



Antifungal activity, brine shrimp cytotoxicity and phytochemical screening of *Gladiolus watsonoides* Baker (Iridaceae)

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ABSTRACT

Background: Plants and plant products are by far the most utilized by humans as source of medicine. They are used by various cultures in various ways. Although the beginning of the usage was by trial and error, accessibility may have also contributed to some potentially medicinal plants not being accessed and utilized, hence lack of ethnomedicinal usage in history. *Gladiolus watsonoides* Baker (Iridaceae) is one such plant with high antifungal and cytotoxic activities yet no previous reports have been recorded on its usage, biological activities and phytochemicals. The aim of this study was to investigate the antifungal activity, cytotoxicity and the phytochemical components in this plant. **Method:** Organic and aqueous extracts were tested for antifungal activity against *Candida albicans*, *Aspergillus niger* and *Cryptococcus neoformans* by disc diffusion method. Toxicity tests were done using brine shrimp larvae – nauplii and phytochemical screening for the presence of selected classes of compounds conducted by standard chemical procedures. **Results:** Corms from Organic extracts showed moderate to high antifungal activity against the three test opportunistic fungi. The aerial extracts were not active against *Aspergillus niger* and *Cryptococcus neoformans*. Varied toxicity levels were noted against the nauplii. Amongst the classes of compounds screened, only steroids were absent in corms while glycosides, anthraquinones and steroids were absent in the aerials. **Conclusion:** Further analysis need to be undertaken before more conclusions can be made on the potential significance of this plant as lead to antifungal drug development.

KEY WORDS: *Gladiolus*, Antifungal, Cytotoxicity, Phytochemicals, Drug development

1. INTRODUCTION

Use of plants to treat a wide range of diseases by different cultures continues to date. Many of the plants used in ethnomedicine have also been followed for their biological activity, in most cases there is usually a relationship between the use of plants in ethnomedicine and the biological activity results¹. A lot of research has been invested in such, however there are plants in the wild with no known reports of usage in ethnomedicine but may have potential natural products with significant biological activities². Use of plants in traditional medicine is an ancient thing and the human race got to discover medicinal and food plants through trial, error and experience. The plants used also may have depended on their availability and place of occurrence; some other plants with various biological activities may have occurred in areas not easily accessible hence may have never been accessed for use in ethnomedicine.

Gladiolus watsonoides is one such plant with high antifungal activity but no reports at all on ethnomedicine, biological activities and phytochemical components. It is a herb, erect with hairless leafy stems ending in a spike of up to 15 uniformly bright glossy red flowers. Mainly found in wet soils in bamboo alpine zones 2400m-4200m above the sea level³.

Opportunistic fungal infections remain a threat to millions of lives of those individuals with compromised immune systems. Increasing incidence by infections such as Candidiasis, aspergillosis, and cryptococcosis have been reported due to growing numbers of immunocompromised cases related to HIV/AIDS, cancer, diabetes, old age, organ transplant and other surgical procedures^{4,5,6}. These infections by the three most common opportunistic human fungal pathogens have high occurrences as follows: *Candida albicans* (20-40%), *Aspergillus fumigatus* (50-90%), and *Cryptococcus neoformans* (20-70%)⁷. Despite the high incidence and mortality rates caused by these fungal pathogens, there are limited numbers of antifungal drugs used to treat them. In addition to this, most of the drugs are unaffordable, some results into rapid development of resistance especially due to non compliance, and adverse side effects^{4,5,8,9,10}. An

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alternative source, probably from plants may therefore be necessary. Besides, plants with biological activity and no known reports for usage in ethnomedicines should be documented and investigated as in ethnomedicines are worth documented for further investigation of useful anti-infective agents. This present study investigated the antifungal activity, cytotoxicity and the nature of the major chemical classes present in *Gladiolus watsonoides* Baker (iridaceae).

2. MATERIALS AND METHOD

2.1 Collection of the plant materials

Gladiolus watsonoides was collected from Aberdares National Park, Kenya through Wandare route towards Satima in the year 2012. The plant was identified using keys in³, voucher specimens in the Nairobi University herbarium and a plant taxonomist from the School of Biological Sciences, University of Nairobi. The aerial parts were separated from the corms, shredded into small pieces and dried at room temperature. Once dry, the samples were ground into powder and kept in a dry condition awaiting extraction for bioassays and phytochemical screening.

2.2 Preparation of crude extracts

30g of each of the powdered samples were extracted using water and a mixture of dichloromethane & methanol in the ratio 1:1 four times at 24 h intervals. Each of the extract was filtered and concentrated under reduced pressure at 40°C to obtain crude extracts. Dry aqueous extracts were obtained by use of a freeze drier. These were then stored in airtight containers at 4°C awaiting bioassays and phytochemical screening.

2.3 Preparation for antifungal activity

Stock solution of each sample was prepared by dissolving 0.5g of dried crude in 10ml of water in the case of aqueous extracts and Dimethylsulphoxide (DMSO) in the case of the organic solvent extracts. This was at first serially diluted such that there were five different concentration levels in which the tests were done. These were 5mg/100µl, 2.5mg/100µl, 1.25mg/100µl, 0.625mg/100µl, 0.3125mg/100µl. Samples which showed activity with the lowest concentration set were further tested at much lower concentrations at 0.15625mg/100µl, 0.07813mg/100µl and 0.03902mg/100µl. The aim of setting different concentrations was to establish the minimum inhibitory concentrations.

2.4 Determination of antifungal activity

Antifungal activity of the crude extracts was determined against three opportunistic fungal pathogens; *Candida albicans*, *Aspergillus niger* and *Cryptococcus neoformans*. All the fungi were first sub-cultured in Sabourauds Dextrose Agar growth media (SDA). Broth suspensions of the organisms were prepared using distilled water and pep-

tone media. The tests were done using disc diffusion method according to¹¹. Commercially prepared sterile discs impregnated with 100µl of the solution of sample at each concentration, were aseptically transferred into petri dishes with about 25ml SDA media, freshly inoculated with the test organism. Dimethylsulfoxide (DMSO) and water which were used to re-dissolve the crude extracts, served as the negative controls while Amphotericin B served as the positive control. The plates were prepared in replicates, sealed with parafilm to avoid contamination and incubated at 37°C for *Candida albicans* and *Cryptococcus neoformans* while those of *Aspergillus niger* were incubated at 25°C. The antifungal activities were evaluated by measuring the diameter of the zones of inhibition using a transparent ruler after 24 hours, 48 hours and 72hours^{12,13}.

2.5 Toxicity tests using brine shrimp

2.5.1 Hatching of the brine shrimp larvae

Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water prepared by dissolving 10g of sea salt (sigma chemicals, Co, UK) in 500ml of distilled water. This was added to a culture tank which had two partitions. One part was darkened, while the other was left exposed. A pinch of brine shrimp eggs was added to the darkened portion and warmed with a 60 watt lamp to provide warmth necessary for hatching. The observation was made after 24 hours and successful hatching confirmed by the presence of visible several actively moving nauplii in the culture tank. The partition wall had small holes through which the larvae (nauplii) moved to the exposed side of the tank.

2.5.2 Brine shrimp assay

This stock solutions of 1000ppm were prepared by dissolving 3mg of each extract in 3ml of DMSO in the case of organic extracts and water in the case of aqueous extracts. This stock solution 1000ppm was serially diluted to prepare other concentrations such as 500ppm, 100ppm, 10ppm and 5ppm. 10 live nauplii were transferred using a micro pipette into vials with these set concentrations. This experiment was set in triplicates (three vials per sample per concentration). Water and DMSO served as the negative controls while Rotenone served as the positive control. The observations were done after 24 hours. Cytotoxic activity was determined by recording the number of dead nauplii (immobile) in each vial per sample per concentration. This was then used to calculate the mortality mean, percentages and LD₅₀ values.

2.6 Qualitative Phytochemical analysis

Chemical tests were conducted on organic solvent extracts and powdered plant materials of both aerial and corm parts following standard chemical procedures according to^{14,15,16}. The phytochemicals analysed were alkaloids, flavonoids, saponins, tannins, glycosides, steroids, anthraquinones and terpenoids.

2.6.1 Screening procedure

2.6.1.1 Test for saponins

5ml of distilled water was added to 0.5g of powdered plant material in a vial . The mixture was shaken and heated in water bath for 2min. The presence of a stable froth indicated the presence of saponins.

2.6.1.2 Test for steroids

Two methods were used to determine the presence of steroids in the plant extracts. I) 0.5g of powdered plant material was mixed with 2ml acetic anhydride in a boiling tube and then cooled in ice for five minutes , 2ml concentrated sulphuric acid was added slowly along the wall of the test tube. Colour change from violet , to blue , to green was an indicative of the presence of steroids. II) concentrated sulphuric acid was slowly added to 2g of plant extract . Effervescence followed by appearance of a clear reddish brown colour at the interface was an indication of a steroidal ring.

2.6.1.3 Test for tannins

10 ml distilled water was added to 0.5g of powdered plant material in a test tube. The was boiled for 3min and filtered using Whatman filter paper No. 1. Ferric chloride was added and the mixture observed for dark or dirty green precipitate which indicated the presence of tannins.

2.6.1.4 Test for terpenoids

0.5g of powdered plant material was added in a boiling tube and 2ml chloroform carefully added, 3ml concentrated sulphuric acid was added drop wise. Presence of a reddish brown colouration at the interface showed positive results for the presence of terpenoids.

2.6.1.5 Test for glycosides

0.5 g of the ground plant material was added to a boiling tube. 10ml distilled water was added and stirred. This was filtered and 2ml of the filtrate hydrolyzed with few drops of concentrated hydrochloric acid, a few drops of ammonia solution was added to the mixture . Five drops of this solution was put a side in a separate test tube and 2ml of benedicts reagent added and boiled. Reddish to brown precipitate was an indicative of the presence of glycosides.

2.6.1.6 Test for flavonoids

0.5 g of the extract was heated with 10ml ethyl acetate over a steam bath for 3 min, the mixture was filtered and 4ml of the filtrate was shaken with 1ml dilute ammonia (50%) . Presence of a yellow colouration indicated the presence of flavonoids.

2.6.1.7 Test for alkaloids

0.5g of the powdered plants parts was added into a boiling tube . 5ml of 2% sulphuric acid was added, mixed and filtered . Few drops of Drangedorff's reagent was added to the filtrate. Presence of an orange red precipitate indicated the presence of alkaloids.

2.6.1.8 Test for anthraquinones

1g of powdered plant material added into a boiling tube and boiled with 2ml of 10% hydrochloric acid for 5min. The mixture was filtered and the filtrate cooled. The filtrate was partitioned against equal volume of chloroform and the chloroform layer transferred into a clean test tube. Equal volume of 10% ammonia solution was added into the chloroform layer, shaken and allowed to separate. The separated aqueous layer was observed for a delicate rose pink colour which showed the presence of anthraquinones.

3. RESULTS

3.1 Antifungal activity

The two different parts of the plant exhibited varied antifungal activity against one or more of the test fungi. In general, corms showed much higher antifungal activity than the aerial parts; however this activity of the corms was outweighed by the activity of the control drug Amphotericin B (Table 1).

Table 1. Minimum Inhibitory Concentrations (MIC in µg/µl) values of the crude organic extracts of *Gladiolus watsonoides* against the test fungi

	CA	AN	CN
Corms	0.3907	6.25	3.125
Aerials	0.7813	NA	NA
Amp B	<0.3907	<0.3907	<0.3907

Key; CA= *Candida albicans* ; AN= *Aspergillus niger* CN= *Cryptococcus neoformans* NA= No activity detected

The aerial parts were not active against *A.niger* and *C.neoformans* whereas the corms showed activity against the three fungi with highest activity revealed against *C.albicans* and the least activity revealed against the *A.niger* as shown by the Minimum inhibitory concentrations in Table 1.

3.2 Brine shrimp assay

In general, both the plant parts showed toxicity to nauplii; artificial sea water which was a negative control was not toxic whereas rotenone, a positive control was highly toxic to the brine shrimp larvae (Table 2).

Table 2. LD₅₀ values of the toxicity by the crude extracts of *G watsonoides*

	LD ₅₀	+ SE
Corms _{Aq}	27.4503	0.1602
Corms _{Org}	0.5492	0.9491
Aerials _{Aq}	57.4512	0.2743
Aerials _{Org}	2.6952	0.3518
Sea water only	2771.8354	0.7657
Sea water + DMSO	147.6151	0.2816
Rotenone	0.4911	1.2426

Key ; Aq denotes aqueous extract ; Org denotes Organic extract

3.3 Phytochemical analysis

Corms of *Gladiolus watsonoides* were richer in phytochemicals than the aerial parts. All the phytochemicals tested were detected in the corms except steroids.

Table 3: Phytochemical constituents from organic extracts of corms and aerial parts of *Gladiolus watsonoides*

Phytochemicals	Corms	Aerial/leaves
Flavonoids	++	++
Saponins	+++	+++
Alkaloids	++	+
Terpenoids	+++	+
Steroids	-	-
Tannins	+++	+++
Glycosides	++	-
Anthraquinones	+	-

Key: +++: strong presence, ++: moderate presence, +: weak presence, -: not detected

The aerial parts showed presence of alkaloids saponins, flavonoids, terpenoids and tannins while steroids, glycosides and anthraquinones were not detected (Table 3).

4. DISCUSSION

Crude organic extracts of *Gladiolus watsonoides* had growth inhibiting effects on one or more of the test fungi (Table 1). The corresponding data on the aqueous extract is not shown since the activity exhibited was too little. Inactivity or less activity showed by water extracts may have been due to the presence of bioactive elements at a much lower concentration or the active elements may have been soluble only in organic solvent and therefore not present in water extracts^{17, 18, 19}.

Crude organic extracts from corms and aerials showed varied activity. Extracts from corms showed the highest activity especially against the three fungi whereas the aerial parts were active against *Candida albicans* only. Although some reports revealed *Candida albicans* to be insensitive to most extract treatments¹⁸. In this study it was the most sensitive to the extracts' exposure. The relatively high antifungal activity of this plant against *Candida albicans* and *Cryptococcus neoformans* may be of interest since they are yeasts which are known to be less susceptible to the available antifungal agents mainly due to their continuously new emergence and general multidrug resistance²⁰.

The insensitivity of *Aspergillus niger* and *Cryptococcus neoformans* to the aerial extracts may have been due to the cell wall composition and site of action of the active ingredient being different from that of *C.albicans*^{21,22}, another explanation for the observation may have been that the active elements against these two fungi were either at lower undetectable levels or absent in the aerials. Still the extracts may have been active against other fungi not tested in the study.

G. watsonoides was found to be rich in phytochemicals (Table 3). Corms showed presence of all the tested classes of secondary metabolites except the steroids whereas anthraquinones, steroids and glycosides were not detected in the aerials. This may explain the varied antifungal and cytotoxic activities observed (Table 1 and Table 2). Biological activities by this plant may have been attributed to the vast presence of secondary metabolites detected. Alkaloids, mainly found in root barks, corms and rhizomes have been reported to possess antifungal, antimalarial, antibacterial and anticancer properties^{1,23,24,25}. Terpenoids, flavonoids and tannins have also been reported to have among others antimicrobial activity and is used in preparations of drugs used against diarrhea^{26, 27, 28}.

Although not detected in this plant, cytotoxic effects of steroids have been utilized in their usage as arrow poisons They have also been investigated for their antibacterial activity^{27, 29}. Saponins, glycosides and athraquinones have been reported to have anticancer, antifungal and antioxidant activities^{26, 30, 31}.

Toxicity tests on this plant in brine shrimp larvae revealed varied toxic levels (Table 2). This may have been attributed to presence of secondary metabolites. So far in this study, its not known which active ingredient exerted the antifungal and the cytotoxic activities. This plant therefore should be considered for further analysis.

5. CONCLUSION

Study on *Gladiolus watsonoides* revealed antifungal and cytotoxic activities along with a wide range of classes of compounds. In order to possibly consider this plant for medical relevance, further analysis on the active elements need to be undertaken before more conclusions can be made on its significance as potential lead to antifungal drug development.

Conflict of interest

All authors have none to declare

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