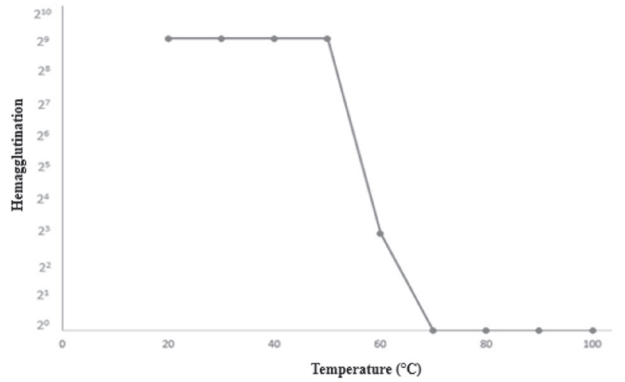

Figure 5. Effect of pH on the hemagglutinating activity of ASL.

Physicochemical characterization of ASL

Fungal lectins, especially those isolated from mushrooms exhibit profound variation in their physicochemical properties (Hassan et al. 2015; Singh et al. 2020). The molecular weight of ASL (16.6 kDa) is similar to the weight reported for *A. blazei* lectin (16 kDa) (Kawagishi et al. 1988). ASL maintained 100% activity up to 50 °C, however, lost 66% of the activity at 60 °C and completely inactivated at 70 °C (Figure 4). ASL is moderately thermostable, as mushroom lectins are reportedly usually stable at a moderate temperature with gradual loss of activity as the temperature increases (Alborés 2014; Wang et al.

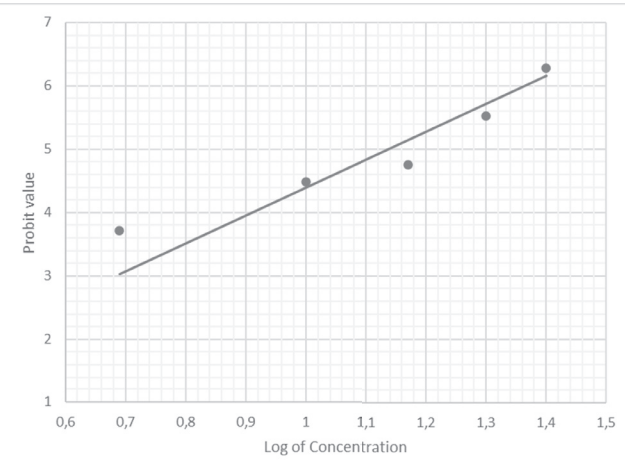

Figure 6. Probit graph for determination of LC_{50}.

2019). However, this result contradicts with the study of Zhao et al. (2011), who reported *A. arvensis* lectin was thermostable up to 90 °C. Several reports have shown that the thermostability of lectins differs, as lectins usually undergo unprecedented conformational changes under harsh temperatures, which might culminate in inactivation mostly owing to the destabilization of interactions necessary for their native conformation (Wang et al. 1996; Singh and Saxena 2013). ASL was stable at acidic pH with maximum activity within pH 2–5, with a 41% loss of activity within pH 7-9 and 50% loss at pH 11 (Fig. 5). Stability of ASL at acidic pH shown was similar to that reported for *Gymnopilus spectabilis* lectin (Alborés et al. 2014).

ASL was completely inactivated by 50 mM EDTA, however, when different metal salts were added to the assay medium, $BaCl_2$ restored 33% of the lectin activity suggesting ASL might be a metalloprotein. The result obtained for ASL contradicts the report on *A. arvensis* lectin (AAL), which was unaffected by divalent ions and enhanced by trivalent ions (Zhao et al. 2011).

Larvicidal study

Chemical method of insect control is one of the major mechanisms of mitigating diseases propagated by these organisms with long-term effects linked with insect resistance, together with detrimental effects on the ecosystem, and human health, paving way for exploration of safer and eco-friendly natural alternatives such as lectins (Gautam et al. 2013; Shaurub et al. 2015; Demok et al. 2019; Satoto et al. 2019; Kumar et al. 2020).

As a distinctive component of the innate defense system of their host, mushroom lectins have a unique ability to recognize diverse carbohydrate-associated structures that mediate most of their varying biological activities (Singh and Saxena 2013; Sabotič et al. 2016). Previous studies on entomotoxic lectins have suggested the fatality induced by these proteins usually involves multiple complementary mechanisms including induction of apoptosis and interaction with specific carbohydrates/glycan structures of vital enzymes, especially those engaged in metabolism and detoxification (de Oliveira et al. 2016; Napoleão et al. 2018). In fact, because of their multivalent assembly, fungal lectins can cross-link cell surface glycoconjugates or trigger distinct oligomerization of glycosylated signaling receptors, which may affect their turnover (Hamshou et al. 2012). Morphological damages of the gut initiated by excessive proteolysis due to specific binding to peritrophic matrixes or epithelial cells of target insects have also been connected to their insecticidal/larvicidal activity (Coelho et al. 2009). As reported by Coelho and co-workers, *Moringa oleifera* lectin increased lumen volume and disrupted the midgut epithelium of *Aedes aegypti* larvae and peritrophic membrane of *Anagasta kuehniella* larvae, ultimately resulting in gut cell death (Coelho et al. 2009; de Oliviera et al. 2017).

Entomotoxic lectins have also been reported to alter critical target insects' biological functions such as pupation, survival, larval development, and adult emergence (Kaur et al. 2009). *Dioscorea batatas* lectin was reported to alter the development of *Helicoverpa armigera* larvae by interacting strongly with vital intracellular structures (Ohizumi et al. 2009).

The entomotoxic lectins vary in physicochemical characteristics, and there are many potential targets because of their multivalent highly stereospecific binding with diverse arrays of insect glycan structures, however, differences in their spatial arrangement together with the proportion of carbohydrate recognition domain may expound the disparity in toxicity mechanisms elicited by these quintessential bioactive proteins (Fitches et al. 2012; Yang et al. 2014; Rani et al. 2017; Singh et al. 2020).

Conclusion

This study concluded that a lectin purified from *A. semotus* showed potent larvicidal activity against *C. quinquefasciatus,* which suggests its application as an alternative and eco-friendly larvicide/insecticide in the control of mosquitoes to mitigate mosquitoes-borne diseases and mortalities. However, a better comprehension of the action mechanism used by the lectin might help further research to investigate the possibilities of biotechnological applications of mushroom lectins in agriculture against phytophagous insects.

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