J. Plant Production, Mansoura Univ., Vol. 3 (3): 545 - 556, 2012

PHYTOCHEMICAL AND MOLECULAR STUDIES ON NARCISSUS IN EGYPT

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ABSTRACT

This study was carried out in National Research Center in Dokki, Giza, Egypt in collaboration with Botany department, Faculty of Science, Mansoura University during 2007-2009 seasons. This study first, aims to find Egyptian wild narcissus bulbs for scientific records and genetic fingerprinting as an Egyptian species. Unfortunately the bulbs almost disappeared from about two decades under the pressure of tourism and ordinary cultivation in north coast of the country. Hopeless looking for any evidence it may be still found, but our search in many areas in Egypt fails to find any bulb. And so, the study continues based on another two species of narcissus foreign one *Narcissus tazetta* cultivated for many years in Egypt and a commercial variety from flowers producer. The other aim of our study was to carry out phytochemical screening to estimate the natural components of *Narcissus tazetta* plants and data showed that many constituents were found in the form of alkaloids including flavonoids, tannins, sterols and triperpenes. Finally, molecular studies using RAPD-PCR were carried out to fingerprint the two species of narcissus using ten 10-mer primers, indicating that unique fingerprinting bands as produced by primers number 1, 2 and 3 could clearly discriminate between the two species for example, fragments with approximately molecular size of 2764 bp, 2344 bp, 1114 bp, 978 bp and 792 bp were detected in *Narcissus tazetta* profile while, fragments with approximately molecular size of 1787 bp, 1996 bp, 1592 bp and 583 bp were detected in commercial narcissus profile.

Keywords: Narcissus, RAPD-PCR, Phytochemical screening, secondary metabolites.

INTRODUCTION

Narcissus is the Latin name for a group of hardy, mostly spring-flowering, ornamental bulbs. There are several narcissus species that bloom in the autumn. Daffodil is the common English name for all narcissuses. The botanic name of the genus is narcissus. They are mostly native to the Mediterranean region, but a few species are found through central Asia to China. Plants belonging to the Amaryllidaceae family are well known for the presence of an exclusive group of alkaloids with a wide range of biological activities (Bastida *et al.,* 2006). Narcissus plants have many medicinal uses due to they contain many active constituents as secondary metabolites as alkaloids, many researchers proved that, narcissus could be used as anti-viral, anti-fungal, anti-tumor and many other uses (Natalia *et al.,* 2010).

Since ancient times, Egyptians have celebrated the many flowers that grow in their land. Whether they were inscribed on monuments to the Pharaohs or offered as gifts of love, flowers have always been a large part of Egyptian life. A wide variety of flowering plants are grown in the fertile Nile

Valley and exported throughout the world. Egypt has a long history of using flowers for both decoration and as religious symbols. In the past, the Nile river valley was lusher and less desert like. Narcissus has also been grown in Egypt since very ancient times. The remains of narcissus bulbs were found on the neck of the mummy of Ramesses II.

In Egypt, for many generation narcissus species always found in north coast in barley field which irrigated with rainfall and so, used to be found in winter, but in last two decades due to the expansion in tourism projects and popular increase in this area and fields now irrigated with water during all of the year, this led to an increase in water content in soil and make the bulbs to be decayed and so, narcissus almost disappeared under the pressure of tourism and ordinary cultivation in north coast of country.

MATERIALS AND METHODS

Plant material:

1- Collect a known *narcissus* variety *Narcissus tazetta* (Plate 1-a) foreign but cultivated for many years in Egypt.
2- Collect a commercial variety from flowers producer in Egypt (Plate 1-b).
3- Looking for a wild *narcissus* variety in Egypt in many probable areas especially in north coast.

Phytochemical screening:

The dried powdered samples of methanolic (MeOH) *Narcissus tazetta* extract were separately screened for the following tests:

1. Test for the presence of carbohydrates and/or glycosides (Conalez, *et al.,* 1962)
2. Test for the presence of Tannins (Clauss, 1961)
3. Test for the presence of alkaloids and/or nitrogenous bases (Fulton, 1932)
4. Test for the presence of flavonoids (Geissman, 1962)
5. Test for the presence of saponins (Harbone, 1973)
6. Test for the presence of unsaturated sterols and/or triperpenes (Wall *et al.,* 1954)
7. Test for the presence of coumarins (Farnsworth, 1966)

Molecular characterization using (RAPD-PCR) analysis

DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle, (1990). Half gram of fresh samples was ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 5 ml preheated CTAB buffer, and incubated at 65°C for 1 hour with occasional shaking. The suspension was then mixed with 1/3 volume of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or sedimentated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and resuspended in 0.5 ml TE buffer. The enzyme, RNAse A (20µg) was added to

the resuspended mixture to digest any contaminating RNA and the tube was incubated at 37°C for 30 min. To remove the enzyme and other contaminating protein, phenol/chloroform extraction was performed. Ten 10-mer primers (Operon technologies Inc., Alameda, California) randomly selected were used in RAPD analysis (table 1).The amplified pattern was visualized on a UV transilluminator and photographed.

Table (1): Primers used in RAPD-PCR experiment.

Primer	Sequence 5'- 3'	Annealing Tm °C / Sec
K1	5'TGGCGACCTG3'	36°C
K2	5'GAGGCGTCGC3'	
K3	5'CCCTACCGAC3'	
K4	5' TCGTTCCGC3'	
K5	5'CACCTTTCCC3'	
Op 4	5'GGACTGGAGT3'	
Op 5	5'TGCGCCCTTC3'	
Op 7	5'GGTGACGCAG3'	
Op 9	5'TGGGGGACTC3'	
Op11	5'GTAGACCCGT3'	

RESULTS AND DISCUSSION

I. Phytochemical studies:

Narcissus species: In this study collection of two narcissus species bulbles, one is *Narcissus tazzetta* (Plate 1-a) and the other is a commercial species from one of flower producers (Plate 1-b) but, we failed to found an Egyptian wild species from many areas of Egypt from Sinaii to Matrouh and through pass to north coast even in the proteted area as El-omaed in Matrouh.

Plate (1) *a- Narcissus tazetta, b- Narcissus* (commercial)

Phytochemical screening: The results of the phytochemical screening of MeOH *Narcissus tazetta* extract are shown in (Table 2) which revealed that carbohydrates and/or glycosides and sterols and/or triterpenes are present in plant extract under investigation. Condensed tannins and hydrolyzable

tannins are present in the plant extract. Flavonoids and other alkaloids are present in a very considerable amounts in plant extract. Saponins and coumarins are absent from the plant extract under investigation.

In conformity of the present results, (Roberts and Wink, 1998; Wink, 2007; 2008) reported that Plants including *Narcissus sp*. produce plenty of secondary metabolites, the alkaloids are one of the most diverse groups of secondary metabolites found in search plants and have a series of structure type. Many alkaloids have shown their powerful toxicity on animals or humans. Most of the lethal alkaloids fall into the class of neurotoxins (Wink, 2008). And some act on the other different organs. For example, pyrrolizidine alkaloids which occur in Senecio plants will cause hepatic disease within a few weeks in horses and cattle (Wink, 1993; Elliott *et al.,* 2005). Alkaloid applications can be found in different areas. Some alkaloids are still used in modern medicine today as natural or modified compounds. (Aniszewsky, 2007).

Table (2): Results of phytochemical screening of MeOH *Narcissus tazetta* extract

Chemical Constituents	Plant extrcat
1.Carbohydrates and/or glycosides	+ ve
2. Tannins	
a.Condensed tannins	+ ve
b.Hydrolysable tannins	+ ve
3. Alkaloids and/or nitrogenous bases	++ ve
4. Flavonoids	++ve
5. Sterols and/or triterpenes	+ ve
6. Saponins	- ve
7. Coumarins	- ve

++ ve denotes the presence of the constituentsin execess amount,
+ ve denotes the presence of the constituents suitable amount and
– ve denotes the absence of the constituents.

Liu *et al.*, (2006) found that the extraction of *Narcissus tazetta* var. chinensis are effective against two insects *Sitophilus zeamais* (Motschulsky) and *Tribolium castaneum* (Herbst*),* and added that the extraction from *N. tazetta* var. chinensis strongly decreased the survival rate of the following tumor cell lines: HL-60, K562, KT1/A3, and A3R. *N. tazetta* var. chinensis has been used as an herbal medicine for the treatment of different diseases in China for thousands of years, Hung and Tsai, (1962).

Abdallah, (1993) reported the isolation of lycorine, pseudolycorine, galanthamine, haemanthamine, tazettine, pretazettine and the new alkaloid narcisine from *Narcissus tazetta* bulbs. Labrana *et al.,* (1999) isolated eleven alkaloids from whole plants of *Narcissus bujei*. Chu and Bun, (2004) represents the first report of purification of a glutamine-rich antifungal peptide (nartazin) from family Amarylliaceace. The peptide, designated as nartazin, was purified from the bulbs of the Chinese daffodil *Narcissus tazetta* var. Piozzi, *et al.,* (1968) extracted two compounds from fresh bulbs of many varieties of daffodils have been extracted. Although their structures are related to many Amaryllidaceae alkaloids, the compounds showed no basic

properties since the nitrogen is amidic in character. The first substance, named narciclasine, shows a strong antimitotic activity and has been assigned structure VIII or its mirror image. The second compound, named narciprimine, has no antimitotic activity and has been given structure XII (Piozzi, *et al.,*1968).

Flavonoids are phytochemicals characterized by a wide range of biological activities, including antioxidant activity, the ability to modulate enzyme or cell receptor activity patterns, and to interfere with essential biochemical pathways (Osmak *et al.,* 2009). Flavonoid compounds distribute widely in vascular plants and bryophytes, and containes about 5,000 kinds have been reported as naturally occurring substances. Many biological activities of the flavonoids were found until now. They include pollinator attractants, oviposition stimulants, feeding attractants and deterrents, allelopathy and phytoalexins. This paper reviews function and activity of flavonoids against plants and other organisms, (Tsukasa, 2003).

II. Molecular studies:

RAPD-analysis: Random amplified polymorphic DNA (RAPD) has been used in this study to identify species-specific markers and establish RAPD-PCR fingerprints to three collections of narcissuses, *N. tazetta*, commercial unknown species and the Egyptian wild bulbs but, unfortunately the Egyptian one could not been found any where and so, the analysis carried out for the first two species only.

The use of random primers in a PCR is a powerful tool that reveals extensive DNA polymorphism, and it has been valuable in genetic analysis (Barakat and Elham, 2004). The ten primers used in this study display a strong amplification with distinct bands. The RAPD markers generated by these primers revealed characteristic profiles for each species of narcissus in terms of number and position of RAPD bands (table 3 and figures 1&2).

Table (3): Summary of data obtained by RAPD-PCR analysis using ten 10-bp long primers for identification of the two species of narcissus.

Primer No.	Primmer sequence 5'- 3'	% of GC	No. of bands		Approx. band size bp
			T	C	
1	5'GGACTGGAGT3'	60	4	2	2464 - 583
2	5TGCGCCCTTC3'	70	1	4	2344 - 641
3	5'GGTGACGCAG3'	70	4	1	2274 - 574
4	5'TGGCGACCTG3'	70	2	2	2711 - 820
5	5'CCCTACCGAC3'	70	1	1	1751
6	5'GAGGCGTCGC3'	80	2	3	990 - 542
7	5' ATCGTTCCGC3'	60	3	4	2413 - 415
8	5'CACCTTTCCC3'	60	6	5	1284 - 243
9	5'TGGGGGACTC3'	70	2	2	990 - 395
10	5'GTAGACCCGT3'	60	2	5	1139 - 395

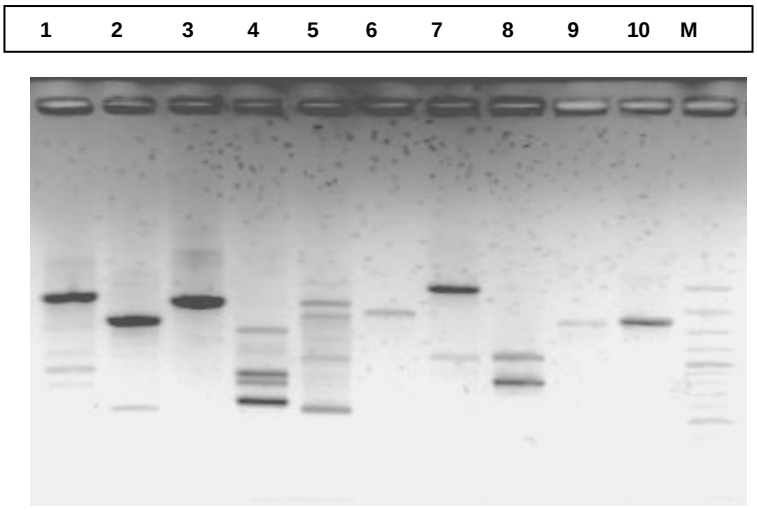

Fig. (1): RAPD fingerprints of two species of narcissus using five random primers.
Lane 1,3,5,7 and 9 represent *Narcissus tazetta* profile
Lane 2, 4, 6, 8 and 10 represent commercial narcissus
M = marker, Lane 1, 2 primer op- 4,
Lane 3, 4 primer op-5, Lane 5, 6 primer op-7
Lane 7, 8 primer k1, Lane 9, 10 primer k3

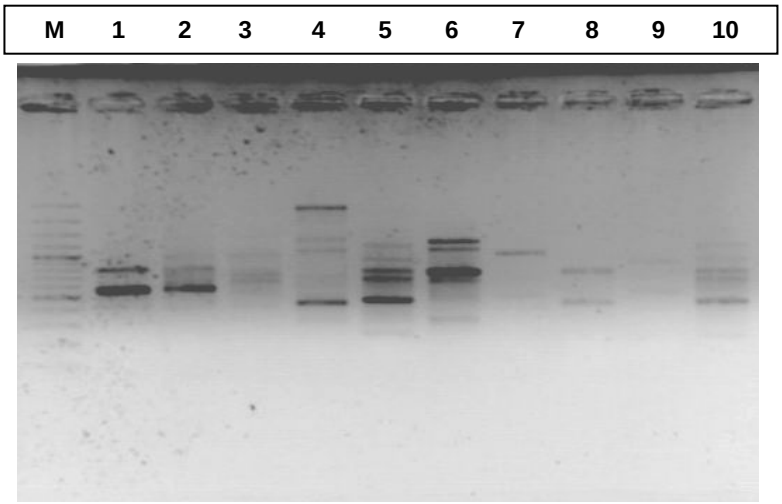

Fig. (2): RAPD fingerprints of two species of narcissus using five random primers.
Lane 1,3,5,7 and 9 represent *Narcissus tazetta* profile
Lane 2, 4, 6, 8 and 10 represent commercial narcissus
M = marker, Lane 1, 2 primer K2
Lane 3, 4 primer k4, Lane 5, 6 primer k6
Lane 7, 8 primer op-9, Lane 9, 10 primer op-11

A maximum of 7, 7 and 11 DNA bands were scored in RAPD profile generated by the primers Op 11, K4 and K5 for both varieties respectively (Table, 3), the size of the amplified fragments ranged from about 2711 bp to 243 bp (table, 5). These primers generated 56 polymorphic bands with 33 unique bands and 23 non-unique bands (table 4) and no monomorphic bands appeared. Thirty three unique bands and 23 non-unique bands were identify and distributed between the two species was illustrated in (table 4) and (figs. 1 & 2, the polymorphism percentage were 100% for each primers for both varieties. Identical size bands observed indicate genetic similarity (Barakat and Elham, 2004), high levels of polymorphism were observed within the two species.

Table (4): Number of amplification, polymorphic, monomorphic products and percentage of polymorphism generated by ten DNA primers used for identifying the two species of narcissus.

Primer No.	Total No. of bands	No. of monomorphic bands	No. of polymorphic bands		Polymorphic bands size with bp		% polymorphism
			Unique bands	Non-Unique bands	Unique bands	Non-Unique bands	
1	6	--	6	--	2464 - 583	--	100
2	5	--	5	--	2344 - 641	--	100
3	5	--	5	--	2274 - 574	--	100
4	4	--	2	2	2711 - 820	1131	100
5	2	--	--	2	--	1751	100
6	5	--	2	3	569 - 542	990 814	100
7	7	--	4	3	2413 - 415	990 758 610	100
8	11	--	6	5	1284 - 243	1139 879 758 722 610	100
9	4	--	1	3	458	990 722 395	100
10	7	--	2	5	623 - 467	1139 879 814 722 395	100
Total	56	--	33	23			100

Three primers 1, 2 and 3 produce unique bands only while, the primers number 5 produces only two non-unique bands, the other six bands (4, 6, 7, 8, 9, and 10) produce unique and non-unique bands. All primers produce 56 bands all were polymorphic, ranged from 2 bands (primer 5) up to 11 bands (primer 7) (table 3). Unique bands as produced by primers number 1, 2 and 3 could clearly discriminate between the two species for example, fragments with approximately molecular size of 2764 bp, 2344 bp, 1114 bp, 978 bp and 792 bp were detected in *Narcissus tazetta* profile while, fragments with approximately molecular size of 1787 bp, 1996 bp, 1592 bp and 583 bp were detected in commercial narcissus profile.

The present results are in consistent with those obtained by large number of investigations who reported that RAPD analysis is an effective tool in identifying the different species and varieties (Barakat and Elham, 2004). Terzi, (1997) used random decamers primers to produce stable RAPD markers in samples of 13 barley, 10 oat, and 12 triticale commercial varieties. He reported that RAPD markers provide a valuable addition to classical morphological markers for varieties identification and for the estimation of genetic similarities among genotypes.

Rossetto *et al.,* (1997) used random primers to identify species-specific markers for some species of *Eucalyptus*. Liu *et al*., (2000) used four random primers to compare genetic characters of trispecific cotton cultivars. Cheng *et al*., (2002) reported that RAPD analysis is an effective tool in identifying of *Hibiscus cannabinus* L. varieties and determining their genetic relationship. Barakat and Elham, (2004) successfully used four random primers and RAPD similarity analysis to differentiate between two species of *Gossypium*, *Gossypium hirsutum* and *Gossypium barbedense*.

El-Didamony *et al*., (2004) explore the use of the RAPD-PCR technique to fingerprint a virulent and an avirulent isolate of *Ralstonia solanacearum* and three phages to determine if it is possible to distinguish them using this technique. Abdelmigid, (2006) used two techniques of RAPD analysis with three tissues obtained from alfalfa *(Medicago sativa*) to evaluate the effective protocol for genetic fingerprinting analysis and the difference between each procedure.

Zouhar *et al*., (2007) used the production of the appropriate collection of species-specific RAPD markers, their conversion to specific PCR-based assay for the rapid and sensitive identification of *Ditylenchus dipsaci* normal type of biological races in plant organs and tissues of majority plant hosts in Central Europe. Recently, Colling *et al.,* (2010) used random amplified polymorphic markers (RAPD) to analyze the genetic structure of *Narcissus pseudonarcissus*.

Fritsh *et al.,* (1993) and Williams *et al.,* (1990) demonstrated the importance of GC content of primers on the PCR yield of detectable amplified products. It is of interest, in the present study, to mention that no correlation between primer Guanine –Cytosine (GC) content and the clarity of banding pattern was noted (see table 3). In accordance with the present results, Marillia and Scoles, (1996) and Barakat and Elham, (2004) demonstrated the importance of GC content of primers on the PCR yield of detectable amplified products, they noticed that no correlation between primer GC content and the clarity of banding pattern.

Table (5): Molecular size in bp of the amplified DNA products generated by ten random primers used to for identifying the two species of narcissus.

Mol. Size of bands bp	Primer 1		Primer 2		Primer 3		Primer 4		Primer 5		Prime 6		Primer 7		Primer 8		Primer 9		Primer 10	
	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
2711							+													
2464	+																			
2413														+						
2344			+																	
2274					+															
1996						+														
1898					+															
1787		+																		
1751									+	+										
1592				+																
1387														+						
1284																+				
1231															+					
1172	+																			
1139																+				+
1131							+	+												
1116														+						
1114					+															
990												+	+				+			
978	+																			
916				+																
879															+					+
820								+												
814											+	+							+	
804				+																
792	+																			
758													+		+					
722																+		+		+
673															+					
641				+																
623																				+
610													+			+				
583		+																		
574					+															
569												+								
542											+									
467																			+	
458																	+			
436															+					
415														+						
395																		+		+
318																+				
243															+					

T = *Narcissus tazetta*, C = commercial narcissus species

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دراسات كيموحيوية علي النرجس فى مصر

محمد نجيب عبد الغنى [1]، فوزيه اسماعيل محمد [2]، زينب محمد العشرى [2] و حمدى محمود عبد الرحمن[2]

1-قسم النبات - كليه العلوم - جامعه المنصوره

2-قسم الخليه والوراثه - المركز القومى للبحوث - القاهره

تم هذا لبحث فى معامل المركز القومى للبحوث بالجيزه بالتعاون مع قسم النبات-كليه العلوم-جامعه المنصوره

اجرى هذا البحث

(اولا) بهدف جمع ابصال نبات النرجس صنف(*Narcissus tazetta*) المصرى والذى تواجد بها الاف السنين وبخاصه فى السواحل الشماليه حيث تم العثور على بقاياه فى مقبره رمسيس الثانى وسجله العديد من العلماء لكن فى السنوات الاخيره وتحت ضغط الزحف السكانى و الانشاءات السياحيه والرى الدائم طوال السنه بديلا عن الرى الشتوى الموسمى كاد يؤدى الى انقراض هذا النبات الجميل المنظر والمفيد طبيا.

ولقد تعذر ايجاد ايه ابصال خلال فتره البحث وتم استخدام صنفين اخرين من النرجس هما صنف تجارى غير معرف تصنيفيا و اخر استقدم من الخارج (هولندا) وتمت زراعته فى البيئه المصريه لعده سنوات (*Narcissus tazetta*).

(ثانيا) عمل تحليل كيمياتى لمكونات الصنف (*Narcissus tazetta*) ولقد وجد به العديد من المكونات المستخدمه طبيا او التى يمكن استخدامها حيث وجد ان اكبر المكونات الموجوده هى القلويدات والفلافونيدات وكذللك وجدت التربينات الثلاثيه والتانينات لكن بكميه اقل مما يؤكد تجارب اخرى عديده عن وجود هذه المكونات التى لها استخدامات طبيه عديده مقاومه للخلايا السرطانيه و الاورام وكذلك مرض النسيان او الزهايمر.

(ثالثا) اجريت مقارنه بالبيولوجيا الجزيئيه بين الصنفين باستخدام تقنيه RAPD-PCR باستخدام عشره بادئات محدوده النيوكليوتيدات للتمييز بينهما جينيا.

قام بتحكيم البحث

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