

Indexed, Abstracted and Cited: [ISRA Journal Impact Factor](#), [International Impact Factor Services \(IIFS\)](#), [Directory of Research Journals Indexing \(DRJI\)](#), [International Institute of Organized Research and Scientific Indexing Services](#)

World Journal of Biology and Medical Sciences

Published by Society for Advancement of Science®

ISSN 2349-0063 (Online/Electronic)

Volume 1, Issue- 3, 56-63, July-September, 2014



WJBMS 1/1/03/2014

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A Double Blind Peer Reviewed Journal

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RESEARCH PAPER

Received: 19/05/2014

Revised: 20/08/2014

Accepted: 21/08/2014

Laboratory Test for Flavonoids and Carbohydrates in Wild Species *Gladiolus imbricatus*

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ABSTRACT

The genus Gladiolus contains about 250 species. Gladiolus imbricatus, a rare species spread in the hill region of Europe, is resistant to abiotic and biotic stress being one of the most cold-tolerant in the genus. Moreover it contains high vitamin C and minerals in the leaves and the flowers are considered as edible. The aim of our study was to develop in vitro technologies for this plant. Initiation of in vitro cultures was started from wounded seeds or excised mature embryos. In this study biologically active compounds in plant material and to develop in vitro technologies for G. imbricatus. Physico-chemical methods of analysis established the presence of polysaccharides, flavonoids, terpenoids and vitamin C mainly and fewer aromatic and quinones compounds.

For medium-term conservation maintenance of the in vitro cultures in low temperature in the dark proved to be the best in reducing the growth rate of the shoots after 3, 6 and 12 months. The recovery of plant growth was evaluated after 12 months, by the transfer to normal growth conditions. 25% of the plants were recovered after one year storage. Besides the importance for biotechnology, the in vitro techniques described here might be used, after molecular analysis of genetic stability, for the restoration of natural populations into the habitats where this species became extinct. The aim of this paper was to establish reproducible in vitro culture techniques for the rare wild species G. imbricatus.

Keywords: *G. imbricatus, Chemical Composition, Physico-chemical methods, Aromatic and quinones compounds and In Vitro Techniques.*

INTRODUCTION

Polymorphic; the perianth in the north and west of the range is reddish-purple, but in the south and east it is frequently violet-purple. Some plants may produce axillary branches. The width of the wing of the seed is also variable. Plants with seeds with vestigial wings have been described from the Ukraine as *G. apterus* Klokov in Kotov & Barbarich, Fl. RSS Ucr. 3: 407 (1950). Abnormally large specimens, with additional leaves, superficially resembling *G. communis*, are occasionally found throughout the range of *G. imbricatus*.

The majority of the species (150) are originally from South Africa, which is considered as the diversification center for the cultivars (Ohri and Khoshoo, 1985). Today's gladioli are hybrids of different wild species or hybrids of subsequently obtained hybrids.

About three quarters of the world's population relies on herbal medicines for

health care [Premanathan et al., 2000]. A requirement in medicinal drugs from a plant material remains very high. It is connected with a number of their advantages, namely: possibility of the long-term use, soft therapeutic action, availability and non-toxicity. Secondary metabolites are essentially produced and extracted from plants grown in the field under influence of seasonal variations. As a result of reduction of natural supplies of medical plants, perspective is an alternative biotechnological method of production of BAC from the in vitro culture.

Gladiolus imbricatus belonging to Iridaceae family. Grows mainly in dry grasslands at the border of bushes at an altitude between 100 and 1450 m. *G. imbricatus* are almost the most cold-tolerant species of the genus. *G. imbricatus* growing in Ukraine in the Carpathians, in Polissia and Roztochchja (Figure 1).



Fig 1. Wild species *G. imbricatus*



Fig 2. Distribution of *G. imbricatus* in world.

According to literature data they contain in their composition mainly glycosides, vitamins and essential oils and fewer aromatic and quinones compounds. In ethnomedicine the plant is used as anesthetic and lactogenic agent, have bactericidal, astringent, tonic, sedative actions. Infusion of corm used to treat allergies. Chemical composition requires more detailed study [Rakosy-Tican, 2000].

To identify and evaluate the therapeutic potential of medicinal herbs, isolation of active components and structural elucidation of these compounds is very essential in medicinal chemistry and natural product research.

The aim of this paper was to establish phytochemical composition and reproducible in vitro culture techniques for the rare wild species *G. imbricatus*. The resulting ethanolic and water extracts was filtered and concentrated and then tested for various BAC by conventional methods - polysaccharides, flavonoids and glycosides, terpenoids, vitamin C, aromatic and

quinones compounds were commonly found.

MATERIAL AND METHODS

Collection of plant materials the raw material for the study was *G. imbricatus*. The plants have been collected from natural habitat, after flowering period. The plant was air-dried without access to sunlight. Then they were finely crushed and used for extraction.

Extraction of plant materials

Plant extract was prepared by Soxhlet extraction method. Powdered plant material was uniformly packed into a thimble and extracted with different solvents separately. Solvents used were chloroform and hexane, and 70% ethanol and hot water. The process of extraction continues till the solvent become colorless [Aiyegroro, and Okoh, 2010]. The resulting extracts were filtered and concentrated by vacuum distillation in a water bath at 40 °C. Output of extracts ranged from 7-19%. Dried extract was kept in refrigerator at 4°C

for their future use in phytochemical analysis. Phytochemical screening procedure the presence of BAC determined using pharmacopoeial methods [State Pharmacopoeia of Ukraine (2011)] as well, as by thin layer chromatography (TLC) [Harbone, 1998].

Test for flavonoids

Shinoda test four pieces of magnesium fillings (ribbon) are added to the 1 ml ethanolic extract followed by few drops of concentrated hydrochloric acid. A pink or red color indicates the presence of flavonoid [Andrew, (2002)]. Colors varying from orange to red indicated flavones, red to crimson indicated flavonoids, crimson to magenta indicated flavonones. Ferric chloride test to 2 ml of the extract, few drops of 10% ferric chloride solution were then added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.

Sodium hydroxide test To 2 ml of the ethanolic extract 10% aqueous sodium hydroxide was later added to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids.

Test for Quinones

About 0.5 g of plant extract was taken and added 1 ml of extract and 1 ml of con. H₂SO₄ was added formation of red colour shows the presence of quinones.

One drop of ethanolic test solution is placed on a filter-paper, followed by 1 drop of 0-2 percent ethanolic phenylacetone nitrile solution and 1 drop of 0.1 N sodium hydroxide. A positive response is indicated by the appearance of a blue or violet stain edged by a yellow ring.

Molisch's test for Carbohydrates

Few drops of Molisch's reagent were added to the portion of sample dissolved in

distilled water; this was then followed by addition of 1 ml of conc. H₂SO₄ by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet colour at the interphase of the two layers was a positive test [Yisa (2009)].

Test for terpenoids (Salkowski test) Five ml of each extract was mixed in 2 ml of chloroform, and concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids [Ody, P. (1993)].

Thin Layer Chromatography

TLC of flavonoids Extract was subjected to TLC analysis, to find the presence of flavonoids to support the chemical test. Solution of quercetin was used as a standard. A volume of 1 ml of solutions of standards and investigated extracts was spotted on the plates with a thin layer of silikahel. Plate is placed in a chamber with a solvent system butanol: acetic acid: water in volume ratio 3:1:1. After uplift the front of the solvent to a height of 10-12 cm and dried chromatograms exhibit with concentrated ammonia solution, spots of flavonoids were observed under UV light (yellow color). TLC of Carbohydrates 0.02 ml of water extract was applied to the base line of the chromatogram "Silufol". Plate is placed in a chamber with a 90% solution of ethanol and chromatographed. The chamber previously saturated by solvent vapors for 30 min. After passing the solvent front about 12 cm plate removed from the chamber and air dried. Spots were visualized by spraying successively the chromatogram with 20% thymol solution and dilute sulfuric acid and heating at 100-150°C for 4 min. The chromatogram shows the basic spot color orange.



Fig 3. Plant growth, after 3 month, for low temperature, low light or normal growth.

TLC of vitamin C 0.5 g of powdered material placed in a flask, add 5 ml of distilled water, and stirred for 15 minutes after the infusion is filtered. Filtrate was applied to the plate "Sylufol", near sample ascorbic acid. Plate is placed in a chamber with a solvent system: ethyl acetate - ice acetic acid (8:2). After chromatography plate was dried in air. Chromatogram spraying 0.04% water solution of Na₂ 6-dichloroindophenol. Ascorbic acid is shown with white spots on a blue background. TLC of Quinones Silufol UV-254 was used for analytical TLC. The components were visualised under UV/Vis light at 254 and 365 nm in a UV cabinet and by using a solution of phosphomolybdic acid (20 g) and ceric sulphate (2.5 g) in 500 ml of sulphuric acid (5%), followed by heating. Quinones were detected by spraying the plate with a solution of 5% KOH in ethanol followed by heating for 5 minutes at 105°C.

In vitro corm development

In this study, fresh corms of *G. imbricatus* were used as explant. For pre-treatment, the corms were cleaned in running water

and outer scales were removed. Corms were surface-sterilized with 70% EtOH for 17 min and with 4.5% Sodiumhypochlorite for 20 min, consecutively, and washed three times with sterilized distilled water [4]. The experiments were maintained on solidified basal medium Murashige & Skoog which contained, mineral salts and vitamins (100 mg/L myo-inositol, 2 mg/L 1glisin, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 30 g/L sucrose, 8 g/Lagar) and 2,5 mg/L α - naphthaleneacetic acid (NAA) and 0,3 mg/L kinetin. The pH of medium was adjusted to 5.8 before autoclaving for 15 min. For callus cultures, sterilized corms were divided into two group. First group of corms was cut into 4 mm transverse slices, and the second group was cut into 4 mm longitudinal slices. Callus cultures were grown at a temperature of 24 ± 2 °C with illumination provided by cool white florescent lamps at 40 μ E with a 16-h light period and subcultured. Subculturing was periodically carried out at 4 weeks intervals. Five callus lumps were inoculated

in each 250 ml flask, three flasks in each treatment. All experiments were repeated three times. Cultured at a temperature of 23-25°C in the dark. The frequency callus development assessed in percentage by the number of explants that gave callus from the total eksplants.

RESULTS AND DISCUSSION

Phytochemical analysis

The phytochemical screening of the *G. imbricatus* extracts showed the presence of flavonones, carbohydrates, vitamin C and also aromatic and quinones compounds as shown in table1.

Table 1. Qualitative analysis of the phytochemicals of the *G. imbricatus*.

Compounds	Extracts			
	Ethanol	Chloroform	Aqueous	Hexane
Flavonoids	+	+	-	+
Quinones	+	+	-	+
Vit. C	-	-	+	-
Carbohydrates	-	+	+	+
Terpenoids	-	-	-	-

+ = Presence; - = Absence

Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. For example, flavonoids have been reported to have antiviral, anti-allergic, anti-platelet, anti-inflammatory and antitumor activities [Rice-Evans, 1997]. Carbohydrates, especially pectin, increase resistance to the effects of many allergens. Quinone compounds have various bioactivities and play important roles in the nature. Among a lot of quinone compounds including naturally occurring compounds, especially the naphthoquinone derivatives have wide variety of bioactivities. Also potency as anti-inflammatory agent, anti-allergic agent, anti-asthma medicine, anti-dragon gore medicine, anti-psoriasis medicine, bronchodilator, thrombus prevention and hypotension are found in their derivatives. They can be also utilized

for the infection drugs as anti-fungal agents, anti-virus medicines and antibacterial agents against gram-positive bacteria, gram-negative bacteria, etc. In vitro corn development Maximum formation of callus (94-95%) was detected at 4 weeks. With the introduction of explants in vitro conditions first signs of callus genesis appeared after 1-2 weeks of cultivation. The resulting callus had a bright, slightly yellowish color, characterized by dense texture and low intensity of growth. A visual analysis callusnyh cultures were signs of morphogenesis, callus different relative homogeneity structure. Further research has to be determine the content of BAC of callus cultures and it's quantitative analysis, and comparing them with the content and biological activity in extracts from medicinal plants *G. imbricatus*. The above data show that *G. imbricatus* has medicinal properties

and can be recommended for clinical study and introduction of public health practice.

Table 2. The percentage of germination, mean leaf and root growth and mean leaf number per plant in *Gladiolus imbricatus*

Parameter	Wounded seed	Mature embryo
Percentage of germination (% \pm SD)	75 \pm 5	50 \pm 6
Mean leaf length (cm \pm SD)	3.26 \pm 0.7	1.84 \pm 0.4
Mean root length (cm \pm SD)	4.8 \pm 0.3	5.0 \pm 0.2
Mean number of leaves (\pm SD)	2.66 \pm 0.8	1.75 \pm 0.7

CONCLUSION

Extracts obtained by the above method is a yellow liquid with aromatic. Here a phytochemical analysis of various solvent extract of *G. imbricatus* was conducted. Phytochemical analysis of the plant *G. imbricatus* showed the presence of many BAC, such as flavonoids, carbohydrates, vitamin C and aromatic and quinones compounds. *G. imbricatus* studied here can be a potential source of useful drugs exploiting the antimicrobial, antioxidant and antitumor activities of these plants. The wild plant harbours higher values of the active ingredients than the ornamental plant which needs further research. Advanced studies are being conducted on these plants in order to isolate, identify, characterize and elucidate the structure of the BAC. The results on in vitro culture will help the stable maintenance in vitro with lower costs along with further possible use of callus for research BAC and biological activity. The wild rare species *G. imbricatus* was for the first time successfully cultured in vitro, multiplied and induced to form both well-developed corms and embryogenic callus. The results can be compared with those previously reported for *Gladiolus* cultivars or other wild species.

regenerated in vitro from wounded seeds or mature embryos (germination on MS $\frac{1}{2}$ medium with 3% (w/v) activated charcoal; the growth of the shoots is recorded on MS-3 medium after one month).

The plant material collected from the field in late summer was heavily contaminated but mature seeds proved to be a suitable starting material for in vitro culture initiation. This was in contrast to gladioli cultivars where usually corms and corm buds were giving good results (Ziv, 1970).

ACKNOWLEDGEMENTS

Authors express thanks to State Fund of Fundamental Researches Semnan University of Iran for providing of research.

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