

In vitro cytotoxicity of extracts and fractions of *Calotropis procera* (Ait.) roots against human cancer cell lines

Madhulika Bhagat, Jatinder Singh Arora¹, Ajit Kumar Saxena²

School of Biotechnology, University of Jammu, Jammu-180 006, ¹Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, 143005, ²Pharmacology Division, Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi-180 001, India

This study was designed to determine the antiproliferative activity of three extracts (alcoholic, hydro-aqueous and aqueous) and their fractions from the root part of *Calotropis procera* using human oral (KB) and central nervous system (SNB-78) cancer cell lines as a model system. KB and SNB-78 cells were cultured in the presence of extracts and fractions at various concentrations (10, 30 and 100 µg/ml) for 48 h, and the percentage of cell viability was evaluated by the sulforhodamine-B (SRB) assay. Our result indicates that out of the three extracts of *C. procera* (root), alcoholic extract had shown greater potential for growth inhibition followed by hydro-aqueous extract at three different concentration of 10 µg/ml, 30 µg/ml and 100 µg/ml in a dose-dependent manner, whereas aqueous extract was found to be least active against both oral and CNS human cancer lines. On evaluation of the fractions prepared from alcoholic and hydro-aqueous extracts, it was observed that chloroform fraction from alcoholic extract was antiproliferative for oral (KB) cancer cell line and *n*-butanol fraction from alcoholic extract was antiproliferative for CNS cancer cell line than remaining fractions at three different concentration of 10 µg/ml, 30 µg/ml, 100 µg/ml in a dose-dependent manner. Thus, our result indicates that the root part of *C. procera* possess *in vitro* cytotoxicity against oral and CNS human cancer cell lines. Further investigations are required to obtain the clinically important lead molecules for the drug development.

Key words: Cytotoxicity, *Calotropis procera*, fractions, human cancer cell line extracts

INTRODUCTION

Cancer remains the major cause of morbidity and mortality through the world and still the management of the cancer is not up to the mark. Traditionally, the disease is best managed by surgical removal, chemotherapy and radiotherapy. However, in view of the side effects due to drugs used in chemotherapy of different cancers, herbal medicine is becoming very popular among cancer patients.^[1] Humankind has for centuries used many species of medicinal plant to treat several diseases and these are also known to produce wealth of phytochemicals.^[2] In recent years, secondary plant metabolites, previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents.^[3] Over one and a half million practitioners of the Indian System of Medicine in the oral and Codified streams use medicinal plants in preventive, promotive and curative applications. However, in order to make these remedies acceptable, there is a need to scientifically evaluate to identify active principle. In the traditional Indian Medicinal system, the Ak plant or *Calotropis procera* (Ait.) R.Br. (*Asclepiadaceae*)

has been used for a variety of disease conditions that include its use in treatment of leprosy, ulcers, piles and tumours.^[4] The root extract of *C. procera* has been found to produce a strong cytotoxic effect on COLO 320 tumour cells.^[5] The chloroform-soluble fraction of its roots, ethanolic extract of its flowers and aqueous and organic extracts of its dried latex also exhibit a strong anti-inflammatory activity in a animal model of acute and chronic inflammation.^[6] In the search of a potential anticancer agent and to validate the cytotoxic claim of this plant, we tried to explore its cytotoxic potential against human cancer cell lines.

MATERIAL AND METHODS

Plant Collection

Roots of *C. procera* were collected locally from Parmandal area of Jammu in the month of December and were authenticated at the source by the taxonomist of the Institute. A voucher specimen was deposited at the herbarium of the Institute vide IIIM collection No.17600, Acc. No. 194731.

Preparation of Plant Extracts

The dried powdered plant material (500g) was percolated with 95% ethanol, and then concentrated to dryness (27.0g) under reduced pressure to yield alcoholic extract. Hydro-

Address for correspondence: Dr. A. K. Saxena, Scientist, Division of Pharmacology, Indian Institute of Integrative Medicine, Canal Road, Jammu-180016, India. E-mail: aksaxena2003@yahoo.com

Received: 15-12-2008; **Accepted:** 27-06-2009; DOI: 10.4103/0973-8258.62165

alcoholic extract was prepared by percolating another lot of dried powdered plant material (500g) with 50% ethanol and then concentrating it to dryness (43.0 g) under reduced pressure. For the preparation of aqueous extract, the dried powdered plant material (500 g) was heated with distilled water (1.5 L) on steam bath for 2 h, the supernatant was decanted and filtered through celite powder and the process was repeated four times; pooled extract was concentrated on rotavapour and dried in a lyophilizer and 53.8 g extract was obtained.^[7]

Preparation of Fractions

The alcoholic extract was fractionated sequentially with *n*-hexane, chloroform, *n*-butanol and water. The dried alcoholic extract (20 g) was macerated with *n*-hexane (4 × 500 ml). The combined solvent portion was evaporated under reduced pressure to yield hexane fraction (1.46 g). The residue was further macerated with chloroform (4 × 500 ml). The combined organic layer was evaporated under reduced pressure to yield chloroform fraction (1.73 g). The residue obtained was dissolved in distilled water (1 litre) and partitioned between *n*-butanol and water. The process was repeated four times (4 × 500 ml), the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield *n*-butanol fraction (8.55 g). The aqueous part was concentrated under reduced pressure to give aqueous fraction (6.4 g).

The hydro-alcoholic extract was fractionated sequentially with chloroform, *n*-butanol and water. The dried hydro-alcoholic extract (10 g) was macerated with chloroform (4 × 100 ml). The combined organic layer was evaporated under reduced pressure to yield chloroform fraction (0.34 g). The residue obtained was dissolved in distilled water (200ml) and partitioned between *n*-butanol and water. The suspension was taken in a separating funnel and extracted with *n*-butanol (4 × 100 ml). The combined *n*-butanol extract was evaporated to dryness under reduced pressure below 50°C to yield (1.12 g) *n*-butanol fraction. The aqueous fraction was filtered, centrifuged at 1000 rpm for 20 min and finally dried on freeze dryer and yield was 0.68 g.

The aqueous extract fraction was fractionated sequentially with *n*-butanol and water. The dried aqueous extract (10 g) was suspended in water (200 ml). The suspension was taken in a separating funnel and extracted with *n*-butanol (4 × 100 ml). The combined *n*-butanol extract was evaporated to dryness under reduced pressure to yield (1.02 g) *n*-butanol fraction. The aqueous fraction was filtered, centrifuged at 1000 rpm for 20 min and finally dried on freeze dryer to yield (0.56 g) aqueous fraction.

Human Cancer Cell Lines and Positive Control

The KB (oral) and SNB-78 (CNS) human cancer cell line were

obtained from National Center for Cell Science, Pune, India, and cultured in DMEM and RPMI- 1640 medium (pH 7.4), respectively. The media were supplemented with Fetal Calf Serum (10%), penicillin (100 units/ml), streptomycin (100 µg/ml) and glutamine (2 mM).

In vitro Cytotoxic Assay

In vitro cytotoxicity of extracts and fractions was determined using sulforhodamine-B (SRB) dye as previously described.^[8] In brief, the stock solution (20 mg/ml) of the alcoholic, hydro-alcoholic and aqueous extracts was prepared in dimethyl sulfoxide (DMSO), dimethyl sulfoxide–water (1:1) and hot water, respectively, and was further diluted with the growth medium containing all the supplements as mentioned above and gentamicin to obtain desired concentration. The stock solution of hexane, chloroform and butanol fractions was prepared in dimethyl sulfoxide in which aqueous fraction was dissolved in distilled water. Positive controls, paclitaxel and adriamycin were prepared in dimethyl sulfoxide (DMSO, 10⁻² M) and then diluted with a medium containing all the supplements and gentamicin to obtain desired concentrations of 1 × 10⁻⁵ M and 1 × 10⁻⁶ M. The cells were grown in tissue culture flasks in a growth medium at 37°C in an atmosphere of 5% CO₂ and 95 % relative humidity in a CO₂ incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in the growth medium. The cells with more than 97% viability (Trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 µl of cell suspension (10⁵-2 × 10⁵ cells/ml depending upon mass doubling time of cells) was transferred to a well of 96-well tissue culture plate. The cells were incubated for 24 h. The test materials (100 µl) were then added to the wells and cells were further allowed to grow for another 48 h. The cell growth was stopped by gently layering 50 µl of 50% trichloroacetic acid. The plates were incubated at 4°C for an hour to fix the cells attached to the bottom of the wells. Liquid of all the wells were gently pipetted out and discarded. The plates were washed five times with distilled water and air dried. Sulforhodamine B (100 µl, 0.4% in 1% acetic acid) was added to each well, and the plates were incubated at room temperature for 30 min. The unbound SRB was quickly removed by washing the wells five times with 1% acetic acid. Plates were air dried, Tris-HCl buffer (100 µl, 0.01 M, pH 10.4) was added to all the wells, and the plates were gently stirred for 5 min on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm. Suitable blanks and positive controls were also included. Each test was done in triplicate. The value reported here in are mean of two experiments.

RESULTS AND DISCUSSION

In the present work, three different solvents, alcoholic,

hydro-alcoholic (1:1) and water were used to prepare the extracts from roots of the *C. procera*. The extractive yield was considerably more in aqueous extract (10.76 %) followed by hydro-alcoholic extract (8.6 %) and alcoholic extract (5.4 %). Further, extract were fractionated using different solvent of increasing polarity; the extractive yield of the *n*-butanol fraction from alcoholic extract had showed maximum yield [Table 1]. Likewise, *n*-butanol fraction showed maximum yield in case of hydro-alcoholic and aqueous extracts.

The cytotoxic study conducted earlier had revealed its cytotoxic potential against COLO 320 tumour cells.^[5] We have evaluated cytotoxicity against oral (KB) and central nervous system (CNS) (SNB-78) cancer cell lines. The percentage growth inhibition for the three extracts *viz.*, alcoholic, hydro-alcoholic and aqueous at 10, 30 and 100 µg/ml was 46, 67 and 73% for alcoholic extract, 33, 52 and 69% for hydro-aqueous extract, and 29, 43 and 64% for aqueous extract against the oral human cancer cell line [Figure 1]. Thus, the alcoholic extract had most pronounced cytotoxicity against the oral human cancer cell line.

In order to obtain better insight into the nature of active principles responsible for the cytotoxic activity, the crude extracts were fractionated using the solvent of increasing polarity. Cytotoxicity of these fractions prepared from the three extracts showed that among all the fractions, chloroform fraction of the alcoholic extract showed maximum 23, 45 and 70% growth inhibition at 10, 30 and 100 µg/ml against the oral human cancer cell line in comparison to the rest of the fractions [Figures 2 and 3]. However, fractions of aqueous extract were found to be least effective (data not included).

Similarly, for CNS (SNB-78) human cancer cell line out of the three extracts, alcoholic extract was most active and showed 35, 50 and 70% growth inhibition at 10, 30 and 100 µg/ml followed by hydro-aqueous extract and aqueous extract was least active [Figure 4]. When fractions of all the three extracts were evaluated for *in vitro* cytotoxicity, it was found that the *n*-butanol fraction of alcoholic extract showed 50% growth inhibition at 100 µg/ml, which is highest among all the fractions studied for alcoholic extract

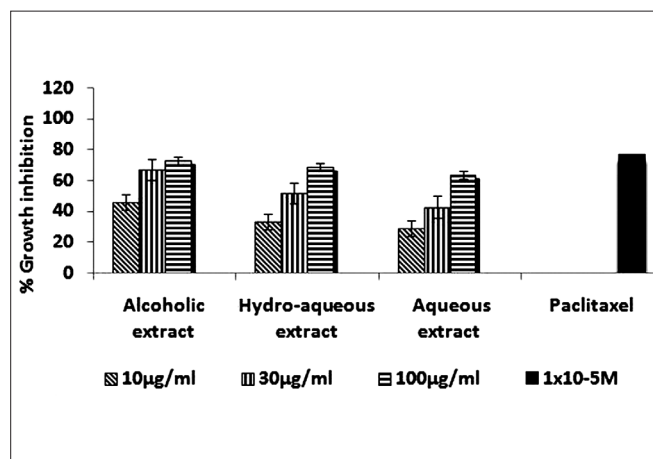


Figure 1: *In vitro* cytotoxicity of the extracts of *Calotropis procera* (root) against oral human cancer cell line

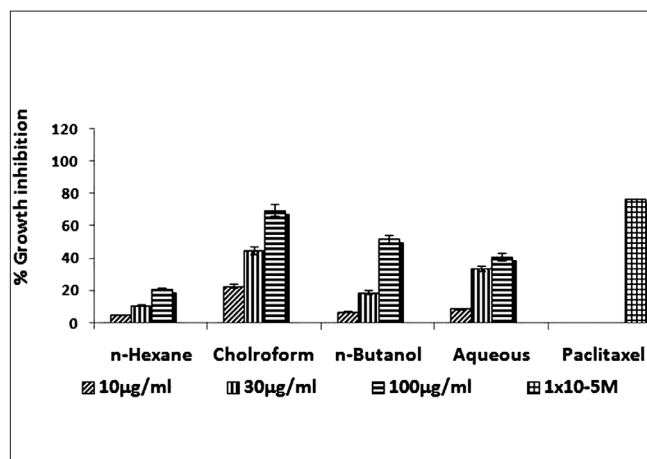


Figure 2: *In vitro* cytotoxicity of *Calotropis procera* (root) fractions from alcoholic extracts against KB (oral) cancer cell lines

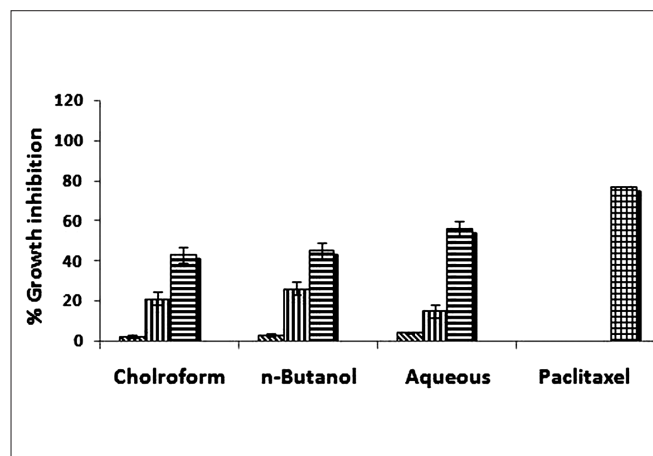


Figure 3: *In vitro* cytotoxicity of *Calotropis procera* (root) fractions from hydro-alcoholic extracts against KB (oral) cancer cell lines

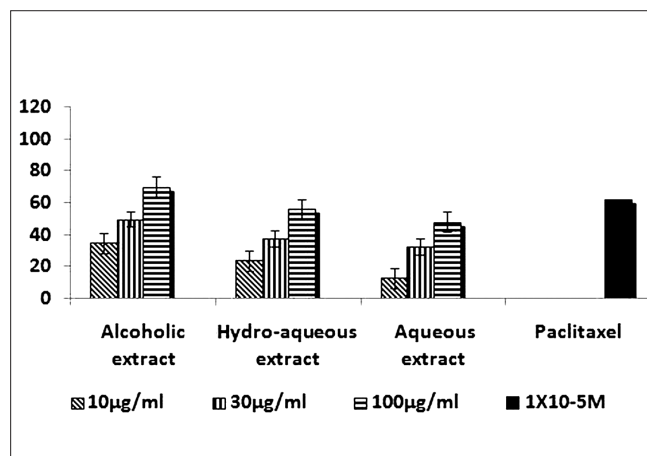


Figure 4: *In vitro* cytotoxicity of the extracts of *Calotropis procera* (root) against CNS human cancer cell line

Table 1: Extractive yield (percentage) of extracts and fractions of *Calotropis procera*(root part)

| Extract | Percentage yield | Fraction | Percentage yield |
|--------------------------|------------------|------------|------------------|
| Alcoholic | 5.4 | n-hexane | 7.3 |
| | | Chloroform | 8.65 |
| | | n-butanol | 42.75 |
| | | Aqueous | 32 |
| Hydro-alcoholic (1:1) | 8.6 | Chloroform | 3.4 |
| | | n-butanol | 11.2 |
| | | Aqueous | 6.8 |
| Aqueous | 10.76 | n-butanol | 10.2 |
| | | aqueous | 5.6 |

[Figure 5]. The *n*-butanol extract of hydro-alcoholic extract showed maximum effect against CNS human cancer cell line [Figure 6]. Fractions of aqueous extract were found to be least effective (data not included).

All the three extracts (alcoholic, hydro-alcoholic and aqueous) of *C. procera* root have shown anti-proliferative activity against both oral and CNS cancer cell lines, but alcoholic extract was found to possess more potential and aqueous extract had least activity. The cancer growth inhibition by these extracts was cell line and concentration dependent. The studies also indicated that the chemical constituents of *C. procera* responsible for cytotoxic activity are present in alcoholic extract and are non-polar in nature. The chloroform fraction (alcoholic extract) was most active for oral cancer cell line, whereas *n*-butanol fraction (alcoholic extract) was most active for CNS cancer cell line as compared to the rest of the fractions of alcoholic, hydro-alcoholic and aqueous extracts. The aqueous fractions were found to be least effective. Cytotoxic studies of the fractions further indicated that the active constituents are non-polar and present in chloroform fraction for oral cancer cell line, while active constituents for CNS cancer cell lines are polar in nature and present in *n*-butanol fraction. Various studies had reported that root bark of *C. procera* has shown high *in vivo* tolerance of tumour growth and prolonged survival in the human xenograft models of nude mice.^[9] Latex possesses chemopreventive action in *in vivo* and cytotoxic potential in cancer cell lines.^[10] Chemical constituents reported from the extracts and fractions of leaves and roots are alkaloids, flavonoids, tannins, steroids, terpenoids, saponins and glycosides.^[11] Three cytotoxic chemical constituents reported are calotropin, usharin and calotoxin.^[12] These biologically active compounds may be responsible for the cytotoxicity against cancer cell lines. Further isolation and identification of the active compounds in the crude alcoholic extracts and fractions are recommended.

CONCLUSION

The study clearly demonstrated the *in vitro* cytotoxic potential

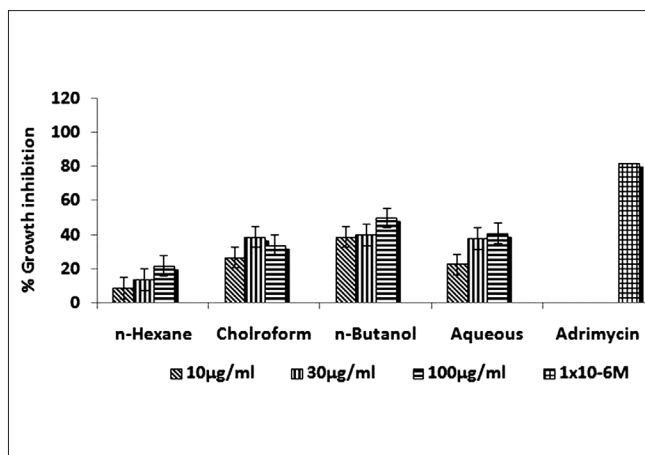


Figure 5: *In vitro* cytotoxicity of *Calotropis procera* fractions from alcoholic extracts against CNS human cancer cell lines

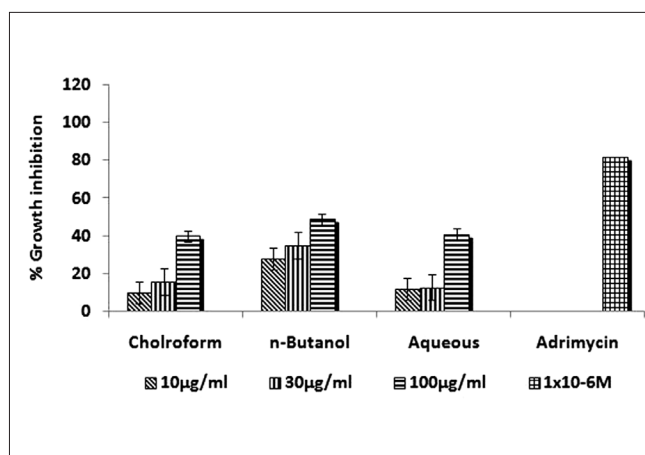


Figure 6: *In vitro* cytotoxicity of *Calotropis procera* fractions from hydro-alcoholic extracts against CNS human cancer cell lines

of the root part of the *C. procera*. Here, we have investigated and also validated cytotoxic potential of the *C. procera* (root part) against KB and SNB-78 human cancer cell lines. The activity lies in the chloroform fraction of the alcoholic extract for the oral human cancer cell lines and *n*-butanol fraction of the alcoholic extract for the CNS cancer cell line. Further investigation is required for isolating active constituent responsible for the cytotoxicity. Some constituents from *C. procera* may serve as a novel powerful antitumour after further detailed investigation. Moreover, other biological activities on different cell lines which are correlated to traditional treatments of *C. procera* should also be investigated.

ACKNOWLEDGMENTS

Authors are grateful to National Centre for Cell Science, Pune (India) and National Cancer Institute, Frederick, MD, U.S.A for providing human cancer cell lines. Authors also acknowledge the help provided by Dr B K Kapahi and Dr V K Sharma for this paper. This paper is the part of the Ph.D Thesis work of Ms. Madhulika Bhagat.

REFERENCES

1. Yates JS, Mustian KM, Morrow GR, Gillies LJ, Padmanaban D, Atkins JN, *et al.* Prevalence of complementary and alternative medicine use in cancer patients during treatment. *Support Care Cancer* 2005;13:806-11.
2. Cragg GM, Boyd MR, Khanna R, Kneller R, Mays TD, Mazan KD, *et al.* International collaboration in drug discovery and development: the NCI experience. *Pure Appl Chem* 1999;71:1619-33.
3. Krisharaju AV, Rao TV, Sundararaju. Assessment of bioactivity of Indian medicinal plants using Brine shrimp (*Altenaria salania*) lethality assay. *Int J Appl Sci Engg* 2005;2:125-34.
4. Kumar VL, Arya S. Medicinal uses and pharmacological properties of *Calotropis procera*. In: Govil JN, ed. *Recent Progress in Medicinal Plants*. Texas: Studium Press 2006;11:373-88.
5. Smit HF, Woerdenbag HJ, Singh RH, Meulenbeld GJ, Labadie RP, Zwaving JH. Ayurvedic herbal drugs with possible cytostatic activity. *J Ethanopharmacol* 1995;47:75-84.
6. Arya S, Kumar, VL. Antiinflammatory efficacy of extracts of latex of *calotropis procera* against different mediators of inflammations. *Mediators inflamm* 2005;14:228-32.
7. Chairungsrilerd N, Takeuchi K, Ohizumi Y, Ohta T, Nozoe S. Mangostanol, a prenyl xanthone from mangostana. *Phytochemistry* 1996;43:1099-102.
8. Bhahwal AS, Kumar A, Gupta P, Sharma M, Sethi VK, Saxena AK, *et al.* Cytotoxic and apoptotic activities of novel amino analogues of boswellic acids. *Bioorg Med Chem Lett* 2007;17:6411-6.
9. Van Quaquebeke E, Simon G, Andre A, Dewelle J, Yazidi ME, Bruyneel F, *et al.* Identification of a novel cardenolide (2''-oxovoruscharin) from *Calotropis procera* and the hemisynthesis of novel derivatives displaying potent *in vitro* antitumor activities and high tolerance: structure-activity relationship analysis. *J Med Chem* 2005;48:849-56.
10. Choedon T, Mathan G, Arya S, Kumar VL, Kumar V. Anticancer and cytotoxic properties of the *Calotropis procera* in a transgenic mouse model of hepatocellular carcinoma. *World J Gastroenterol* 2006;12:2517-22.
11. Hassan FL, Bilbis FL, Ladan MJ, Umar RA, Dangoggo SM, Saidu Y, *et al.* Evaluation of antifungal activity and phytochemical analysis of leaves, roots and stem barks extracts of *Calotropis procera* (Asclepiadaceae) *Pak J Biol Sci* 2006;9:2624-9.
12. Atal CK, Setui PD. Isolation, properties and Kinetics studies of calotropis. *Planta Med* 1962;10:77.

Source of Support: Nil, **Conflict of Interest:** None declared.

Staying in touch with the journal

1) Table of Contents (TOC) email alert

Receive an email alert containing the TOC when a new complete issue of the journal is made available online. To register for TOC alerts go to www.greenpharmacy.info/signup.asp.

2) RSS feeds

Really Simple Syndication (RSS) helps you to get alerts on new publication right on your desktop without going to the journal's website. You need a software (e.g. RSSReader, Feed Demon, FeedReader, My Yahoo!, NewsGator and NewzCrawler) to get advantage of this tool. RSS feeds can also be read through FireFox or Microsoft Outlook 2007. Once any of these small (and mostly free) software is installed, add www.greenpharmacy.info/rssfeed.asp as one of the feeds.