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ORIGNAL ARTICLE

Potent Immunomodulatory and Antimicrobial Activities of Gazania linearis: Linking Bioactivity to its Phytochemical Profile

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Conflict of Interest
All the authors have no conflict of interest

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Abstract
Gazania linearis is an ornamental plant native to South Africa, occasionally cultivated in Pakistan as a garden plant. The phytochemical screening on the plant indicated the existence of cardiac glycosides, anthraquinones, flavonoids, saponins, and tannins. The dichloromethane plant extract (GLWPD) employed highest TPC contents; 274 mg GAE/g of DE while methanolic extract (GLWPM) moderately retained TFC contents; 74 mg QE/g of DE. The plant extract: GLWPD displayed the promising antimicrobial activity against a bacterial strain named *Pseudomonas aeruginosa* with 74.50% inhibition and a fungal strain named *Microsporum canis* with 72.80% inhibition. Both GLWPD and GLWPM extracts showed a significant antioxidant potential against DPPH with 61.07±0.12% and 80.34±2.10% inhibition at IC_{50} values; 53.10±1.71 and 40.38±2.53µg/mL respectively. GLWPM also indicated a notable immunomodulatory activity in oxidative burst assay with 71.3% ROS inhibition at IC_{50} value of 22.0±0.3µg/mL after comparison with the standard drug: ibuprofen. From this study, *Gazania linearis* is proved to have considerable antimicrobial, antioxidant and immunomodulatory properties due to the presence of medicinally active secondary metabolites.

Keywords: *Gazania linearis*, Phytochemical screening, antimicrobial activity, antioxidant activity, Oxidative burst assay.

Introduction

Since ancient times, plants have been a vital source of medicine, offering all-natural cures for a wide range of illnesses. Plant-based remedies have long been used in traditional medical systems in the various regions of the world like Ayurveda in Indo-Pak region, Traditional Chinese Medicine (TCM) in China, and Indigenous practices across Africa and America (1). Numerous plants derived compounds have been discovered, purified and are being utilized in pharmaceuticals because of modern science advancements, helping to create life-saving medications. Alkaloids, flavonoids, saponins, terpenoids, and glycosides are among the many phytochemicals found in the medicinal plants that contain anti-inflammatory, antioxidant, antimicrobial and anticancer properties (2). Furthermore, by lowering side effects and encouraging holistic healing, plant-based medications present viable substitutes for the synthetic drugs (3).

The genus: Gazania of Asteraceae family contains 25 South African native herbaceous species. Several species including *Gazania linearis* (Thunb.) Druce is grown as ornamental plants in various regions especially in Pakistan. They produce large, bright orange and yellow composite

flowers that resemble daisies (4). In traditional medicine, various gazania species have been traditionally used to treat toothaches and prevent miscarriages. They have also been used in purgative preparations, particularly when combined with aloes (5). There aren't many studies that assess Gazania's biological effects. In few studies, the antimicrobial, phytotoxic, cytotoxic, antioxidant and hepatoprotective effects of *Gazania rigens* were evaluated (6,7). In a previous chemical investigation, the *n*-hexane extract of *Gazania linearis* rhizome led to the isolation of one steroidal glucoside, six terpenoids type compounds. The *n*-hexane *Gazania linearis* extract and two purified compounds named Santamarine and *β*-amyrin exhibited moderate cytotoxic potential against two cell lines: MCF-7 and COLO-205 (4). Keeping in view, the objective of the current study was to examine the phytochemicals and determine the antimicrobial, antioxidant, and immunomodulatory activities exhibited by the whole plant of Gazania linearis.

EXPERIMENTAL

Plant Collection, Authentication, and Extract Preparation:

The plant: *Gazania linearis* was selected from the botanical garden of Bahauddin Zakriya University, Multan (Pakistan). The plant was identified from a taxonomist of department of Botany, Institute of Pure and Applied Biology, B.Z.U, Multan and a voucher No: http://legacy.tropicos.org/Image/100511674?projectid=32 was allotted to this plant. *Gazania linearis* whole plant was allowed to dry under shaded conditions for 15 days, grinded by a grinder and finally weighed. Sequential extraction of 1000 g of dried plant material was performed using dichloromethane (L090000 by Sigma-Aldrich) and methanol (34860 by Sigma-Aldrich) via maceration. The solvents were removed by evaporation under pressure using a rotary evaporator to obtain concentrated dichloromethane (GLWPD) and methanolic (GLWPM) extracts, respectively.

Preliminary phytochemical analysis

The standard tests were performed to detect different secondary metabolites identified in Gazania linearis including alkaloids, cardiac and anthraquinone glycosides, flavonoids, saponins, and tannins (7).

Total Phenolic Contents determination (TPC)

After mixing, 100µL of the tested sample with 10µL of a 10% diluted FCR (FolinCiocalteu) reagent, the mixture was passed by incubation for 10 minutes, after incubation 90 µL of 15% w/v aqueous Na_2CO_3 solution was added, and then further incubated at 37°C for 90 minutes. The absorbance was measured using 750 nm wavelength and the whole data were stated as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE) using gallic acid as the standard. The Total Phenolic Content (TPC) was measured using the calibration curve prepared within the concentration range of 0–100 µg. (8). Triplicates of the assay were performed.

Total Flavonoid Contents determination (TFC)

Quercetin was used as the standard in the calculation of TFC using calibration curve, in methanol at a concentration of 1 mg/mL with aliquots varying from 0–100 µL. After 5-minute standing period, the test material about (100µL) mixed with 25µL $NaNO_2$ solution (1%) and then added followed by 10µL $AlCl_3$ (10%) and 35µL NaOH (4%) solutions respectively. At absorbance was recorded at 510 nm wavelength using methanol as solvent. All the data is stated as milligrams of quercetin equivalent per gram of dry plant extract (mg QE/g DE). (8).

Antibacterial activity (Agar well diffusion method)

As the test microorganisms, following strains of bacteria were employed: *Shigella sonnei*, *Salmonella typhi*, Escherichia coli (NCTC 10418), Staphylococcus aureus (NCTC 6571), Bacillus subtilis (NCTC 8236), and Pseudomonas aeruginosa (ATCC 10145). The nutrient agar plates were covered with the bacterial culture inoculum, which contained 106 cfu/ml. Six wells measuring 8 mm in diameter were aseptically pressed into the agar medium by using a sterile cork borer. 100 µL of plant extract was added in each well using the concentrations range from 0–1 mg/mL and allowed to diffuse for 2 hours at atmospheric temperature. The inoculated plates were then incubated at 37°C for 24 hours. In this investigation, both ampicillin and ciprofloxacin were used as standard antibacterial drugs and after incubation the % inhibition was measured as the entire procedure was accomplished in triplicate, and the results were presented as the mean of ± standard deviation (SD). (9).

Antifungal Assay (Agar well diffusion method)

As the microorganisms tested, the following strains of fungus were used: *Aspergillus flavus* (PTCC 5018), *Candida albicans* (ATCC 90028), *Fusarium Lini*, *Microsporum canis* (PTCC 5069), and *Trichophyton rubrum***.** The three antifungal drugs including miconazole, Amphotericin-B and clotrimazole respectively were used as standard drugs (9). The

remaining experimental procedure was conducted in accordance with the methodology described for the antibacterial effect.

Antioxidant activity (DPPH radical scavenging assay)

A test solution of 10µL was combined with 90µL of a 100µM methanol DPPH solution in a 96-well plate to yield a total volume of 100µL. The solution was thoroughly blended and incubated for 30 minutes at 37°C. Subsequently, the decrease in absorbance was analyzed at 517 nm using a microplate reader, and the results were calculated using the following formula for % inhibition as; Percentage Inhibition = (Abs. of Control - Abs. of Test / Abs. of Control) × 100

Immunomodulatory Activity: Oxidative Burst Assay using Chemiluminescence Technique:

Immunomodulatory potential was evaluated using an oxidative burst assay based on the chemiluminescence method. 25 µL of diluted whole blood was mixed with calcium- and magnesium-supplemented Hank's Balanced Salt Solution (HBSS++) and incubated, the procedure was repeated three times using 25 µL of test samples at variable concentrations of 1, 10, and 100 µg/mL. Control wells contained HBSS++ only and cells to serve as baseline reference.

The prepared 96-well microplate was placed inside a luminometer equipped with a thermostat chamber and incubated at 37ºC for 15 minutes to allow adequate interaction between the blood cells and the test compounds. After the incubation period, 25 µL of serum-opsonized zymosan (SOZ) was introduced into each well to stimulate the oxidative burst response. This was succeeded by adding 25 µL of luminol, a chemiluminescent probe used to detect reactive oxygen species (ROS).

The luminometer was then used to measure the ROS levels in each well, expressed with reference to the relative light units (RLU). The intensity of luminescence corresponded to the level of ROS production, providing insights into the immunomodulatory effects of the test compounds. (8).

Results and Discussion

Phytochemical screening

The extracts were subjected to preliminary phytochemical screening is a starting key; it reflects the nature of various secondary metabolites and medicinal components present in the plants. The testing of secondary metabolites on the plant indicated the existence of cardiac glycosides, anthraquinone glycosides, saponins, flavonoids and tannins as summarized in the table 1.

Table 1: Results of secondary metabolites detection in *Gazania linearis*

Extracts	Alkaloids	Cardiac glycosides	Anthraquinones	Saponins	Flavonoids	Tannins
GLWPD	-	+	-	-	+	+
GLWPM	-	+	+	+	+	-

(+) shows presences of secondary metabolites, (−) shows absence of secondary metabolites.

Total Phenolic and Total Flavonoid Contents

Total phenolic content (TPC) and total flavonoid content (TFC) of Gazania linearis extract were calculated by using Folin–Ciocalteu's reagent. The results were stated as gallic acid equivalents (GAE) for TPC and quercetin equivalents (QE) for TFC. The results indicated that GLWPD plant extract showed highest TPC: 273.79±1.32 and low TFC: 51.31±0.21 while GLWPM exhibited appreciable TPC: 124.11±2.51 and moderate TFC: 74.11±0.89. The GLWPD and GLWPM extracts F/P ratio was 0.18 and 0.69 respectively as presented in the table 2.

Table 2: Total Phenolics and Total Flavonoids Contents of *Gazania linearis* extracts

Extracts	TPC (mg GAE/g of DE)	TFC (mg QE/g of DE)	Flavonoids/Phenolics (F/P ratio)
GLWPD	273.79±1.32	51.31±0.21	0.18
GLWPM	124.11±2.51	74.11±0.89	0.69

GAE: Gallic acid Equivalent QE: Quercetin Equivalent DE: Dry extract

Antibacterial Activity

The extract; GLWPD showed a promising activity against *Pseudomonas aeruginosa* with 74.50% inhibition, moderate against *Staphylococcus aureus* (53.35% inhibition) and *Bacillus subtilis* (41.25% inhibition) respectively but remained inactive against

Escherichia coli and *Salmonella typhi*. On the other hand, GLWPM was found to be active against all bacterial strains with significant potential against *Staphylococcus aureus* (79.45% inhibition), *Bacillus subtilis* (67.30% inhibition), intermediate activity against *Escherichia coli* (51.75% inhibition), and lower activity against *Pseudomonas aeruginosa* (32.40% inhibition) and *Salmonella typhi* (18.50% inhibition) respectively after comparison with the standard drug; ciprofloxacin (Table 3).

Table 3. Antibacterial activity of *Gazania linearis* extracts.

Samples	**Antibacterial activity (%Inhibition at 3mg/ml concentration)**				
	Bacillus subtilis	***Escherichia coli***	***Salmonella typhi***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
GLWPD	41.25 %	0 %	0 %	74.50 %	53.35 %
GLWPM	67.30 %	51.75%	18.50 %	32.40 %	79.45 %
Ciprofloxacin (standard)	92.60 %	91.45 %	89.12 %	97.87 %	93.34 %

Antifungal Activity

Among the all-fungal strains, GLWPD retained the best activity against *Microsporum canis* with 72.80% inhibition and lowest against *Aspergillus niger* with 15% inhibition. The plant extract; GLWPM exhibited the moderate potential against two fungal strains; *Aspergillus niger* and *Candida albicans* with 44.70% and 30.50% inhibitions respectively and both the extracts are inactive against remaining all fungal species (Table 4).

Table 4. Antifungal activity of *Gazania linearis* extracts.

Samples	**Antifungal activity (%Inhibition at 0.4mg/ml concentration)**				
	Aspergillus niger	***Candida albicans***	***Fusarium lini***	***Microsporum canis***	***Trichophyton rubrum***
GLWPD	15.00 %	0 %	0 %	72.80 %	0 %
GLWPM	44.70 %	30.50%	0 %	0 %	0 %
Amphotericin B	94.70 %	–	–	–	–
Clotrimazole	–	91.50 %	–	–	–
Miconazole	–	–	95.35 %	94.80 %	94.80 %

Antioxidant activity (DPPH scavenging assay)

Antioxidant activity was evaluated using most common DPPH scavenging assay. The activity of the plant extracts was compared with the standard drug; BHT that indicated 88.53±0.91% inhibition with IC_{50} value of 20.23±1.23µg/mL. The plant extracts; GLWPD and GLWPM scavenged 61.07±0.12% and 80.34±2.10% DPPH with their IC_{50} values; 53.10±1.71 and 40.38±2.53µg/mL respectively at dose of 50µg/mL. The results are presented in the table 5 below.

Table 5. Anti-oxidant activity of *Gazania linearis* extracts.

Samples	**Dose (µg/mL)**	**%DPPH scavenging**	**IC_{50} values (µg/mL)**
GLWPD	50	61.07±0.12	53.10±1.71
GLWPM	50	80.34±2.10	40.38±2.53
BHT (standard)	50	88.53±0.91	20.23±1.23

Immunomodulatory activity (Oxidative burst assay)

The oxidative burst assay was employed to assess the immunomodulatory activity of Gazania linearis extracts. The extract; GLWPM showed the excellent

potential; 71.3% ROS (Reactive Oxygen Species) inhibition with IC_{50}; 22.0±0.3µg/mL while GLWPD exhibited 40.10% inhibition. The plant extracts results were compared with the standard; Ibuprofen that indicated 78.10% inhibition at IC_{50} value of 12.1±0.1µg/mL. All results are expressed in the table 6.

Table 6: Immunomodulatory activity of *Gazania linearis* extracts.

Extract/standard	Concentration (µg/mL)	%Inhibition	IC_{50} (µg/mL)
GLWPD	75	40.1%	-
GLWPM	75	71.3%	22.0±0.3
Ibuprofen	75	78.1%	12.1±0.8

Discussion:

Even with the use of various advanced modern research techniques and instruments, the primary screening tests to identify the phytochemicals are still the simplest, accurate, reliable and cost-effective methods. In natural product research, phytochemical screening is an essential initial step that directs subsequent bioactive compound isolation, purification, and pharmacological investigations (11). The present phytochemical screening results are consistent with earlier reports on Gazania linearis as well as other species of the genus *Gazania* i.e., *Gazania rigens* (4,6,7). Plant extracts with high concentration of total phenolic content (TPC) and total flavonoid content (TFC) exhibit robust bioactivity, rendering them useful in the fields of medicine, food, cosmetics, and agriculture (12). Significant amount of total phenolic content (TPC) and total flavonoid content (TFC) were detected in both dichloromethane (GLWPD) and methanolic (GLWPM) plant extracts.

(GLWPM) gave the direction to evaluate antimicrobial, antioxidant and immunomodulatory potential of *Gazania linearis*. The flavonoids and phenolics play a crucial role in defense mechanism, including antibacterial and antifungal activities and are proven to have antioxidant and immunomodulatory potential (8,10, 12). The widespread overuse and misuse of antibiotics worldwide, particularly in Pakistan, has contributed to the emergence of resistance in various microorganisms.

Therefore, attempts are underway to address the problem of widespread antibiotic resistance and investigate alternate sources of antimicrobial agents, including medicinal plants (13). The antimicrobial results of *Gazania linearis* are consistent with the Gazania species like *Gazania rigens* that displayed the prominent antibacterial effect against gram-positive bacteria like *S. aureus*, gram negative bacteria; *E. coli* and *P. aeruginosa* and a fungal strain i.e. *C. albicans* (5, 6).

Free radicals contribute to many degenerative diseases by altering DNA, breaking its bases and strands, attacking the mitochondrial electron transport chain's NADH+-dehydrogenase complex, peroxiding LDL, and interfering with the synthesis of ATP (14). Free radicals have been found to perform a substantial role in the etiology of a number of ailments, involving Alzheimer's disease, amyotrophic lateral sclerosis, neoplastic diseases, respiratory disorders, cardiovascular diseases, cognitive & memory loss, arthritis, Parkinson's disease and uraemia (10,14). Interest in nutrition and food science has increased in recent decades due to natural diets high in phenolic and flavonoid compounds that have antioxidant potential. To fight these kinds of illnesses, plant extracts and compounds that have been separated from flavonoids and phenolics have been studied (8,12).

The primary mechanism underlying antimicrobial and tumoricidal effects involves the excessive release of reactive oxygen species (ROS). As these reactive oxygen species are also implicated in various epigenetic processes, they play a significant role in cellular regulation and response mechanisms. Factors that are responsible for the initiation, advancement, and modification of breast and cervical cancers, oxidative stress is closely associated with carcinogenesis. According to recent research, using immune-modulatory medications to treat cancer patients has improved results. In order to combat cancer, it is crucial to continue researching the use of different treatment combinations to boost immunity against the disease (15).

Conclusion

The research study concluded that *Gazania linearis* contains medicinally active secondary metabolites and are rich in phenolic and flavonoid contents, most likely contributing to antimicrobial, antioxidant and immunomodulatory potential. Further research is required to isolate and purify such compounds primarily responsible for these activities.

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