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Research Article

An evaluation of the phytochemical screening and antifungal activities of various parts of *Allium neapolitanum* Cirillo

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Abstract

The current research work is concerned with the identification of secondary metabolites through phytochemical screening as well as the evaluation of antifungal potential by well diffusion method of the ethanolic and methanolic extracts of the leaves, flowers and bulbs of *Allium neapolitanum* Cirillo belonging to Family Liliaceae. The results of phytochemical screening showed the presence of tannins, phlobatannins, flavonoids and terpenoids in the leaves, flowers and bulbs; glycosides in the flowers and leaves; reducing sugars in the leaves and bulbs; anthraquinones and saponins in the bulbs only while fixed oils and fats in the leaves of *Allium neapolitanum*. For *Fusarium* species, among the ethanolic extracts, the highest inhibition 126.05%, 135.2% and 152.75% was shown by bulbs followed by leaves (82%, 83.33% and 116.4%) and flowers (80.01%, 77.8% and 85.43%) at 6 µl/ml, 12 µl/ml and 18 µl/ml concentrations. Similarly, among the methanolic extracts, the highest inhibition was also shown by bulbs (114.04%, 118.5% and 127.3%), followed by flowers (80.01%, 96.3% and 107.25%) at 6 µl/ml, 12 µl/ml and 18 µl/ml concentrations respectively. However, leaves did not show any activity at the given concentrations. For *A. niger* and *Alternaria* species, no activity was observed at all. Findings from this study confirmed that the plant extracts can be used as natural fungicides to control pathogenic fungi, thus reducing the dependence on the synthetic fungicides.

Keywords: Antifungal activities; Ethanolic and methanolic extracts; Fungal strains; Phytochemical screening

Introduction

The earlier decade has verified a vast recovery in the demand and utilization of herbal plant species. The useful therapeutic effects of herbal products are mainly caused by the combination of secondary metabolites

found in them, called phytochemicals. Phytochemicals are naturally occurring and biologically active chemical compounds, mostly found in nuts, grains, seeds, fruits, tea and vegetables which prevent diseases and improve human health. Herbal medicines have been used traditionally around the world in the cure and prevention of different kinds of diseases as these are a rich source of bioactive phytochemicals or bionutrients **[1]**. Phytochemicals have a major role in preventing incurable chronic diseases such as cancer, diabetes and coronary heart diseases. The disease preventing functions that constitute the major classes of phytochemicals are detoxifying agents, antioxidants, neuropharmacological agents, dietary fibers, anticancer and immunity-potentiating agents. Some of the phytochemicals have more than one function **[1]**. Though phytochemicals are non-essential elements and therefore not necessarily required for sustaining human life, yet possess significant characteristics, inhibit or to fight against several diseased conditions. Because of these properties, numerous research activities have been conducted to report the health benefits of phytochemical constituents **[2]**. The medicinal value of a plant lies in some chemical substances that produce a particular physiological action on the human body. The most important of these bioactive constituents of plants include tannins, terpenoids, flavonoids, alkaloids, steroids and carbohydrates **[3]**. Knowledge of the chemical constituents of plants is desirable, not only for the recognition of therapeutic agents, but also because such information may be helpful in discovering new sources **[4]**. Successful assurance of biologically active compounds from plant material depends on the type of solvent used in the extraction method **[5]**. The active constituents in plants are the chemicals that have a medicinal effect on the body. These constituents and their actions within the body are also referred to as their pharmacology. They have been classified into 16 main groups: anthocyanins, cardiac and cyanogenic glycosides, flavonoids, phenols, tannins, alkaloids, anthraquinones, coumarins, saponins and glucosilinates **[6]**. Recently, frequent studies have revealed that certain natural foods could counteract the development of several diseases **[7]**. The genus *Allium* is an essential dietary source of antioxidant phytochemical compounds which have the ability to scavenge free radicals: organosulfer compounds, phytoalexin (e.g. allixin), flavonoids, trace of elements such as calcium, zinc, germanium, selenium and volatile oil having sulphur constituents **[8]**. Numerous phytochemicals have been obtained from different species of this genus, but only a few (allinase and ajoene) are active as pesticides. The phenol and sulphur containing allelopathic compounds of genus *Allium* may be used for weeds and insects management in agricultural practices **[9]**. Besides the commonly known onion and garlic, many others such as scallion, wild garlic, leek and shallot etc are generally cultivated for their edible and dietary use as well as folklore medicine **[10]**. The aim of the current study is to identify the secondary metabolites in the leaves, flowers and bulbs of the selected plant and also the antifungal potential of the ethanolic and methanolic extracts of *Allium neapolitanum* Cirillo.

Materials and methods

Collection and drying of plant materials

The procedures followed by and Nisa et al. and Malik et al. **[11, 12]** was used for the collection of *Allium neapolitanum* - Cirillo from the botanical garden of Islamia College Peshawar in the month of April 2018 and was identified by Dr. Barkatullah and Dr. Naveed Akhtar (Assistant Professors, Department of Botany, Islamia College Peshawar).

Extraction

The procedure used by Malik et al., Nisa et

al. and Fatima et al. **[11-13]** was followed for the extraction of plant materials.

Detection of tannins

A small quantity of each extract was dissolved in 5 ml distilled water. The solution was heated on spirit lamp and then filtered with the help of filter paper. A small quantity (few drops) of ferric chloride ($FeCl_3$) was added to each filtrate using a dropper. Tannins were detected by the appearance of a dark green coloured solution.

Detection of anthraquinones

0.5 g of each extract was boiled with 10% HCL solution for a few seconds on spirit lamp. 10% HCL solution was prepared by using the following formula:

$C_1V_1 = C_2V_2$

$V_1=C_2V_2 /C_1$

V_1=10 (100)/36.5 (HCL at. mass)

V_1=27.939 ml HCL (for 100 ml water)

V_1=27.939/2=13.6ml HCL (for 50 ml water)

V_1=13.6 /2=6.84 ml HCL (for 25 ml water)

V_1=6.84 /2=3.4 ml HCL (for 12.5 ml water)

V_1=3.4 /2=1.42 ml HCL (for 6 ml water)

So, 10% HCL solution was prepared by dissolving 1.42 ml HCL in 6 ml distilled water.

Each mixture was then filtered and allowed to cool. Equal volume of chloroform (i.e. 1.42 ml $CHCL_3$ dissolved in 6 ml distilled water) was added to each of the resultant filtrates. Finally, a few drops of 10% NH_3 (ammonia solution) were added to each mixture and then boiled on spirit lamp.

10% ammonia solution was also prepared according to the following formula.

$C_1V_1=C_2V_2$

$V_1=C_2V_2 / C_1$

V_1=10 (100)/17.031 (at. mass of ammonia)

V_1=58.71 ml ammonia (for 100 ml water)

V_1=3.66 ml ammonia (for 6 ml water)

So 10% ammonia solution was prepared by dissolving 3.66 ml ammonia in 6 ml distilled water. The presence of anthraquinones was confirmed by the appearance of rose-pink coloured solution.

Detection of glycosides

A small quantity of each extract was hydrolyzed with 3 ml HCL and neutralized with 3 ml NaOH solution (the NaOH solution was prepared by dissolving 1.25 g of NaOH in 31 ml distilled water) followed by the addition of a small quantity (few drops) of Fehling solutions A and B to each mixture. Glycosides were detected by the appearance of the red colored precipitate.

Detection of reducing sugars

Small quantity of each extract was shaken with about 4 ml distilled water with the help of shaker and then, filtered. Each of the resultant filtrates was heated with a small quantity (few drops) of Fehling solutions A and B for a few seconds on spirit lamp. The presence of reducing sugars was confirmed by the appearance of orange-red coloured precipitate.

Detection of saponins

Using a shaker, about 0.5 g of each extract was shaken with 5 ml distilled water and the solution was heated to boil on spirit lamp. The presence of saponins was indicated by the appearance of frothing (creamy miss of small bubbles).

Detection of flavonoids

About 0.5 g of each extract was mixed with 3 ml diluted sodium hydroxide (NaOH) solution, followed by the addition of 3 ml hydrochloric acid (HCL) to each of them. Flavonoids were detected by the appearance of a yellow coloured solution.

Detection of phlobatannins

0.5 g of each extract was dissolved in a small quantity of distilled water and then filtered. Each filtrate was heated with 2% hydrochloric acid (HCL) solution on spirit lamp.

2% HCL solution was prepared according to the formula:

$C_1V_1=C_2V_2$

$V_1=C_2V_2 / C_1$

V_1=2 (100) / 36.6 (at. mass of HCL)

V_1=5.47 ml HCL (for 100 ml water)

V_1=1 ml HCL (for 12 ml water)
So, 2% HCL solution was made by dissolving 1 ml HCL in 12 ml of distilled water. The appearance of red precipitate demonstrated the presence of phlobatannins.

Detection of terpenoids

About 0.5 g of each extract was dissolved in 2 ml of chloroform ($CHCL_3$) and 3 ml of concentrated sulphuric acid was added to each of them. Terpenoids were detected by the appearance of reddish brown coloration.

Detection of fixed oils and fats

A small quantity of each extract was pressed between two filter papers. The appearance of oil stains on filter paper confirmed the presence of fixed oils and fats.

Nutrient broth media

Nutrient broth was used for the growth and multiplication of fungal strains.

Preparation of nutrient broth media

For three fungal strains, nutrient broth was made by dissolving 0.78 g nutrient broth in 60 ml of distilled water (1.3 g in 100 ml distilled water). It was properly shocked and boiled for one minute to completely dissolve the medium. The nutrient broth was then sterilized along with three flasks in the autoclave at 121°C for about 45 minutes **[14]**.

Inoculation of fungal strains

After sterilization, about 20 ml of nutrient broth was poured into each of the sterilized flask and then allowed to cool at room temperature for a few minutes and labeled. After cooling, fungal strains were inoculated into each of the labeled flask with the help of a streaking loop. The streaking loop was heated on a spirit lamp after inoculation of each strain to avoid contaminations. After inoculation, the flasks were covered with aluminum foil and then kept in an incubator for 24 hours. The process of inoculation was carried out in a laminar flow hood.

Dilution of nutrient broth

After 24 hours, fresh nutrient broth was prepared according to the above procedure and sterilized along with 3 flasks in an autoclave for about 45 minutes. After sterilization, about 10 ml of freshly prepared nutrient broth was taken in each of the sterilized flask and each was mixed with 10 ml taken from previously prepared inoculated nutrient broth. The antifungal activity was then performed on this mixture of fresh and previously inoculated nutrient broth **[11, 14]**.

Potato-dextrose agar (PDA)

PDA consists of potato infusion and dextrose. Potato infusion provides a nutrient base for the luxuriant growth of most fungi and dextrose serves as a growth stimulant.

Preparation of PDA

For ethanolic and methanolic extracts of flowers, leaves and bulbs of *Allium neapolitanum*, PDA was prepared by dissolving 61.4 g of PDA in 1575 ml distilled water (39 g in 1000 ml or 1 L distilled water). It was shacked well and boiled for a few minutes until the medium was completely dissolved. The completely dissolved medium along with 63 petri dishes was then sterilized in the autoclave machine at 121°C for about 45 minutes **[15]**.

Preparation of DMSO solution

About 0.50 g of each extract was dissolved in 3000 µl/ml dimethylsulphoxide (DMSO) and then properly shocked. It was to be used later in antifungal test.

DMSO (Negative Control)

DMSO was used as a negative control.

Clotrimazole (positive control)

It is a broad spectrum antifungal which is used against most of the fungi. It was taken as a standard or positive control.

Antifungal test

After sterilization, about 25 ml of medium was poured into each of the sterilized petri dishes and allowed to cool and solidify at room temperature. Fungal strains were cultured on the media with the help of cotton buds. After swabbing, wells were made in the media with the help of a sterilized cork borer. Three wells were made in each petri dish. The cork borer was heated on a spirit lamp for

each fungal strain to avoid contaminations. The DMSO solution was poured in the wells with the help of a sterilized micropipette. Three concentrations of DMSO of each extract were used, i.e. 6 µl/ml, 12 µl/ml and 18 µl/ml. This whole activity was performed in a laminar flow hood. The petri dishes were then transferred carefully to the incubator and kept there for about 24 hours **[13, 14]**.

Measurement of zone of inhibition

The zone of inhibition was measured by measuring the diameter of clear region in millimeters with the help of a scale **[13, 14]**.

Results and discussion

In the current study, the whole plant of *Allium neapolitanun* was screened out for detection of important phytochemical constituents as well as to determine the antifungal potential. Phytochemical screening is important to find out the nature of drugs. Various phytochemical tests were conducted to confirm the presence or absence of important phytochemical constituents such as tannins, anthraquinones, glycosides, reducing sugars, saponins, flavonoids, phlobatannins, terpenoids, fixed oils and fats in the flowers, leaves and bulbs of *Allium neapolitanum* (Table 1) using standard procedures **[11, 14]**.

Many medicinal plants and their extracts contain different phytochemicals with biological activity that can be of valuable therapeutic index. Much of the protective effect of medicinal plants has been attributed by phytochemicals, which are the non-nutrient compounds **[13]**. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. The phytochemical screening of the plant parts of *Allium neapolitanum* showed that the leaves, flower and bulb contain most of the secondary metabolites analyzed. They were shown to possess alkaloids, phenolic compounds, flavonoids glycosides, steroids, tannins and triterpenoids.

The results showed the presence of secondary metabolites either in all parts or in some parts of *Allium neapolitanum*. The flowers, leaves and bulbs showed positive results for tannins, phlobatannins, terpenoids and flavonoids (Table 1). A research work conducted by Arify *et al*. **[16]** reported the presence of saponins, terpenoids, flavonoids, amino acids and proteins, volatile oils and cardiac glycosides in *Allium sativum*.

Table 1. Phytochemical screening of methanolic extracts of flowers, leaves and bulbs of *Allium neapolitanum*

S. No	Secondary metabolites	Chemicals used	Flowers	Leaves	Bulbs
1	**Tannins**	Distilled water, $FeCl_3$	+	+	+
2	**Phlobatannins**	Distilled water, HCl	+	+	+
3	**Terpenoids**	$CHCl_3$, H_2SO_4	+	+	+
4	**Flavonoids**	HCl, NaOH	+	+	+
5	**Glycosides**	HCl, NaOH, Fehling Solution A and B	+	+	-
6	**Reducing sugars**	Distilled water, Fehling Solution A and B	-	+	+
7	**Saponins**	Distilled water	Negligible	-	+
8	**Anthraquinones**	HCl, Chloroform ($CHCl_3$), Ammonia (NH_3)	-	-	+
9	**Fixed oils and fats**	No chemicals required	-	+	-

+ indicates the presence of secondary metabolites, _ indicates the absence of secondary metabolites

The flowers and leaves showed positive while the bulbs showed negative results for glycosides. The leaves and bulbs showed positive while the flowers showed negative results for reducing sugars. A research work carried out by Ameh *et al*. **[17]** reported the presence of carbohydrates, glycosides and proteins in high concentrations, alkaloids, saponins, reducing sugars, oils and steroids in medium concentrations while flavonoids and acidic compounds in low concentrations in the methanolic extract of bulb of *Allium sativum.* However, Singh **[18]** confirmed the presence of carbohydrates, reducing sugars, lipids, flavonoids, ketones, alkaloids and tannins in *Allium sativum.*

The bulbs showed positive while the flowers and leaves showed negative results for anthraquinones and saponnins. The fixed oils and fats were found only in the leaves of *Allium neapollitanum.* A research work carried out by Singh and Kumar **[19]** explored the presence of alkaloids, carbohydrates, reducing sugars, flavonoids and glycosides in the aqueous extract, tannins and phenolic compounds in the methanoilc extracts while saponins, amino acids, proteins, terpenoids and steroids in both extracts of *Allium cepa*. Bhandari et al. **[20]** reported the presence of steroids, terpenoids, flavonoids, reducing sugars and glycosides in *Allium wallichii.* Similarly Adawia et al. reported the presence of phenols, flavonoids, tannins, saponins, steroids and terpenoids in the bulbs and aerial parts of wild plants belonging to family Liliaceae. Begum and Yaseen **[21]** also reported the presence of steroids, flavonoids, glycosides, phenolic compounds, alkaloids, resins, oils, carbohydrates, and proteins in various extracts of *Allium cepa.*

Antifungal activity

The antifungal potential of the ethanolic and methanolic extracts of leaves, flowers and bulbs of *Allium neapolitanum* was tested against three fungal strains such as *Fusarium* species, *Aspergillus niger* and *Alternaria* species. A research work conducted by Eltaweel [**22]** demonstrated the antimicrobial potential of the methanolic and aqueous extracts of bulbs of *Allium cepa* against *S. aureus* using well diffusion method.

In case of *Aspergillus niger* and *Alternaria* species, no activity was observed at neither of the given concentrations (6 µl/ml, 12 µl/ml and 18 µl/ml) which indicate *Allium neapolitanum* is inactive against these fungal strains. These results are not concides with the reports of other researcher **[21, 23]**.

Clotrimazole was used as a positive control which showed antifungal activities with zone of inhibition ranging from 16 mm to 18 mm.

The Table 2 indicates antifungal potential of ethanolic extracts. While (Table 3) represents the antifungal potential of methanolic extracts of the leaves, flowers and bulbs of *Allium neapolitanum* against *Fusarium* species. Three different concentrations 6 µl/ml, 12 µl/ml and 18 µl/ml were used against *Fusarium* species. Leaves, flowers and bulb ethanolic extracts the highest zone of inhibition was 21.33 ± 0.58 mm, 15.66 ± 0.58 mm and 28 ± 2 mm found with 18 µl/ml concentration followed by 12 µl/ml and 6 µl/ml concentrations with inhibition zone. However, the highest inhibition zone was observed with ethanolic extract of bulbs while lowest 13.33 ± 1.53 mm inhibition zone was occurred with flowers extract. This indicates that ethanolic extract of bulbs contain phytochemical compounds that largely inhibit the growth of fungi campared to other leaves and flowers. These results were also supported the work of Virmani *et al*. **[23]** who concluded that the growth of Aspergillus fumigates was inhibited by the ethanolic extracts of garlic bulb. Furthermore, Yin and Cheng **[24]** reported water soluble extracts of garlic bulb had an inhibitory effect against *Aspergillus niger* and *Aspergillus flavus*.

Table 2. Antifungal potential of ethanolic extracts of the leaves, flowers and bulbs of *Allium neapolitanum* against *Fusarium* species

Extract (ethanolic)	Concentration (µl/ml)	Zone of inhibition (mm)	Inhibition %
Leaves	6	13.66 ± 1.53	82
	12	15 ± 1.732	83.33
	18	21.33 ± 0.58	116.4
Flowers	6	13.33 ± 1.53	80.01
	12	14 ± 1	77.8
	18	15.66 ± 0.58	85.43
Bulbs	6	21 ± 7.81	126.05
	12	24.33 ± 1.15	135.16
	18	28 ± 2	152.8
+ve conttrol (Clotrimazole)	6	16.66 ± 0.58	
	12	18 ± 2.64	
	18	18.33 ± 1.154	
-ve control (DMSO)			

Table 3. Antifungal potential of methanolic extracts of the leaves, flowers and bulbs of *Allium neapolitanum* against *Fusarium* species

Extract (methanolic)	Concentration (µl/ml)	Zone of inhibition (mm)	Inhibition %
Leaves	6	Nil	Nil
	12	Nil	Nil
	18	Nil	Nil
Flowers	6	13.33 ± 0.58	80.01
	12	17.33 ± 1.53	96.3
	18	19.66 ± 0.58	107.25
Bulbs	6	19 ± 1	114.04
	12	21.33 ± 0.58	118.5
	18	23.33 ± 0.58	127.3
+Ve control (Clotrimazole)	6	16.66 ± 0.58	
	12	18 ± 2.64	
	18	18.33 ± 1.154	
-Ve control (DMSO)			

However, in methanolic extracts the highest inhibition zones 19.66 ± 0.5 mm, 8, 23.33 ± 0.58 mm were observed in flowers and bulbs extracts with 18 µl/ml concentrations followed by 12 µl/ml and 6 µl/ml concentrations. These results indicated that the inhibition was concentration dependent. The higher the concentration higher will be the inhibition zone. Many of the previous research works indicated the broad spectrum antifungial activities of *Allium neapolitanum* plant parts extracts and this may be attributed due to the presence of different phytochemical compounds. However, no results were found in all three concentrations of leaves extracts. These results indicated that the metanolic extracts of *Allium neapolitanum* leaves are inactive against *Fusarium* species. Similar results were also reported by Riaz *et al*. **[23]**. Furthermore, ethanol is a best solvent for plant extracts compared to methanol as ethanol showed potential against fungal strain at lowest 6 µl/ml concentrations and at higher (18 µl/ml) concentration showed greater inhibition zone compared to methanol extracts. These results also supported by the reports of Virmani *et al*. **[23]**. The ability of garlic to inhibit the

growth of fungi could be attributed to the fact that garlic contains many antimicrobial compounds [**25]**. The mechanisms of action of phytochemicals against microorganism vary and depend on these phytochemicals **[26]**.

Conclusion and recommendations

The results of antifungal tests of the ethanolic and methanolic extracts of the leaves, flowers and bulbs of *Allium neapolitanum* suggested that the organic solvents (ethanol and methanol) possess the ability to extract the important phytochemicals. However, the ethanolic and methanolic extracts of bulbs showed best antifungal activities followed by flower extracts while leaves methanolic extract has no potential to control the fungal growth at given concentrations. Moreover, the antifungal activities were found to be increased with increasing concentrations and that is why best results were shown at the concentration of 18 µl/ml as compared to 6 µl/ml and 12 µl/ml. As *Allium neapolitanum* is a rich source of important phytochemicals as well as possessing antifungal potential against most of the tested fungal strains so that is why this plant may be used in antibiotics as well as for medicinal purposes. Moreover, in addition to ethanol and methanol, other organic solvents may also be used for extraction and also use different concentrations in order to get varying results.

Authors' contributions

Conceived and designed the experiments: MS Khan & MA Sajad, Performed the experiments: S Malik, Analyzed the data: Barkatullah, ZU Nisa, MA Sajad, G Saddiq & S Muhammad Contributed materials/ analysis/ tools: MS Khan, M Shah & N Aziz Wrote the paper: S Malik, MA Sajad & ZU Nisa.

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