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Original Research Article

In vitro antioxidant anti inflammatory and cytotoxicity activities from Hexane extract of *Bryonopsis laciniosa* fruits

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Abstract

Bryonopsis laciniosa also known as "Shivlingi" annual climber with bright red fruits and is reported to be highly medicinal in India. As a folk medicine, the plant is used in treatment of broad range of diseases and disorders. In the present study, Hexane extract of B. laciniosa fruits were used to evaluate in vitro anti inflammatory, antioxidant and Cytotoxicity (towards MCF-7 cell line) activities. In vitro anti inflammatory activity by inhibition of protein denaturation, antioxidant assays like DPPH, ABTS, H_2O_2 and FRAP were used to measure the antioxidant capacity of the hexane extracts and cytotoxicity activity using MCF-7 breast cancer cell line. Hexane extract showed the effective antioxidant activity in all assays compared to ascorbic acid and BHT. The results for In vitro anti inflammatory activity of hexane extract and Dichlofenac drug were equivalent, hexane extract showed promising activity for inhibition of protein denaturation assay. The cytotoxicity activity from hexane extract was noticeable against MCF-7 cell line. The overall results show potential application of Bryonopsis laciniosa fruits suggesting their potential application as a health-promoting functional ingredient or natural preservative in foods.

Keywords: Bryonopsis laciniosa fruits; antioxidant; anti inflammatory activity; cytotoxicity

Introduction

The adverse effects free radicals and oxidative stress on public health have become a serious concern in last decades which is mainly caused due to unhealthy life style and increased pollution. Under stress, the concentration of reactive oxygen species (ROS) (e.g., superoxide anion radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide) in our bodies is higher than the concentration of enzymatic antioxidants (e.g., superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid, vitamin E and glutathione) [1]. This imbalance in the body leads to damage to bio molecules like lipids, proteins, carbohydrates and DNA, and consequently induces degeneration, destruction and toxicity of various organic molecules [2]. The increased accumulation of free radicals initiate the development of major diseases, including atherosclerosis, cardiovascular disease, cataracts, rheumatoid arthritis, inflammatory disorders, anemia, asthma, cancer, and Parkinson's and Alzheimer's diseases, as well as ageing [3] [4]. To overcome this problem, scientists suggest using of natural plant sources as a diet rich in antioxidant compounds. Medicinal plants are rich in antioxidants traps free radicals and therefore act as a type of preventive medicine [5]. The increasing research on antioxidants and the protective role of phytoconstituents have grabbed full attention and favored the development of the functional food and plant based medicine market. The plant kingdom offers a wide range of natural antioxidants. However, little is known about the practical usefulness of most of them. Many herbal beverages, frequently used in folk medicine, have antioxidant and pharmacological properties linked with the presence of phenolic compounds, especially flavonoids [6][7].

Bryonia laciniosa syn Bryonopsis laciniosa (N.O. Cucurbitaceae) is a plant commonly known as 'Shivlingi' in India. The leaves and fruits are reported to be highly medicinal value [8]. The whole plant has a bitter taste, and is a general tonic with mild

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laxative effect. The plant is also used against snake bite (part not specified). Leaves are commonly used for hepato-depurative disorders (internal use) and decoctions of the whole plant for skin inflammations (external and topical use). However, local healers and key informants avoid using plant extracts in humans for long periods, so as to prevent a certain degree of toxicity and adverse effects, which can vary considerably according to the method of preparation, doses and physical condition of the individual. Apart from leaves, Fruits are used as aphrodisiac, tonic. Sharp, cutting, lancinating or tearing pain, inflammation with muscular tension is cured by this plant [9] . In contrast, the fruits of the B.laciniosa have more potential pharmacological activities in comparison with other parts of the plants (Stem, Leaves and Seeds). In this study, the antioxidant activity in vitro anti inflammatory properties and cytotoxicity were evaluated and compared.

Material and Methods

Chemicals

Chemicals, such as 1,1 diphenyl-2-picrylhydrazyl (DPPH), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, 98% purity), potassium ferricyanide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), ferric chloride, and aluminium chloride, were purchased from Sigma Chemicals (Steinheim, Germany). Solvents such as methanol, chloroform and hexane were purchased from Merck (Germany). Butylated hydroxytoluene (BHT) and ascorbic acid were purchased from HiMedia (India). All chemicals and solvents used in the study were of analytical grade.

Plant material

Fruits of B. laciniosa L plants (5 kg) were collected from the Gulbarga University campus during the month of December 2013.

Extract preparation

The collected fruits of B. laciniosa were washed under running tap water to remove dust particles and dried in the shade for 72 h. The air-dried fruits were coarse powdered and stored in an air-tight dark glass bottle at 25 ˚C for further use. The powdered fruits (100 g) were successively extracted by three different solvents (350 ml each), from non-polar to polar, i.e., hexane, chloroform and methanol, using Soxhlet extraction. The successive extracts of different solvents were dried, weighed and stored at -4˚ c for further use.

Evaluation of in vitro anti inflammatory activity

In vitro anti-inflammatory activity of the hexane extract was tested by the method of Sangita Chandra et al. (2012) [10] with a slight modification. The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's eggs), 2.8 mL of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of the hexane extract (final concentrations 62.5, 125, 250, 500, and 1000 μg/ml). An equal volume of double-distilled water served as a control. The mixtures were incubated at 37 ±2 ˚C in an incubator for 15 min and then heated at 70 ˚C for 5 min. After cooling, their absorbance was measured at 660 nm (UV-1800 spectrophotometer, Shimadzu), with the vehicle used as a blank. Diclofenac sodium was treated similarly for determination of absorbance and used as a standard at the final concentrations of 62.5, 125, 250, 500, and 1000 μg/ml. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ Inhibition} = (Vt / Vc - 1) \times 100,$$

where Vt = absorbance of the test sample, Vc = absorbance of the control.

The extract/drug concentration giving 50% inhibition (IC_{50}) was determined by plotting the percentage of inhibition relative to the control against treatment concentrations.

In vitro antioxidant activity

The free radical scavenging activity of the hexane extract was determined by using various in vitro assays such as DPPH, ABTS, Reducing power and Hydrogen peroxide assay.

DPPH radical scavenging activity

Free radical scavenging activity of the hexane extracts were determined by using a stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). DPPH is a free radical of violet colour. The antioxidants in the sample scavenge the free radicals and turn it into yellow colour. The change of colour from violet to yellow is proportional to the radical scavenging activity.

DPPH radical scavenging activity was assayed by the method of Braca et al. (2002) [11] with a slight modification. Briefly, the assay contained 0.9 ml of DPPH solution (0.004% w/v) was prepared in 95% methanol and various concentrations of hexane extracts and standards with the stock solutions (20 mg/ml) in the same solvent and made up to 1 ml with ethanol. The contents were mixed well immediately and then incubated for 30 min at room temperature (24 –30˚ C). The degree of reduction of absorbance was recorded in UV–Vis spectrophotometer at 517 nm. Ethanol (95%), DPPH solution and ascorbic acid (AA) were used as blank, control and reference standard respectively.

DPPH radical scavenging activity (%) = (Ac − As)/Ac × 100,

Where Ac is the absorbance without the samples and As is the absorbance in the presence of the samples.

ABTS radical scavenging activity

ABTS radical cation decolonization activity was assayed by the method of Thoo et al. (2013) [12] and Surveswaran et al. (2007) [13] with a slight modification. ABTS radical cations were generated by reacting 7 mm ABTS with 2.45 mm potassium per sulfate (1:1). The mixture was left to stand for 12 to 16 h in the dark at room temperature. The ABTS radical cation solution (100 μL) was then diluted with ethanol (3.9 ml) to give an absorbance of 0.700 ±0.02 at 734 nm. Different concentrations of the hexane extract were mixed with diluted ABTS radical cation solution (1 ml). The mixture was vortexed and left to stand at room temperature for 6 min. The absorbance of the resulting solution was measured at 734 nm using a UV-visible spectrophotometer. Ascorbic acid and BHT were used standard reference for ABTS radical scavenging activity. The ABTS radical scavenging activity was calculated according to the following equation:

ABTS radical scavenging activity, % = (Ac − As)/Ac × 100,

Where Ac is the absorbance without samples and As is the absorbance in the presence of the samples.

Ferrous reducing-antioxidant power (FRAP)

Total reducing power of the hexane extract was estimated according to Oyaizu (1986) [14] and Oh et al. (2013) [15] with a slight modification. In FRAP, potassium ferric cyanide is reduced to ferrous cyanide by antioxidants present in the sample. The hexane extract of varying concentration (50 to 800 μl) were mixed with 0.25 ml phosphate buffer (0.2 mm, pH 6.6) and 1% potassium ferricyanide (0.25 ml). The reaction system was closed and incubated at 50° C in a water bath for 20 min. After the incubation period, 0.25 ml of 10% trichloroacetic acid was added to the assay system and the contents were mixed well. The mixture was centrifuged at 5,000 rpm for 5 min. The supernatant (0.5 ml) was mixed with an equal volume of distilled water and 0.1 ml of ferric chloride solution (0.1%, w/v). The colour developed was read at 700 nm using UV-Visible spectrophotometer and the results were compared with Ascorbic acid and BHT as a standard reference, the readings were expressed as the mean absorbance value.

Hydrogen peroxide-scavenging activity

The Hydrogen peroxide-scavenging activity of hexane extract was determined by the method of Cetinkaya et al. (2012) [16] .

Hydrogen peroxide solution (1 mm) was prepared with 50 mm phosphate buffer (pH 7.4). Different concentrations of the hexane extract (1 ml) were allowed to react with 0.6 ml of the hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The scavenging activity of chloroform extract was compared with Ascorbic acid and BHT. The hydrogen peroxide scavenging activity was calculated according to the following equation:

Hydrogen peroxide scavenging activity, % = (Ac — As)/Ac) × 100,

Where Ac is the absorbance without the samples and As is the absorbance in the presence of the samples.

Preparation of cell line

The cytotoxicity assay was carried out using MCF-7 cells (human breast carcinoma). The cell line was procured from the National Centre for Cell Sciences (NCCS, Pune, India). Stock cells were cultured in DMEM supplemented with 10% inactivated foetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in a humidified atmosphere at 5% CO_2, 37 °C until reaching confluence. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96-well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

MTT cell viability screening

Cell viability was assessed by measuring the amount of insoluble formazan formed in live cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt [17] (Francis and Rita, 1986). Cell suspensions at 1 × 10^5 cell/ml were seeded in 96 well microtiter plates. Hexane extract in different concentration were added into the wells. MTT reagent was added after 72 h exposure followed by dissolution of formed formazan crystal using DMSO. Optical density was read with micro plate reader at 540 nm. The dose-response curve is plotted and concentration which gave 50% inhibition of cell growth (IC_{50}) is calculated. Concentration that inhibits 50% of cell viability was used as a parameter for cytotoxicity.

Statistical analysis

Data were expressed as mean ± SD. Analyses of variance (ANOVA) were done for the comparison of results using Fischer's test. Statistical significance was set at $p < 0.05$.

Results & Discussion

In vitro anti inflammatory activity

In the present study the protein denaturation bio assay was selected for in vitro assessment of anti inflammatory properties of Hexane extract of B. laciniosa fruits. Denaturation of tissue proteins is one of the well-documented causes of inflammation and arthritic diseases. The production of auto-antigens in certain arthritic diseases may be due to denaturation of tissue proteins in vivo [18] [19]. The in vitro anti-inflammatory effect of the Hexane extract was evaluated for its ability to prevent denaturation of egg albumin. The results are summarized in Graph 1. The hexane extract compared with Diclofenac sodium showed similar anti inflammatory activity. The difference was further confirmed by comparing the respective IC_{50} values. The hexane extract showed an IC_{50} value of 362.53 ±0.67 μg/ml, whereas that of diclofenac sodium was found to be 320.83 ±1.17μg/ml. Therefore, from the findings of this preliminary experiment it can be concluded that hexane extract of B.laciniosa have a noticeable anti inflammatory effect against inhibition of protein denaturation in vitro.

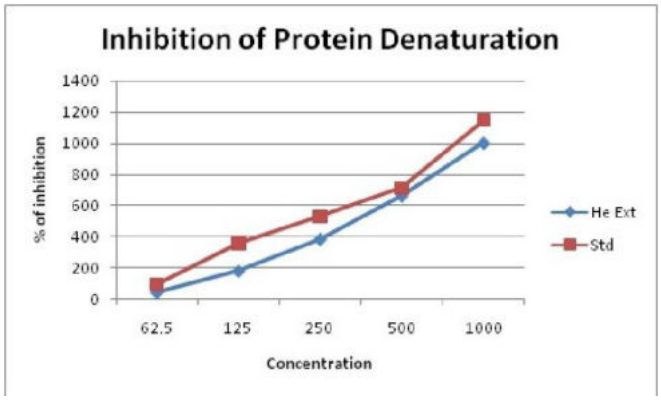

Figure 1 In vitro anti-inflammatoryactivity of Hexane extract and Standard (Diclofenac sodium) Values are means ± SD(n = 3).

Antioxidant assays

Phenolic compounds exhibit their antioxidant activity through their radical scavenging effects. Radical scavenging activity is very important owing to the deleterious role of free radicals in biological systems and generally proceeds via hydrogen atom transfer or donation of electrons [20] .

DPPH radical scavenging activity

In this study, the hexane extracts showed tendency to quench the DPPH free radicals, as indicated by the concentration dependent increase in percentage inhibition. The results revealed that the fruit extracts had the higher DPPH radical scavenging ability similar to Ascorbic acid and BHT. The scavenging activity of Hexane extract, Ascorbic acid and BHT were 87.22 ± 1.33, 99.05 ± 0.29 and 95.08 ± 0.57 respectively are shown in Graph 2. The IC_{50} values (concentration of the extract that was able to scavenge half of the DPPH radical) for the hexane extract, ascorbic acid and BHT were 223.5 ±1.2, 31.66 ±2.3 and 28.3 ±2.4 μg/ml respectively are presented in Table 1. Thus, by comparing IC_{50} values of DPPH assay, BHT showed an effective antioxidant activity followed by ascorbic acid and Hexane extract. By comparing overall results, hexane extract has the potential free radical trapping ability by donating protons and can serve as a source of free radical inhibitors or scavengers possibly acting as primary antioxidants, along with the reference standards that have similar scavenging activities.

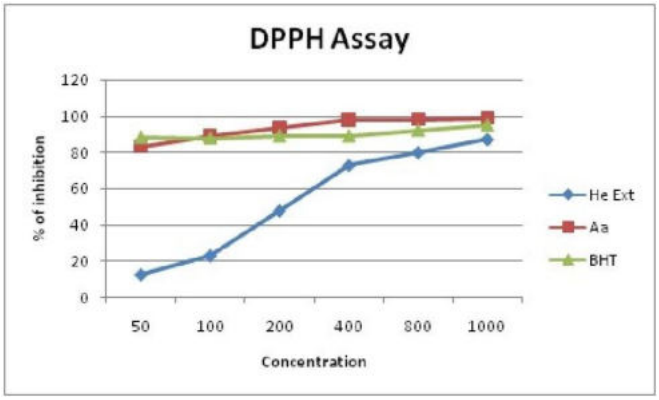

Figure 2 DPPH radical scavenging activity of Hexaneextract, Ascorbic acid and BHT.Values are means ± SD (n = 3).

ABTS radical scavenging activity

To determine free radical scavenging activity hexane extract ABTS radical scavenging assays are performed to estimate the free radical scavenging activity of a sample and are based on the reduction of these radicals. The hexane extract exhibited the highest radical scavenging activity when compared to Ascorbic acid and BHT. The ABTS radical scavenging activity of the hexane extract, Ascorbic acid and BHT was 79.77 ± 0.44, 69.21± 0.34 and 54.83 ± 0.52 % respectively are shown in Graph 3. IC_{50} values for hexane extract, ascorbic acid and BHT were 342.81 ± 0.67, 507.6 ±2.05 and 401 ±2.35 μg/ml, respectively (Table 1). The results clearly imply that the hexane extracts inhibit ABTS radical or scavenge the radical in a dose dependent manner. ABTS•+ radical is generated from oxidation of ABTS•+ by potassium persulphate, is a good tool for determining the an-

Table 1 IC50 values of DPPH, ABTS, Hydrogen Peroxide andAbsorbance of Reducing power of hexane extract of B.laciniosa fruits.

Samples	DPPH (μg/mL)	ABTS (μg/mL)	H2O2 (μg/mL)	Reducing power (Abs at 700 nm)
Hexane Extract	223.5 ±1.2	342.81 ± 0.67	186.91±0.53	0.827±0.02
Ascorbic acid	31.66 ±2.3	507.6 ±2.05	73.66 ±0.94	1.33±1.24
BHT	28.3 ±2.4	401 ±2.35	66.6 ±0.9	2.03±1.06

tioxidant activity of hydrogen-donating and chain breaking antioxidants [21]. This assay is applicable for both lipophilic and hydrophilic antioxidants. The radical scavenging activity of the hexane extracts of B.laciniosa were estimated by comparing the percentage inhibition of formation of ABTS•+ radicals with that of Ascorbic acid and BHT. The extracts exhibited the highest radical-scavenging activities when reacted with the ABTS radicals.

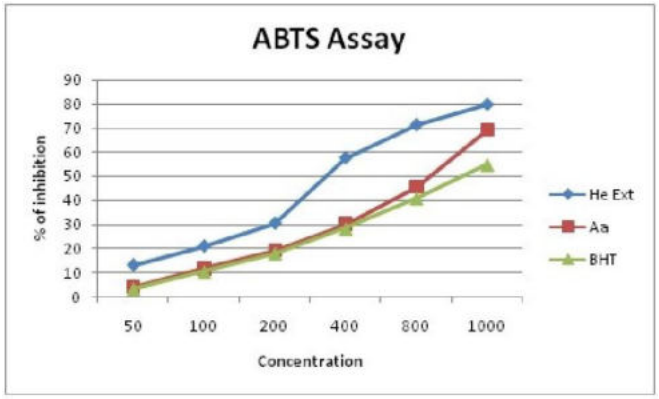

Figure 3 ABTS radical scavenging activity of Hexaneextract, Ascorbic acid and BHT. Values are means ± SD(n = 3).

Hydrogen peroxide scavenging activity

The hydrogen peroxide radical-scavenging activity of the hexane extracts of B.laciniosa was estimated by comparing the percentage inhibition of formation of peroxyl radicals with that of Ascorbic acid and BHT. Hydrogen peroxide scavenging activity of hexane extracts, Ascorbic acid and BHT are presented in Graph 4. Hexane extracts showed moderate inhibition against peroxyl radical which was less in comparison with Ascorbic acid and BHT (78.82 ± 0.53, 93.94 ± 0.09 and 95.54 ± 0.73). The results clearly indicate that hexane extracts of B. laciniosa are highly potent in neutralizing hydrogen peroxide radicals. IC50 values of hexane extract, ascorbic acid and BHT were 186.91±0.53, 73.66 ±0.94 and 66.6 ±0.9 μg/ml respectively are shown in Table 1. H2O2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results showed that B. laciniosa fruit extract have an effective H2O2 scavenging activity.

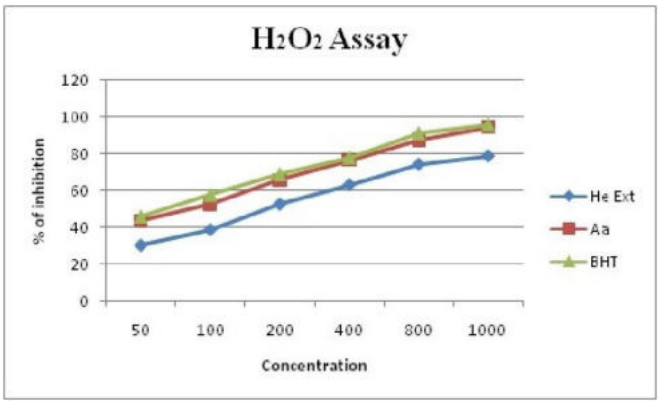

Figure 4 Hydroxyl radical scavenging activity of Hexaneextract, Ascorbic acid and BHT. Values are means ± SD(n = 3).

Ferrous reducing-antioxidant power (FRAP)

In the reducing power assay, the presence of antioxidants in the extract of B.laciniosa would result in the reduction of Fe3+/ferricyanide complex to its form. The reducing power of compound may serve as a significant indicator of its potential antioxidant activity [22] . The ferric reducing power of the hexane extract was determined by comparing with that of Ascorbic acid and BHT. The increased absorbance values of the extracts at 700 nm indicate an increase in reductive ability. Absorbance values of hexane extracts are presented in Graph 5. The reducing power of BHT was found to be significantly higher than those of Ascorbic acid and hexane extract. The reducing powers of hexane extract, Ascorbic acid and BHT were 0.827±0.02, 1.33±0.04 and 2.03±0.06 respectively. The reducing power increased with the increase of their concentrations. This data imply that these extracts have significant ability to react with free radicals to convert them into more stable nonreactive species and to terminate radical chain reaction.

Cytotoxicity assay

The search for new potential bio-active compounds in medicinal plant is a realistic and promising strategy for prevention and cure of many deadly diseases. In the present study B.laciniosa fruits was evaluated for cytotoxic activity on MCF-7 cell line using MTT (3-(4, 5-dimethylythiazol-2- yl)-2, 5-diphenyl-2H-tetrazolium hydrobromide) assay. The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays.

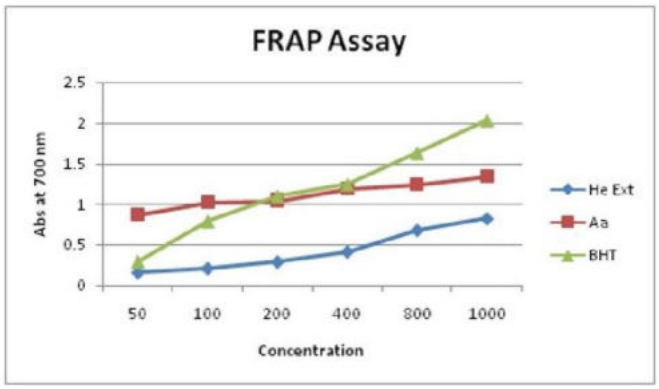

Figure 5 Reducingpower of Hexane extract, Ascorbic acid and BHT. Values are means ± SD(n = 3).

In the present study cytotoxicity of hexane extract at different concentration (62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, and 1000 μg/ml) were presented in Table 3. The cytotoxicity of hexane extract was found to be dose dependent and increases with increased concentrations. The maximum cytotoxic activity was found to be 75.25±2.4 % at 1000 μg/mL concentration with an IC50 value of 453.33 ± 1.6 μg/ml.

As per our earlier findings, the hexane extract of B. laciniosa fruits consist of many secondary metabolites like carbohydrates, alkaloids, terpenoids, steroids, tannins and phenols. The cytotoxicity is due to these different secondary metabolites which may have a different pharmacological activity.

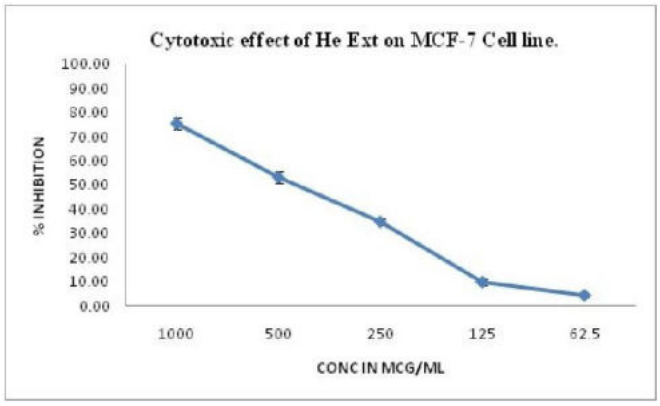

Figure 6 Cytotoxicity Assay of hexane extract analyzed byMTT assay on MCF-7 Cell line measuring the absorbance of the reaction at 540nm.Values are means ± SD (n = 3).

Conclusions

We have demonstrated that the hexane extracts of Bryonopsis laciniosa L. showed potent anti inflammatory, radical scavenging activity and Cytotoxicity against Breast cancer. Hexane extract can be explored for its applications in the prevention of free radical related diseases. The overall activity is due to the type of phenolics groups present and may be some unidentified antioxidants anti inflammatory and anti cancer compounds. The hexane extract of B.laciniosa allows us to conclude that extracts is good candidates for further studies of activity-monitored fractionation to identify their active components.

Conflict of interest statement

The authors have declared that there is no conflict of interest.

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