Phytochemical studies and cytotoxicity evaluations of *Colchicum tunicatum* Feinbr and *Colchicum hierosolymitanum* Feinbr (Colchicaceae): two native Jordanian meadow saffrons

By: Feras Q. Alali; Khaled Tawaha; Tamam El-Elimat; Rana Qasaymeh; Chen Li; Jason Burgess; Yuka Nakanishi; David J. Kroll; Mansukh C. Wani; Nicholas H. Oberlies

Alali, F.; Tawaha, K.; El-Elimat, T.; Qassaymeh, R.; Li, C.; Burgess, J. P.; Nakanishi, Y.; Kroll, D. J.; Wani, M. C. and Oberlies, N. H. (2006) Phytochemical studies and cytotoxicity evaluations of *Colchicum tunicatum* Feinbr and *Colchicum hierosolymitanum* Feinbr (Colchicaceae): Two native Jordanian meadow saffrons. *Natural Product Research.* 20, 558-566.

Abstract:

As a part of our continuing investigation of Jordanian *Colchicum* species, the biologically active components of *Colchicum hierosolymitanum* Feinbr and *Colchicum tunicatum* Feinbr (Colchicaceae) were pursued. The brine shrimp lethality test (BSLT) was used to direct the fractionation and isolation of active components. Five and four known colchicinoids were isolated and characterized from *C. tunicatum* and *C. hierosolymitanum*, respectively. The known colchicinoids, reported for the first time from these two species are: (-)-colchicine (I), 3-demethyl-(-)-colchicine (II), (-)-cornigerine (III), β-lumicolchicine (IV), and (-)-androbiphenyline (V) from *C. tunicatum*, and (-)-colchicine (I), 2-demethyl-(-)-colchicine (VI), (-)-cornigerine (III), and *β*-lumicolchicine (IV) from *C. hierosolymitanum*. The chemical structures of the isolated compounds have been elucidated using a series of spectroscopic and spectrometric techniques principally; 1D-NMR (^{1}H and ^{13}C) and low resolution EI-MS and APCIMS. All pure compounds were evaluated for cytotoxicity against three human cancer cell lines; MCF-7 human breast carcinoma, NCI-H460 human large cell lung carcinoma, and SF-268 human astrocytoma. (-)-Colchicine (I) and (-)-cornigerine (III) were found to be the most bioactive of the identified compounds with EC_{50} values in the range of 0.016-0.097 μM.

Article:

1. Introduction

Jordan acts as a flora bridge between the continents of Asia, Africa, and Europe, and it is situated at the junction of four phyto-geographical areas, the Mediterranean, the Irano-Turanean, the Saharo-Arabian, and the Tropical or Sudanian 1,2. Due to its position at these crossroads of climatic and botanic regions, Jordan has a rich and somewhat unique biodiversity of wild plants 3,4.

The genus *Colchicum* belongs to the family Colchicaceae, which comprises 19 genera, and 225 species 5. In Jordan, at least nine species of *Colchicum* can be found in the wild, namely: *Colchicum brachyphyllum* Boiss. & Haussk. ex Boiss., *C. crocifolium* Boiss., *C. hierosolymitanum* Feinbr, *C. ritchii*. R. Br., *C. schimperi* Janka, *C. stevenii* Kunth, *C. tauri* Siehe ex Stef., *C. triphyllum* Kunze, and *C. tunicatum* Feinbr 1,6,7.

The marked medicinal and/or toxic properties of some species of the genus *Colchicum* have been known for more than 2000 years. Dioscorides was aware of their toxic properties; writings of Arab investigators in the tenth century recommended their use in gout 8,9.

Colchicine (**I**), the major alkaloid of *C. autumnale*, which was first isolated in 1820 by Pelletier and Caventou 10, is still used today for the treatment of gout 11, and in a number of proinflammatory disorders, such as familial Mediterranean fever 12, and Behcet's disease 13. Colchicine (**I**) was found to posses potent antitumor

activity in clinical investigations. However, its use as an antineoplastic agent is limited, due to lack of tumor selectivity and high toxicity 14,15. Among colchicine analogs, demecolcine, is used for treatment of myelocytic leukemia and malignant lymphoma 16. Colchicine is also used in biological and breeding studies to produce polyploidy, or multiplication of the chromosomes in the cell nucleus 9.

In our continuing studies on Jordanian *Colchicum* species 17-19, the colchicinoids of *C. hierosolymitanum* and *C. tunicatum* were pursued, as, to the best of our knowledge, these two species have not been investigated previously for bioactive constituents.

Colchicum tunicatum is one of the first native geophytes to flower (September-October). It is characterized as a perennial herb, with underground corms, covered by thick, dark brown-black scales, growing at marginal lands and clay-like soil of desert 1, 7, 20.

Colchicum hierosolymitanum is characterized as a perennial herb, which flowers in autumn (October--November). It is distinguished by large, underground corms, which have a conical, almost snail-like appearance, and are covered by onion-like scales. It is found growing in mountains in heavy soil 1,7.

2. Results and discussion

Guided by the brine shrimp lethality test (BSLT), five pure known compounds were isolated from roots, corms, stems, and leaves of *C. tunicatum* for the first time. These compounds can be categorized into three classes: colchicine-type: (-)-colchicine (**I**) (2.320 g, 0.1% w/w), 3-demethyl-(-)-colchicine (**II**) (109.18 mg, 0.009% w/w), and (-)-cornigerine (**III**) (37.78 mg, 0.003% w/w); β-lumiderivatives: β-lumicolchicine (**IV**) (16.72 mg, 0.002% w/w); and allocolchicine-type: (-)-androbiphenyline (**V**) (7.61 mg, 0.0005% w/w). The corms of *C. hierosolymitanum* yielded four known compounds, which were new to the species, and that can be categorized into two classes: colchicine-type: (-)-colchicine (**I**) (325 mg, 0.05% w/w), 2-demethyl-(-)-colchicine (**VI**) (20 mg, 0.003% w/w), and (-)-cornigerine (**III**) (5 mg, 0.001% w/w); and β-lumiderivatives: β-lumicolchicine (**IV**) (35 mg, 0.005% w/w). The structures of the isolated compounds were elucidated using a series of spectroscopic and spectrometric techniques principally; 1D-NMR (^{1}H and ^{13}C) and low resolution EI-MS and APCIMS, and by comparisons of these spectral data with those reported in the literature 19,21-24 (figure 1).

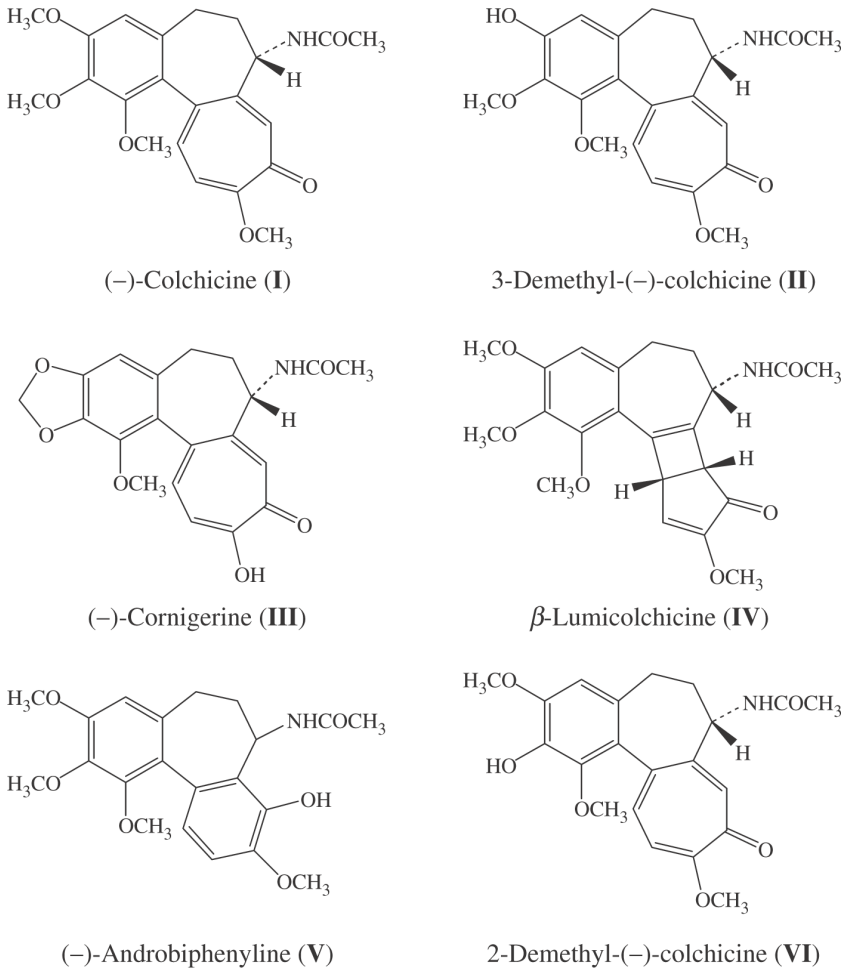

Figure 1. The structures of colchicinoids from *C. tunicatum* (**I**-**V**) and *C. hierosolymitanum* (**I**, **III**, **IV**, and **VI**).

Liquid--liquid fractions A-E, which were obtained according to the method of Šimánek and coworkers 25,26, from both species and for all plant parts were tested for toxicity against the BSLT (table 1). Fraction C, the most active fraction, was pursued for further purification. Compounds **I**--**VI** were tested for general toxicity against the BSLT, and for anticancer activity against the human cancer cell panel. Three cancer cell lines were selected: MCF-7 human breast carcinoma, NCI-H460 human large cell lung carcinoma, and SF-268 human astrocytoma. In the BSLT (table 2), compounds **I** and **III** were by far the most toxic, with LC_{50} values of 3.7 and 7.7 μg mL^{-1}, respectively, with all other colchicinoids being approximately an order of magnitude less toxic. Indeed, this structure-activity relationship held against the human cancer cell panel, with **I** (EC_{50} value: 0.016-0.025 μg mL^{-1}) and **III** (EC_{50} value: 0.061-0.097 μg mL^{-1}) exhibiting activity on the same order of magnitude as the positive control, camptothecin. This positive correlation between the two bioassays illustrates and adds to the power of the BSLT as a simple, rapid, bench-top, in-house screening bioassay capable of identifying cytotoxic compounds. However, the other compounds should not be discounted for their cytotoxic activity, as the BSLT is limited in its predictive capacity to distinguish between strong-to-moderate and weak potency cytotoxic compounds. The BSLT represents a quick initial screen for potent cytotoxins, but a finer level of discrimination for anticancer activity required the human cancer cell panel. Similar results with respect to the BSLT and the human cancer cell panel were noted in an earlier study of a related species of *Colchicum* 19.

Table 1. The BSLT results for *C. tunicatum* and *C. hierosolymitanum* liquid fractions according to the scheme of Šimánek and coworkers ,.

Plant part	Fraction name	LC_{50}	Confidence intervals 95%
C. tunicatum			
Leaves	A	696.8	1822.3-379.0

Table 1. The BSLT results for *C. tunicatum* and *C. hierosolymitanum* liquid fractions according to the scheme of Šimánek and coworkers ,.

Plant part	Fraction name	LC_{50}	Confidence intervals 95%
	B	433.4	898.4-246.3
	C	3.1	5.6-1.5
	D	74.6	133.7-41.9
	E	218.1	382.4-128.5
Corms	A	262.9	591.7-137.8
	B	22.9	35.6-14.6
	C	3.0	5.5-1.3
	D	20.0	36.3-10.9
	E	15.2	26.0-8.9
Stems	A	231.3	392.1-139.3
	B	47.0	74.3-30.0
	C	3.1	5.9-1.3
	D	7.3	14.5-3.2
	E	6.7	12.5-3.3
Roots	A	539.6	1454.9-278.5
	B	14.7	25.3-8.3
	C	2.8	5.8-1.0
	D	93.3	158.2-54.5
	E	15.7	28.9-8.3
C. hierosolymitanum			
Corms	A	117	56.2-251
	B	9	2.9-22
	C	0.33	0.066-0.97
	D	16.2	3.7-61.3
	E	0.46	0.07-1.6

Table 2. Human cancer cell panel and BSLT results for compounds I-VI.

	Human cancer cell panel[a]			
Compound	**MCF-7**	**H460**	**SF268**	**BSLT[b]**
Note: nt = not tested. [a]Cytotoxicity results are expressed as EC_{50} values (μM; concentration to inhibit growth by 50%) derived from single experiments using 11 concentration data points, each run in triplicate.				
[b]BSLT results are expressed as LC_{50} values (μg mL^{-1}; concentration to kill 50% of the brine shrimp) derived from single experiments using four data points, each run in triplicate.				
[c]Positive controls.				
(-)-Colchicine (**I**)	0.016	0.030	0.025	3.7
3-Demethyl(-)-colchicine (**II**)	0.18	0.51	0.28	74.6
(-)-Cornigerine (**III**)	0.061	0.059	0.097	7.7
β-Lumicolchicine (**IV**)	1.5	2.1	3.2	74.6
(-)-Androbiphenyline (**V**)	0.20	0.26	0.37	48.8
2-Demethyl(-)-colchicine (**VI**)	nt	nt	nt	26.2
Camptothecin[c]	0.072	0.0069	0.059	nt

Table 2. Human cancer cell panel and BSLT results for compounds I-VI.

	Human cancer cell panel[a]			
Compound	**MCF-7**	**H460**	**SF268**	**BSLT[b]**
(-)-Colchicine[c]	nt	nt	nt	2.8

3. Materials and methods

3.1. General

All NMR experiments were performed in $CDCl_3$ with TMS as an internal standard; a Bruker DPX-300 instrument was utilized for the ^{1}H-, ^{13}C-NMR. The APCIMS were determined on an Applied Biosystems/MDS Sciex API 150 EX single quadrupole LC/MS system (Applied Biosystems, Foster City, CA); EI-MS 70 eV on VG-7070 E LREI. The HPLC was performed on a Lachrom® MERCK-HITACHI (Tokyo, Japan), equipped with quaternary gradient L-7150 pump, L-7455 Diode-Array Detector, L-7200 auto-sampler, and D-7000 Interface. The preparative HPLC column was a Hibar® MERCK, pre-packed column RT 250-25, Lichrosorb® RP-18 (7 µm). Column chromatography was carried out using silica gel 60 (0.06-0.2 mm; 70-230 mesh) and TLC utilized silica gel 60 with gypsum and pigment addition for UV visualization (both from Scharlau Chemie S.A., Barcelona, Spain). The TLC spots were visualized by UV (VILBER LOURMAT, 4 W--254 nm Tube) or made visible by spraying the developed plates with 5% phosphomolybdic acid in EtOH.

3.2. Plant material

Corms, stems, leaves, and roots of *C. tunicatum* were collected during its vegetating stage from al-Mafraq in the Northern part of Jordan in January 2003. The collected materials were identified by Prof. Dawud Al-Eisawi, plant taxonomist, Faculty of Agriculture, Jordan University, Amman, Jordan. Flowers, stems, and corms of wild *C. hierosolymitanum* were collected from Erhaba, Irbid, in the Northern part of Jordan in October 2001. The collected materials were identified by Prof. Ahmad El-Oqlah, plant taxonomist, Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid, Jordan. Voucher specimens of *C. tunicatum* (PHC #101) and *C. hierosolymitanum* (PHC #102) were registered and deposited at the Herbarium Museum of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.

The plant raw material of *C. tunicatum* was divided into four parts: corms, stems, leaves, and roots; while for *C. hierosolymitanum* it was divided into three parts: flowers, stems, and corms. The clean-divided plants parts were air-dried at room temperature with the corms sliced into small pieces to speed up their dryness. After drying, their exact weights were recorded. The plant parts were grounded to powder using a laboratory mill. Powdered material was maintained at room temperature (22-23°C), protected from light until required for analysis.

3.3. Extraction and fractionation

The ground plant materials of *C. tunicatum* (633 g leaves, 171 g stems, 1433 g corms, and 83 g roots) and *C. hierosolymitanum* (650 g corms) were extracted exhaustively with MeOH and then filtered (MeOH fraction), and the filtrate was dried under reduced pressure via a rotary evaporator. Fractionation of this crude alkaloid extract was carried out based on the method of Šimánek and coworkers 19,25,26. Briefly, MeOH fractions, 153 g leaves, 100 g stems, 527 g corms, and 12 g roots of *C. tunicatum* and 80 g corms of *C. hierosolymitanum* were dissolved in 120, 37, 350, 20, and 300 mL 5% acetic acid, respectively. The acidic solutions were then

subjected to extraction (three times) with 450, 300, 900, 75, and 450 mL petroleum ether each time, respectively (Fraction A). The aqueous acid residues were extracted three times with 450, 300, 900, 75, and 350 mL diethyl ether each time, respectively (Fraction B). The acidic aqueous solutions were then made alkaline (pH 9) with 10% NH_4OH, and then re-extracted four times with 750, 600, 1500, 150, and 450 mL dichloromethane each time, respectively (Fraction C). The basic aqueous solutions were adjusted to pH 12 with sodium hydroxide, and extracted three times with 450, 300, 900, 75, and 300 mL diethyl ether each time, respectively (Fraction D), and finally extracted three times with 450, 300, 900, 75, and 300 mL dichloromethane, respectively (Fraction E). All these fractions were brought to dryness (in vacuum) and the exact weights were recorded as the following:

				Fractions[a] (g)				
Plant part	**Fresh weight (g)**	**Dry weight (g)**	**MeOH extract (g)**	**A**	**B**	**C**	**D**	**E**
[a]Based on the method of Šimánek and coworkers 25,26.								
C. tunicatum								
Leaves	1949	633	153	17.01	1.27	1.36	0.09	0.24
Stems	526	171	100	2.61	0.63	0.83	0.26	0.22
Corms	4413	1433	527	12.95	2.5	4.64	0.05	0.34
Roots	255	83	12	0.71	0.21	0.12	0.02	0.03
C. hierosolymitanum								
Corms	2000	650	80	4.68	1.36	1.17	0.03	0.05

Open glass column chromatography of fractions C, 1.169 g leaves, 0.668 g stems, and 4.47 g corms of *C. tunicatum* and 1.0 g corms of *C. hierosolymitanum* were carried out on silica gel 60, 0.06-0.2 mm, 70-230 mesh, packed in *n*-hexane. Elution of the columns was conducted using 100% hexane gradually enriched with dichloromethane. The columns were then eluted with solvent mixtures of dichloromethane--methanol of increasing polarity. Eluted fractions were followed by TLC, and similar fractions were combined into pools. The pools were subjected to further purification using semi-preparative HPLC, using a gradient solvent system of CH_3CN and 3% acetic acid in water (10 : 90-60 : 40 over 30 min) with a 10 mL min^{-1} flow rate, monitoring at 245 nm, and injecting between 75 and 175 mg of material dissolved in 2 mL of MeOH and mobile phase in a 1 : 1 ratio. The purities of the isolated compounds were checked by TLC developed with either $CHCl_3$: MeOH [9 : 1] or CH_2Cl_2 : acetone : diethylamine [12 : 6 : 2].

3.4. Structural elucidation of active components

The structures of the purified compounds were elucidated using a series of spectroscopic and spectrometric techniques principally: 1D-NMR (^1H and ^{13}C) and low resolution EI-MS and APCIMS, and by comparing with the reported spectral data in the literature 19. An authentic standard material of (-)-colchicine, Sigma, was available.

3.5. Brine shrimp lethality test

The BSLT was performed as described previously 27,28.

3.6. Human cancer cell panel

A panel of three human cancer cell lines was selected 19: MCF-7 human breast carcinoma (Barbara A. Karmanos Cancer Center, Detroit, MI), NCI-H460 human large cell lung carcinoma (American Type Culture Collection, Manassas, VA), and SF-268 human astrocytoma (NCI Developmental Therapeutics Program, Frederick, MD) cell lines were all adapted and maintained in RPMI-1640 medium supplemented with fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA) at 10% (v/v) and the antibiotics, penicillin G (100 U mL^{-1}) and streptomycin sulfate (100 μg mL^{-1}) in a humidified 5% CO_2 atmosphere kept at 37°C. Strict attention was paid to using cells in the logarithmic phase of cell growth, and fresh cell stocks were expanded at the end of 20 passages to maintain continuity of results during fractionation and compound purification.

Cell suspensions were first prepared at densities of 3000 (MCF-7), 1500 (NCI-H460), or 10,000 (SF-268) cells per 50 μL of medium for each well of 96-well culture dishes and plated in triplicate for each drug concentration. Plant extracts, fractions, and pure compounds were dissolved in DMSO initially at 4 mg mL^{-1}, then diluted in culture medium at twice the intended final concentration. Fifty microliters of each 2 × drug solution was then added to wells containing an equal volume of each cell suspension. For initial screening and fractionation samples, cells were exposed to fractions at final concentrations of 2 and 20 μg mL^{-1}; for EC_{50} determinations, pure compounds were diluted serially in half-log steps. In all cases, the final DMSO concentration was ≤0.5%. Blank wells and wells with media but no cells were included for background correction since trichloroacetic acid (TCA)-precipitated proteins from serum alone results in some background sulforhodamine B (SRB) absorbance. After a three-day continuous exposure, cells were fixed by addition of 25 μL of cold 50% (w/v) TCA to the growth medium in each well at 4°C for 1 h, then washed five times with water. The TCA-fixed cells were then stained for 30 min with 50 μL of 0.4% (w/v) SRB in 1% (v/v) acetic acid followed by five rinses with 1% (v/v) acetic acid to remove unbound dye. The fixed, stained plates were air-dried and bound dye then solubilized by incubation with 100 μL of 10 mM Tris base for at least 5 min. Absorbance was measured at 540 nm using a Tecan Ultra multiplate reader. The percent cellular survival was calculated as the fractional corrected absorbance of drug/extract-treated samples relative to control cells treated with vehicle alone: (sample OD_{540} - media blank OD_{540}/mean control OD_{540} - media blank OD_{540}) ×100. For EC_{50} calculations, survival data were evaluated by variable slope curve-fitting using Prism 4.0 software (GraphPad, San Diego, CA).

4. Conclusions

C. tunicatum Feinbr and *C. hierosolymitanum* Feinbr (Colchicaceae), two unexplored native Jordanian meadow saffrons, were studied phytochemically. Directed by the BSLT, five and four known compounds were isolated and identified from *C. tunicatum* and *C. hierosolymitanum*, respectively. These compounds were tested for general toxicity against the BSLT and for cytotoxicity against a human cancer cell panel. (-)-Colchicine (**I**) and (-)-cornigerine (**III**) were found to be the most bioactive.

Acknowledgements

The authors thank Prof. Dawud Al-Eisawi, Biology Department, Faculty of Science, University of Jordan, Amman, Jordan and Prof. Ahmad El-Oqlah, Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid, Jordan for identifying the plant material. The authors from Jordan acknowledge the kind financial support of the Deanship of Scientific Research, Jordan University of Science and Technology, Irbid, Jordan. This work was also supported in part by the Fogarty International Center of the National Institutes of Health with co-funding from the National Institute of Allergy and Infectious Diseases, the National Institute of Drug Abuse, and the National Science Foundation under grant number R21 TW006628 for the International Cooperative Biodiversity Groups. C.L. and N.H.O. gratefully acknowledge partial support via a Research Scholar Grant to N.H.O. from the American Cancer Society (RSG-02-024-01-CDD).

References

- 1. Al-Eisawi, DM (1998) *Field Guide to Wild Flowers of Jordan and Neighbouring Countries* pp. 1-12. Jordan Press Foundation Al Rai , Amman
- 2. Feinbrun-Dothan, N. (1986) *Flora Palestina* Maps 1 and 2, The Israel Academy of Sciences and Humanities , Jerusalem
- 3. Al-Khalil, S. (1995) *Int. J. Pharmacog.* **33** , p. 317.
- 4. Abu-Irmaileh, B. and Afifi, FU (2000) *Dirasat, Med. Biol. Sci. (in Arabic)* **27** , p. 53.
- 5. Nordenstam, B. Kubitzki, K. (ed) (1998) *The Families and Genera of Vasular Plants* **3** , pp. 175-185. Springer-Verlag , Berlin
- 6. Oran, SA and Al-Eisawi, DM (1998) *Dirasat, Med. Biol. Sci. (in Arabic)* **25** , p. 84.
- 7. Feinbrun-Dothan, N. (1986) *Flora Palestina* pp. 26-31. The Israel Academy of Sciences and Humanities , Jerusalem
- 8. Tyler, VE, Brady, LR and Robbers, JE (1988) *Pharmacognosy* pp. 242-243. 9th Edn, Lee & Febiger , Philadelphia
- 9. Trease, GE and Evans, WC (1989) *Trease and Evans' Pharmacognosy* pp. 600-603. 13th Edn, Bailliere Tindall , London and Philadelphia
- 10. Pelletier, PJ and Caventou, JB (1820) *Ann. Chim. Phys.* **14** , p. 69.
- 11. Terkeltaub, RA (2003) *N. Engl. J. Med.* **349** , p. 1647.
- 12. Drenth, JP and van der Meer, JW (2001) *N. Engl. J. Med.* **345** , p. 1748.
- 13. Sakane, T. and Takeno, M. (2000) *Expert. Opin. Investig. Drugs* **9** , p. 1993.
- 14. Eigsti, OJ and Dustin, JP (1955) *Colchicine in Agriculture, Medicine, Biology, and Chemistry* pp. 255-265. The Iowa State College Press , Ames
- 15. Kneedler, WH (1945) *J. Amer. Med. Assoc.* **129** , p. 272.
- 16. Samuelsson, G. (1992) *Drugs of Natural Origin: A Textbook of Pharmacognosy* pp. 241-244. Swedish Pharmaceutical Press , Stockholm
- 17. Al-Fayyad, M., Alali, F., Alkofahi, A. and Tell, A. (2002) *Nat. Prod. Letts.* **16** , p. 395. [informaworld]
- 18. Alali, F., Tawaha, K. and Qasaymeh, RM (2004) *Phytochem. Anal.* **15** , p. 27.
- 19. Alali, FQ, El-Elimat, T., Qandil, A., Alkofahi, A., Tawaha, K., Burgess, JP, Nakanishi, Y., Kroll, DJ, Navarro, HA, Falkinham III, JO, Wani, MC and Oberlies, NH (2005) *J. Nat. Prod.* **68** , p. 173.
- 20. Halevy, AH (1985) *Handbook of Flowering* **VI** , pp. 234-242. CRC Press , Boca Raton, Florida
- 21. Meksuriyen, D., Lin, LJ, Cordell, GA, Mukhopadhyay, S. and Banerjee, SK (1988) *J. Nat. Prod.* **51** , p. 88.
- 22. Freyer, AJ, Abuzarga, MH, Firdous, S., Guinaudeau, H. and Shamma, M. (1987) *J. Nat. Prod.* **50** , p. 684.
- 23. Tojo, E., Abuzarga, MH, Freyer, AJ and Shamma, M. (1989) *J. Nat. Prod.* **52** , p. 1163.
- 24. Hufford, CD, Capraro, HG and Brossi, A. (1980) *Helv. Chim. Acta* **63** , p. 50.
- 25. Santavy, F., Preininger, V., Šimánek, V. and Potesilova, H. (1981) *Planta Med.* **43** , p. 153.
- 26. Sutlupinar, N., Husek, A., Potesilova, H., Dvorackova, S., Hanus, V., Sedmera, P. and Šimánek, V. (1988) *Planta Med.* **54** , p. 243.
- 27. Meyer, BN, Ferrigni, NR, Putnam, JE, Jacobsen, LB, Nichols, DE and McLaughlin, JL (1982) *Planta Med.* **45** , p. 31.
- 28. McLaughlin, JL and Rogers, LL (1998) *Drug Inf. J.* **32** , p. 513.