

# “Preliminary Phytochemical Screening, DNA Protection, Antioxidant and Anti-proliferative Effect of Seed Extracts of *Bixa orellana* L.”

Yogesh kumar, Dinesh babujestadi and Latha Periyasamy\*

Department of Biochemistry and Molecular Biology, Pondicherry University, Puducherry-605014, India

## Address for Correspondence

Assistant Professor  
Department of  
Biochemistry and  
Molecular Biology,  
Pondicherry  
University, R.V. Nagar,  
Kalapet, Puducherry-  
605014 India.

E-mail:  
[lathaperiyasamy6@gmail.com](mailto:lathaperiyasamy6@gmail.com)

## ABSTRACT

**Objective:** To screen the bioactive compound of *Bixa orellana* L. seed extract and evaluating the antioxidant potential, reducing power, DNA protection ability and anti-proliferative activity against Hep3B cancer cell line. **Methods:** *Bixa orellana* L. seed extracts of different solvents i.e. petroleum ether (PE), chloroform (CE), acetone (AE), methanol (ME) and water (WE) extract were screened for the phyto-bioactive compounds. Rapid free radical antioxidant screening and hydroxyl radical scavenging assay were done by DPPH Dot blot method and by using safranin respectively. Reducing power assay was done by ferric chloride method. DNA damage protective ability was evaluated by Fenton reagent method and anti-proliferative ability against Hep3B cells were carried out by MTT (Thiazolyl blue tetrazolium bromide) assay. **Results:** The screening of bioactive compound shows the presence of alkaloids, saponins, flavonoids, phytosterols, diterpenes, carotenoids, tannins and phenolic compounds. All extracts scavenged the DPPH free radicals indicated by discoloration of purple color background to yellow-white spot. All extract showed the reducing power ability in a dose dependent manner by reducing the ferric chloride. Hydroxyl radical scavenging and DNA protection ability was maximum for PE extract. PE extract shows the anti-proliferative activity against Hep3B cell line and IC<sub>50</sub> values were found to be 78.11 µg/ml and 70.86 µg/ml for 24 and 48h respectively. **Conclusions:** These results suggest that *Bixa orellana* seeds have a good antioxidant potential, DNA protective ability and anti-proliferative effect. Further studies are necessary for detailed chemical characterization and better understanding of their biological activities of the most active ingredients.

**Keywords:** Antioxidants, *Bixa orellana*, Anti-proliferative, DNA damage, Phytochemical screening, DPPH dot-blot.

## INTRODUCTION

From ancient times medicinal plants are used for the treatments of various diseases. In recent year's awareness are more towards natural sources due to various side effects of chemical drugs. Medicinal plants are less expensive, safe and rarely have side effects. *Bixa orellana* belongs to family Bixaceae, commonly known as annatto, economically important as it is a natural source of bixin and used in food and cosmetics from historic times<sup>1</sup>. It is also a source of carotenoid derivatives, terpenoids, tocotrienols, arenes and flavonoids<sup>2,3</sup>. Reactive oxygen species (ROS) are generated as a consequences of cellular metabolism. Cancer cell demand high level of ROS to maintain their high proliferation rate<sup>4</sup>. Carotenoids modulates the genetic damage caused by chemical or mutagens. Antioxidants are the molecules which help to eliminate the toxic compound from the body and decrease the risk of several chronic diseases such as cancer, heart diseases and macular degeneration<sup>5,6</sup>. Seeds, roots and leaves were used to treat inflammatory conditions, fevers and parasitic diseases. Aqueous extract of seeds of *Bixa orellana* has reduced the hyperlipidemia effect in male Swiss mice<sup>7</sup>. The present study is a focus on the phytochemicals present in seed of *Bixa orellana*, antioxidant properties, free radical scavenging activity, anti-proliferative potential and DNA protection ability from oxidative damage.

## MATERIALS AND METHODS

### Chemicals

DPPH was purchased from Sigma Aldrich, Bangalore, India. DMEM medium, fetal bovine serum (FBS) and other chemicals used in the work were purchased from HIMEDIA Pvt. Ltd, Bombay, India. The chemicals used were of analytical grade.

Hep3B cell line were purchased from the National Center for Cell Science (NCCS) Pune, India.

### Plant material collection and authentication

The seeds of *Bixa orellana* were purchased from a local herbal shop in Pondicherry and later it was authenticated by Professor N. Parthasarathy of Department of Ecology and Environmental Sciences, School of Life Sciences, Pondicherry University, Puducherry-605014, India.

### Cell lines and culture conditions

Hep3B cells were cultured in DMEM, high glucose containing 10% FBS and maintained at 5% CO<sub>2</sub> at 37 °C. The medium was supplemented with antibiotic penicillin 100U/ml and streptomycin 10µg/ml.

### Preparation of *Bixa orellana* seed extracts

The seeds were made into powder through a mechanical grinder and 76 gram of the seed powder soxhletted sequentially with 250ml of each solvent namely petroleum ether, chloroform, acetone and methanol. For water extract, residues were heated at 80°C with 250ml of water for 2hours. Further, it was filtered through Whatman no. 1 filter paper and evaporated through rotavapor. The extract was stored in the fridge for further studies. Percent of yield was calculated as below:

$$\% \text{ extract yield} = (W_1/W_2) \times 100.$$

Where W<sub>1</sub> is the weight of the extract obtained after soxhletion and W<sub>2</sub> is the total weight of powder taken for the extraction.

### Phytochemical screening

Phytochemical test was carried out for five different extracts i.e. petroleum ether (PE) extract, chloroform extract (CE), acetone extract (AE), methanol extract (ME) and water extract (WE) by standard procedure<sup>8</sup>.

### The rapid Dot-Blot staining assay for antioxidant activity by 1, 1-diphenyl -2-picryl hydrazyl (DPPH)

Different concentration (5, 10, 20, 30 and 50µg) of the extract was spotted on the Aluminum TLC plate (silica gel 60 F254 Merck, India). TLC plate was air dried and stained by keeping the plate upside down for 10 Sec in 0.4mM DPPH solution in methanol. The plates were incubated for 10 min in the dark. The active antioxidant constituents of the extract were detected as white spot against a purple background. Ascorbic acid was used as standard<sup>8</sup>.

### Total antioxidants by phosphomolybdenum method

The total antioxidant activity of the extract was evaluated by the phosphomolybdate method<sup>9,10</sup>. An aliquot of 0.1 ml of sample solution was mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. Tubes were capped and incubated at 95°C for 90 min. The tubes were allowed to cool to room temperature and the absorbance was taken at 695 nm against a blank. Ascorbic acid was used as a standard. The total antioxidant capacity was expressed as a milligram equivalent of ascorbic acid.

### Reducing power assay

The reducing power of the extract was evaluated according to the method of Aiyegoro and Okoh<sup>11</sup>. Briefly, 1 ml of the various concentration of the extract were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of K<sub>3</sub>Fe (CN)<sub>6</sub> (1%w/v). The resulting mixture was incubated at 50°C for 20 min in water bath, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was later centrifuged at 3000 rpm for 10 min. 2.5 ml of upper layer was collected, mixed with distilled water (2.5 ml) and 0.5 ml of FeCl<sub>3</sub> (0.1%, w/v). The absorbance was then measured at 700 nm against blank.

Butylated hydroxyanisole (BHA) was used as standard.

### Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay determined by Sun *et al*<sup>12</sup>. The reaction mixture consists of 0.8 ml of 6% v/v H<sub>2</sub>O<sub>2</sub>, 1ml of 10 mmolL<sup>-1</sup> of ferrous ammonium sulphate-EDTA complex, 0.1 ml of safranin (0.5mg/ml) and 1ml of different concentration of extract was added. It was incubated for 1 hour in the dark. After that it was incubated at 40°C for 30 min, and absorbance was taken at 520nm. The change in absorbance indicates the hydroxyl radical scavenging capacity of extracts. The scavenging rate was expressed as:

Scavenging rate (%) =  $(A_0 - A/A_0) \times 100$ , where A<sub>0</sub> is the absorbance of control and A is the absorbance of the extracts.

Ascorbic acid was used as positive control.

### In vitro cytotoxicity activity (MTT) assay

The cytotoxicity of the petroleum ether seed extract against the Hep3B cells was determined using MTT (Thiazolyl blue tetrazolium bromide) assay adapted from Mossmann<sup>13</sup>. Cells were seeded into 96-well plates at 10,000 cells/well and treated with different concentration of the PE extracts. After 24h and 48 h of treatment period, 20 µl of MTT (5mg/ml) was added to each well and incubated for 4h in dark at 37°C, the formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm using a micro plate ELISA reader (Molecular Devices Inc., Sunnyvale, CA, USA). All experiments were performed in three replicates. Percentage cytotoxicity of the cell was calculated using the following formula:

$$\% \text{ cell survival} = [(A_C - A_T) / A_C] \times 100$$

Where A<sub>c</sub> = absorbance of the control cells, A<sub>T</sub> = absorbance of the extract treated cells.

### DNA damage protection assay

Genomic DNA was isolated from Hep3B cells. 2µg of DNA was mixed with 5µl of 1mM FeCl<sub>3</sub>, 5µl of 0.1mM Tris-buffer (pH 7.4). Then, 10 µl of 2% different extract was added. After adding 5µl of 5mM H<sub>2</sub>O<sub>2</sub>, the mixture was incubated at 37°C for 30 min. After the incubation, test samples were analyzed on 0.8% agarose gels at 100 V with 1xTAE buffer at room temperature for 30 min. Quercetin (5mM) was used as positive control<sup>14</sup>. Gels were scanned on a syngene gel documentation system.

### Statistical analysis

The experimental results are expressed as mean ± SD of triplets. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey HSD with the help of SPSS 16 for windows. A value of  $p \leq 0.05$  was considered statistically significant. The IC<sub>50</sub> values were calculated by linear regression analysis.

## RESULTS AND DISCUSSION

Plants are a rich source of antioxidants. It protects the cells from various oxidants that has potential to cause damage to cells that may lead to cancer. Antioxidants depending upon solubility may be classified as water soluble or lipid soluble. Water soluble antioxidants protect the cell cytoplasm and blood plasma whereas lipid soluble antioxidants protect the cell membrane from lipid peroxidation<sup>29,30</sup>. The percentage extractive value for all five extracts were calculated. ME extract gave maximum yields whereas yields of AE extract was low. PE extract, CE extract and WE extract gave the approximately same yields (Table 1).

### Phytochemical analysis

A preliminary phytochemical screening was done for all the five extracts prepared. The PE extract found to contains phytosterols. CE extract gave positive results

for the presence of alkaloids, phytosterols, diterpenes and carotenoids. Similarly AE extract found positive for alkaloids. ME extract showed the presence of alkaloids and carbohydrates whereas water extract found to contains carbohydrates, saponins, flavonoids, tannins and phenolic compounds and diterpenes (Table 2).

### Antioxidant activity of *Bixa orellana* seed by Dot-blot assay

Natural antioxidants present in medicinal plants are responsible for inhibiting the harmful consequences of oxidative stress. Many plants extract exhibit efficient antioxidant properties due to their active phytoconstituents, including phenolics and related compounds<sup>28</sup>. The antioxidant capacity of *Bixa orellana* seed extract was evaluated semi-quantitatively by a rapid DPPH Dot-blot staining method. DPPH is a stable free radical and accepts electron or hydrogen radical to become stable diamagnetic molecule<sup>28</sup>. The DPPH free radicals were scavenged by anti-oxidants present in the plant. The discoloration of Purple color of DPPH reagent to yellow-white spots by *Bixa orellana* seed extract revealed the free radical scavenging property of the extract (Figure 1). All extract has shown positive response towards DPPH scavenging activity.

### Determination of total antioxidant activity

The phosphomolybdenum method is based on the reduction of molybdenum by the antioxidants and the formation of a green molybdenum (v) complex, which has maximum absorption at 695nm. The total antioxidant capacity observed in the extract of *Bixa orellana* is expressed as a milligram equivalent of ascorbic acid per gram of the extract (Figure 2). Chloroform extract has shown maximum total antioxidant activity (311.12mg of ascorbic acid per gram of chloroform extract). PE, AE, ME and WE

extract has shown 71.88mg, 92.56mg, 21.12mg and 32.92 mg of ascorbic acid per gram of the respective extract. The measurement of antioxidant activity reflects the meaningful comparison of antioxidants compounds and for the diagnosis and treatment of oxidative stress associated ageing, cancer and neurodegenerative diseases<sup>32</sup>.

#### Reducing power assay

Electron transfer ability of a compound determines its reducing power capacity and serves as a significant indicator of its potent antioxidant potential by reducing  $\text{Fe}^{3+}$ /ferricyanide complex to its form<sup>28</sup>. In this assay according to potential of the extract, the yellow color of a test solution changes to green and blue. Greater is the absorbance at 700nm greater is the reducing power. Figure 3 represents the reductive capabilities of the different extract. The extract was observed for the increase in reducing power while increasing concentration, but in comparison with standard butylated hydroxyanisole it was very low. The order of reducing ability of the extract was  $\text{CE} > \text{AE} > \text{PE} > \text{WE} > \text{ME}$  extracts. The result showed the reducing capacity of the extract, which is an important parameter for a compound to be a good antioxidant<sup>27</sup>.

#### Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive oxygen species since it can pass easily through the cell membrane and it induces damage to the several biomolecules such as DNA, lipids and protein<sup>15,16</sup>. The scavenging effect of extract and quercetin are shown in figure 4. All extract except WE, scavenged the hydroxyl radical formed in a dose dependent manner. The IC<sub>50</sub> values for quercetin, PE, CE, AE and ME were found to be 40.81µg/ml, 40.68µg/ml, 52.4681µg/ml, 53.38µg/ml and 42.5781µg/ml respectively. Hydroxyl radical scavenging activity was maximum for PE extract and effectiveness

was found to be as that of quercetin followed by ME, CE and AE extract. The hydroxyl radical scavenging activity may be attributed to the presence of different phytochemical constituents in the extracts.

#### Anti-proliferative activity

Various plants have been found to inhibit tumor growth. The anti-proliferative assay by MTT determines the integrity of mitochondria and reflects the viability of the cells<sup>31</sup>. The results of MTT assay showed that the petroleum ether extract of *Bixa orellana* seed inhibited the proliferation of Hep3B liver cancer cell in time and dose dependent manner (Figure 5). The respective Inhibitory concentration 50 (IC<sub>50</sub>) was 78.11 µg/ml and 70.86 µg/ml for 24h and 48h of treatment. Phytosterol is known to have anti-cancer potential and retarded the growth and spread of breast cancer cell in mice<sup>18,33</sup>. The presence of phytosterols in the petroleum ether extract of the seed of *Bixa orellana* may be responsible for anti-proliferative activity of the Hep3B cancer cell line.

#### DNA damage protection assay

Hydroxyl radicals formed by Fenton reaction induces breaks in DNA strands. Different seed extracts of *Bixa orellana* was evaluated to assess the prevention of oxidative DNA damage by hydroxyl ( $\bullet\text{OH}$ ) radicals. Combined treatment with  $\text{FeCl}_3$  and  $\text{H}_2\text{O}_2$  in the absence of plant seed extract led to the oxidative damage of the genomic DNA producing base radicals and sugar radicals. The maximum prevention of DNA damage shown by the PE extract. CE, AE, WE extract also prevented the DNA from oxidative damage to some extent but ME extract does not protect the DNA at a 200µg / ml concentration (Figure 6). DNA protection activity from Fenton reaction may be attributed to the presence of various antioxidant phytochemical constituents which were reported to neutralize different free



radical agents like superoxide, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl (OH) and peroxy (ROO) by diverse mechanisms including electron donation as reducing agent<sup>24</sup>.

## CONCLUSIONS

Plants are a good source of bioactive compounds with negligible side effects. These bioactive compounds play a significant role in terminating the generation of free radical chain reactions<sup>17</sup>. *Bixa orellana* is a plant of interest from historic times due to different culinary and medicinal use. Hence this study was carried out to evaluate the *Bixa orellana* seed for antioxidants, anti-proliferative and DNA protective ability from oxidative stress. The qualitative phytochemical analysis of different solvent extract has revealed the presence of alkaloids, carbohydrates, tannins, phenolic compounds, carotenoids, flavonoids, diterpenes and Phytosterols. Flavonoids are the most important natural phenolic and they possess a broad spectrum of chemical and biological activities including free radical scavenging properties<sup>19</sup>. Flavonoids reduce free radicals by up-regulating, quenching and have a variety of biological activities, such as antioxidative<sup>20</sup>, antidepressant<sup>21</sup>, anti-inflammatory<sup>22</sup> and cancer preventive<sup>23</sup>. The presence of these phytochemicals such as tannins, flavonoids, carotenoids and phenols in *Bixa orellana* seed extract may give credence to its local medicinal usage for different ailments. Tannins have been used traditionally for the treatment of diarrhea, hemorrhage and detoxification<sup>25,26</sup>.

From this study, it can be concluded that different seed extracts of *Bixa orellana* demonstrated good antioxidant, reducing power, free radical scavenging activity, DNA protection ability as well as anti-proliferative properties. Further studies are needed for isolation, structural elucidation, and screening of active principles. This study provides experimental evidence and supports the folkloric use of this plant.

## ACKNOWLEDGMENTS

The authors acknowledge the infrastructural facility provided by DBT-IPLS, Pondicherry University.

## REFERENCES

1. Giuliano G, Al-Babili S, von Lintig J. Carotenoid oxygenases: cleave it or leave it. *Trends in plant science*. 2003; 8(4):145-9. Epub 2003/04/25.
2. N. Frega, M. Mozzon, Bocci F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *Journal of the American Oil Chemists' Society*. 1998; 75(12):1723-7.
3. Isaac J.O. Jondiko, Pattenden G. Terpenoids and an apocarotenoid from seeds of *Bixa orellana*. *Phytochemistry*. 1989; 28(11):3159–62.
4. Sosa V, Moline T, Somoza R, Paciucci R, Kondoh H, ME LL. Oxidative stress and cancer: an overview. *Ageing research reviews*. 2013; 12(1):376-90. Epub 2012/11/06.
5. Lampe JW. Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies. *The American journal of clinical nutrition*. 1999; 70(3 Suppl): 475S-90S. Epub 1999/09/09.
6. Stanner SA, Hughes J, Kelly CN, Buttriss J. A review of the epidemiological evidence for the 'antioxidant hypothesis'. *Public health nutrition*. 2004; 7(3):407-22. Epub 2004/05/22.
7. Ferreira JM, Sousa DF, Dantas MB, Fonseca SG, Menezes DB, Martins AM, et al. Effects of *Bixa orellana* L. seeds on hyperlipidemia. *Phytotherapy research: PTR*. 2013; 27(1): 144-7. Epub 2012/03/28.
8. Mandal S, Patra A, Samanta A, Roy S, Mandal A, Mahapatra TD, et al. Analysis of phytochemical profile of Terminalia arjuna bark extract with anti-oxidative and antimicrobial properties. *Asian Pacific journal of tropical biomedicine*. 2013; 3(12):960-6. Epub 2013/10/08.
9. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of

- antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical biochemistry*. 1999; 269(2):337-41. Epub 1999/05/01.
10. Wan C, Yu Y, Zhou S, Liu W, Tian S, Cao S. Antioxidant activity and free radical-scavenging capacity of *Gynura divaricata* leaf extracts at different temperatures. *Pharmacognosy magazine*. 2011; 7(25):40-5. Epub 2011/04/08.
  11. Aiyegoro OA, Okoh AI. Preliminary phytochemical screening and in vitro antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. *BMC complementary and alternative medicine*. 2010; 10:21. Epub 2010/05/18.
  12. Sun X, Hao L, Ma H, Li T, Zheng L, Ma Z, *et al*. Extraction and in vitro antioxidant activity of exopolysaccharide by *Pleurotus eryngii* SI-02. *Brazilian journal of microbiology*: [publication of the Brazilian Society for Microbiology]. 2013; 44(4):1081-8. Epub 2014/04/02.
  13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*. 1983; 65(1-2):55-63. Epub 1983/12/16.
  14. Jiang Y, Han W, Shen T, Wang MH. Antioxidant Activity and Protection from DNA Damage by Water Extract from Pine (*Pinus densiflora*) Bark. *Preventive nutrition and food science*. 2012; 17(2):116-21. Epub 2012/06/01.
  15. Gutteridge JM. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *The Biochemical journal*. 1984; 224(3):761-7. Epub 1984/12/15.
  16. Spencer JP, Jenner A, Aruoma OI, Evans PJ, Kaur H, Dexter DT, *et al*. Intense oxidative DNA damage promoted by L-dopa and its metabolites. Implications for neurodegenerative disease. *FEBS letters*. 1994; 353(3):246-50. Epub 1994/10/24.
  17. Basma AA, Zakaria Z, Latha LY, Sasidharan S. Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta* L. *Asian Pacific journal of tropical medicine*. 2011; 4(5):386-90. Epub 2011/07/21.
  18. Bradford PG, Awad AB. Phytosterols as anticancer compounds. *Molecular nutrition & food research*. 2007; 51(2):161-70. Epub 2007/02/03.
  19. MF Ghafar KP, KK Weng, A Ismail. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. *African Journal of Biotechnology*. 2010; 9(3):326-30.
  20. Moure As, JMC, DF, JMD, Ânguez, *et al*. Natural antioxidants from residual sources. *Food Chem*. 2001; 72: 145-71.
  21. Butterweck V, Jurgensliemk G, Nahrstedt A, Winterhoff H. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. *Planta medica*. 2000; 66(1):3-6. Epub 2000/03/08.
  22. Crespo ME, Galvez J, Cruz T, Ocete MA, Zarzuelo A. Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS. *Planta medica*. 1999; 65(7):651-3. Epub 1999/11/27.
  23. Le Marchand L. Cancer preventive effects of flavonoids-- A review. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapy*. 2002; 56(6):296-301. Epub 2002/09/13.
  24. Esra Maltas SY. Evaluation of Phytochemicals and Antioxidant Activity of *Ginkgo biloba* from Turkey. *Pharmacologia*. 2012; 3(4):113-20.
  25. D.E. Okwu INE. Evaluation of The Phytonutrients and Vitamins Content of Citrus Fruits. *International Journal of Molecular Medicine and Advance Sciences*. 2006; 2(1):1-6.
  26. Afolayan A J MBO. Ethnobotanical study of medicinal plants used as anti-obesity remedies in Nkonkobe Municipality of South Africa. *Pharmacognosy Journal*. 2010; 2(11):368-73.
  27. Omidreza Firuzi, Antonio Lacanna, Rita Petrucci, Giancarlo Marrosu, Luciano Saso. Evaluation of the antioxidant activity of flavonoids by “ferric reducing antioxidant power” assay and cyclic voltammetry. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 2005; 1721(1-3): 174–184.
  28. Ramasamy Subramanian, Palanivel Subbramaniyan and Vairamuthu Raj.

- Antioxidant activity of the stem bark of *Shorea roxburghii* and its silver reducing power. *SpringerPlus*. 2013; 2(28).
29. Silvia Vertuani, Angela Angusti and Stefano Manfredini. The Antioxidants and Pro-Antioxidants Network: An Overview. *Curr Pharm Des*. 2004; 10(14):1677-94.
  30. HELMUTSIESI. Impaired physiological society symposium: endothelial and smooth muscle cell function in oxidative stress. *Experimental Physiology*. 1997, 82, 291-295.
  31. Aziz DM. Assessment of bovine sperm viability by MTT reduction assay. *Anim Reprod Sci* 2006; 92; 1–8.
  32. Apak, Reşat; Güçlü, Kubilay; Özyürek, Mustafa; Bekdeşer, Burcu; Çekiç, Sema; Bener, Mustafa; Çelik, Esin. Antioxidant activity/capacity assays, cuprac assay and its importance to human health. *Turkish Journal of Biochemistry*. 2012. 37(S1), p53.
  33. Awad AB, Downie A, Fink CS, Kim U. Dietary phytosterol inhibits the growth and metastasis of MDA-MB-231 human breast cancer cells grown in SCID mice. *Anticancer Res*. 2000 Mar-Apr; 20(2A):821-4.

**Table 1.** Extractive value of different solvent extract of *Bixa orellana* seed

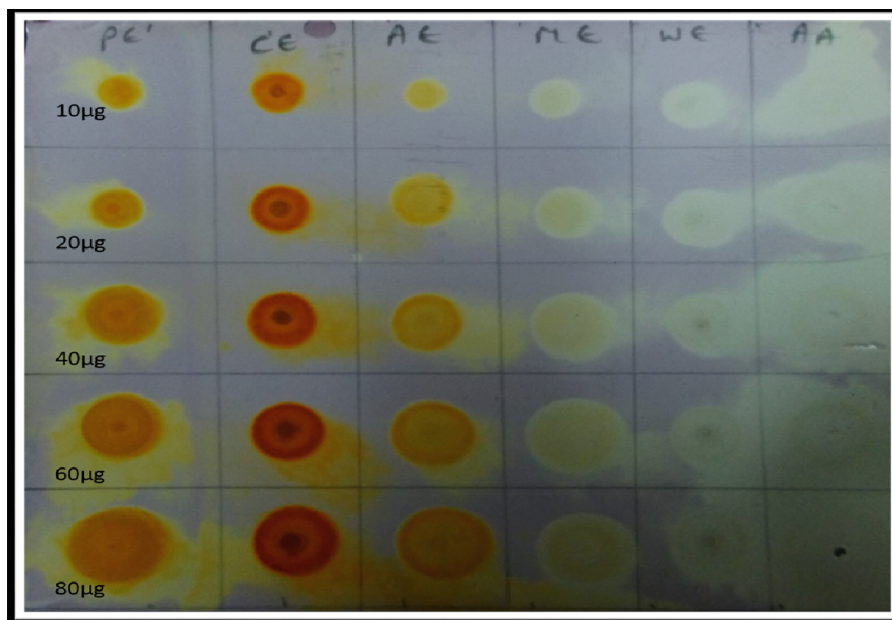
Extract	Petroleum ether (PE)	Chloroform (CE)	Acetone (AE)	Methanol (ME)	Water (WE)
Extractive value	4.23%	4.20%	0.68%	8.57%	4.42%



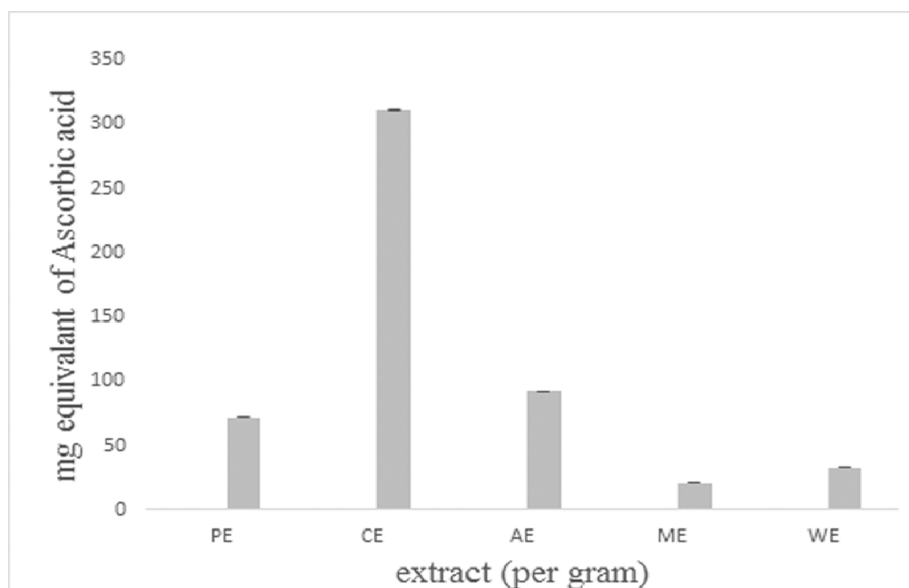
**Table 2.** Preliminary phytochemical analysis of different solvent extract of seeds of *Bixa orellana*

Name of the phytoconstituents	Test name	Name of the extracts				
		Petroleum-ether (PE)	Chloroform (CE)	Acetone (AE)	Methanol (ME)	Water (WE)
Alkaloids	Mayer's test	-	+	+	-	-
	Wagner's test	-	+	+	-	-
	Hager's test	-	+	+	+	-
	Dragendorff's test	-	+	+	+	-
Carbohydrate	Benedict's test	-	-	-	-	+
	Fehling's test	-	-	-	+	-
Saponins	Foam test	-	-	-	-	+
Flavonoids	Alkaline reagent test	-	-	-	-	+
	Lead acetate	-	-	-	-	-
Tannins and phenolic compounds	Lead acetate test	-	-	-	-	+
	Ferric chloride test	-	-	-	-	+
Phytosterol's	Salkowski's test	-	+	-	-	-
	Liebermann-Burchard's test	+	+	-	-	-
Detection of Diterpene	Copper acetate test	-	+	-	-	+
Test for carotenoids	With sulphuric acid	-	+	-	-	-

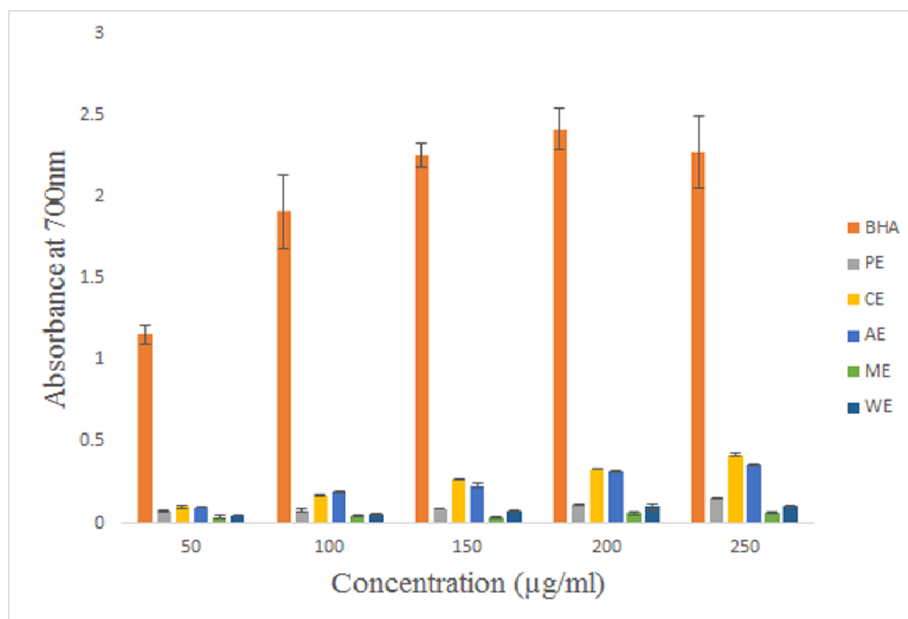
Positive (+) and Negative (-) sign represent the presence and absence of the phytoconstituents respectively.



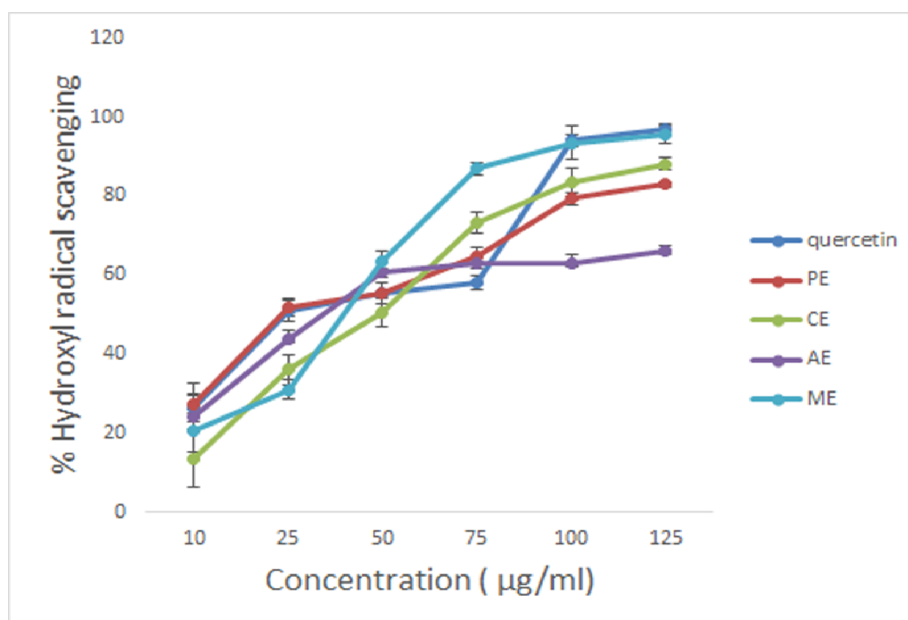
**Figure 1.** Dot-blot assay for antioxidant activity of *Bixa orellana* extract by DPPH. Different solvent extract i.e. Petroleum ether (PE) extract, chloroform extract (CE), acetone extract (AE), methanol extract (ME), water extract (WE) were spotted and ascorbic acid (AA) was used as standard



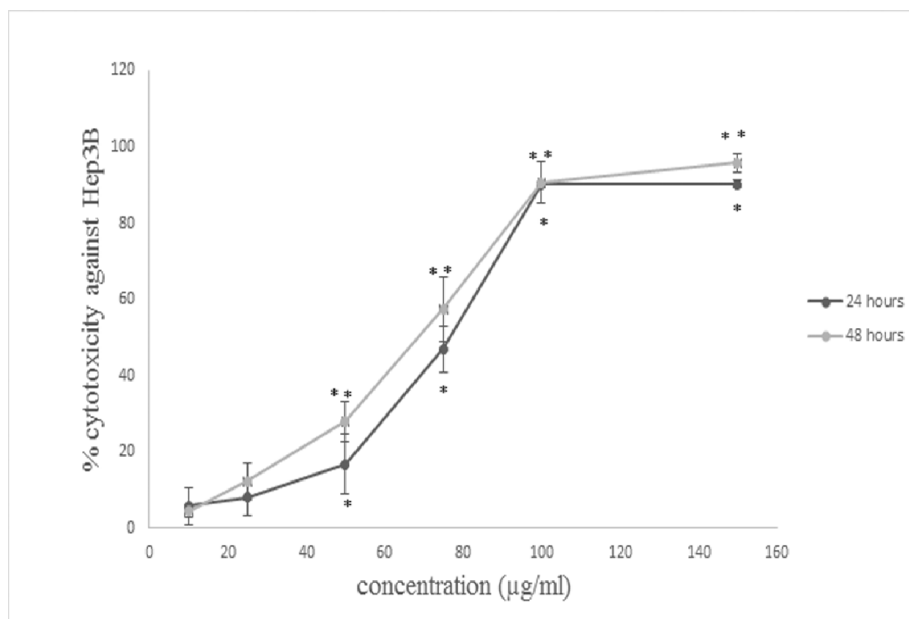
**Figure 2.** Total antioxidant capacity of the different extract compared to ascorbic acid



**Figure 3.** Reducing power of the *Bixa orellana* seed extract

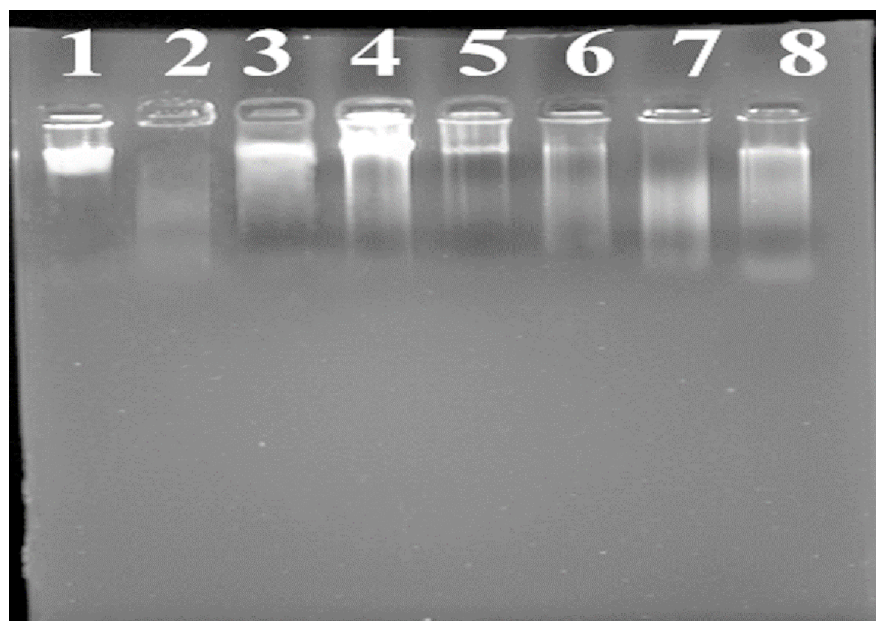


**Figure 4.** Hydroxyl radical scavenging activity of *Bixa orellana* seed extract



**Figure 5.** *In vitro* anti-proliferative effect of petroleum ether extract of seeds of *Bixa orellana*

Hep3B cells were exposed to PE extract for 24h (\*) and 48h (\*\*) and inhibitory concentration 50 were 78.11 µg/ml and 70.86 µg/ml respectively. The data are expressed as percentage cytotoxicity and represent the means  $\pm$  S.D. of three independent experiments. The values were compared to control by one way ANOVA (using SPSS 16 for windows) at a p value  $\leq$  0.05.



**Figure 6.** DNA damage protection assay of *Bixa orellana* extract by Fenton reaction

Lane 1: untreated control DNA (2µg); lane 2: DNA (2µg) + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 3: DNA (2µg) + quercetin (5mM) + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 4: DNA (2µg) + 10µl 2% PE extract + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 5: DNA (2µg) + 10µl 2% Chloroform extract + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 6: DNA (2µg) + 10µl 2% acetone extract + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 7: DNA (2µg) + 10µl 2% methanol extract + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM); lane 8: DNA (2µg) + 10µl 2% water extract + FeCl<sub>3</sub> (1mM) + H<sub>2</sub>O<sub>2</sub> (5mM).