

**ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF  
*HERITIERA FOMES* LEAVES****Bhupendra Nath Dwivedy<sup>1\*</sup>, Prashant Dabral<sup>1</sup>, Rajeev Kumar<sup>1,2</sup>**<sup>1</sup>Gyani Inder Singh Institute of Professional Studies, Dehradun, Uttarakhand, India.<sup>2</sup>Jodhpur National University, Jodhpur, Rajasthan, India.Article Received on  
27 June 2014,Revised on 22 July 2014,  
Accepted on 17 August 2014**\*Correspondence for  
Author****Bhupendra Nath Dwivedy**Gyani Inder Singh Institute of  
Professional Studies,  
Dehradun, Uttarakhand, India.**ABSTRACT**

Antioxidants have been widely used in the food industry to prolong shelf life. However, there is a widespread agreement that some synthetic antioxidants such as butyl hydroxyl anisole and butyl hydroxyl toluene (BHA and BHT, respectively) need to be replaced with natural antioxidants because of their potential health risks and toxicity. Thus, the search for antioxidants from natural resources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting

peroxidation. Antioxidants that scavenge free radicals play an important role in prevention of cardiovascular disease, aging, cancer, and inflammatory disorders.

**KEY WORDS:** Antioxidant, Heritiera Fomes, DPPH, Ascorbic Acid.**1 INTRODUCTION**

Antioxidants are any substance that delay or inhibits oxidative damage to a target molecule. At a time one antioxidant molecule can react with single free radicals and are capable to neutralize free radicals by donating one of their own electrons, ending the carbon-stealing reaction. Antioxidants prevent cell and tissue damage as they act as scavenger.<sup>[1]</sup> Cell produce defense against excessive free radicals by their preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. A variety of components act against free radicals to neutralize them from both endogenous and exogenous in origin.<sup>[2]</sup> These include:

1. Endogenous enzymatic antioxidants.
2. Non enzymatic, metabolic and nutrient antioxidants.
3. Metal binding proteins like ferritin, lactoferrin, albumin and ceruloplasmin.

Free radicals are produced under certain environmental conditions and during normal cellular function in the body. These molecules are missing an electron, giving them an electric charge. To neutralize this charge, free radicals try to steal an electron from, or donate an electron to, a neighboring molecule. This process, called oxidation, creates a new free radical from the neighboring molecule. The newly created free radical, in turn, searches out another molecule and steals or donates an electron, setting off a chain reaction that can damage hundreds of molecules. Antioxidants halt this chain reaction. Some antioxidants are themselves free radicals, donating electrons to stabilize and neutralize the dangerous free radicals. Other antioxidants work against the molecules that form free radicals, destroying them before they can begin the domino effect that leads to oxidative damage.<sup>[3]</sup>

## **2 Qualitative assay**

### **2.1 Principle**

A Suitably diluted stock solutions were spotted on pre-coated Silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract. The plates were dried at room temperature and were sprayed with 0.02% 2, 2-diphenyl-1-picryl hydrazyl (DPPH) in ethanol. Bleaching of DPPH by the resolved bands was observed for 10 minutes and the color changes (yellow on purple background) were noted.<sup>[4]</sup> DPPH forms deep pink color when it is dissolved in ethanol. When it is sprayed on the chromatogram of the extract, it forms pale yellow or yellow color which indicates the presence of antioxidants.<sup>[5]</sup>

### **2.2 Thin Layer Chromatography (TLC) Technique**

Thin-layer chromatography (TLC) is a chromatographic technique that is useful for separating organic compounds. Because of the simplicity and rapidity of TLC, it is often used to monitor the progress of organic reactions and to check the purity of products. The main purpose of this technique is to detect polar, non-polar and medium polar groups present in the plant extract under UV light at short (254nm) and long (360nm) wavelength.<sup>[6]</sup>

### 2.3 Solvent system used

Groups	Solvent system	Ratio
Polar	Chloroform : Ethanol : Water	40:10:1
Medium polar	Chloroform : Ethanol	5:1
Nonpolar	n- Hexane : Ethyl acetate	2:1

## 3 Methods

### 3.1 TLC plate preparation

Commercially available TLC plate is cut into appropriate size of 10 cm long and 8 cm wide.

### 3.2 Sample application

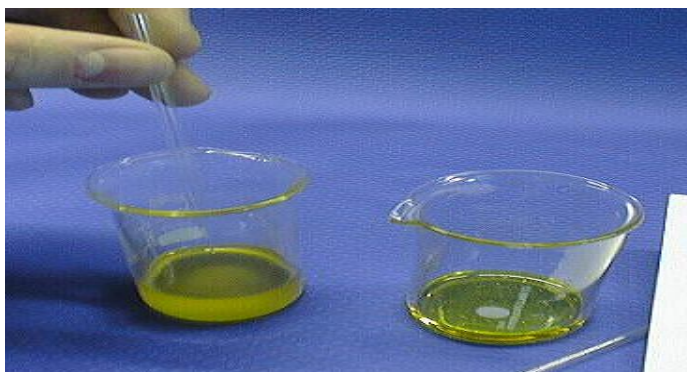
A fine capillary tube was used as spotter for sample application in the TLC plates.

A very little amount of plant extract was taken in a small vial and diluted suitably with ethanol.

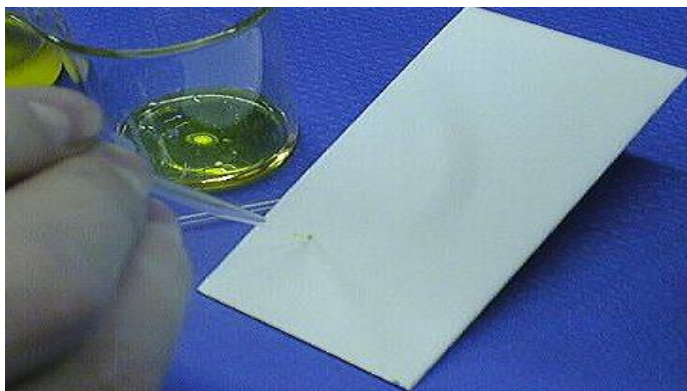
The sample was spotted in uniform size (about 0.3 cm) on TLC plates.

The sample was applied several times in each spot to get better chromatogram.

Each spot was dried before applying another volume of solution to the same spot.



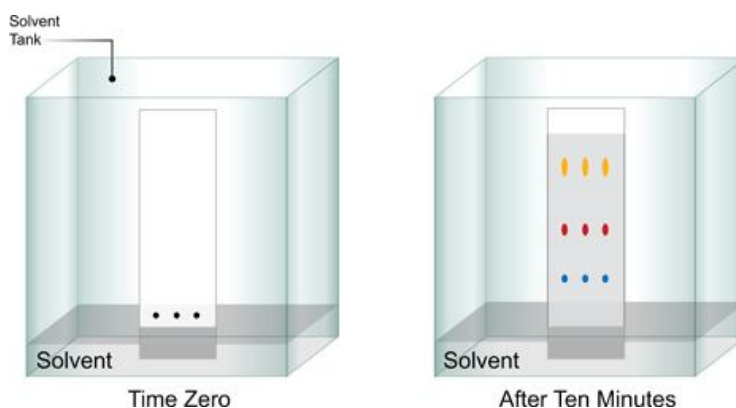
**Fig.1.1: Capillary tube as sample spotter.**



**Fig. 1.2: Spotting of sample**

### 3.3 Development of chromatogram

The chromatogram was developed by ascending technique.



**Fig. 1.3: A typical chromatogram by ascending technique.**

1. Three solvent systems in the ratio mentioned before were kept in three jars due to the presence of different groups of compound in the plant extract.
2. The plates were placed in each jar in such a manner that the sample spots were just above the solvent surface.
3. Filter papers were kept into each jar by wetting with respective solvent systems to keep those jars saturated and the jars were closed tightly.
4. The solvents were allowed to move up the plates by capillary action until they have traveled a distance of about 7-8 cm from the point of application of the sample on a 10 cm plate.
5. The plates were then removed from the jars and dried with a current of air suitably.
6. After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun. And the other plate was sprayed with 10%  $\text{H}_2\text{SO}_4$ .

Again two plates were spotted by the process mentioned above and chromatogram was run in solvent system chloroform and methanol (5:1). After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun. And the other plate was sprayed with 10%  $\text{H}_2\text{SO}_4$ . Following the similar manner again two plates were spotted by the process mentioned above and chromatogram was run in solvent system n-hexane and acetone (2:1). After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun. And the other plate was sprayed with 10%  $\text{H}_2\text{SO}_4$ .

### 3.4 UV positive components detection

After drying the plates, three plates from three solvent systems were observed visually under UV light and various regions were marked.

After these plates were sprayed with 10%  $\text{H}_2\text{SO}_4$  and dried on hot plate at 110-120°C for some time.<sup>[7]</sup>

### 3.5 Antioxidant positive components detection

Remaining three plates from three solvent systems were observed visually under UV light and various regions were marked.

After these plates were sprayed with 0.02% DPPH and dried on hot plate at 110-120°C for some time.<sup>[8]</sup>

### 3.6 Observation

The observed plates are given below which were viewed under UV detector both in short (254 nm) and long (360 nm) wavelength. When the plates were viewed under UV detector a lot of colored and fluorescent positive components were found in short and long wavelengths respectively. After applying DPPH on the TLC plate, yellow color on purple background was observed which indicated the presence of antioxidant components in the extract of *H. fomes*.



**Sample**

(Chloroform: Ethanol: = 5:1 as solvent)



**Standard (Ascorbic acid)**

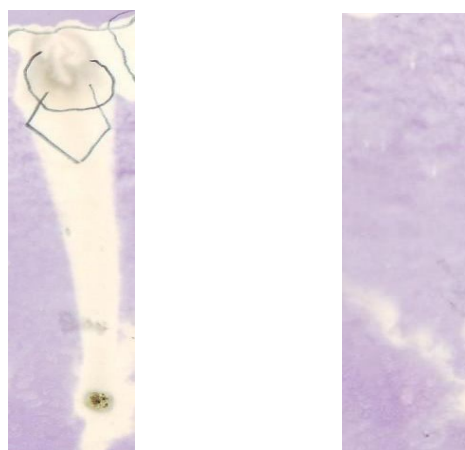


**Sample**

(Chloroform: Ethanol: Water= 40:10:1 as solvent)



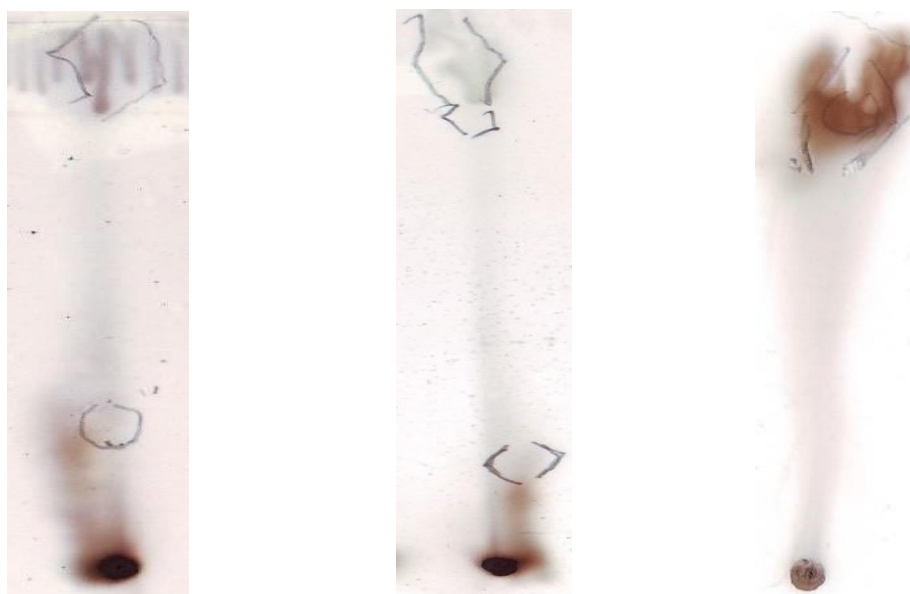
**Standard (Ascorbic acid)**



**Sample                      Standard (Ascorbic acid)**

(n- hexane : ethyl acetate = 2:1 as solvent)

**Fig.1.4: Comparison of TLC plate for *H. fomes* leaves with Standard (Ascorbic acid) after applying DPPH.**



**Sample¹**

**Sample²**

**Sample³**

**Sample¹:** (Chloroform: Ethanol: = 5:1 as solvent)

**Sample²:** (Chloroform: Ethanol: Water = 40:10:1 as solvent)

**Sample³:** (n- hexane : ethylacetate = 2:1 as solvent)

**Fig.1.5: TLC plates of *H. fomes* leaves after applying 10% H<sub>2</sub>SO<sub>4</sub>.**

#### 4 Quantitative analysis

##### 4.1 Principle

The anti-oxidant potential of the ethanolic extract was determined on the basis of their scavenging activity of the stable 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical. DPPH



is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. The aliquots of the different concentrations (1-500 µg/ml) of the extract were added to 3 ml of a 0.004% w/v solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC<sub>50</sub> (Inhibitory conc. 50%) was determined. IC<sub>50</sub> value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals.<sup>[9]</sup> The formula used for % inhibition ratio is-

$$\% \text{ inhibition} = (\text{Blank absorbance} - \text{Sample absorbance} / \text{Blank absorbance}) \times 100$$

## 4.2 Method

### 4.2.1 Preparation of solution

At first 10mg extract of *H. fomes* was measured by electronic balance and mixed with 20 ml of ethanol (99-100%) to prepare 500 µg/ml solution of extract as stock solution.

Another six concentrations of solutions were prepared by proper dilution method. These concentrations were 200 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, 5 µg/ml, 1 µg/ml. The following technique was followed to prepare different concentrations of solutions from stock solution:

$$\frac{\text{Volume which we have to take from stock solution}}{\text{Desired concentration}} \times \text{Desired volume} = \frac{\text{Desired concentration}}{\text{Supplied concentration}}$$

In the same way, various concentrations of ascorbic acid solutions were prepared.

20 mg DPPH powder was measured by electronic balance and mixed with 500 ml of ethanol (99-100%) to prepare 0.004% DPPH solution. It should be kept in cool, dry and dark place.<sup>[10]</sup>

### 4.2.2 Working procedure

1. Seven test tubes were taken and labeled for 500 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, 5 µg/ml, 1 µg/ml.
2. Then 2 ml of solution of each concentration was taken into test tubes designed for each concentration.
3. 6 ml of DPPH solution was then added to every test tube and kept for 30 minutes at dark place.
4. In the same manner solutions of ascorbic acid were taken in seven test tubes and DPPH solution was added and kept for 30 minutes at dark place.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.

- 7 After 30 minutes, absorbance of each test tube was determined by UV spectrophotometer at 517 nm. Noted that before taking absorbance UV spectrometer reading was nullified with blank solvent i.e. ethanol.
- 8 The readings were noted down carefully. Then % of inhibition was calculated as-  

$$\% \text{ inhibition} = [(\text{Blank absorbance} - \text{Sample absorbance}) / \text{Blank absorbance}] \times 100$$
- 9  $IC_{50}$  was determined from % inhibition vs. conc. graph.

## 5 RESULT AND DISCUSSION

The DPPH antioxidant assay is based on the ability of 2,2-diphenyl-1 picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm, and also for visible purple yellow color. When DPPH accepts an electron donated ion by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the change in the absorbance. In the TLC-based qualitative antioxidant assay using DPPH assay, *H. fomes* leaves showed the free radical scavenging properties indicated by the presence of strong yellow spot on a purple background on the TLC plate.

UV spectrophotometer reading of absorbance for Blank solution:

1 <sup>st</sup> reading	2 <sup>nd</sup> reading	Average
0.815	0.813	0.814

**Table 5.1: DPPH Scavenging Assay of Ascorbic acid.**

Conc. (ascorbic acid) ( $\mu\text{g} / \text{ml}$ )	Log Conc.	Absorbance 1 <sup>st</sup> reading	Absorbance 2 <sup>nd</sup> reading	Absorbance Average	% inhibition
1	0	0.730	0.732	0.731	10.19
5	0.699	0.657	0.655	0.656	19.41
10	1	0.502	0.503	0.503	30.20
50	1.699	0.060	0.063	0.062	92.38
100	2	0.033	0.032	0.033	95.94
200	2.301	0.028	0.028	0.028	96.56
500	2.699	0.025	0.025	0.025	96.92



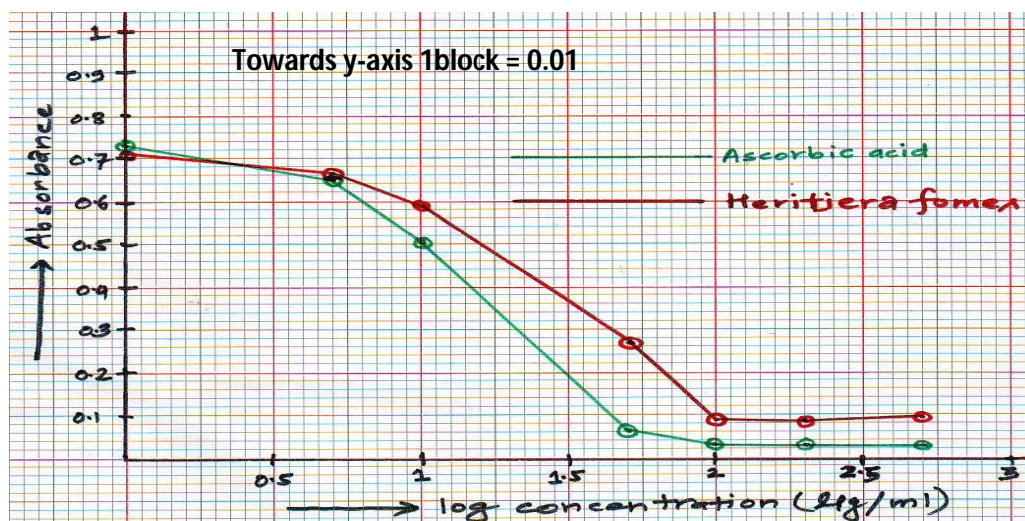


Fig.1.5: Absorbance Vs Log Concentration of Ascorbic acid and *H. fomes*

Table 5.2: DPPH Scavenging Assay of *H. fomes* leaves

Conc. (ascorbic acid) ( $\mu\text{g} / \text{ml}$ )	Log Conc.	Absorbance 1 <sup>st</sup> reading	Absorbance 2 <sup>nd</sup> reading	Absorbance Average	% inhibition
1	0	0.720	0.719	0.7195	11.60
5	0.699	0.661	0.662	0.6615	18.73
10	1	0.596	0.597	0.5965	26.72
50	1.699	0.263	0.267	0.265	67.45
100	2	0.099	0.100	0.0995	87.77
200	2.301	0.095	0.096	0.0955	88.26
500	2.699	0.097	0.098	0.0975	88.022

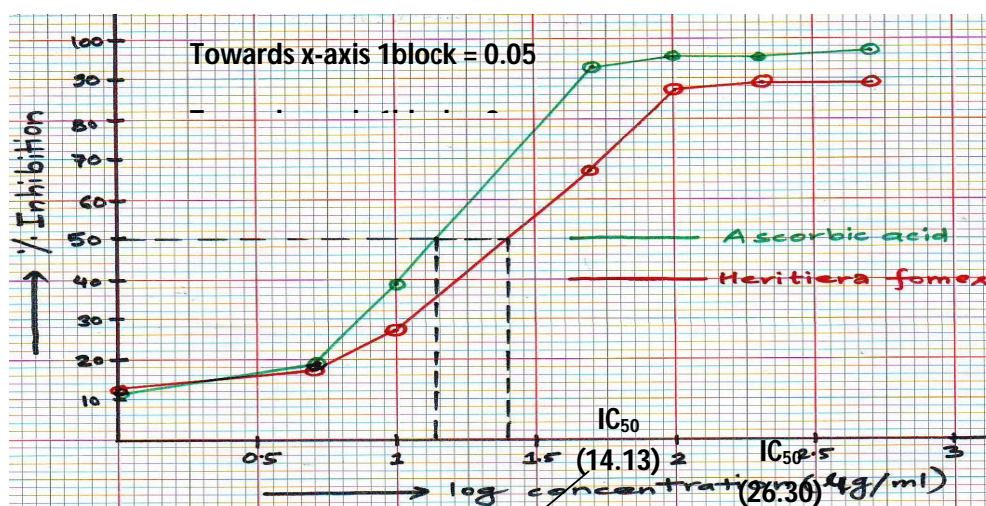


Fig.1.6: Comparison of % inhibition Vs log concentration graph for standard (Ascorbic acid) Vs *H. fomes* leaves ( Sample).

## 6 CONCLUSION

Antioxidants have been widely used in the food industry to prolong shelf life. However, there is a widespread agreement that some synthetic antioxidants such as butyl hydroxyl anisole and butyl hydroxyl toluene (BHA and BHT, respectively) need to be replaced with natural antioxidants because of their potential health risks and toxicity. Thus, the search for antioxidants from natural resources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting peroxidation. Antioxidants that scavenge free radicals play an important role in prevention of cardiovascular disease, aging, cancer, and inflammatory disorders. Recent researches have shown that the antioxidants of plant origin with free-radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress. Plant extracts and phytoconstituents found effective as radical scavengers and inhibitors of lipid peroxidation. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant. In the TLC-based qualitative antioxidant assay using DPPH assay, *H. fomes* leaves showed the free radical scavenging properties indicated by the presence of strong yellow spot on a purple background on the TLC plate. In the quantitative assay, *H. fomes* leaves displayed a very free radical scavenging activity in the DPPH assay ( $IC_{50} = 26.30$  approx.  $\mu\text{g/ml}$ ) which is comparable to that of ascorbic acid ( $IC_{50} = 14.13$  approx.  $\mu\text{g/ml}$ ), a well-known standard antioxidant.

## REFERENCES

1. Jacob R.A., 1995, The integrated antioxidant system, Nutrition Research, 15: 755-766.
2. Prakash A., 2001, Antioxidant Activity, Analytical progress report of Medallion Laboratories, 19 (2): 1-2.
3. Sadhu S. K., Okuyama E., Fujimoto H. and Ishibashi M., 2003, Separation of *Leucas aspera*, a medicinal plant of Bangladesh, guided by prostaglandin inhibitory and antioxidant activities. *Chem Pharm Bull.* 51(5): 595-598.
4. Badami S., Gupta M. K., Suresh B., 2003, Antioxidant activity of the ethanolic extract of *Striga orobanchioides*. Journal of Ethnopharmacology Volume 85, Issues 2-3, Pages 227-230.
5. Dudonne S., Vitrac S., Coutiere P., Woillez M., Merillon J.M., 2009, Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial

- interest using DPPH, ABTS, FRAP, SOD and ORAC assays. J. Agric. Food Chem. 57: 1768-1774.
6. Cioffi G., D'Auria M., Braca A., Mendez J., Castillo A., Morelli I., De Simone F., De Tommasi N., 2002, Antioxidant and Free Radical Scavenging Activity of Constituents of the Leaves of *Tachigalia paniculata*, J. Nat. Prod. 65: 1526-1529.
  7. Ramchoun M., Harnafi H., Alem C., Benlys M., Elrhaffari L., Amrani S., 2009, Study on antioxidant and hypolipidemic effects of polyphenol rich extract from *Thymus vulgaris* and *Lavendula multifida*, Pharmacognosy Research, 1: 106-112.
  8. Dash D.K., Yeligar V.C., Nayak S.S., Ghosh T., Rajalingam D., Sengupta P., Maiti B.C., Maity T.K., 2007, Evaluation of hepatoprotective and antioxidant activity of *Ichnocarpus frutescens* (Linn.) R.Br. on paracetamol-induced hepatotoxicity in rats, Tropical Journal of Pharmaceutical Research, 6: 755-765.
  9. Yildirim A., Oktay M., Bulaloul V., 2001, The antioxidant activity of the leaves of *Cydonia vulgaris*, Turkish Journal of Medical Sciences, 31: 23-27.
  10. Nagulendran K., Velavan S., Mahesh R., Begum V.H., 2007, *In vitro* antioxidant activity and total polyphenolic content of *Cyperus rotundus* rhizomes, E-Journal of Chemistry, 4: 440-449.