Phytochemical screening and antibacterial activity of *Cyclamen persicum* Mill tuber extracts

Raed Alkowni[1]*, Shehdeh Jodeh[2], Fatima Hussein[3] and Nidal Jaradat[3]
[1]Department of Biology and Biotechnology, An-Najah National University, Nablus, Palestine
[2]Department of Chemistry, An-Najah National University, Nablus, Palestine
[3]Department of Pharmacy, An-Najah National University, Nablus, Palestine

Abstract: The emerging drug resistance bacteria increased the demand on the discovery of antibiotics from natural sources. This research was aimed to study the antibacterial reactivity; as well as the phytochemicals, of the wild type of *Cyclamen persicum*, using nine different extraction methods where four solvents (Methanol, Ethanol, Hexane; and Water) were involved with varied extraction periods ranged from 2 up to 10 hours. The antibacterial activity of crude methanol extract (CME) was found as the best method of extraction, with particular emphasis on the method with prolonged extraction time of (10 hrs). The antibacterial activities of produced CME were determined by using agar diffusion method against two of gram-positive bacteria and two gram-negative ones. The CME treated Mueller-Hinton-Agar plates, were exhibited antibacterial effects against the gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) by showing of inhibition zone after overnight incubation, while nothing was noticed on those of gram negative ones (*Pseudomonas aeruginosa* and *Escherichia coli*). These results that proved the antibacterial activity of the *Cyclamen persicum* tubers were positively tested the Saponin glycosides from plant. In addition to that, methanol solvent could be the useful method for extractions of Cyclamen and can be used in any developing drugs against pathogenic gram positive bacteria.

Keywords: *Cyclamen persicum* tuber crude extracts, antibacterial activity, methanolic extracts, Pharmacognosy.

INTRODUCTION

Cyclamen (*Cyclamen persicum* Mill.) belongs to the family *Primulaceae*, comprises 22 species (Grey-Wilson, 2003). It is grown widely in Mediterranean countries (Srouji 2003; Plant Resources of the USSR, 1986) and had several medicinal, nutritional and ornamental benefits (Primorac *et al.*, 1985). The storage organ of the cyclamen has no papery covering and roots may develop from the top, sides or bottom depending on the species. For example *Cyclamen hederifolium* roots were developed from top or sides of the tubers, while *Cyclamen persicum* and *Cyclamen coum* from bottom (Phillips and Rix, 1989). The sizes of the tubers were varied from 2 cm (i.e. *Cyclamen parviflorum*) up to 24 cm as in *Cyclamen hederifolium* (Kupchan *et al.*, 1967).

In vitro, extracts from cyclamen tubers were reported to exhibit cytotoxical (Leporatti, and Ivancheva, 2003), spermicidal (Foubert *et al.*, 2008), and antimicrobial activities (Fennell *et al.*, 2004). Early investigation on different *Cyclamen* species resulted in isolation of saponin glycosides (Mahasneh and El-Oqlah, 1999).

In addition to Saponins, Piperidine alkaloid and sterols were isolated from *cyclamen coum* tubers (Mahasneh, and El-Oqlah, 1999). The anthocyanin and flavonoids pigments of many Cyclamen cultivars had been reported for their effects in pigmentation (Çalıs *et al.*, 1997). Besides, Triterpene glycoside and Repandoside were isolated from *Cyclamen repandum* tubers to give a significant reduction of the cancer cell lines number including *HeLa* (human cervical cancer cells), H-446 (human lung cancer cells), HT-29 (human colon carcinoma cells), and U937 (human leukemia cells), at concentrations between 1 and 50M as compared to control (Yayli N, Baltaci 1997). In this tale, medical researches indicated the putative analgesic; anti-inflammatory and antimicrobial activities for cyclamen tubers extracts (Guarrera, 2005; Loi, *et al.*, 2004). Other researches had been carried recently for the use of their tubers as source of activated carbon for pharmaceutical adsorption (Jodeh *et al.*, 2015).

In this research study, nine deferent extraction methods were screened for detection of six phytochemicals from *Cyclamen persicum* tubers and the crude extracts were tested for their anti bacterial activity on medical bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*) which are belonging to Gram positive and Gram negative groups. The obtained results could be used for medical purposes as a new product added to pharmacognosy.

MATERIALS AND METHODS

Plant material collections

The wild type of *Cyclamen persicum* was collected from different areas in northern part of the West Bank/Palestine

**Corresponding author:* e-mail: ralkowni@najah.edu

during the summer period (July and September) of 2012. The collected plants were botanically identified by the pharmacognosist Dr. Nidal Jaradat at the Pharmacognosy laboratory, Department of Pharmacy, An-Najah National University and furthermore by Herbarium of the Pharmaceutical Chemistry and Technology Division (Laboratory of Pharmacognosy) where the voucher specimen number for *Cyclamen persicum* is (Pharm-PCT-777). The collected tubers were sun dried, cut into small pieces and ground to powder.

Chemicals extraction of cyclamen tubers powder

Nine different extraction methods were trialed on Cyclamen persicum tubers powders, in order to test six of phytochemicals (Alkaloid; Phenolic Compounds; Saponin Glycosides; Tannins; Starch; and Flavonoids) and measure their efficacy of antimicrobial. Briefly the aqueous extraction method (W); about 150g of Cyclamen tubers powder was soaked in 1L of hot distilled water as solvent and refluxed with continuous magnetic stirring for about 4 hours at 40ºC by using heater and stirrer [Heidolph OB2000] equipment. Then the resulting extract was concentrated using a rotary evaporator (Heidolph VV2000),with the water bath set at 40°C, till a gummy yellow crude extract was obtained and then stored at 4ºC temperature in air tight container.

Beside aqueous extraction method, other three organic solvents (Methanol (M), Ethanol (E), and Hexane (H)) were achieved in separate or in combinations extractions. The first extractions were made with the solvent Methanol using the simple refluxing method for obtaining crude methanol extract (CME). Briefly, (150g) of Cyclamen tubers powder were suspended in 1L of Methanol (99%) as solvent and refluxed with continuous magnetic stirring for about 4 hours at 40ºC. The resulting extract was concentrated using a rotary evaporator with the water bath set at 40°C, till a gummy yellow crude extract was obtained and then stored at 4ºC temperature in air tight container. Other two extractions were done but one with of Ethanol (99%) for crude ethanol extraction method; and the other one of Hexane for crude hexane extraction method.

Furthermore, another two extracts were made by using first either Methanol or Hexane for 4 hours changed then with either Hexane (MH) or Methanol (HM) consecutively for another 2 more hours. Another two more extracts were produced using either Methanol (MLL) or Hexane (HLL) for 4 hours in the Soxhlet extractor followed by a liquid-liquid-extraction with the other solvent for another 2 more hours.

The last extraction method (MLt) which was the novelty of this research, by prolonging the methanol extraction for 10 hours in order to study the effect of the extraction period on the phytochemical analysis and antibacterial reactivity.

Chemical testing of extracts from cyclamen tubers powder

The extracted cyclamen tubers powder was tested for the presence of six phytochemicals as listed in the table 1.

Microbial selection and antibacterial activity tests

Four types of bacteria; *Staphylococcus aureus* and *Bacillus subtilis* as gram positive bacteria and *Pseudomonas aeruginosa* and *Escherichia coli* for gram negative; which have medical pathogenesis were subjected to antibacterial tests by *C. persicum* extracts. All of these bacteria were obtained from previously collected and identified sources in the Labs of Faculty of Science; An-Najah University. The agar well diffusion method (Magaldi *et al*., 2004) was used for anti-bacterial assays, where the inhibition zone was measured as an indicator of the extract anti-bacterial activity. Mueller-Hinton-Agar (MHA) was used for gram positive and MacConkey-Agar (MCA) for gram negative ones. The agar media was previously prepared and sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes; before media were poured into sterile Petri dishes plates (9cm), and left to dry before they were immediately used or stored at 4°C.

The bacterial inoculates were prepared by transfer morphologically similar colonies with a sterile loop to growth liquid media (MHA or LB) and incubated at 35°C with shaking until the visible turbidity was equal to or greater than the 0.5 McFarland standard. After adjustment of the suspension density to be equal of 0.5 McFarland standards (10^7-10^8 CFU/ml), the surface of agar media plate was inoculated by streaking with cotton-tipped swab after it was dipped into the inoculum suspension. The streaking was repeated two times, to ensure an even distribution of inoculums. Then, by using the micropipette; 50µl of the extracts (dissolved in 10% DMSO) were added to their corresponding wells. The inhibition zone diameter was measured after 24h of an incubation period at 37°C.

Several aliquots of the extracts were applied as (100% (pure); 50%; 25%; 10%; 5%) each by adding 10% of DMSO. The 50µl of each extract aliquots were tested to determine the bactericidal concentration sensitivity for each extraction method.

RESULTS

Initially *Cyclamen persicum* tubers had been pulverized and tested for their phytochemical contents following the corresponding procedure for each (table1). Primarily investigation of different *Cyclamen* species resulted in isolation of Saponin Glycosides (Altunkeyik *et al*., 2012; El Hosry *et al*., 2014).

Table 1: List of Phytochemical tests of *C. persicum* crude powder extraction

Test Name	Method	Phytochemical
Wagner's	Extract was treated with Wagner's reagent (Iodine in Potassium Iodide)	Alkaloid
Ferric Chloride	Extract was treated with 3-4 drops of ferric chloride solution	Phenolic Compounds
Frothing	The extract was shaken with 5 ml of distilled water in a test tube	Saponin Glycosides
Gelatin	1% gelatin solution containing sodium chloride was added to the extract	Tannins
Iodine	One gram of extract was treated with the reagent of the starch (iodine)	Starch
Flavonoids	2 g of methanolic extract was added to 1 ml concentrated sulfuric acid and 0.5g of Magnesium, for 3 min t	Flavonoids

The results of cyclamen phytochemical screening tests revealed the presence of Saponin Glycosides, Starch and Phenolic, but none of Alkaloid; Tannins; or Flavonoids were detected (table 2).

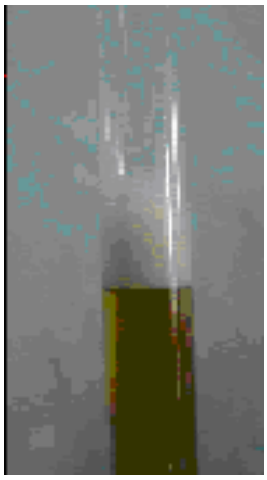

Fig. 1: Frothing test for Saponin glycoside detection

Regarding the experiment design, nine different extracts were obtained (M, E, W, H, MH, HM, MLL, HLL, MLt). After that, the solvents from all the extractions were removed with vacuum rotary evaporator and the remaining extracts were collected. The yields of extracts from *Cyclamen persicum* tubers were found to be up to 9% (w/w). This was calculated according to the following equation:

{Yield of the crude extract (wt%)= (weight of extract/ weight of CT powder) × 100%}

The Frothing test showed positive results only with M (only Methanol), HM (first Hexane, then Methanol), E (only Ethanol), W (only Distilled Water) and MLt (10h long extraction Methanol).

The liquid-liquid-extraction followed the Soxhlet extraction showed strange behavior of flocculation (MLL and HLL), whereas the extracts with Hexane, either pure Hexane extraction, or followed with Methanol extraction, or the fluid-fluid extracts (H, HM, MLL and HLL) showed lipidic characteristics and yellowish color. The extracts made with Methanol, Ethanol and Water showed a high density, brownish color and caramelized-like sugar. The antibacterial activities were tested using the well diffusion method on MHC plates previously swabbed with either one of *S. aureus* or *B. subtilis*, and on MCH swabbed with either one of *E. coli* or *P. aeruginosa*. After 24h incubation at 37ºC, the *in vitro* inhibition zone as a result of anti-bacterial activity against the bacteria was measured in millimeter using transparent ruler. The Hexane-based extracts didn't affect the bacteria (H and HM). The same results obtained from liquid-liquid extracts the bacteria (MLL and HLL). The water-based extract (W) had no effect and thus it was used as the control for evaluation of the best extraction method.

Table 2: Active ingredients according to phytochemical screening tests

Phytochemical test	Result
Alkaloid	–
Starch	+
Saponin glycoside	+
Flavonoid	–
Tannin	–
Phenolic compounds	+

The extracts (M, MH, E and MLt), showed significant inhibitions, were then subjected to further dilutions tests to measure the minimum inhibitory and bactericidal concentrations sensitivity.

The methanol extract that followed by Hexane (MH) did not show higher antibacterial activity than the normal methanol one (M), suggesting that there was an advantage in removing fats with Hexane before Methanol extraction. It is also true for avoiding methanol shorter extraction period (2 hrs instead of 4 hrs) to have better anti-bacterial effect as in (HM) extraction method.

Although ethanol-based extracts (E) showed just a slight activity, the best results were obtained for both methanol extracts (M and MLt) showed an inhibition zone of (11; 13 mm in diameter) on media inoculated with *S. aureus* respectively, and (10; 13 mm in diameter) on *B. subtilis.* Surprisingly none of the extractions but not of gram negative ones. In fact, the gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) did not exhibit any affected from any of the extracts. Thus, the methanol crude extracts remain the best extraction method of choice for antibacterial activity against gram positives.

Table 3: Anti-bacterial assays on gram-positive bacteria with M and MLt extracts at different dilutions. All dilutions had been made with distilled water and only 50µl volume of the extracts was applied.

Extract Bacteria	M (Methanol extraction /4 hrs)		MLt (Methanol extraction /10 hrs)	
	dilution factor	Inhibition diameter [mm]	dilution factor	Inhibition diameter [mm]
S. aureus	Pure	11	Pure	13
	1:1	9	1:1	11
B. subtilis	Pure	10	Pure	13
	1:1	-	1:1	11
			1:4	10

For those with significant results, different dilution series were applied; several dilutions of the methanolic crude extracts were tested for their sensitivity to Gram positive bacteria. The results indicated that pure methanol extracts from cyclamen tubers were active against gram positive *Staphylococcus aureus* bacteria as well as *Bacillus subtilis* bacteria on (MHA) agar plate. High sensitivity was given to the MLt extracts (methanol extracts with period longer than 4h). *Bacillus subtilis* was exhibited significant inhibitory activities in dilutions of 25% rather than *S. aureus* (table 3).

DISCUSSION

Cyclamen persicum is one of the most widespread herbal plants worldwide including Palestinian wilds. It was described as one of the most important Palestinian traditional herbal medicine beside its uses for food (Saad and Said, 2011). Their importance encouraged researchers to propagate them *in vitro* (Stefan, 2005; Qubaj, 1999; Abu-Qaoud, 2004; Al-Majthoub, 1999).

Although Cyclamen *spp*. had been reported to exhibit antifungal activity (Someya *et al.*, 2000); this study aimed to assess the antibacterial activity of the *Cyclamen persicum* against several medical pathogenic bacteria to be another source used for pharmaceutical treatments and pharmacognosy. Two groups (Gram negative and Gram positive) bacteria were subjected to this study in order to evaluate their responses to cyclamen crude extracts.

The Frothing test showed positive results with Methanol extraction methods of (CME), Hexane, followed by Methanol extraction method (HM), Ethanol extraction (E), aqueous extraction method (W) and with 10 hrs prolonged methanol extraction method. Frothing test, positively detected of Saponin Glycosides by the presence of 1/2 cm foam which lasted in the test tube for 10 min (Fig.1), confirming the results mentioned before the isolation of Saponin from cyclamen plants (Altunkeyik *et al*., 2012; El Hosry *et al*., 2014)

These detected phytochemicals from all Cyclamen tubers extracts were in agreement with previous reports which indicated that Cyclamen species contain Saponin which also could have anti-inflammatory and antibacterial activities (Mahasneh and El-Oqlah, 1999, Speroni *et al.*, 2007). In fact, Saponin anti-inflammatory effects were investigated *in vitro* (Speroni *et al.*, 2007). Not all Saponin's have antibacterial activity, even though, many of them were considered as part of plants' defensive systems (Kluytmans *et al.*, 1997); for example, medicagenic and zanhic acid saponins isolated from alfalfa plants did not show any activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtitles*, *Pseudomonas aeruginosa* and *Mycobacterium intracellular* (Aarestrup *et al.*, 2000). However, some saponins such as Ivy saponin, Spirostanol saponin, Asterosaponin from starfish, and Yucca saponin showed more antimicrobial activities against gram positive bacteria (*Staphylococcus aureus*) but not on gram-negative bacteria (*Escherichia coli*) at the same concentration (Mahato *et al.*, 1988).

The cyclamen tuber extracts were exhibited an inhibitory activity to the growth of the Gram-positive bacteria but surprisingly not to the growth of the Gram-negative bacteria. Although some researchers reported that Cyclamen tubers extracts had antimicrobial activities against *Pseudomonas aeruginosa*, *Salmonella choleraesuis* bacteria (Loi, *et al.*, 2004), the obtained results of this research were in agreement with many other researchers whom explaining that Gram negative bacteria had shown more resistant to many plant extracts previously studied due to their cell membrane structure that forms protective coat for their microbes (Vlietinck *et al.*, 1995; Rabe and Van Staden, 1997; Nostro *et al.*, 2000). As Saponin was reported as the main phytochemical component of the *C. persicum* tuber extracts, this research would recommend the importance of *Cyclamen persicum* (wild type) for pharmacological use and research to purify the active ingredient and to be subjected to furthermore standardized drug assays.

CONCLUSION

Cyclamen persicum tubers powders were found to contain both Saponin Glycosides and phenolic compounds as active ingredients. The methanol extracts were found to be the best extraction method compared with others extraction methods; with an advantage to the prolonged period of extraction (10h prolonged Methanol extraction).

The antibacterial activities of this extract were exhibited antibacterial activity against gram positive bacteria but not on gram negative ones. Further investigation could be carried on the Phenolic compounds for their antioxidant activity. The cyclamen plant could be recommended as one of the medicinal plants for pharmaceutical uses.

REFERENCES

Aarestrup FM, Agerso P, Ahrens JC, Jorgensen M Madsen and Jensen LB (2000). Antimicrobial susceptibility and presence of resistance genes in Staphylococci from poultry. *Vet. Microbial.*, pp.353-364.

Altunkeyik H, Gülcemal D, Masullo M, Alankus-Caliskan O, Piacente S and Karayildirim T (2012). Triterpene saponins from *Cyclamen hederifolium*. *Phytochem*, **73**: 127-133.

Abu-Qaoud H (2004). Direct Regeneration in Cyclamen Persicum Mill. Using Seedling Tissues. *An-Najah Univ. J. Res*. (N. Sc.), Vol. 18(2),

Al-Majthoub M (1999). M.Sc. *In vitro* propagation of wild cyclamen (*Cyclamen persicum* Mill). College of Agriculture, Jordan University of Science and Technology, Irbid, Jordan.

Çalıs I, Yürüker A, Tanker N, Wright AD and Sticher O (1997). Triterpene saponins from *Cyclamen coumvar. coum*. *Planta. Med.*, pp.166-170.

El Hosry L, Di Giorgio C, Birer C, Habib J, Tueni M, Bun SS, Herbette G, De Meo M, Ollivier E and Elias R (2014). In vitro cytotoxic and anticlastogenic activities of saxifragifolin B and cyclamin isolated from *Cyclamen persicum* and *Cyclamen libanoticum*. *Pharm. Biol.*, **52**(9): 1134-1140.

Fennell CW, Lindsey KL, Gaw LJ, Sparg SG, Stafford, GI, Elgorashi EE, Grace OM and Van J (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *J. Ethnopharmacol.*, **94**: 205-217.

Foubert K, Theunis M, Apers S, Vlietinck AJ and Pieters L (2008). Chemistry distribution and biological activities of 13, 28-epoxy-oleanane saponins fromthe plant families Myrsinaceae and Primulaceae. *Curr. Org. Chem.*, pp. 629-642.

Grey-Wilson Ch (2003). Cyclamen: A Guide for Gardeners, Horticulturists and Botanists Hardcover. Timber Press, Incorporated

Guarrera PM (2005). Traditional phytotherapy in Central Italy (Marche, Abruzzo, and Latium). *Fitoterapia*, pp.1-25.

Jodeh Sh., Fatima Abdelwahab a, Nidal Jaradat b, Ismail Warad Wade Jodeh (2015). Adsorption of diclofenac from aqueous solution using Cyclamen persicum tubers based activated carbon (CTAC). *JAUBAS*, Doi:10.1016/j.jaubas.2014.11.002

Kluytmans J, van Belkum A and Verbrugh H (1997). Nasal carriage of *Staphylococcus aureus*: Epidemiology, underlying mechanis ms and associated risks. *Clin. Microbiol. Rev*., **10**(3): 505-520.

Kupchan SM, Hemingway RJ, Knox JR, Barboutis SJ, Werner D and Barboutis MAJ (1967). Tumor inhibitors. XXI. Active principles of Acer negundo and *Cyclamen persicum*. *Pharm. Sci*., **56**(5): 603-608.

Leporatti ML and Ivancheva S (2003). Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. *J. Ethnopharmacol.*, **87**: 123-124.

Loi MC, Poli F, Sacchetti G, Selenu MB and Ballero M (2004). *In vitro* modulation of tracheal smooth muscle reactivity by extracts of some Senegalese medicinal plants. *Fitoterapia*, pp.277-295.

Mahasneh AM and El-Oqlah AA (1999) Antimicrobial activity of extracts of herbal plants used in the traditional medicine of Jordan. *J. Ethnopharmacol*.. **64**(3): 271-276.

Mahato SB, Sudip SK and Poddar G (1988). Triterpenoid saponins. *Phytochem*, **27**(10): 3037-3067.

Magaldi S, Mata-Essayag S, Hartung de Capriles C, Perez C, Colellaa MT, Olaizola C and Ontiveros Y (2004) Screening methods to determine antibacterial activity of natural products. *Braz. J. Microbiol.*, **38**: 369-380.

Nostro A, Germano MP, D'Angelo V, Marino A and Cannatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Lett. Appl. Microbiol*. **30**: 379-384.

Phillips R and Rix M (1989). The Random House Book of Bulbs, New York, pp.255-257.

Plant Resources of the USSR (1986). Families *Peoniaceae Thymelaceae* [in Russian], *Nauka, Leningrad*, pp.169-170.

Primorac M, Sekulovic D, Antonic S Pharmazie (1985). *In vitro* determination of the spermicidal activity of plant saponins. *Pharmazie*, **40**: 585-590.

Qubaj T (1999). Effect of acid or base treatments on rooting of semi-hard wood cut tings of 'Nabali" olive cuttings. M.Sc. Thesis, University of Jordan, Amman, Jordan.

Rabe T and Van Staden J (1997). Antibacterial activity of South African plants used for medicinal purposes. *J Ethnopharmacol.*, **56**: 81-87.

Saad B and Omar Said (2011). Greco-Arab and islamic herbal medicine traditional system, ethics, safety, efficacy, and regulatory issues. John Wiley & Sons, Inc., p.564.

Someya N, Kataoka N, Komagata T, Hirayae K, Hibi T and Akutsu K (2000). Biological control of cyclamen soilborne diseases by *Serratia marcescens* strain B2. *Plant Dis*., **84**: 334-340.

Speroni E, Cervellati R, Costa S, Dall'Acqua S, Guerra MC, Panizzolo C, Utan A and Innocenti G (2007). Analgesic and antiinflammatory activity of *Cyclamen repandum* S. et S. *Phytother. Res.*, **21**(7): 684-689.

Srouji ES (2003). Cyclamens From Galilee: Memoirs of a Physician From Nazareth. iUniverse.

Stefan A, Rensing, Daniel Lang, Eik Schumann, Ralf Reski and Annette Hohe EST (2005). Sequencing from *Embryogenic Cyclamen persicum* cell cultures identifies a high proportion of transcripts homologous to plant genes involved in somatic Embryogenesis. *J. Plant Growth Regul.*, **24**(2): 102-115.

Vlietinck AJ, van Hoof L and Totte J *et al.* (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J. Etyhnopharmacol.*, **46**: 31-47

Yayli N and Baltaci C (1997). A novel glycoside linked piperidine alkaloid from *Cyclamen coum*. *Turk. J. Chem*, **21**: 139-143.