

Original Article

V. NEGUNDO, L. CAMARA AND B. VARIEGATA PLANTS LEAF EXTRACT EXHIBIT CONSIDERABLE IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITIES

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ABSTRACT

Objective: The study was planned to investigate antioxidant and anticancer activities with the preliminary phytochemical analysis of methanolic extracts of *Vitex negundo* (*V. negundo*), *Lantana camara* (*L. camara*) and *Bauhania variegata* (*B. variegata*) plants leaf extracts.

Methods: Phytochemical evaluation was performed for all the extracts, as per the standard methods. *In vitro* antioxidant activities were performed by using DPPH (2,2-Diphenyl-1-Picrylhydrazyl), ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) and FRAP (Ferric reducing antioxidant power assay) method and compared with standard antioxidants. The anticancer activity of plant extract was assessed using MTT colorimetric assay.

Results: The study of preliminary phytochemical proved the existence of alkaloids, flavonoids and phenolic types of phytochemicals in high amount. Methanolic extract of *L. camara* shows minimum IC₅₀ value for DPPH assay (48.75±2.34 µg/ml) and FRAP assay (274.66±3.65 µg/ml). In ABTS assay *B. variegata* extract exhibit minimum IC₅₀ value (60.48±3.01 µg/ml). Lower the IC₅₀ value of extract, higher the effectiveness of the plant. Methanolic extract of all plants methanolic extracts showed anticancer activity against SH-SY-5Y cells (human neuroblastoma cell) but *V. negundo* was more effective against SH-SY-5Y cells with IC₅₀ value (209 µg/ml) compared to remaining extracts.

Conclusion: The current finding accomplished the *in vitro* activities, so that plant could be a superior source of antioxidant and anticancer drugs. But further *in vivo* assessment was needed before adding it into the pharma industry.

Keywords: Antioxidant activity, Anticancer activity, Phytochemical screening, *V. negundo*, *L. camara* and *B. variegata*

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INTRODUCTION

Traditional plants are essential resource of valuable drugs and continue to be extensively used to treat many diseases. Discovery of new beneficial agents from plant source has been targeted in recent years. Compounds from natural sources have the beneficial effect which attributes among others, to the elevated level of bioactive compounds [1]. The bioactive molecules known as secondary metabolites produce from different parts of plant that are involved in defense mechanism of plants. Antioxidant component contains moieties which can scavenge free radicals and have reducing power, thus it protects human body from oxidative stress [2]. Antioxidants derived from plants are less toxic, more practical and economical and hence there is emerging interest in natural antioxidants from plant origin [3]. Phenolics and flavonoids are secondary metabolites from plants acts as antioxidant and according to earlier reports they have powerful free radical scavenging activity [4]. A broad variety of antioxidants are projected to be used in the treatment of various diseases from both natural and artificial origin [5]. From the last few decades of life, incidences of many cancers have increased exponentially with time. In the human population globally over six million public die because of cancer each year, representing it to be the leading single reason of death in human population [6]. Many plant sources are used to derive anticancer medicine and about 60% of the medicines are derived from plant origin for example, camptothecin from *Cuscutareflxa verma* and taxol from *Taxus brevifolia* [7]. Plant derived drugs having low side effects cause apoptosis and cytotoxicity precise to the cancer cells. The cytotoxic effect of medicinal plants and their phyto-constituents against cancerous cell lines are the evidences of positive helpfulness of medicinal plants for diversity of ailments [8].

V. negundo Linn. (Family Verbenaceae) is a significant plant of the Ayurveda. All parts but leaves of *V. negundo* is generally used in the indigenous system of medicine. The leaves are used for the

treatment of eye-disease, skin-ulcers, toothache, rheumatoid arthritis, and inflammation, enlargement of the spleen, gonorrhoea, and bronchitis. Against Ehrlich ascites tumor cells, *V. negundo* plant's leaf extract shown antiasthmatic [9] and anticancer activity [10]. *L. camara* Linn, (Verbenaceae) has been used to cure a broad range of diseases in many parts of the world [11]. *L. camara* was used to treat fevers, colds, rheumatism, asthma and high blood pressure [12]. The result of pharmacological study reported that extracts from leaves of *L. camara* showed strong antioxidant activities [13]. Bark of *B. variegata* (Leguminosae) is traditionally used as stimulant to cure ulcers and also useful in skin diseases. In the treatment of snake poison the roots are used as an antidote [14]. This plant is also used in folklore medicine to treat several diseases [15]. The current study, therefore reports the phytochemical composition along with their antioxidant and anticancer activities from *V. negundo*, *L. camara* and *B. variegata* plants leaf extracts.

MATERIALS AND METHODS

Plant materials

The fresh sample of selected medicinal plant *V. negundo*, *L. camara* and *B. variegata* were collected in August 2013 under the supervision of a botanist from Pune, India. Then all Plant specimens were authenticated and submitted in organ form to Dr. Subhash Sadhu Deokule, Department of Botany, University of Pune, India. Authentication of all plant specimens was done using macroscopic, microscopic, histochemical and phytochemical parameters.

Preparation of plant extracts

Leaves of selected plants were collected, washed under running tap water and dried by blotting. For preparing dried powder of leaf extract, washed leaves were dried at 40 °C in hot air oven for about 4-5 d and ground to make fine powder. 10 g of powder was taken with 100 ml of methanol and incubated at 20 °C for the cold

percolation extraction. Then the methanolic extract was concentrated by rotary evaporator under reduced pressure (at 30-40 °C) filtrate obtained was concentrated and labeled as ME (methanolic extract). Then yield was calculated for extraction method. The dried extracts were stored at -20 °C in airtight bottles for further studies. 10 mg of each extracts were dissolved in 1 ml dimethyl sulfoxide (DMSO) and were diluted with DMEM medium. Then, test solutions were sterilized using 0.22 µm Syringe filters (Axiva, Scichem biotech) and used as a stock solution for further experiments.

Phytochemical screening

Phytochemical evaluation was performed for all the extracts, as per the standard methods [16].

Chemicals and reagents

Following were the chemicals used in the present study. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Gibco. Dimethyl Sulfoxide (DMSO) and methanol purchased from Merk Ltd., Mumbai, India. Antibiotics were purchased from Hi-Media Laboratories Ltd., Mumbai.

Antioxidant activity

DPPH free radical scavenging activity

Required quantity of trolox was dissolved in methanol and stock solution of the sample was prepared by dissolving 10 mg of dried methanolic extract in 1 ml of methanol to give a concentration of 10 mg/ml. The 1.5 ml DPPH solution was added to 3 ml methanol and for control reading at the 517 nm absorbance was taken immediately. Different concentration of test sample was added and made 2 ml using methanol for dilution. Then 1.5 ml DPPH solution was added to each test tube. Using UV-visible spectrophotometer absorbance was taken at 517 nm after 15 min. Methanol was used as blank. The free radical scavenging activity (%) was measured using the following formula:

% DPPHscavengingactivity(%)

$$= \frac{\text{Control absorbance} - \text{sample absorbance} \times 100}{\text{Control absorbance}}$$

ABTS assay

The ABTS assay was performed according to the protocol of Arnao *et al.*, with slight modification [17]. Different quantities (10, 20, 40, 80 and 100 µg) of the phyto-extracts were tested. Absorbance was taken at 734 nm. In the ABTS assay, IC₅₀ values for the percentage of ABTS radical scavenging were calculated.

% ABTS scavenging activity(%)

$$= \frac{\text{Control absorbance} - \text{sample absorbance} \times 100}{\text{Control absorbance}}$$

FRAP assay

Antioxidant activity was determined according to the method by Yusufoglu *et al.* [18]. 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide was added to all test tubes. 100, 200, 300, 400 and 500 µg/ml concentration of the plant extract was

used for the study. This makes a total 1 ml of plant extract. In different eppendorf tubes 1 ml plant extracts were added test tubes and this mixture was incubated for 20 min at 50 °C. Then 2.5 ml of TCA was added to all tubes and then tubes were centrifuged for 10 min at 3000 rpm. After centrifuging, 2.5 ml of supernatant liquid was collected and 2.5 ml of distilled water and 0.5 ml of FeCl₃ was added to all test tubes. UV absorbance was recorded at 700 nm.

Cell lines and culture medium

SH-SY-5Y (Human neuroblastoma cell) cell culture was procured from National Center for Cell Sciences (NCCS) Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated FBS, 1% (v/v) Penicillin Streptomycin solution and amphotericin (5 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent. Medium was changed after every 2-3 d. The cells were grown in 25 cm² culture flask and all experiments were carried out in 96 well plates (Himedia).

Determination of cell viability by MTT assay

The capability of cells to endure toxic concentration is the principle behind all cytotoxicity assays. This assay is based on the theory that dead cells or their products do not reduce tetrazolium. The MTT assay depends on the mitochondrial activity per cell and amount of cells present. Mitochondrial enzyme succinate dehydrogenase cleave tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) [19]. 1 × 10⁶ cells were plated in 96-well plates to check the toxicity of extracts towards SH-SY-5Y (human neuroblastoma cells). After adherence of cells, the medium was removed and replaced by media having the plant extracts. In 5% CO₂ incubator the plates were incubated for 24h at 37 °C. Colorimetric assay with the tetrazolium salt MTT was used to determine cell viability. Absorbance of the formed purple formazan was measured at a wavelength of 570 nm. Results were expressed as percentage cellular viability of the extracts.

% Cell viability

$$= \frac{\text{O. D of control sample} - \text{O. D. of treated sample} \times 100}{\text{O. D of control sample}}$$

Statistical analysis

Data represented in this study were based on means ± standard deviation (SD) of three identical experiments done in triplicates. Statistical significance was determined by analysis of variance (ANOVA). P value ≤ 0.05 was considered statistically significant. LD₅₀ value represented the concentration of the test samples that caused 50% inhibition.

RESULTS

There has been revival of great interest in medicinally important plants. This is because of increased awareness of the limitations of ability synthetic pharmaceutical products to control major diseases.

Extraction yield of plant

Table 1 show the extraction yield of different plants which was calculated using below formula. The highest yield was obtained in *V. negundo* plant and the lowest yield was obtained in *B. variegata*.

Yield of plant extracts (%)

$$= (\text{weight of dried sample} / \text{weight of original sample}) \times 100$$

Table 1: Extraction yield (%) from methanolic extracts of *V. negundo*, *L. camara* and *B. variegata* plants leaf extracts

S. No	Plant name	Yield (%)
1	<i>V. negundo</i>	8.12
2	<i>L. camara</i>	7.54
3	<i>B. variegata</i>	5.65

Phytochemical analysis

The results of the phytochemical composition of all extracts are given in table 2.

The result shows that all extracts contain alkaloids, phenol and flavanoids in high amount and saponin in moderate amount and carbohydrate in low amount. Phytosterols is present in methanolic extract of *L. camara* only.

Table 2: Phytochemical analysis from methanolic extracts of *V. negundo*, *L. camara* and *B. variegata* plants leaf extracts

Chemical test	<i>V. negundo</i>	<i>L. camara</i>	<i>B. variegata</i>
Alkaloids			
Mayer's test	+++	+++	+++
Wagner's Test	+++	+	++
Dragendorff's test	++	-	++
Carbohydrates			
Molisch's test	-	+	++
Fehling's test	+	-	++
Benedict test	+	-	+
Phytosterols			
Salkowaski test	-	+	-
Phenols			
Ferric Chloride Test	+++	+++	+++
Flavonoids			
Ammonia test	++	+++	++
Saponins			
Foam test	++	++	+

-- Absence, += Presence, ++ = Moderate Presence, +++ = More Presence

Antioxidant activity

Given the complexity of the oxidation process and the diverse nature of antioxidants, below methods (DPPH, ABTS and FRAP) were used to evaluate the antioxidant activity of extracts from different plants.

DPPH assay

In DPPH assay, plant extracts were able to reduce the stable free radical DPPH to the yellow colour 1, 1-diphenyl-2-picrylhydrazyl due to their hydrogen donating property [20]. Concentration of the sample necessary to decrease the initial concentration of DPPH by 50% (IC_{50}) under the experimental condition was calculated. A lower IC_{50} value denoted a higher antioxidant activity. IC_{50} values of methanolic extracts of *L. camara*, *B. variegata*, *V. negundo* leaves and standard trolox were 48.75 ± 2.34 $\mu\text{g/ml}$, 68.94 ± 3.12 $\mu\text{g/ml}$, 309.71 ± 1.23 $\mu\text{g/ml}$ and 14.26 $\mu\text{g/ml}$, respectively (fig. 1). A lower IC_{50} value denoted a higher antioxidant activity.

ABTS assay

A concentration-dependent assay was carried out with the methanolic extracts of selected plants and the results are presented in (Fig.2). The IC_{50} value of plants in the order: BHA (8.68) > *B. variegata* (60.48 ± 3.01) > *L. camara* (70.43 ± 2.48) > *V. negundo* (72.15 ± 2.32) respectively. The scavenging effect was increased with increasing concentration. These results present a direct association of the antioxidant activity with BHA.

Ferric-reducing activity

The FRAP antioxidant activity was employed to determine the reducing potential of methanolic extract of selected plants. The IC_{50} value of the *V. negundo*, *L. camara* and *B. variegata* leaves methanolic extract was mentioned in table 3. BHA was used as a standard. There is a direct relationship between the methanolic concentration of plant extract, BHA and reducing power, where the increase of concentration of plant extract and BHA causes an increase in the reducing capacity (fig. 3).

Cytotoxicity assay

Methanolic extract of *V. negundo*, *L. camara* and *B. variegata* leaves were tested on SH-SY-5Y cells at different concentration (500, 400, 300, 200, 100 and 50 $\mu\text{g/ml}$). Cytotoxicity was concentration dependent for SH-SY-5Y cells. The highest cytotoxicity was observed in methanolic extract of *V. negundo* followed by *L. camara* and *B. variegata*. IC_{50} value for *V. negundo* was (209 $\mu\text{g/ml}$) followed by *L. camara* (273.64 $\mu\text{g/ml}$) and *B. variegata* (318 $\mu\text{g/ml}$) (fig. 4).

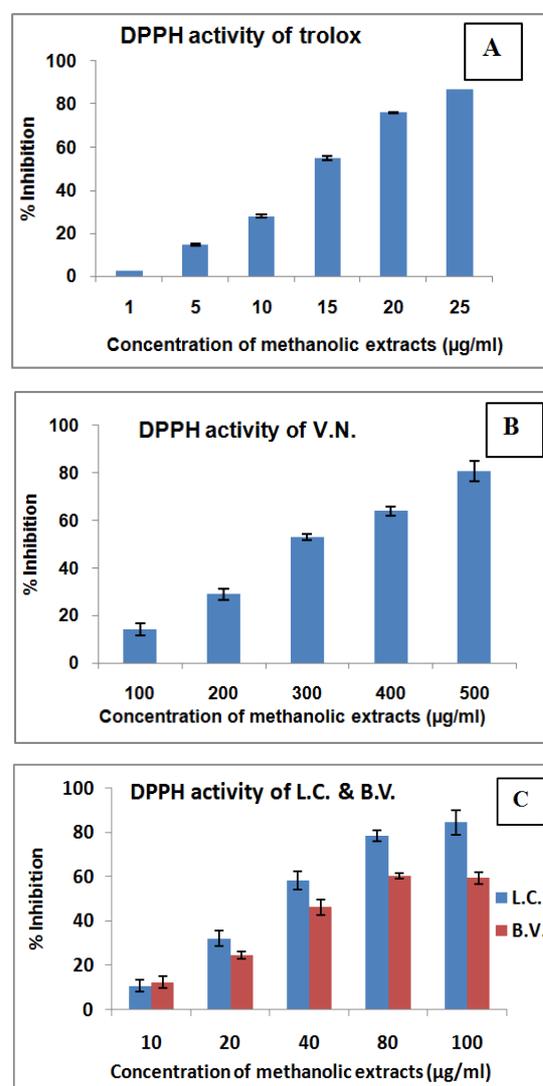


Fig. 1: (A) DPPH scavenging activity of Trolox used as standard. (B) DPPH scavenging activity from methanolic extract of *V. negundo* (V. N.). (C) DPPH scavenging assay from methanolic extracts of *L. camara* (L. C.) and *B. variegata* (B. V.). The results are expressed as mean \pm SD of three replicates

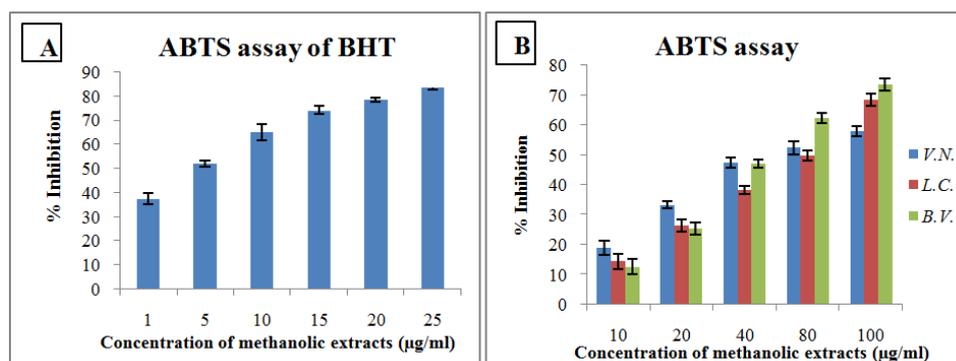


Fig. 2: (A) ABTS activity of standard BHT. (B) ABTS activity from methanolic extracts of the *V. negundo* (V. N.), *L. camara* (L. C.) and *B. variegata* (B. V.). Results are expressed as mean±SD of three replicates

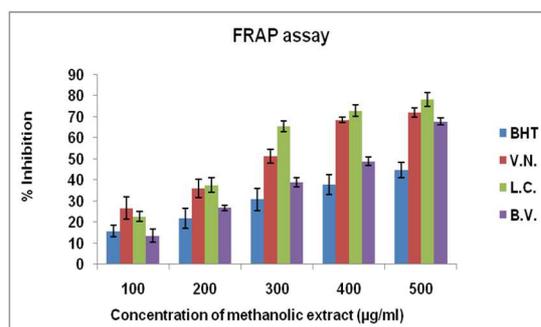


Fig. 3: FRAP activity from methanolic extracts of the *V. negundo* (V. N.), *L. camara* (L. C.), *B. variegata* (B. V.) and standard BHT. The results are expressed as mean±SD of three replicates

Table 3: Antioxidant effect of methanolic extract of selected plants

Plants name	DPPH IC ₅₀ value (µg/ml)	ABTS IC ₅₀ value (µg/ml)	FRAP IC ₅₀ value (µg/ml)
<i>V. negundo</i>	309.71±1.23	72.15±2.32	303.2±1.95
<i>L. camara</i>	48.75±2.34	70.43±2.48	274.66±3.65
<i>B. variegata</i>	68.94±3.12	60.48±3.01	384.4±3.14
Standard	Trolox= 14.26	BHT= 8.68	BHT= 548.48

Values are expressed as mean±SD, IC₅₀ value of methanolic extracts of plants for various antioxidant systems with respective standard. Each value represents mean value±SD of triplicate samples analysis, SD: standard deviation.

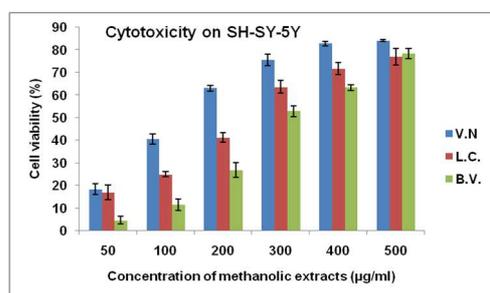


Fig. 4: Cytotoxicity activity from methanolic extracts of the *V. negundo* (V. N.), *L. camara* (L. C.) and *B. variegata* (B. V.) against SH-SY-5Y cells. Cells were incubated for 24 h with various concentrations of methanolic extracts of selected plants. Cell proliferation was examined using MTT assay. All experiments performed in triplicate

DISCUSSION

Plants are known to possess pharmacological activities and persist to serve as a possible source of drugs and some plant based drugs

are in extensive clinical use [21]. The present study describes the phytochemicals, antioxidant and cytotoxic activities of *V. negundo*, *L. camara* and *B. variegata* leaves extracts.

The phytochemical study showed the presence of alkaloids, phenolics and flavonoids, which are well-known to disclose a broad variety of biological activities like antioxidant, cytotoxic, anticancer, hepatoprotective, antimicrobial, neuroprotective, antiviral and cardioprotective, properties [22]. All these natural phytochemicals possess antioxidant activity [23-25]. Biochemical activities included antioxidant parameters such as DPPH, ABTS and FRAP activities. Plants have been thoroughly evaluated for their antioxidant potential, thereby confirming their roles as potent precursors for developing effective medicines that protect against oxidative stress [26]. For screening antioxidant activity of plant extracts, DPPH assay was most widely used method [27]. With the addition of plant extracts, DPPH was reduced to diphenylpicryl hydrazine in a concentration dependent manner. The number of available hydroxyl groups can be correlated with reduction in the number of DPPH molecules. *V. negundo*, *L. camara* and *B. variegata* plant extracts showed significantly higher inhibition percentage (stronger hydrogen donating ability). The DPPH scavenging potential of *V. negundo*, *L. camara* and *B. variegata* plant extracts might be due to its reducing actions. Reducing capacity is due to presence of

reductase, which involved in prevention of binding of metal ions, chain initiation and radical scavenging [28].

The ABTS+chemistry implicates nonstop production of ABTS+ radical mono cation. It is converted to its radical cation by the addition of potassium persulfate and absorbs the light at 734 nm [29]. ROS are also formed due to metal ion mediated oxidative stress and which leads to various diseases. Metal ion induced free radical production also leads to DNA damage and lipid peroxidation [30]. The antioxidant power of BHA was considerably more evident linking to the selected plants. According to result methanolic extracts of *V. negundo*, *L. camara* and *B. variegata* have significant free radical scavenging activity and thus shows the significant antioxidant potential. In general, the ferric-reducing test is mainly employed to see the capacity of natural antioxidant in giving an electron or hydrogen [31]. According to earlier reports, there is a direct relation between ferric-reducing ability and antioxidant potential of some secondary bioactive molecules. For that reason, it is fully believed that the maximum absorbance at 700 nm indicates a large reducing power [32]. FRAP assay was used to find out the reducing ability from methanolic extracts of selected plants, in which the reduction of the ferricyanide complex to the ferrous form occurred due to plant extracts. Antioxidant activity was increased when concentrations of test extracts were increased, representing concentration dependent response (fig. 3) [33]. The FRAP activity in the *V. negundo*, *L. camara* and *B. variegata* suggested the presence of richer flavonoids and phenolic phytochemicals [34]. Plant-derived compounds have been a significant resource of numerous clinically helpful anticancer drugs. These include vinblastine, the camptothecin derivatives, vincristine, topotecan and paclitaxel. Ethanolic and aqueous extract of leaves of *V. negundo* shows antitumor effect against Dalton's Ascitic Lymphoma (DAL) in Swiss Albino mice [35]. Pour et al., (2011) reported that the methanolic extract of *L. camara* was studied for its cytotoxicity and oral acute toxicity [36]. Against Dalton's ascetic lymphoma, leukemia and many cancer cell lines, *B. variegata* stem was shown to have cytotoxic activity. It was previously studied that *B. variegata* contains flavones which are extra selective against ovarian cancer cell lines [37]. Secondary molecules present in the plant might be accountable for showing anticancer effect. According to earlier reports phenolic or flavonoids interacts with proteins and other important biological pathways in the cells that make them lethal to the cell or behave as growth inhibitors [38].

CONCLUSION

The study showed that the phyto-constituents present in plants have ability to act as antioxidant as a result of their powerful radical scavenging activities. The presence of alkaloids, phenolic and flavonoids compounds are accountable for both antioxidant and cytotoxic activities. Based on the findings of the study, it can be concluded that *V. negundo*, *L. camara* and *B. variegata* leaves have an inhibitory effect on SH-SY-5Y cells. This study has provided confirmation that *V. negundo*, *L. camara* and *B. variegata* are potential source of antioxidants and cytotoxic activities. This study provides useful information to researchers for future utilization of this plant for pharmaceutical purposes.

AUTHOUR CONTRIBUTION

All authors contributed extensively to the work presented in this paper. Nutan Badgular designed and performed experiments, analyzed data and wrote the paper. Dr. Kinnari Mistry supervised development of work, helped in data interpretation and manuscript evaluation. Dr. Kinnari Mistry acted as corresponding author. Dr. Jagdish Patel helped in cell culture related assay.

CONFLICT OF INTERESTS

Authors declare that they do not have any conflict of interest

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