INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 23, No. 4, p. 46-54, 2023

RESEARCH PAPER OPEN ACCESS

# Antibacterial Properties and Cytotoxicity of *Geodorum Densiflorum* Rhizome Lectin: A Promising Solution to Combat Antibiotic Resistance

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**Key words:** Lectin, *Geodorum densiflorum*, Antibacterial, Toxicity

http://dx.doi.org/10.12692/ijb/23.4.46-54 Article published on October 03, 2023

## Abstract

Antibiotic resistance is a pressing issue in modern medicine, posing a significant concern in the 21st century. Microorganisms have developed new resistance mechanisms that spread widely, endangering our ability to treat various infectious diseases and increasing nosocomial infections. Lectins, which exhibit broad antibacterial activity, work based on carbohydrate specificity, varying in effectiveness depending on the plant species from which they are derived from. They offer a potential alternative to combat antibiotic resistance. However, further research is needed for clinical application. In our study, we explored the physiological effects of GDL, a lectin weighing around 12 ± 1 kDa, sourced from *Geodorum densiflorum* (Lam.) rhizomes. We assessed its antibacterial activity against six bacterial strains (*Staphylococcus aureus*, *Shigella boydii*, *E. coli*, *Shigella sonnei*, *Agrobacterium sp.*, and *Shigella dysentery*). GDL displayed varying levels of growth inhibition, with minimal inhibitory concentrations ranging from 40 to 320 µg/mL. Additionally, we examined the cytotoxicity of *Geodorum densiflorum* lectin using the brine shrimp lethality bioassay. GDL exhibited dose-dependent toxicity towards *Artemia* larvae, with an $LC_{50}$ value of 385 µg/ml. This cytotoxic result suggests that future research on lectin applications from *Geodorum densiflorum* rhizomes may have significant implications in clinical microbiology, paving the way for further in-depth investigations.

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**Introduction**

Contagious bacterial illness resulted fatalities all over the world including less economically developed nations (Nair *et al.*, 2013). Infections remain closely linked to illness and death (Cavalcante *et al.*, 2022). The 2020 World Health Organization report recorded 48.9 million infections in 2017, leading to 11 million sepsis-related deaths worldwide (Cavalcante *et al.*, 2022). These infections, caused by both Gram-positive and Gram-negative bacteria, are responsible for severe human illnesses (Nair *et al.*, 2013). Additionally, bacterial resistance to multiple medications is a significant challenge, increasing hospitalization costs and death rates globally (Frieri *et al.*, 2016).

Antibiotic-resistant pathogens pose a significant contemporary public health concern. The emergence of these resistant strains hasn't been accompanied by the introduction of new clinical compounds (Ling *et al.*, 2015). Furthermore, there is growing attention to the potential toxicity and adverse effects of current drugs in healthcare. While synthetic antibiotics have reduced the presence of these organisms, they bring challenges like drug resistance (Arima *et al.*, 2002), high costs, and potential negative impacts on the host.

Increasing infections and microbial resistance are reducing the effectiveness of antibiotics. Consequently, the pharmaceutical industry is now focusing on developing low-cost, nature-derived drugs with minimal side effects to combat this challenge (Newman and Cragg, 2007).

Plant materials, rich in glycoproteins with inherent toxicity, significantly impact various biological processes (Khatun *et al.*, 2009). Exploring plant-derived phytochemical compounds holds promise for innovative antimicrobial agents (Cavalcante *et al.*, 2022).

The brine shrimp lethality bioassay is a conventional technique for assessing bioactive compounds, revealing both cytotoxic effects and a wide range of pharmacological properties. It uses *Artemia salina*, commonly known as brine shrimp, in laboratory bioassays to determine toxicity levels by calculating the median lethal concentration (Meyer *et al.*, 1982). This method is appealing due to its simplicity and the minimal quantity of toxin required for conducting the test (Sarkar *et al.*, 2010).

Lectins are proteins that possess the capacity to selectively and temporarily attach to monosaccharides and glycoproteins (Sharon and Lis, 1995). They can either attach to or immobilize microorganisms by causing them to clump together or restrict the invasion of pathogens (Holmskov *et al.*, 2003). They have been discovered to possess various beneficial properties, including inhibiting tumor growth (Kabir *et al.*, 2013), combating bacteria and fungi (Juno *et al.*, 2022), viruses (Ahmed *et al.*, 2022), and hindering cell proliferation (Alyousef *et al.*, 2018), and modulating the immune system across a range of biological contexts (Arfin *et al.*, 2022).

The terrestrial orchid *Geodorum densiflorum*, locally called 'Shonkhomul,' is a rare plant in the Orchidaceae family, primarily found in Bangladesh and certain parts of India (Kabir *et al.*, 2019). It has a history of traditional use for various therapeutic purposes, including relieving joint pain, arthritis, diabetes, menstrual irregularities, wound healing, and skin infections (Kabir *et al.*, 2019). Pharmacological studies have shown its potential for antimicrobial activities (Kabir *et al.*, 2019).

While over a hundred lectins have been purified and studied, there is a lack of research on their antibacterial properties and toxicity in relation to brine shrimp (Khatun *et al.*, 2009). Additionally, no reports exist regarding the antibacterial and toxicological investigations of *Geodorum densiflorum* lectin on brine shrimp nauplii.

Therefore, our primary objective was to evaluate the antibacterial and toxicological aspects of *Geodorum densiflorum* lectin from its rhizome for therapeutic use.

**Materials and methods**

*Materials*

Methylthialazole Tetrazolium (MTT) and Tris-HCl were procured from Carl Roth. The QA-cellulose originates from the Japanese company Wako, while the marker proteins are from TaKaRa (Japan). Nutrient broth medium containing Peptone, Sodium chloride, HM peptone and Yeast extract. The biochemical reagents were sourced from Human (Germany), and all other substances and reagents utilized in the experiment were of the purest analytical quality.

*Purification of GDL*

The rhizomes of *Geodorum densiflorum* were acquired from a local grocery store and subsequently refined according to the procedure followed by Kabir *et al* (Kabir *et al.*, 2019). To summarize, we started by creating a homogenate using 30 grams of *Geodorum densiflorum* rhizome segments in Tris-HCl buffer with a pH of 8.2. After centrifuging the homogenate for 25 minutes at 10,000 g and 4°C, we collected the clear supernatant. We then prepared a QA-cellulose-packed column with the same buffer, loaded the column with the supernatant, and collected the fraction that did not bind to the column. This fraction was later dialyzed against water. Lastly, we assessed the purity and hemagglutination activity using the techniques described previously (Kabir *et al.*, 2019).

*Inhibition of bacterial growth*

Various types of pathogenic and non-pathogenic bacterial species were used to perform the growth inhibition test by GDL at different concentrations in nutrient broth media following a method outlined by Kabir *et al* (Kabir *et al.*, 2011b). The bacterial species under investigation included *Shigella boydii*, *Escherichia coli*, *Shigella sonnei*, *Agrobacterium sp.*, *Shigella dysentery*, *and Staphylococcus aureus.* Maintaining all aseptic precautions, 50 µl of nutrient broth media was added to microtiter plates, followed by the inclusion of 320 µg/ml of GDL in the first well. The solution was then serially diluted, and the seventh column was left untreated. Each microtiter plate well was inoculated with freshly grown strains of *Shigella boydii*, *Escherichia coli*, *Shigella sonnei*, *Agrobacterium sp.*, *Shigella dysenteria*, *and Staphylococcus aureus,* and the plates were incubated at $37^0$C for 24 hours. After the incubation, a microtiter plate reader was used to detect the absorbance at 630 nm, and the given formula was used for measuring the percentage of bacterial growth inhibition.

Inhibition percentage = (Control absorbance – GDL-treated bacteria absorbance) / Control absorbance X 100.

*Toxicity test of GDL against brine shrimp nauplii*

To assess the lethality of GDL, nauplii of brine shrimp (*Artemia* nauplii L.) were used. Following a previous study, the *Artemia* cysts were hatched in artificial saltwater (Kabir *et al.*, 2011b). In a nutshell, the process began with hatching *artemia* cysts in artificial seawater at a temperature of 28°C, with continuous exposure to light and aeration. The artificial seawater was prepared by dissolving 38 grams of NaCl in 1 liter of distilled water, and its pH was adjusted to 7.0 using sodium tetraborate. These cysts were then placed in a glass tube at a density of 1 gram of cysts per liter of artificial seawater. After 48 hours, aeration was stopped, and the light source was directed towards the bottom of the tube. The nauplii, being phototropic, moved towards the light at the bottom of the tube, making it easier to separate them from the unhatched cysts. Different concentrations of GDL (30, 60, 120, 250, 500, and 1000 µg/ml) were prepared in artificial seawater, and each vial received 10 *Artemia* nauplii. The total volume in each vial was adjusted to 4 ml by adding artificial seawater.

These experiments were conducted in triplicates, and the negative control group consisted of artificial seawater and *Artemia* nauplii without *Geodorum densiflorum* lectin. After 24 hours, the number of deceased nauplii was counted against a well-lit background using a 3x magnifying hand lens for each concentration, and the $LC_{50}$ values were calculated using Probit analysis, following the method outlined by *Finney* (Finney, 1971).

*Statistical evaluation*

We assessed GDL lethality by comparing the survival of larvae in GDL-treated tubes to a control group. We used Reish and Oshida's (1986) arithmetic graphic method to make this comparison and calculated the $LC_{50}$ (the dose causing 50% shrimp death within 24 hours) using Finney's Probit analysis.

To measure toxicity, we calculated the percentage of mortality using this formula:
% mortality = (Number of dead nauplii / Initial nauplii count) x 100.

We gauged the extract's toxicity by observing a statistically significant decrease in brine shrimp survival compared to the control group.

The Chi-square ($\chi 2$) test assessed this significance, aiming to identify any difference between observed and expected mortality. The hypotheses tested were:

- Null Hypothesis (H0): Observed mortality equals expected mortality.
- Alternative Hypothesis (H1): Observed mortality does not equal expected mortality, at a significance level (alpha) of 0.05.

The results from the experiments were presented in the form of mean values along with their standard deviations (mean ± S.D.). Statistical analysis was conducted using both Dunnett's t-test and one-way ANOVA, and this analysis was carried out using SPSS software version 21, developed by SPSS Incorporated in Chicago, USA.

**Results**

*Purification process of GDL*

The lectin, GDL was isolated and subjected to SDS-PAGE, where it exhibited a sole band with an apparent molecular weight of approximately 12.0 ± 1.0 kDa. (data not shown).

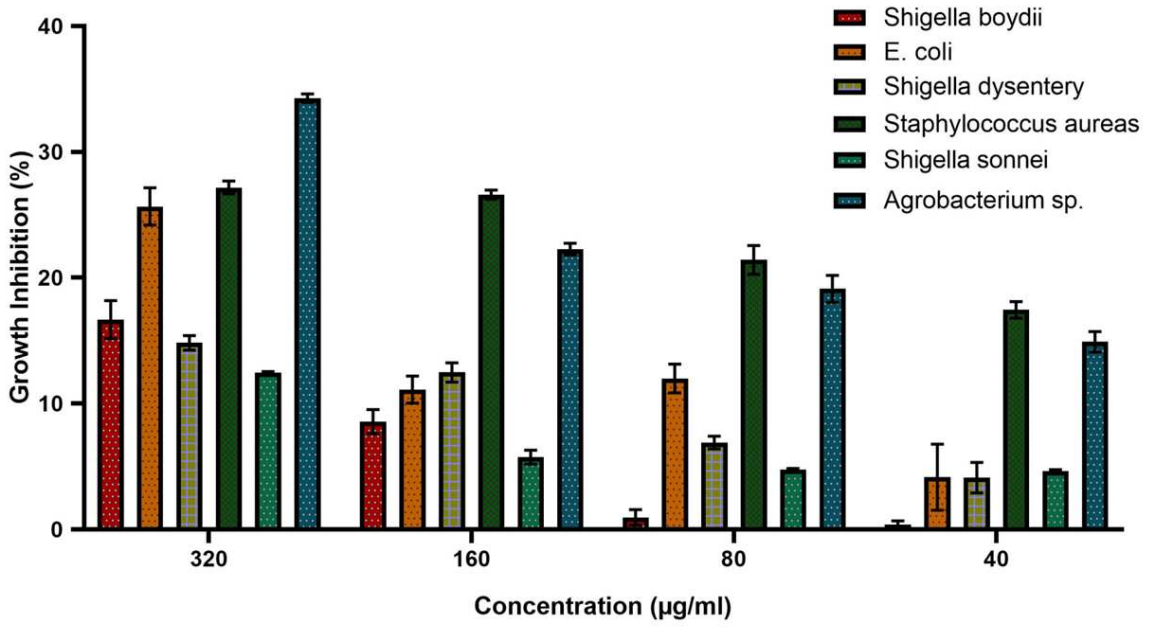


**Fig. 1.** Growth inhibitory effect of GDL against pathogenic and non-pathogenic bacteria. *Agrobacterium sp*. and *Staphylococcus aureus* responded to GDL to the most. Growth of *Shigella boydii* was not affected in lower concentrations whereas the effect against *Shigella sonnei* and *E. coli* were similar at 160, 80 and 40 µg/ml. Data are expressed in mean ± S.D; $p<0.05$.

*Bacterial growth inhibition*

In this work, we evaluated the inhibitory impact of GDL against a spectrum of bacterial species. Our findings demonstrate that GDL effectively inhibited the growth of *Shigella dysentery, E. coli, Staphylococcus aureus*, *Shigella sonnei*, *as well as*

*Agrobacterium sp.* at higher doses of 320 µg/mL and 160 µg/mL. Activity of GDL gradually decreased at lower doses. Furthermore, we observed that *Agrobacterium sp.* showed the highest sensitivity to GDL, followed by *Staphylococcus aureus*, *E. coli, Shigella dysentery*, *and Shigella sonnei*, respectively. GDL's inhibitory effect, on the other hand, against *Shigella boydii* was observed to be more effective at higher doses of 320 µg/mL and 160 µg/mL, while no considerable effect was observed at lower doses (80 µg/mL and 40 µg/mL). Interestingly, *Agrobacterium sp.* and *Staphylococcus aureus* were found to be significantly inhibited by GDL at almost all concentrations tested, indicating their high sensitivity towards GDL, compared to other bacterial strains. (Fig. 1).

*Toxicity assay of GDL against nauplii of brine shrimp*

Nauplii of brine shrimp were used to test GDL's toxicity. GDL was shown to be lethal in a concentration-dependent manner. Using Probit analysis, the $LC_{50}$ for GDL was determined to be 385 µg/ml (Fig. 2 and Fig. 3).

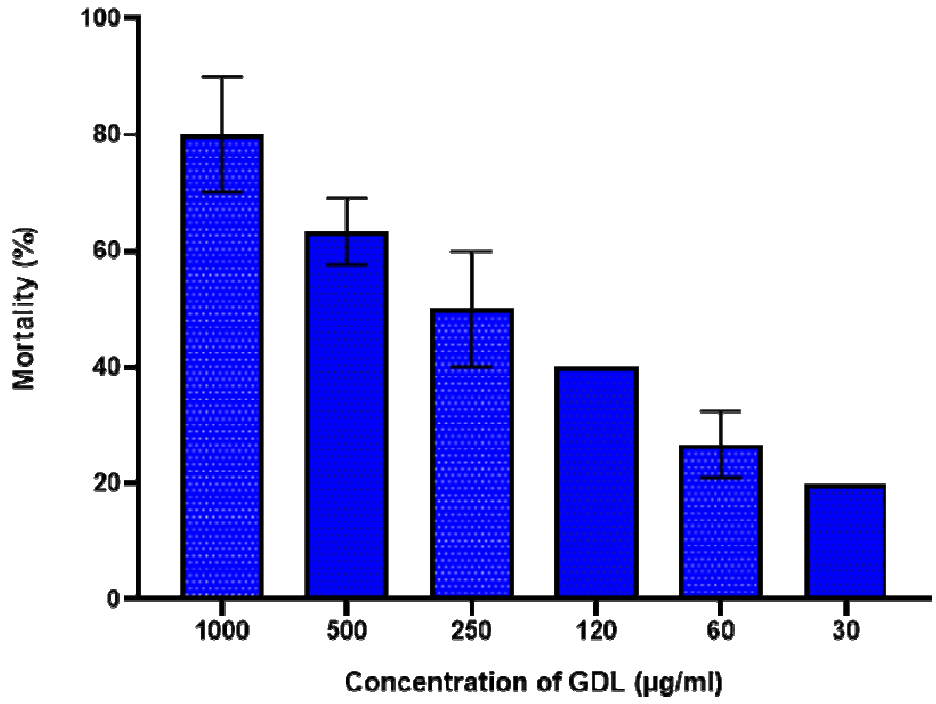


**Fig. 2.** Toxicity of GDL. Percentages of mortality of nauplii of brine shrimp in different concentrations of GDL were expressed in mean ± S. D.; $p < 0.05$.

**Discussion**

The current study successfully purified GDL, a lectin with molecular weight of 12.0 ± 1.0 kDa, from *Geodorum densiflorum* rhizomes, as reported previously (Kabir *et al.*, 2019). Antibacterial activity of GDL was then assessed, revealing two distinct levels of actions: a low inhibitory fraction suggesting bacteriostatic activity while a higher activity is potentially indicative of bactericidal effects (Hubert *et al.*, 1996). Microscopic observations showed that some bacteria became malformed or demonstrated reduced motility during the antibacterial assessment compared to the control. GDL exhibited modest inhibitory impact on gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Shigella boydii*, *E. coli*, *Shigella sonnei*, *Agrobacterium sp.*, and *Shigella dysentery*), with *Agrobacterium sp.* and *Staphylococcus aureus* were the most susceptible. It was reported that the highest antibacterial activity was displayed by *Holothuria scabra* lectin (Gowda *et al.*, 2008), which completely inhibited the growth of both gram +ve bacteria (group

D *Streptococci* and *Staphylococcus sp.*) and gram -ve bacteria *(Serratia sp., E. coli, Proteus sp.,* and *Shigella sp.*).

It is widely recognized that *Shigella* bacteria can produce Shiga toxins, which have been shown to bind with GalNAc (Hasan *et al.*, 2021). In this study, GDL inhibited the growth of three *Shigella* species. Since lectins and toxins are evolutionarily related (Hasan *et al.*, 2021), it is plausible that GDL, like Shiga toxins, bind to GalNAc, thereby inhibiting bacterial growth through lectin-glycan interactions.

*Agrobacterium tumefaciens* causes various plant tumors, including crown gall disease, which has been responsible for the extinction of many plant species. When the transfer-DNA (T-DNA) from the tumor-inducing (Ti) plasmid enters the host plant's genome, crown galls form (Chilton *et al.*, 1980). In our study, we found that GDL significantly reduced the growth of this non-pathogenic bacterium. Although intriguingly, *Geodorum densiflorum* lectin inhibited the proliferation of pathogenic bacteria, more investigation is required to identify the clinical relevance of this protein's antibacterial action in microbiology and other therapeutic contexts.

Various lectins exhibit toxicity, and their toxicity levels against nauplii of brine shrimp have been reported already. Our current data revealed that the mortality rate of nauplii of brine shrimp increased proportionally with lectin concentration. In this study, a 50% mortality rate ($LC_{50}$) for larvae was observed at a concentration of 385 µg/ml for GDL, which is less potent than KRL and the mannose-specific Mulberry seed lectin, which displayed $LC_{50}$ values of 18±6 and 21.87 µg/ml, respectively (Kabir *et al.*, 2011b; Absar *et al.*, 2005;). Furthermore, the $LC_{50}$ values for *Solanum tuberosum* lectin-Deshi (STL-D) and *Solanum tuberosum* lectin-Sheelbilatee (STL-S) were documented as 90 µg/ml and 75 µg/ml (Hasan *et al.*, 2014), respectively. Additionally, the $LC_{50}$ values for *Momordica charantia* lectin and NNTL were found to be 49.7 µg/ml and 120 ±29 µg/ml, respectively (Kabir *et al.*, 2015; Kabir *et al.*, 2011c). Some lectins were reported to be highly toxic against brine shrimp nauplii, like the lectin isolated from leaves of *Moringa oleifera,* which exhibited $LC_{50}$ value of 15.8 µg/mL, 17.78 µg/mL and 14.12 µg/mL for SLL-1, SLL-2 and SLL-3, respectively (Khatun *et al.*, 2009).

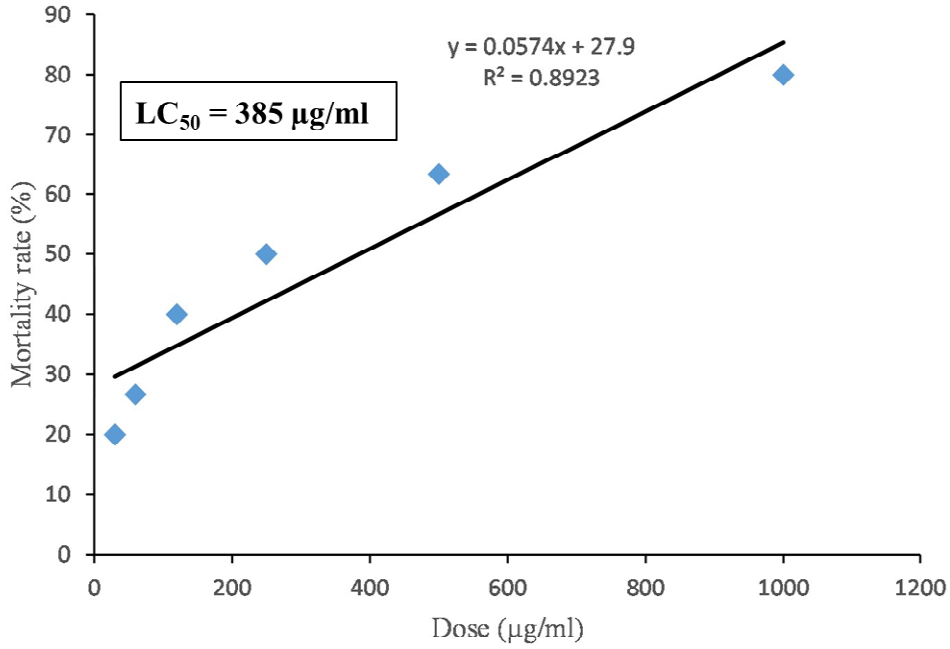


**Fig. 3.** Determination of $LC_{50}$ of *Geodorum densiflorum* rhizome lectin.

Result of our current study also indicates the lower toxicity of GDL when compared to Chitinases from "Shilbilatee" potatoes ($LC_{50}$ is 20 µg/mL) (Kabir *et al.*, 2011a), lectin from *Aplysia kurodai* (Sea Hare)

Eggs ($LC_{50}$ is 63.63 µg/mL) (Swarna *et al.*, 2021), *Moringa oleifera* seed lectin ($LC_{50}$ is 131 µg/ml) (Asaduzzaman *et al.*, 2018), *Amaranthus gangeticus* seed lectin ($LC_{50}$ is 250 µg/ml) (Hasan *et al.*, 2021) and Snake Gourd Seed Lectin ($LC_{50}$ is 261±29 µg/ml) (Kabir *et al.*, 2012). Similar low toxic effect was also found in the case of MytiLec-1 ($LC_{50}$ is 384.53 µg/mL) when compared to GDL (Hasan *et al.*, 2019). A lectin which was less toxic than GDL was a seed lectin from *Trichosanthes dioica* ($LC_{50}$ is 84.0 mg/ml) (Islam *et al.*, 2021). These findings indicate that GDL exhibits milder toxicity among the lectins mentioned above.

## Conclusion

In conclusion, this research investigated the dose-dependent toxic effect of GDL against the nauplii of brine shrimp. The results also demonstrated that GDL displayed varying degrees of growth inhibition against the tested bacteria, with *Agrobacterium sp.* and *Staphylococcus aureus* being the most sensitive. These findings indicate that GDL has the potential to be used in the development of new antibacterial therapeutics.

## Competing Interests

The authors declare that they have no competing interests.

## Funding

The study received no funding.

## Author Contributions

IH and SRK wrote the main study protocol and designed the study. MRM did all the experiments. MRM and SRK performed data analysis. SRK and IH supervised the data collection. MRM wrote the initial draft of the manuscript, which was revised by SRK and IH. MTA and SRK provided administrative and logistics supports. All authors read and approved the final manuscript.

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