Isolation of Ursolic Acid from Bruckenthalia Spiculifolia Followed by HPLC Analysis

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Ursolic acid is a pentacyclic-terpenic compound that has numerous biological activities, such as anti-inflammatory, antioxidative, antiprotozoal, antimutagenic and anticancer properties. This study aims the optimization of the extraction procedure of ursolic acid from Bruckenthalia spiculifolia by maceration and ultrasonic irradiation and the optimization of high performance liquid chromatographic (HPLC) with photodiode array detector method used for the quantification of ursolic acid present in the extract. The influence of several extraction parameters, such as the concentration of extraction solvent, the ratio of liquid to vegetal material, the size of vegetal material, the extraction time and temperature were evaluated. The optimum extraction conditions proved to be for maceration 80 % aqueous ethanol solution, a ratio of liquid to material of 20 : 1 (mL : g), the particle size between 125 - 1250 μm, process time of six days, temperature of 20 °C and for ultrasonic extraction 80 % aqueous ethanol solution, a ratio of liquid to material of 20 : 1 (mL:g), the particle size of 1250 μm, at a temperature of 30 °C, for 30 min.

Keywords: ursolic acid, maceration, ultrasonic irradiation, HPLC

Ursolic acid ($C_{30}H_{48}O_3$) or acid (3β)-3-hydroxyurs-12-en-28-oic is a hydroxy pentacyclic triterpene acid that has a good chemoprotective activity for the human body. It is spread on a large scale, in nature and it can be isolated from different plant extracts, alone or together with sugars. Based on the pharmacological studies, ursolic acid proved to have remarkable bioactive properties such as: anti-oxidative [1-2], anti-inflammatory [1-2], analgesic [2], anti-bacterial [3-4], anti-glycative [5], anti-tumor [6], anti-HIV [7], anti-carcinogenic [8], cholesterol-lowering [9], hepatoprotective [10], cardio tonic [11], sedative and anxiolytic effects, [12], diuretic [13], but also tonic and modulator effects of collagen synthesis [14]. Ursolic acid has been widely used in cosmetics and health foods industry because of its relatively non-toxicity. Because of its anticancer activities, ursolic acid has been the subject of interest for preclinical anti-cancer research [15]. Some of the bioactive effects of ursolic acid are more intense than other reference substances. For example the anti-inflammatory effect of ursolic acid is twice as intense as in the case of indomethacin, a compound which is used as an anti-inflammatory and non-steroidic reference substance [16].

Ursolic acid can be isolated from the leaves or the flowers of Calluna vulgaris (Ericaceae), Salvia officinalis (Lamiaceae), Pyrola rotundifolia (Pyrolaceae), Staphylea holocarpa (Rosaceae), Rosa woodsii (Rosaceae), from the bark of Olearia paniculata (Asteraceae), or Polylepis australis (Rosaceae) and from the fruits of Loquat (Eriobotrya japonica Lindl.) [17]. At present, many studies are mainly focused on the extraction of triterpene acids such as ursolic acid from vegetal species by maceration, with few concerning on the extraction with ultrasounds or microwave assisted extraction. Some of the modern analytical techniques used for ursolic acid analysis include thin layer chromatography (TLC) [18], capillary zone electrophoresis [19], high performance liquid chromatography (HPLC) [20], and gas chromatography (GC) [21]. Due to its ability to handle high molecular masses and thermally unstable compounds and the possibility of using several selective detection systems, HPLC is one of the most used techniques for the determination of triterpene acids from vegetal samples. The aim of this study was focused on two main objectives, the optimization of the extraction procedures of ursolic acid from leafs, stems and flowers of Bruckenthalia spiculifolia by maceration and ultrasounds and the development of a rapid, simple, effective and reproducible HPLC method for the quantification of ursolic acid from the vegetal extract. The effects of several extraction parameters, such as the concentration of extraction solvent, the liquid:vegetal material ratio, the size of vegetal material, the extraction time and temperature were evaluated.

Experimental part

Chemicals and materials

The ursolic acid standard was purchased from Sigma Chemical (USA) and both methanol and acetonitrile of HPLC grade were obtained from Merck (Germany). Ethanol (95 %) used for the extraction was purchased from Sigma Chemical (USA). The stock solution (1000 μg/mL) was prepared by dissolving the ursolic acid in methanol. The calibration standards (50-1000 μg/mL) were prepared from the stock solution by serial dilutions with methanol. Water purified by a Milli-Q Ultrapure water purification system (Millipore, USA) was used for experiments. The ultrasonic extraction was performed with the Elmasonic Versatile Ultrasonic Cleaner (Elma S30-H).

Sampling and sample treatment

Representative samples of healthy plant material (Bruckenthalia spiculifolia) were harvested during June and

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September, from Băişoara area, located in Cluj County in N-W of Romania. The plant material was sampled from the spontaneous flora species, from unpolluted areas, located far away from polluting industrial units, human establishments and access routes between villages. After harvesting, the vegetal material was manually sorted, weighed and placed in cardboard boxes and transported to the laboratory, where it was air dried and manually crushed. The vegetal material was then stored in paper bags at room temperature, away from direct sunlight and very high humidity. The vegetal samples consisted of 100 g of leafs, stems and flowers.

Extraction by maceration and ultrasonic irradiation

The dried and crushed vegetal material was accurately weighed and kept to macerate for 6 days in contact with the solvent (aqueous ethanol solution 95 %) at room temperature (20 - 22°C), in dark glass flasks, and were shaken each day in order to homogenize the content. For the extraction of ursolic acid by ultrasonic irradiation, the dried and crushed vegetal material was mixed with aqueous solutions of different ethanol concentrations, (30 - 95 %) and placed in a glass flask which was immersed into the ultrasonic bath and irradiated. After extraction, the sample was filtered through a 0.45 μm filter and analyzed by HPLC.

Chromatographic analysis

HPLC analyses were performed on a Shimadzu LC equipped with a Shimadzu SPD-DAD photodiode array detector (Shimadzu Corporation, Japan). The separations were carried out at 25 °C on an ODS C18 reversed-phase column (5 μm, 250 × 4.6 mm), by isocratic elution with a mixture of acetonitrile-ultrapure water (90:10 v/v), at a flow rate of 0.8 mL/min. The wavelength detection was set at 210 nm.

Results and discussions

Validation of HPLC method

The analytical method described above was validated for its linearity, sensitivity, precision, and accuracy.

Linearity

The qualitative analysis of ursolic acid was made based on the retention time, while the quantitative analysis was done by the external standard method. The linearity of the HPLC method was established based on the calibration curve by plotting peak areas against analyte concentration (50; 100; 250; 500 and 1000 μg/mL). Each calibration standard was injected three times. The regression equation of the curve was: $y = 3970.13 \cdot x + 28324.57$ and the coefficient of determination (R^2) was 0.9983, proving that the method has a linear relationship between peak areas and concentrations, (fig.1).

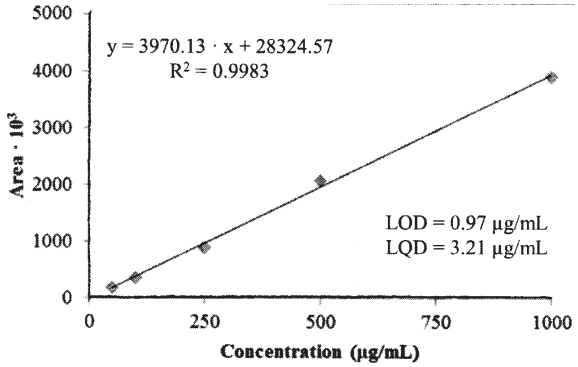


Fig. 1. Calibration curve used for the quantification of ursolic acid

Sensitivity

The sensitivity of proposed method was estimated as the limit of detection (LOD) and quantification (LOQ). LOD and LOQ were calculated based on the signal to noise ratio. LOD (S/N = 3) and LOQ (S/N = 10) were 0.97 μg/mL and 3.21μg/mL, respectively, (fig.1).

Precision

The precision of the quantitative determination of ursolic acid was determined by evaluating the repeatability (intra-day precision) and of the intermediate precision (inter-day precision). For both intra- and inter-day study, three replicates of ursolic acid standard solutions of 100 and 500 μg/mL were analyzed. The precision is expressed as % RSD (relative standard deviation). The results of the analyses showed that the developed HPLC procedure had a good precision, demonstrated by the low RSD which was under 2 % for the intra-day precision and under 3% for the inter-day precision, (table 1).

Accuracy

The vegetal extract was spiked with a standard solution of ursolic acid. The accuracy of the method was determined based on the recovery of the added ursolic acid. The recovery was calculated according to the following formula:

$$recovery[\%] = \frac{detected\ amount - initial\ amount}{spiked\ amount} \cdot 100$$

The method proved to have a high accuracy, since the recoveries of the spiked ursolic acid ranged between 97.50 % to 103.58 %, with a mean value of 99.93 %, (table 2).

Extraction optimization

Ursolic acid was extracted from the vegetal material consisting of a mixture of leafs, stems and flowers of Bruckenthalia spiculifolia by two procedures, maceration and ultrasonic irradiation. The qualitative analysis was performed based on the retention time and the quantification was done by the external standard method, using the calibration curve, (fig.1). Chromatographic analysis of the samples indicates the same retention time.

Conc. (μg/mL)	Intra-Day Precision		Inter-Day Precision	
	Measured Conc. mean[a] ± SD[b] (μg/mL)	RSD[c] (%)	Measured Conc. mean[a] ± SD[b] (μg/mL)	RSD[c] (%)
100	106.34 ± 1.94	1.82	105.06 ± 2.71	2.57
500	505.63 ± 3.81	0.75	507.83 ± 4.23	0.83

[a] average of three determinations; [b] SD = standard deviation; [c] RSD = relative standard deviation

Table 1
PRECISION OF THE HPLC METHOD

Initial amount (µg/mL)	Spiked amount (µg/mL)	Detected amount (µg/mL)	Recovery (%)	Recovery mean ± SD (%)	RSD (%)
125.41	250	371.02	98.24	99.93 ± 2.45	2.45
		384.36	103.58		
		378.23	101.13		
		373.41	99.20		
		369.17	97.50		

Table 2
RECOVERY OF SPIKED URSOLIC ACID

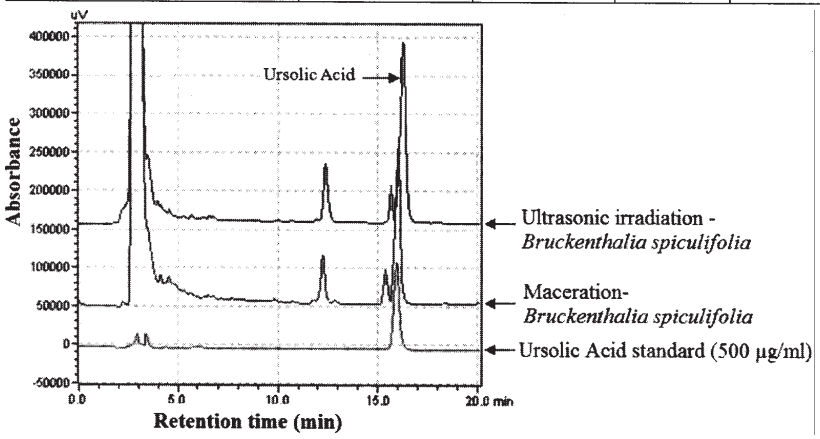


Fig. 2. Overlapping chromatograms corresponding standard solution of ursolic acid and plant extract samples obtained at the optimum process

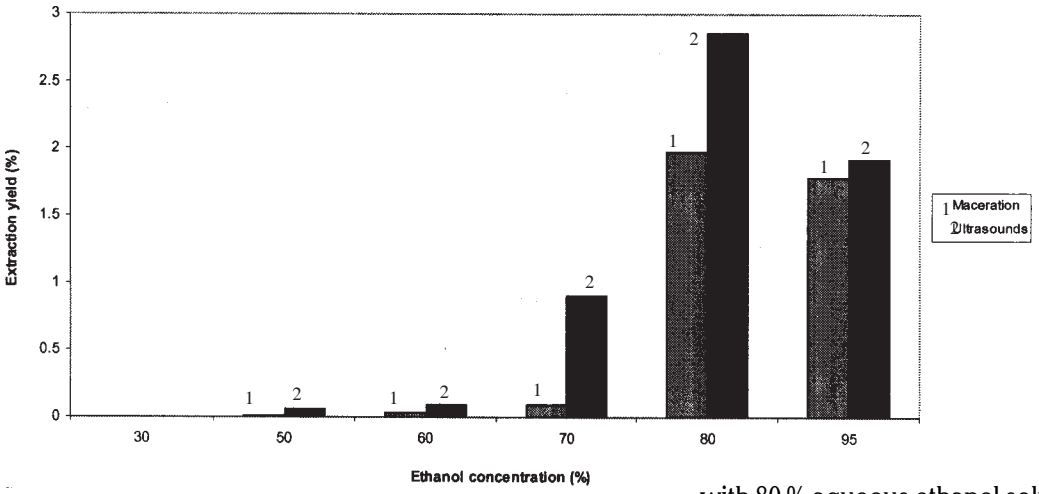


Fig. 3. Effect of aqueous ethanol concentration on the extraction efficiency

The chromatograms of ursolic acid solutions are shown in figure 2.

Effect of ethanol concentration on the extraction efficiency

For testing the effect of ethanol concentration on the extraction efficiency, several tests were performed by using different concentrations (30 - 95%) of aqueous ethanol solutions. The results (fig. 3) showed that the use of aqueous ethanol solution with a concentration of 80 % significantly improves the extraction yield. For maceration the extraction yield reached a maximum level (1.97 %) with 80 % aqueous ethanol solution, while for ultrasounds extraction yield reached a maximum level (2.85 %) with 80 % aqueous ethanol solution.

Effect of ethanol:vegetal material ratio on the extraction efficiency

The effect of liquid:material ratio on the extraction yield was tested by using the following liquid:material ratio 10:1; 20:1; 30:1 and 40:1 (mL:g). The results, (fig. 4) proved that during maceration the yields were higher (up to 2.94 %) at a liquid to material ratio of 20:1, than during ultrasounds

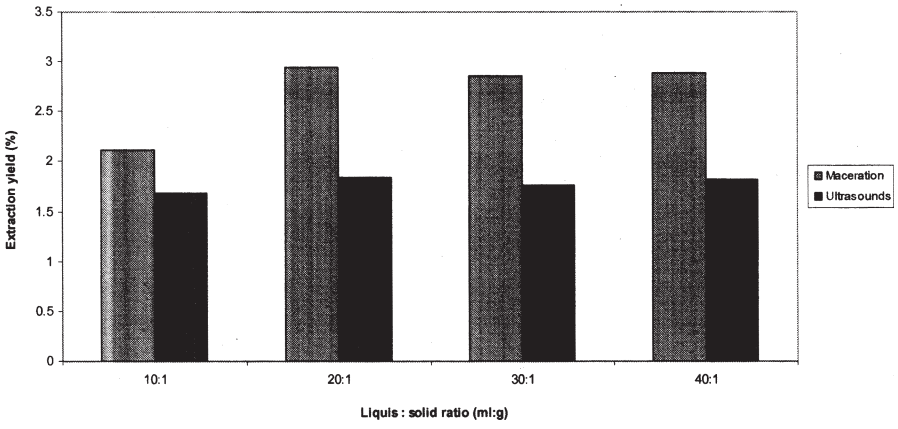


Fig. 4. Effect of ethanol:vegetal material ratio on the extraction efficiency

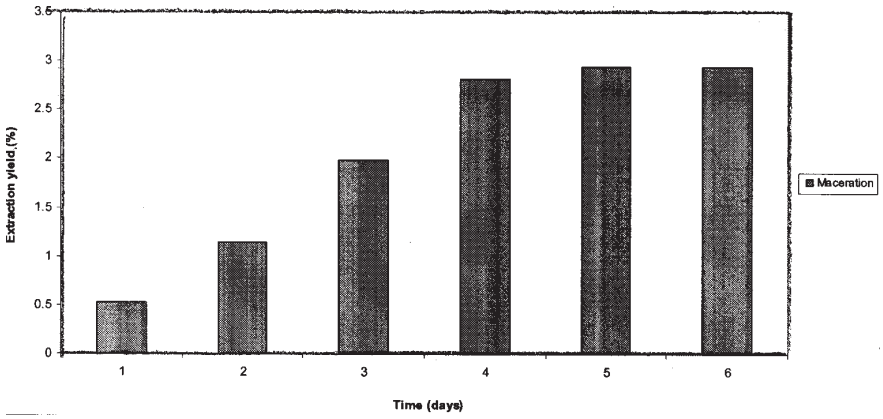
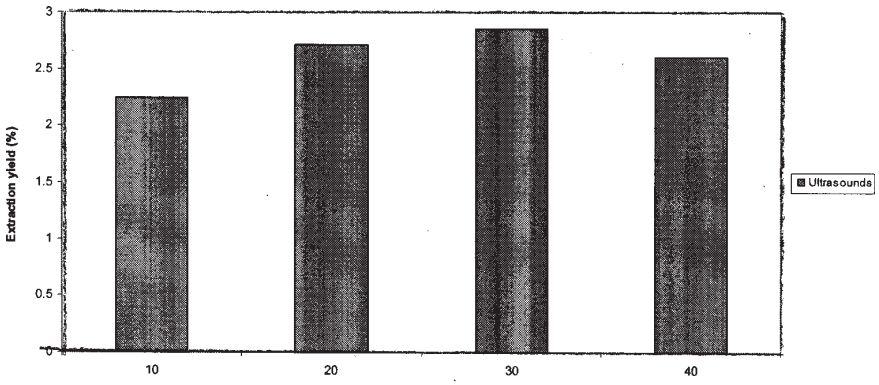


Fig. 5. Effect of time on the extraction efficiency



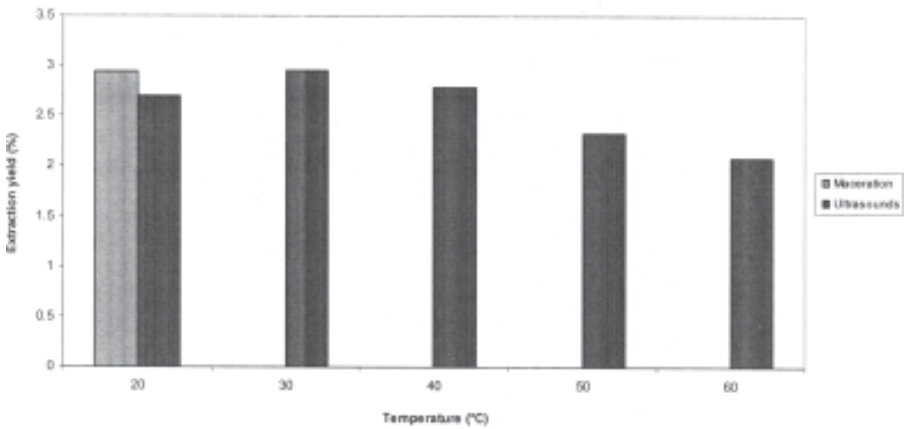


Fig. 6. Effect of temperature on the extraction efficiency

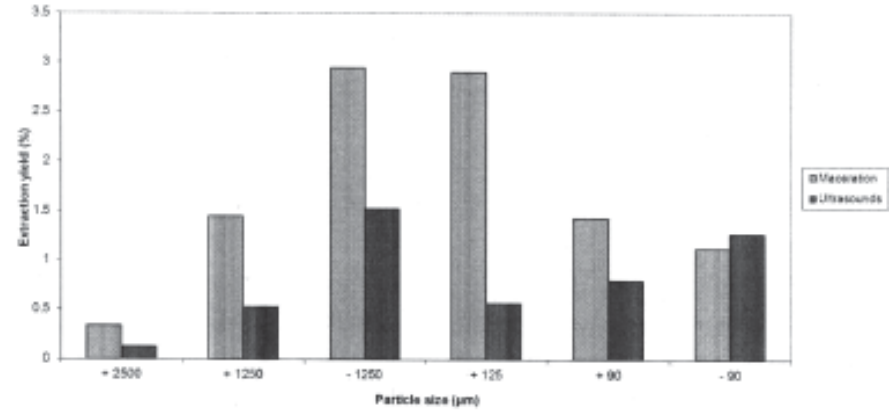


Fig. 7. Particle size effect on the extraction efficiency

extraction, when the yields reached 1.84% at a liquid to material ratio of 20:1.

Effect of time on the extraction efficiency

In order to improve the extraction yields, the influence of time upon the extraction process was tested. The results, (fig.5) showed that there is an increase in the yields of ursolic acid with the increase of irradiating time. A 30 min exposure of ultrasonic irradiation to a solution of 95 % aqueous ethanol has increased the extraction yields at 2.85 %. For maceration, after six days from the beginning of the process, extraction ceases, reaching a yield of 2.94 %.

Effect of temperature on the extraction efficiency

Effects of extracting temperature on the extraction efficiency of ursolic acid were investigated by performing the extraction tests at different temperatures (20, 30, 40, 50 and 60 °C). The results, (fig.6) showed that the extraction yields during ultrasonic irradiation decreased when the temperature increased. The maximum yield for maceration

(2.94%) was registered at 20 °C and for ultrasonic irradiation (2.95%) at 30 °C.

Effect of the vegetal material size on the extraction efficiency

The influence of the size of vegetal material on the yield of extraction has been investigated by selecting six different granulometric fractions, from 90 to 2500 μm. The results, (fig.7) showed that the optimum particle size was between 125 - 1250 μm for maceration and 1250 μm for sonication. The yields were higher during the maceration extraction (2.94 %) than during irradiation extraction (1.52 %).

The results obtained from the survey are comparable with other data reported in the literature [22-23].

Conclusions

Ursolic acid extraction was carried out from Bruckenthalia spiculifolia using two methods, maceration and ultrasonic irradiation. Both methods have been optimized and the results were compared with data from literature. For ultrasonic irradiation the effects of concentration of extraction solvent, the ratio of liquid to vegetal material, the extraction time and temperature were evaluated, the results being comparable with those in the literature. In addition it was studied the influence of the particle size of vegetal material on the extraction. For maceration were not found reliable data matches the extraction from the vegetal material consisting of a mixture of leafs, stems and flowers of Bruckenthalia spiculifolia. The proposed HPLC method proved to be a rapid, precise, reproducible, and sample-saving method, which can be used for quantitative analysis of ursolic acid from vegetal material. The optimum extraction conditions proved to be for maceration: aqueous ethanol solution 80 %, a ratio of liquid to material of 20 : 1 (mL:g), the granulation of plant material between 125 - 1250 μm, temperature of 20 °C for six days, and for ultrasounds: aqueous ethanol solution 80 %, a ratio of liquid to material of 20 : 1 (mL:g), the granulation of plant material 1250 μm and a temperature of 30 °C, for 30 min.

References

1. TSAI S., YIN M., J. Food Sci., **73** (7), 2008, p. 174-178
2. MUNOZ O., CHRISTEN P., CRETTON S., BACKHOUSE N., TORRES V., CORREA O., COSTA E., MIRANDA H., DELPORTE C., J. of Pharma. and Pharmacol., **63** (6), 2001, p. 849-859
3. AZEVEDO M., CAMSARI C., SÁL C., LIMA C., FERREIRA M., WILSON C., Phytother. Res. **24** (S2), 2010, p. 220-224
4. KUREK A., GRUDNIAK A., SZWED M., KLICKA A., SAMLUK L., WOLSKA K., JANISZOWSKA W., POPOWSKA M., LEEUW A., Int. J. G. **97** (1), 2010, p. 61-68
5. WANG Z., HSU C., HUANG C., YIN M., Eur. J. Pharmacol. 628, 2010, p. 255-260
6. H.S. YOUNG, H.Y. CHUNG, C.K. LEE, K.Y. PARK, T. YOKOSAWA, H. OURA, Biol. Pharm. Bull. **17** (7), 1994, p. 990-992
7. KASHIWADA Y., NAGAO T., HASHIMOTO A., IKESHIRO Y., OKABE H., COSENTINO L., LEE K., J. Nat. Prod. 63, 2000, p. 1619-1622
8. RAMOS A., PEREIRA C., COLLINS A., Mutat. Res. **692** (1-2), 2010, 6-11
9. LIN Y., VERMEER M., TRAUTWEIN E., Arterioscl. Throm. Va. **28** (6), E95, 2008, p. 29-37
10. SARAVANAN R., VISWANATHAN P., VISWANATHAN K., PUGALENDI K., Life Sciences, **78** (7), 2006, p. 713-718
11. SOMOVA L., SHODE F., MIPANDO M., Phytomedicine, 11, 2004, p. 121-129
12. PEMMINATI S., GOPALAKRISHNA H., VENKATESH V., RAI A., SHETTY S., VINOD A., PAI P., SHENOY A., J. Appl. Pharm. Sci., **1** (3), 2011, p. 68-71
13. SHIBATA S., J. Korean Med. Sci. 16, 2001, p. 28-37
14. BOTH D., GOODTZOVA K., YAROSH D., BROWN D., Arch Dermatol Res., **293** (11), 2002, p. 569-575
15. NOVOTNY L., VACHALKAOVA A., BIGGS D., Neoplasma, 48, 2001, p. 241-246
16. BARICEVIC D., SOSA S., LOGGIA R., TUBARO A., SIMONOVSKA B., KRASNA A., ZUPANCIC A., J. Ethnopharmacol. 75, 2001, p. 125-;132
17. LIU J., J. of Ethnopharmacol., **49** (2), 1995, p. 57-68
18. SHETTY P., MANGANONKAR K., SANE R., J. Planar Chromatogr., 20, 2007, p. 65-68
19. YANG P., LI Y., LIU X., JIANG S., J Pharm Biomed Anal., **43** (4), 2007, p. 1331-1334
20. TARALKAR S., CHATTOPADHYAY S., J Anal Bioanal Techniques, **3** (3), 2012, p. 1-6
21. JANICSAK G., VERES K., KÁLLAI M., MÁTHÉ I., Chromatographia, 58, 2003, p. 295-299
22. XIA E., WANG B., XU X., ZHU L., Y.SONG, LI H., Int. J. Mol. Sci. **12** (8), 2011, p. 5319-5329
23. XIA E., YU Y., XU X., DENG G., GUO Y., LI H., Ultrasonics Sonochemistry **19** (4), 2012, p. 772-776

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