Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 460–464
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of DPPH free radical scavenging activity by reversed-phase HPLC: A sensitive screening method for polyherbal formulations

D. Chandrasekar, K. Madhusudhana, S. Ramakrishna, Prakash V. Diwan *

Pharmacology Division, Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad 500007, Andhra Pradesh, India

Received 6 May 2005; received in revised form 14 July 2005; accepted 23 July 2005
Available online 16 November 2005

Abstract

The colorimetric method of evaluation of antioxidant activity by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical is with certain shortcomings like failure to indicate antioxidant activity of certain drugs and interference from color pigments of natural products. A specific HPLC method was developed for evaluating the DPPH free radical scavenging activity of commercial polyherbal formulations using a LiChrospher® 100 RP-18e column (250 mm × 4 mm, 5 μM). The mobile phase was a mixture of methanol and water (80:20, v/v) pumped at a flow rate of 1 mL/min. The DPPH peaks were monitored at 517 nm. The method was standardized using known antioxidants such as ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), probucol and α-tocopherol. The 50% radical scavenging activity (IC_{50}) determined by the HPLC method correlated well with that of colorimetry. This HPLC method was applied for the estimation of free radical scavenging activity of Silymarin and a few commercial hepatoprotective polyherbal formulations. While the colorimetric method failed to estimate the free radical scavenging activity of polyherbal formulations, HPLC method was free from interferences and was specific. The HPLC method is sensitive and can be used as a quality control tool for the rapid determination of free radical scavenging activity of variety of products including plant extracts, foods, drugs and polyherbal formulations.

Keywords: Antioxidants; Reversed-phase HPLC; Free radical; DPPH; Polyherbal syrups

1. Introduction

Herbal drugs are rapidly becoming popular in recent years as an alternative therapy. Numerous polyherbal formulations, which are combinations of different herbal extracts/fractions, are used for the treatment of liver diseases. Antioxidants that can protect liver from oxidative damages are included in polyherbal formulations. For developing a satisfactory hepatoprotective herbal formulation, there is a need to evaluate the formulation for desired properties such as antioxidant and hepatoprotective activity. The desired activities of the polyherbal formulations containing different plants/extracts have to be tested again in the formulation form [1]. Hence, there is a need to establish simple and sensitive screening method for antioxidant activity as a means of developing quality control of polyherbal formulations. Stable free radical species such as 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) are often used for the evaluation of the general radical scavenging capabilities of various antioxidants [2]. DPPH, a paramagnetic compound with an odd electron, shows strong absorption band at 517 nm in methanol. The absorbance decreases as a result of color change from purple to yellow due to the scavenging of free radical by antioxidants through donation of hydrogen to form the stable DPPH–H molecule [3]. The colorimetric estimation of DPPH is a simple method but is not applicable to colored foods due to interference by pigments [4]. Also, the DPPH colorimetric method of evaluation may not be of much use to judge the antioxidant activity, as it evidently does not indicate antioxidant activity of drugs such as nimesulide, dapsone and acetylsalicylic acid [5,6]. Yamaguchi et al. [4] developed a DPPH–HPLC method and compared the antioxidant activities of known standard antioxidants and commercial beverages with that of colorimetric method. The applicability of HPLC method

* Corresponding author. Tel.: +91 40 27193753; fax: +91 40 27193753.
E-mail address: diwan@iict.res.in (P.V. Diwan).

0731-7085/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jpba.2005.07.042

was extended to determine the antioxidant activity of crude plant extracts and drugs [5,7,8].

The objective of the present study is to standardize and compare the DPPH–HPLC method with that of colorimetric method using known antioxidants, and its application to determine the antioxidant activity of selected commercial polyherbal hepatoprotective formulations as a means of rapid screening methodology.

2. Materials and methods

2.1. Materials

DPPH (90%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, 97%), probucol and Silymarin were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). L-Ascorbic acid was obtained from Loba Chemie (Mumbai, India) and DL-α-tocopherol 99% from HiMedia Laboratories (Mumbai, India). Methanol (HPLC grade, Qualigens Fine Chemicals, Mumbai, India) and deionized water purified with Nanopure Diamond equipment (Barnstead/Thermolyne, USA) were used. The details of the herbal formulations purchased for analysis are given in Table 1.

2.2. Sample preparation

The DPPH and all standard antioxidants including Silymarin were soluble in methanol. Fresh DPPH stock solution (5 mL) at a concentration of 2.5 mM/mL was prepared on each day of analysis. The stock solutions of trolox, α-tocopherol, probucol, ascorbic acid and Silymarin were prepared in methanol at a concentration of 1 mM/mL and stored at −20 °C. All herbal formulations were diluted in methanol. An aliquot of 100 μL of different concentrations of standard antioxidants or herbal formulations in methanol were added to 100 μL of DPPH in solution (final concentration 250 μM/200 μL). The mixture was vortexed for few seconds and left to stand in the dark for 20 min at room temperature.

2.3. Colorimetric analysis

An aliquot of the mixture was measured for absorbance at 517 nm by a UV–visible spectrophotometer (SPECTRAmax PLUS®, Molecular Devices, USA). The percent radical scavenging activity is determined from the difference in absorbance (A) of DPPH between the control and samples.

$$\text{Radical scavenging (\%)} = \left[\frac{A_{\text{CONTROL}} - A_{\text{SAMPLE}}}{A_{\text{CONTROL}}}\right] \times 100.$$

2.4. HPLC analysis

The sample is filtered through 0.2 μm Nylon membrane filter (Pall Gelman Laboratory, USA) and an aliquot (20 μL) of the sample is injected for HPLC analysis. The blank was prepared by adding 100 μL of methanol to 100 μL of DPPH stock solution (final concentration 250 μM/200 μL) and included before each run. The reversed-phase HPLC system consisted of a Shimadzu HPLC system (LC-10 Ai, Japan) consisting of pump (LC-10 Ai), a system controller (SCL-10AVP), an auto-injector (SIL-10 ADVP) and a diode array detector (SPD-M10 AVP). Data analysis and processing were done by class LC10 software (Version 1.6). Analyses were carried out using a LiChrospher® 100 RP-18e column (250 mm × 4 mm, 5 μM) (Merck, Darmstadt, Germany). Isocratic elution was carried out with methanol/water (80:20, v/v) at a flow rate of 1 mL/min. The DPPH peaks were monitored at 517 nm. The difference in the reduction of DPPH peak area (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample.

$$\text{Radical scavenging (\%)} = \left[\frac{\text{PA}_{\text{BLANK}} - \text{PA}_{\text{SAMPLE}}}{\text{PA}_{\text{BLANK}}}\right] \times 100.$$

3. Results and discussion

3.1. Optimization of mobile phase

As DPPH was soluble in methanol, for HPLC mobile phase optimization different ratios of methanol:water including 50:50, 70:30 and 80:20 (v/v) were examined. When the methanol content was 50%, DPPH eluted beyond 9 min and DPPH retention time was found to decrease on increasing the organic phase content. DPPH eluted as a sharp peak at 6.24 min when the mobile phase ratio was 80:20. The peak was well separated with no interferences, good resolution and acceptable tailing (1.11) as shown in Fig. 1(a) (DPPH final concentration 250 μM/200 μL). The method was specific for DPPH with a run time of 10 min.

Table 1
Commercial Indian polyherbal formulations screened for antioxidant activity by HPLC method

Name of syrups	Main ingredients	Manufacturer	Batch no.
Liv.52®	*Eclipta alba, Phyllanthus niruri, Cichorium intybus, Terminalia chebula, Boehrravia diffusa*	The Himalaya Drug Company, Bangalore, India	21109 CL
New Livfit	*Eclipta alba, Phyllanthus niruri, Rheum emodi, Terminalia purpurea, Cichorium intybus, Tinospora cordifolia, Terminalia chebula, Boehrravia diffusa, Picrorhiza kurroa, Andrographis paniculata*	Dabur Pharmaceuticals, India	3054
Livina	*Terminalia purpurea, Andrographis paniculata, Phyllanthus niruri, Terminalia chebula, Cichorium intybus*	Dey's Manufacturing Ltd., Kolkata, India	LV481
Livomyn®	*Boehravia diffusa, Cichorium intybus, Eclipta alba, Zingiber officinalis, Embelia ribes, Tephrosia purpurea, Tinsospora cordifolia, Picrorhiza kurroa*	Charak Pharma Pvt. Ltd., Mumbai, India	LLS0002

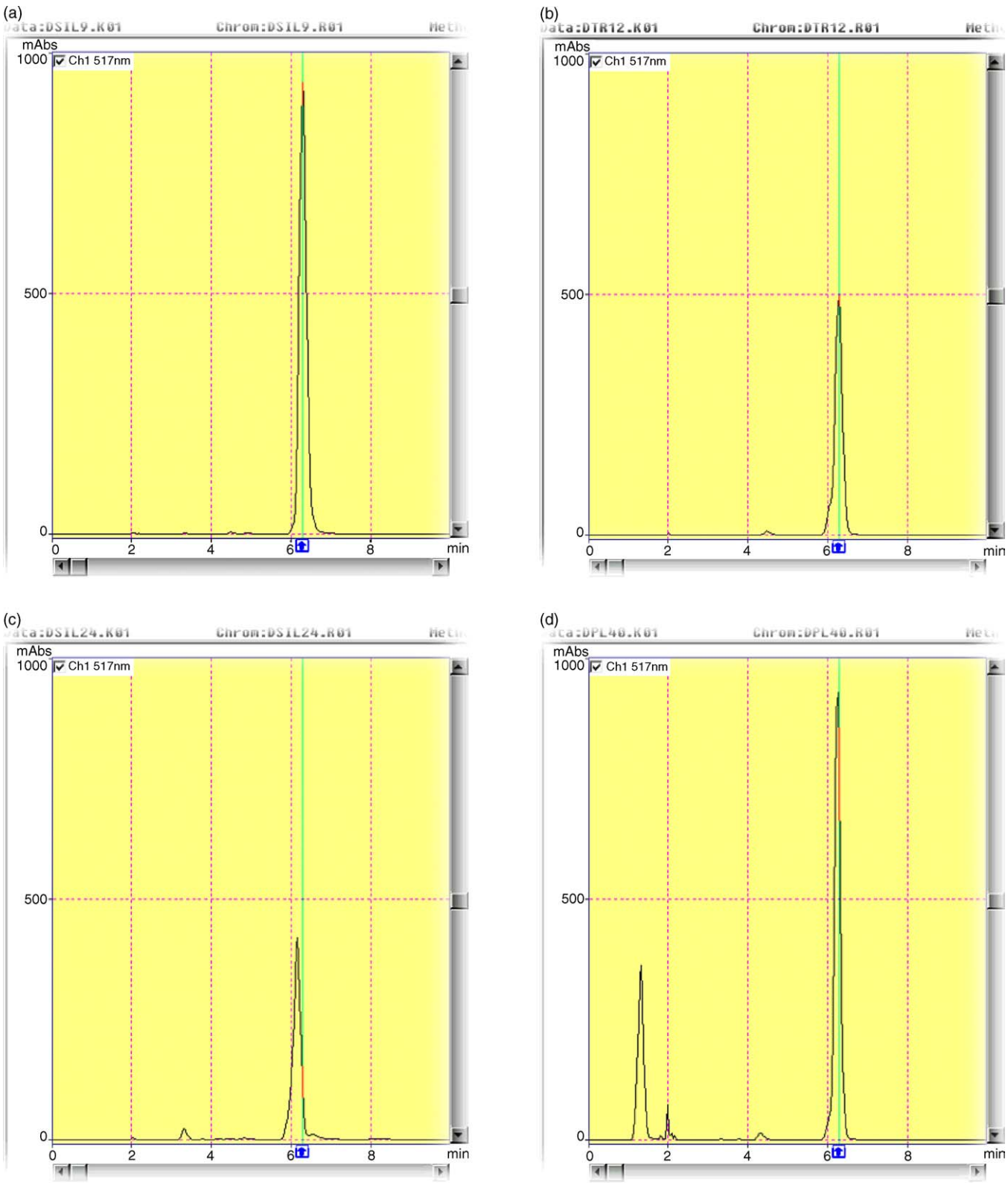

Fig. 1. HPLC chromatograms of DPPH: (a) blank, and after incubation with—(b) trolox 60 μM, (c) Silymarin 120 μM and (d) Livomyn 60 μL. HPLC conditions: column, LiChrospher® 100 RP-18e column (250 mm × 4 mm, 5 μM); mobile phase, a mixture of methanol and water (80:20, v/v); flow rate, 1 mL/min; detection wavelength, 517 nm; injection volume, 20 μL.

Table 2
Repeatability and reproducibility of the retention time and peak area of DPPH blank (250 μM/200 μL) solution ($n = 6$)

	Retention time (min)		Peak area	
	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
Day 1	6.24 ± 0.04	0.56	12779448.67 ± 173900.43	1.36
Day 2	6.21 ± 0.02	0.34	12309323.0 ± 163522.38	1.33
Day 3	6.24 ± 0.05	0.72	12631915.67 ± 303306.01	2.40

3.2. Repeatability and reproducibility

The repeatability and reproducibility of the analytical method was confirmed from the peak area and retention times of the DPPH blank solution. The results as listed in Table 2, indicated good repeatability and inter-day precision with acceptable R.S.D. (<3%).

3.3. Linearity graphs of standard antioxidants

The linearity graphs of all standard antioxidants were constructed by taking the mean value of the triplicate analysis. The linearity of trolox was found to be in the concentration range of 20–100 μM (correlation coefficient, $r^2 = 0.999$), suggesting that trolox can be used as standard for comparison of DPPH free radical scavenging activity (Fig. 1(b)). Linear regression equations for all other standard antioxidants also indicated good correlation in the linearity range of 20–100 μM (Table 3).

3.4. Determination of IC_{50} values

The 50% radical scavenging activity (IC_{50}) of the standard antioxidants and polyherbal formulations is given in Table 4. Based on the IC_{50} values calculated from the linearity curves of various antioxidants, each mole of ascorbic acid, trolox, probucol and α-tocopherol trapped approximately 2.7, 1.8, 2.4 and 2.0 mol of DPPH, respectively. Other investigators reported similar results for ascorbic acid and α-tocopherol [4,9]. The IC_{50} values for ascorbic acid, trolox, probucol and α-tocopherol found by the HPLC method correlated well with that of colorimetric estimation.

Table 3
Linearity graph of standard antioxidants by HPLC method

Standards	Linearity range 20–100 μM	
	Linear regression equation	Correlation coefficient (r)
Trolox	$Y = 1.0557x - 21.621$	0.9992
α-Tocopherol	$Y = 1.059x - 17.311$	0.9932
Probucol	$Y = 0.9982x - 2.6547$	0.9910
Ascorbic acid	$Y = 1.1621x - 9.534$	0.9902

x denotes the concentration (μM/mL) of standard antioxidants and Y denotes the peak area.

Table 4
Free radical scavenging activity of antioxidants and polyherbal formulations

Compound	IC_{50} concentration determined by	
	HPLC method	Colorimetric method
Standard antioxidants (μM)		
Ascorbic acid	46.11 ± 4.78	49.37 ± 3.14
Trolox	67.78 ± 3.78	63.12 ± 2.52
Probucol	52.66 ± 2.59	52.20 ± 3.01
α-Tocopherol	63.57 ± 0.67	61.74 ± 1.28
Silymarin	108.68 ± 0.95	113.78 ± 1.16
Polyherbal formulations (μL)		
Liv.52®	24.09 ± 0.56	214.20 ± 3.24
New LivFit®	22.90 ± 0.53	63.87 ± 1.02
Livina®	37.22 ± 3.26	117.25 ± 5.18
Livomyn®	186.93 ± 7.46	Not detectable

Each value is mean ± S.D. of triplicate analysis.

3.5. Application to polyherbal formulations

The HPLC method was applied for measuring the free radical scavenging activity of colored polyherbal hepatoprotective formulations. In contrast to the standard antioxidants, there was significant difference in the IC_{50} values of polyherbal formulations when determined by both the methods (Table 4). Colorimetric estimation showed no free radical scavenging effect for Livomyn®, whereas HPLC method revealed IC_{50} at same concentrations.

Silymarin is a widely studied and established hepatoprotective drug in various hepatotoxic models involving free radicals [10,11]. It is a mixture of antihepatotoxic flavonolignans from the fruit of *Silybum marianum*. As it is from a single source and in the pure form with no added colorants or additives, it has shown identical IC_{50} values by both the methods (Fig. 1(c)). Liv.52® has proven efficacy in viral hepatitis, alcoholic liver disease and against various hepatotoxic drugs [12,13]. New LivFit® was clinically effective in the treatment of acute hepatitis B [14]. Liv.52®, New LivFit, Livina and Livomyn® contain herbal extracts of known antioxidant and antihepatotoxic herbs such as *Boerhavia diffusa*, *Capparis spinosa*, *Cichorium intybus*, *Achillea millefolium*, *Eclipta alba*, *Tephrosia purpurea*, *Picrorhiza kurroa*, *Phyllanthus niruri*, *Tinospora coridifolia* and *Andrographis paniculata* [15–21].

All these commercial syrups were formulated in flavored syrup base containing color pigments. These pigments will contribute to the observed difference in the IC_{50} values determined by the colorimetry. Also, Livomyn® contains carmoisine as the colorant. This colorant might interfere with the absorbance of DPPH in the colorimetric method leading to the failure in detection of small changes in the DPPH absorbance. Yamaguchi et al. [4] reported similar results for beverages indicating the insensitiveness and nonspecific analysis of DPPH by colorimetry. However, the HPLC method was specific for DPPH and was based on the reduction in DPPH peak area. The superiority of HPLC method was more evident from the detection of small changes in the DPPH absorbance reflected by the peak area even in the presence of mixture of herbal extracts and colorants (Fig. 1(d)).

4. Conclusions

Due to the flooding of the world market with a variety of polyherbal formulations for the ailment of liver disorders, there is an increased demand for rapid standardization methods to avoid the batch-to-batch variations in their efficacies. The DPPH colorimetric method for evaluation of antioxidant activity is with certain shortcomings like failure to indicate antioxidant activity of certain drugs and interference from color pigments. A simple and sensitive HPLC method was developed for screening antioxidant activity of polyherbal formulations. The HPLC method developed was specific for DPPH with an acceptable reproducibility and short run time allowing for rapid determination of radical scavenging activity of several samples. The IC_{50} values determined for the standard antioxidants by the HPLC method agree well with those by colorimetry. The HPLC method was successfully applied for the determination of antioxidant activity of polyherbal formulations and can serve as a quality control tool.

Acknowledgements

The authors thank the Director, IICT, for his encouragement and support for the present investigation. One of the authors, Mr. Chandrasekar, thanks CSIR, New Delhi, for award of SRF.

References

[1] A. Subramoniam, P. Pushpangadan, Ind. J. Pharmacol. 31 (1999) 166–175.
[2] W.B. Williams, M.E. Cuvelier, C. Berset, Lebebsm-Wiss Techn. 28 (1995) 25–30.
[3] O.I. Arouma, B. Halliwell, G. Williamson, in: I. Arouma, S.L. Cuppett (Eds.), Antioxidant Methodology, AOCS Press, IL, USA, 1997, pp. 173–204.
[4] T. Yamaguchi, H. Takamura, T. Matoba, J. Terao, Biosci. Biotech. Biochem. 62 (1998) 1201–1204.
[5] N. Karunakar, M.C. Prabhakar, D.R. Krishna, Arzneim. Forsch. Drug Res. 53 (2003) 254–259.
[6] S.O. Burmistrov, T.I. Oparina, V.M. Prokopenko, Klin. Lab. Diag. 11 (1997) 14–18.
[7] A. Dapkevicius, T.A. van Beek, H.A.G. Niederlander, A.E. de Groot, Anal. Chem. 71 (1999) 736–740.
[8] P. Krishnaiah, V.L.N. Reddy, G. Venkataraman, et al., J. Nat. Prod. 67 (2004) 1168–1171.
[9] I.I. Koleva, H.A.G. Niederlander, T.A. van Beek, Anal. Chem. 72 (2000) 2323–2328.
[10] M. Wang, L. Lagrange, J. Tao, Fitoterapia 67 (1996) 166–171.
[11] V.J. Chrungo, K. Singh, J. Singh, Ind. J. Exp. Biol. 35 (1997) 611–617.
[12] S. Gopumadhavan, S. Jagadeesh, B.L. Chauhan, R.D. Kulkarni, Alcohol.: Clin. Exp. Res. 5 (1993) 1089–1092.
[13] V. Padma, V. Suja, C.S. Shyamala Devi, Fitoterapia 69 (6) (1998) 520–522.
[14] R. Mehrotra, N.K. Natu, R. Dixit, et al., Ind. J. Clin. Pract. 12 (1998) 37–40.
[15] M. Mandal, S. Mukherji, J. Environ. Biol. 22 (2001) 301–305.
[16] C. Gadgoli, S.H. Mishra, Fitoterapia 66 (1995) 319–323.
[17] A.K. Saxena, B. Singh, K.K. Anand, J. Ethnopharmacol. 40 (1993) 155–161.
[18] R. Chander, N.K. Kapoor, B.N. Dhawan, Biochem. Pharmacol. 44 (1992) 180–188.
[19] V.K. Prasanth, S. Shashidhara, M.M. Kumar, B.Y. Sridhara, Pharm. Biol. 39 (2001) 325–328.
[20] B.P. Reddy, V.N. Murthy, V. Venkateshwarlu, C.K. Kokate, D. Rambhau, Ind. Drugs 30 (1993) 338–341.
[21] I.B. Koul, A. Kapil, Ind. J. Pharmacol. 26 (1994) 296–300.