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# FREE RADICAL SCAVENGING ACTIVITY OF VARIOUS EXTRACTS OF *ANISOMELES MALABARICA* (L)

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**Keywords:**

Anisomeles, Antioxidant, DPPH, Ferrous ion, Leaves extracts

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**ABSTRACT:** The present study aimed to determine the antioxidant potential of different solvent extracts of *A. malabarica*. Drug discovery from traditional medicinal herbs has played a significant role in the therapeutic management of cancer and, undoubtedly, most novel clinical applications of medicinal plants and its secondary metabolites and derivatives. The leaves extract showed various folklore and traditional applications in India. The polar and non-polar solvents extracts such as hexane chloroform, ethyl acetate, methanol, and aqueous were subjected to various *in-vitro* antioxidant assays such as DPPH, ABTS, Superoxide, Nitric oxide, Hydroxyl radical and Ferrous ion chelation, Butylated Hydroxy Toluene was used as a standard. The percentage of scavenging and chelation values for antioxidant were obtained and tabulated. Effective concentration ($EC_{50}$) values are calculated using linear interpolation. The result clearly exhibited that the methanol leaves extract possesses more efficient antioxidant properties than the other extracts. This scavenging free radical capacity can be used as a good anti-oxidizing agent in various fields of medicine, the food industry *etc*.

**INTRODUCTION:** Natural products have long been implemented as alternative health care treatment and in discovering modern drugs. Medicinal plants refer to the class of plants applied for therapy or to possess pharmacological actions for humans and animals. Drug discovery from traditional medicinal herbs has played a significant role in the therapeutic management of cancer. Undoubtedly, most novel clinical applications of medicinal plants and their secondary metabolites and derivatives of the medicinal plants over the last five decades have been effectively applied against combating cancer and other diseases [1].

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Recently, much attention has been paid to the extracts obtained from plant species to analyze their biological activities. The discovery of novel secondary metabolites from medicinal plants [2]. The human body possesses numerous antioxidant defense systems. It comprises enzymatic and non-enzymatic pathways that could help maintain a steady equilibrium between prooxidants and antioxidants to ensure well-being [3]. Free radicals initiate oxidative stress, which seeks stability through electron pairing with biological macromolecules such as proteins, lipids, and DNA in healthy human cells and cause protein and DNA damage along with lipid Peroxidation [4].

These changes contribute to cancer, atherosclerosis, cardiovascular diseases, aging and inflammation. In most cases, the mechanism of action and efficiency of herbal extracts are yet to be scientifically validated. Therefore, researchers have carried out significant work to focus their attention on

traditional medicines to develop outstanding drugs against different varieties of microbial infections [5]. Oxidative stress is involved in developing and secondary pathology of various human diseases. Several studies showed evidence that regular consumption of plant foods is associated with lowered risk of incidence of these diseases [6].

Similarly, the health-beneficial effect of plants' food stuff is due to several phenolic compounds and their ability to promote antioxidant effects. Recently, antioxidant activity has been primarily examined in common food plants, such as fruits and vegetables. However, recent studies indicate that other plant categories, such as medicinal plants, possess significant antioxidant efficacy [7].

Vijayalakshmi *et al*., 2012 [8] reported that the whole plant of *Anisomeles malabarica* contains higher amount of flavonoids and phenolic compounds, which correspond to greater antioxidant activity. *In vitro* assays indicate that this plant extract is a better source of natural antioxidants, which might be helpful. The human body has a characteristic barrier system to counter free radicals as proteins, such as catalase, superoxide dismutase, and glutathione peroxidase. Selenium, vitamin C, -carotene, vitamin E, lycopene, lutein, and different carotenoids have been utilized as supplementary antioxidants. Hence, the secondary metabolites of the plant, flavonoids and terpenoids, play an important role in the defense against free radicals [9].

*Anisomeles malabarica* (L) is a medicinal plant that has been used as a folk medicine for anorexia, fevers, swelling, and rheumatism. *In-vitro* radical scavenging assays such as hydroxyl, superoxide anion radicals, 2,2-diphenyl-1-picryl hydroxyl (DPPH) and 2,2'-azinobis-(3-ethyl-enzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays [10]. This study aimed to assess the free radical scavenging ability of *A. malabarica* using various *in-vitro* antioxidant assays. Thus, in this study, a number of *in-vitro* assays (DPPH, ABTS, superoxide, nitric oxide, hydroxyl and ferrous ion chelation) were performed to evaluate the antioxidant properties.

## MATERIALS AND METHODS:

**Reagents Required:** ABTS, 1,1-diphenyl-2-picryl hydrazyl (DPPH), Butylated hydroxyl toluene (BHT), Methanol, Butylated hydroxyl toluene (BHT), Potassium persulphate solution, Nitroblue Tetrazolium Nicotinamide Adenine Dinucleotide, Phenazine Metho Sulphate, Griess reagent, Phosphate Buffer Saline (PBS), Sodium nitroprusside (NPS), Sulphanilic acid, Ascorbic acid, Dimethylsulfoxide (DMSO), Phosphate buffer saline, Tricarboxylic acetic acid, Ethylene diamine tetra acetic acid, Ammonium acetate, Acetyl acetone, Ferrous ammonium sulphate, Glacial acetic acid, NASH reagent, Ferric chloride ($FeCl_2$) and Ferrozine.

***In-vitro* Antioxidant Potential of *A. malabarica*:**

**DPPH Radical Scavenging Activity:** The free radical scavenging ability of leaf extract of *A. malabarica* was determined by measuring the discoloration of DPPH radicals [11]. Different concentrations of samples (20, 40, 60, 80, and 100µg/mL) were added to 3 mL of DPPH solution (0.1 mM), mixed thoroughly, and incubated under dark conditions for 30 min at 20°C. After incubation, absorbance was measured at 517 nm using UV-Vis spectrophotometer by keeping DPPH in methanol solution as a negative control, whereas BHT was used as a positive control. The percentage of radical scavenging was calculated according to the formula:

Scavenging of DPPH (%) = Absorbance of Control – Absorbance of Sample × 100 / Absorbance of Control

**ABTS Radical Scavenging Ability:** The ability of leaves extracts to scavenge ABTS radicals (2, 20 - Azinobis-3-ethyl benzothiazolinee 6 sulfonic acid) was determined [12]. ABTS solution was prepared by mixing 3.75 mM ABTS di-ammonium salt with 1.225 mM potassium persulphate solution, and it was incubated overnight at 30°C for completion of the reaction. ABTS cationic free radical solution was adjusted to the absorbance of 0.6±0.05 at 734 nm. Standard ABTS solution 200 µL was mixed with different concentrations (20 – 100 µg/mL), and the absorbance was read at 734 nm for every 5 min up to 60 min. The ABTS scavenging ability was calculated;

Scavenging of ABTS (%) = Absorbance of Control – Absorbance of sample × 100 / Absorbance of control

**Superoxide Radical Scavenging Activity of *A. malabarica*:** This assay was based on the ability of

leaves extracts to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) [13], with minor modifications. Briefly, 3.0 mL of each reaction mixture (0.05 M phosphate-buffered saline (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT 75 µM) and 1.0 mL of samples (20 – 100 µg/mL) was added. The tubes were kept in front of fluorescent light (725 lumens, 34 watts). The entire reaction was enclosed in a box dark condition. Identical tubes containing reaction mixtures were kept in the dark condition and served as a blank. The absorbance was read at 560 nm after 20 min. The percentage inhibition of superoxide generation was estimated by the following equation:

Scavenging of S02 (%) = Absorbance of Control – Absorbance of Sample × 100 / Absorbance of Control

**Nitric oxide Radical Scavenging Ability of *A. malabarica*:** Nitric oxide scavenging ability of leaves extracts was determined [14]. The reaction mixture 2Ml (sodium nitroprusside 10 mM in 0.5mL phosphate buffer 0.5 M; pH7.4) was added to the different samples' concentrations (20–100µg/mL). After incubation for 60 min at 37°C, Griessreagent (α-napthyl-ethylene diamine (0.1%) and sulphanilic acid (1%) in $H_3PO_4$) was added. The pink chromophore generated during diazotization nitriteions was measured in a spectrophotometer at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability of nitricoxide radical (%) was calculated by following formula:

Scavenging of $N0_2$ (%) = Absorbance of Control – Absorbance of sample × 100 / Absorbance of control

**Hydroxyl Radical Scavenging Ability of *A. malabarica*:** The scavenging ability of hydroxyl radicals (OH•) was measured [15]. Various concentrations of leaves extract (20 – 100µg/mL) was added to 1mL of ion-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5mLofEDTAsolution (0.018%) and 1Ml of DMSO (0.85%v/vin0.1M phosphate buffer, pH7.4). The reaction was initiated by adding 0.5mL of scorbic acid (0.22%) and incubated at 80–90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1mL ice-cold TCA (17.5%w/v). In which, 3mL of Nash reagent (7.5g of ammonium acetate, 0.3mL of glacial acetic acid and 0.2 mL of acetyl acetone was mixed and made up to100mL with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm using UV-Visspectrophotometer. The reaction mixture without sample was used as a negative control whereas; BHT was used as a positive control. Percentage of hydroxyl radical scavenging ability (HRSA) was calculated by the following formula:

Scavenging of -OH (%) = Absorbance of Control – Absorbance of Sample × 100 / Absorbance of Control

**Ferrous Inhalation Assay:** The ability of leaves extract and bioactive compound to chelate the ferrous ion (Fe2+) was investigated [16]. Briefly, 50 µL (2mM) $FeCl_2$ was added to 1 mL of different concentration of samples (20 – 100 µg/mL). The reaction was initiated by the addition of 0.2 mL of 5 Mm ferrozine solution. After which the reaction mixture was vigorously shaken and left at room temperature (28±2°C) for 10 min. The absorbance was measured at 562 nm, $Na_2$ EDTA was used as a positive control and percentage inhibition of ferrozine (Fe2+) complex formation was calculated.

Scavenging of -OH (%) = Absorbance of Control – Absorbance of sample × 100 / Absorbance of Control

## RESULTS:

**DPPH Free Radical Scavenging of Leaves Extracts of *A. malabarica*:** The free radical scavenging of leaves extracts of *A. malabarica* such as hexane, chloroform, ethyl acetate, methanol, and aqueous was evaluated. Among all the extracts tested, the methanolic extract showed superior DPPH radical scavenging activity (92.90±0.36%) with an EC50 value of 34.85µg/mL, followed by ethyl acetate extract (71.78±0.31%) with an $EC^{50}$ value of 53.45, Aqueous (59.78±0.41%) with an $EC_{50}$ value of 60.04µg/mL. Chloroform extract showed only moderate activity at 56.67±0.44% ($EC_{50}$ of 59.34µg/mL), while; hexane extract exhibited the least radical scavenging activity (42.81±0.41%) with an $EC_{50}$ value of 94.03 µg/Ml **Fig. 1**. The standard BHT showed the optimal activity at 64.00µg/mL with an $EC_{50}$ value 40.60 µg/mL. Overall, the crude extracts displayed a dose-dependent free radical scavenging activity.

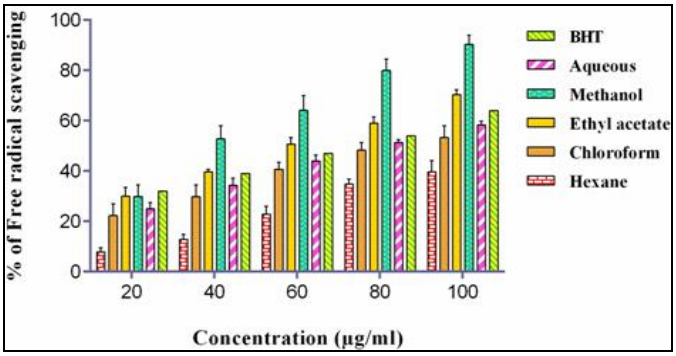


**FIG. 1: DPPH RADICAL SCAVENGING ACTIVITY OF A. *MALABARICA***

**ABTS Radical Scavenging of *A. malabarica:*** ABTS assay is a widely accepted model to determine the total antioxidant activity. Among the various solvent extracts, methanolic extract at 100 µg/mL was found to have the best scavenging activity, 89.21±0.17%, with an $EC_{50}$ value of 49.04µg/mL, followed by ethyl acetate 71.42±0.64% with an $EC_{50}$ value of 62.04 µg/mL, aqueous 69.04 ±0.57% with an $EC_{50}$valueof 79.09µg/mL, hexane 62.09±0.33% with an $EC_{50}$ value of 104µg/mL. In contrast, the chloroform extract showed lowest ABTS radical scavenging activity (57.90±0.17%) with an $EC_{50}$ value of 110.3µg/mL. The extent of reduction or decolorization of the reaction mixture is directly proportional to the increased concentration **Fig. 2.** BHT exhibited the best activity (91.64±0.33%), with an $EC_{50}$ value of 38.11µg/mL, respectively. This result implies that the radical scavenging activity of the extracts might be attributed to their strong proton–donating ability.

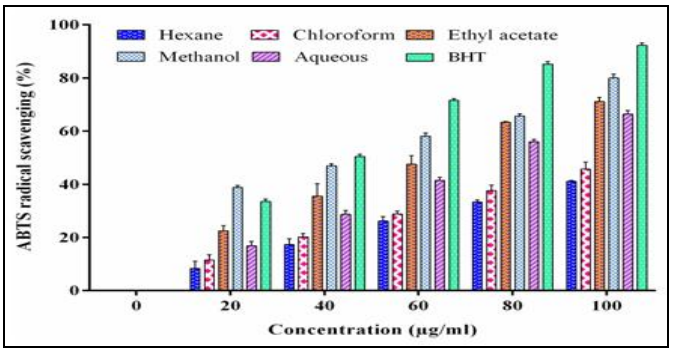


**FIG. 2: ABTS EFFECT ON VARIOUS EXTRACTS OF *A. MALABARICA***

**Superoxide Radical Scavenging Assay:** The superoxide radical scavenging of leaves extracts of *A. malabarica* are illustrated in **Fig. 3**. In which, ethyl acetate extract was the most effective superoxide scavenger (82.13±0.16%) at 100 µg/mL, followed by methanol (68.27±0.23%), Aqueous (44.38±0.11%), and chloroform (41.54±0.42%) with an $EC_{50}$ value of 59.04, 69.04, 100.02 and 103.4 µg/mL, respectively. But, hexane extract exhibited the least scavenging activity (29.91±0.14%), with an $EC_{50}$value 120 µg/mL. BHT exhibited best activity (87.37±0.30%), with an $EC_{50}$ value of 56.04µg/mL, respectively. These results indicated that ethyl acetate and methanol extracts of *A. malabarica* had a notable effect on the scavenging of superoxide radicals.

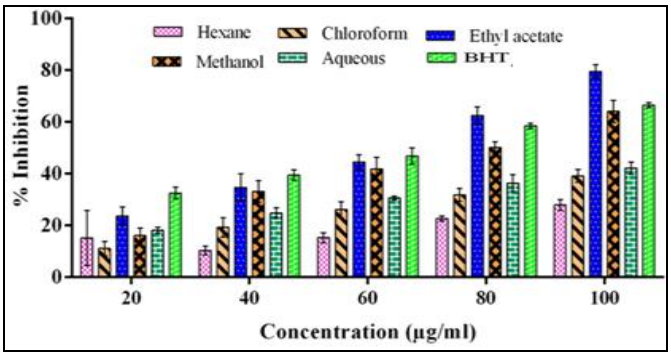


**FIG. 3: SUPEROXIDE RADICAL SCAVENGING OF VARIOUS EXTRACT OF *A. MALABARICA***

**Nitricoxide Radical Scavenging of *A. malabarica:*** The capability of *A. malabarica* on nitric oxide scavenging was increased with an increasing concentration (20 – 100 µg/mL). Among all the extracts, methanol extract possessed the highest radical scavenging activity 85.52 ± 0.28%, followed by ethyl acetate 67.40±0.41% and aqueous extract 62.23±0.39%, with an $EC_{50}$ value 52.50, 63.94 and 83.02 µg/mL, respectively.

However, chloroform and hexane extract showed moderate scavenging activity 58.69±0.17% and 53.43±0.12% with $EC_{50}$ values 93.46, 95.05 µg/mL when compared to standard (BHT 94.39±0.43%) with an $EC_{50}$ value 48.25 µg/Ml **Fig. 4.**

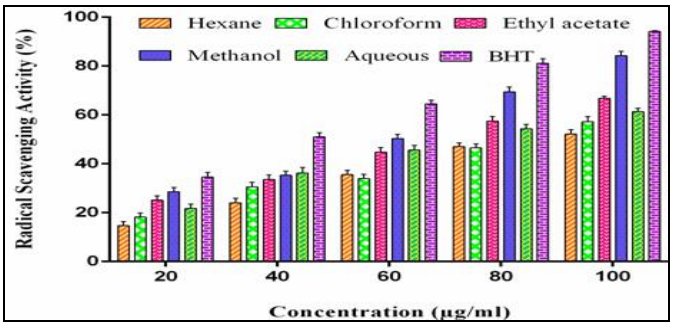


**FIG. 4: NITRIC OXIDE RADICAL SCAVENGING OF VARIOUS EXTRACT OF *A. MALABARICA***

**Hydroxyl Radical Scavenging of *A. malabarica:*** The antioxidant activity of *A. malabarica* was on the basis of their ability to scavenge hydroxyl radical **Fig. 5**. The consistent scavenging activity was obtained in methanolic extract (92.90±0.26%) followed by ethyl acetate (71.78±0.11%) and aqueous (59.78±0.31%), whereas chloroform and hexane extract showed the moderate activity (56.77±0.44 and 42.81±0.53%) at 100 µg/mL. The $EC_{50}$ values of methanol, ethyl acetate, aqueous, chloroform, and hexane ranged from 51.14, 79.10, 95.56, 112.03, and 118.4µg/mL, respectively. The standard BHT scavenged hydroxyl radical sup to 81.04±0.42% ($EC_{50}$58.25 µg/mL). The present study indicated that diterpenescontents were significantly concomitant with antioxidant activity.

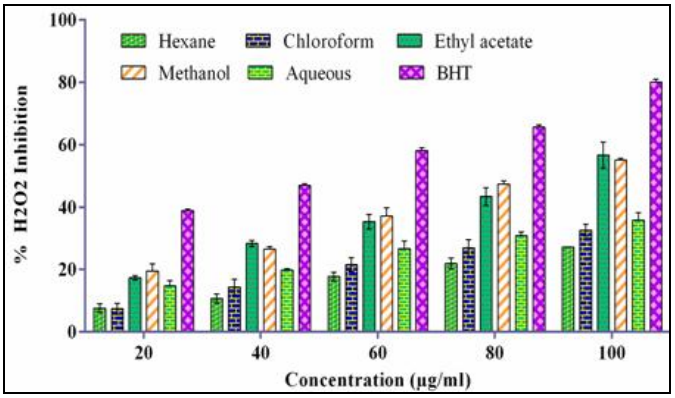


**FIG. 5: HYDROXYL RADICAL SCAVENGING OF VARIOUS EXTRACT OF *A. MALABARICA***

**Ferrousion Chelation of *Anisomeles malabarica:*** The chelating effect of crude extracts was estimated by measuring the iron–ferrozine complex. All the extracts revealed chelating activity in a dose-dependent manner **Fig. 6.** Methanol extract displayed the superior activity (85.52±0.26%), with an $EC_{50}$ value of 52.04 µg/mL, followed by ethyl acetate (67.40±0.24% µg/mL), with an $EC_{50}$ value of 75.89 µg/mL, aqueous extract 62.32±0.22% with an $EC_{50}$ value of 85.03 µg/mL and chloroform 58.69±0.65% with an $EC_{50}$ value 89.03 µg/mL. However, hexane extract displayed the least ferrous ion chelating efficacy (53.43±0.21%) with $EC_{50}$ values of 93.03 µg/mL. The standard BHT scavenged ferrous ions up to 59.12 µg/mL with $EC_{50}$ values of 75.25µg/mL. It was noteworthy that methanol extract displayed the highest antioxidant activity when compared to other crude extracts of *A. malabarica*. $EC_{50}$ values of antioxidant assays were tabulated based upon the ability **Table 1**.

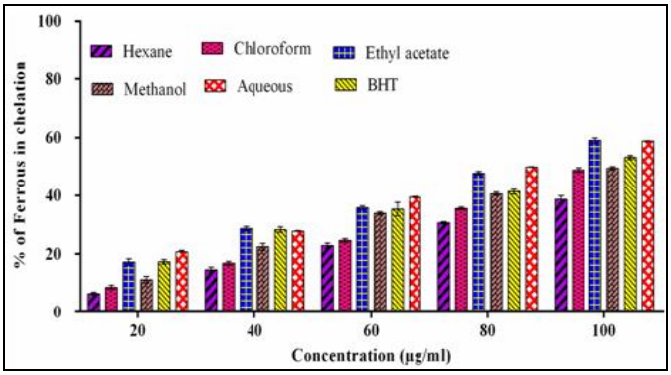


**FIG. 6: EFFECT OF FERROUS ION CHELATION ON VARIOUS EXTRACTS OF *A. MALABARICA***

**TABLE 1: $EC_{50}$ VALUE OF ANTIOXIDANT ASSAYS**

| Assays / Extracts | Hexane | Chloroform | E. Acetate | Methanol | Aqueous | BHT |
|---|---|---|---|---|---|---|
| DPPH | 94.03 | 59.34 | 53.45 | 34.85 | 60.04 | 40.60 |
| ABTS | 104.0 | 110.3 | 62.04 | 49.04 | 79.07 | 38.11 |
| Superoxide | 120.0 | 103.4 | 59.04 | 69.04 | 100.2 | 56.04 |
| Nitric oxide | 95.05 | 93.46 | 63.94 | 52.50 | 83.02 | 48.25 |
| Hydroxyl | 118.4 | 112.03 | 79.10 | 51.14 | 95.56 | 58.25 |
| Ferrous ion | 93.03 | 89.03 | 75.89 | 52.04 | 85.03 | 75.25 |

**DISCUSSION:** The DPPH reaction was very stable at room temperature, producing reliable values in repeated tests. Its odd electron gives a strong absorption at 515 nm in visible spectrophotometer [17]. The decrease in absorbance of DPPH is caused by the reaction between antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum of 517 nm. As antioxidants donate protons to these radicals, the absorbance decreases. The purple color generally fades when an antioxidant is present in the medium [18]. The antioxidant activity is expressed by $EC_{50}$ value, which is defined as the effective concentration of substrate that causes 50% loss of the DPPH & ABTS activity [19]. The crude extracts exhibited highest $EC_{50}$ value (methanol extract 34.85 µg/mL) when compared with BHT standard (40.60µg/mL).

There is an increasing trend to replace synthetic antioxidants, which are of safety concern, with the natural antioxidants available from plant extracts or isolated products of plant origin [20]. The therapeutic benefits of medicinal plants are often related to their antioxidant properties attributed to the extracts' phenolics and flavonoid content [21]. Flavonoids are naturally occurring in plants and are thought to positively affect human health [22]. The perceived antioxidant activity of extracts may be due to the neutralization of ABTS radical [23]. All the extracts of *A. malabarica* possessed stronger ABTS scavenging activity than other research [24].

The hydroxyl radicals are the most toxic among all the reactive oxygen species (ROS) and easily can cross cell membranes at specific sites, react with most biomolecules, and cause tissue damage and cell death [25]. Therefore, it is important to remove the hydroxyl radicals to protect the biological systems [26].

Thus, hydroxyl radical scavenging activity is very important for evaluating the antioxidant activity of various extracts. In the present study, antioxidant activity was determined for the leaves crude extracts showed the highest $EC_{50}$ value in methanol 51.14 µg/mL, and ethyl acetate 79.10 µg/mL, respectively, which is lower than the standard BHT (58.25 µg/mL). Thus *A. malabarica* has a higher ability to scavenge even at lower concentrations, which shows that it is a good candidate for the preparation/additive in the formulation of functional food or nutraceutical. Reactive oxygen species (ROS), including superoxide radicals, have gained significant attention due to the progression of several human diseases and carcinogenesis. So, it is important to eliminate excessive $O_2$- *in-vivo* to prevent important oxidative stress originated diseases [27].

Superoxide radicals are harmful to cellular components and are considered the primary ROS that can further interact with other molecules to generate secondary ROS such as hydroxyl radicals, hydrogen peroxide, and singlet oxygen [28, 29]. Overproduction of ROS leads to an imbalance in intracellular redox status and further, it is associated with oxidative stress. In this study, leaf extracts of *A. malabarica* showed higher $EC_{50}$value in ethyl acetate 59.04 µg/mL and methanol 69.04 µg/mL and standard BHT showed 56.04 µg/mL. It could be useful in drug formulation. Nitric oxide (NO) is a signaling molecule that plays a significant role in the prolongation of inflammation and immunological responses. The mechanism of action of crude extracts or their constituents may be *via* the inhibition of the activity of inducible nitric oxide synthase (iNOs) or by free radical scavenging activities [30, 31]. The recent study of nitric oxide scavenging of *Desmodium gangiticum* showed 30.18% at 200 µg/mL of crude extract (Usha & Muthu Krishnan, 2012) whereas it was 85.52% at 100 µg/mL of methanolic extract. The results clearly denoted that *A. malabarica* possesses superior scavenging ability than the *D. gangiticum*. Generally, iron can stimulate lipid peroxidation by the Fenton reaction and accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl [32].

In addition, other studies have indicated classes of compounds like polysaccharides [33], some peptides, and proteins [34]. Exhibit significant chelating effects. Various authors have claimed that metal chelation plays a minor role in the overall antioxidant activities of some polyphenols [35]. In the present study, crude extracts showed the highest $EC_{50}$ value in methanol (52.04 µg/mL) and ethyl acetate (75.89 µg/mL), respectively, and it is compared with the standard BHT (75.25 µg/mL). Measurement of color reduction allows estimation of the chelating activity of the coexisting chelator [36].

**CONCLUSION:** *Anisomeles malabarica* is an important traditional medicinal plant that possesses a rich source of phytocompounds. The present study results revealed that the *in vitro* antioxidant activities of *A. malabarica* confirmed that methanolic extract exerted the maximum antioxidant activity than the other extracts. The activity was due to highly soluble bioactive metabolites in plant extracts of *A. malabarica*. Based on the results, it may be proposed that the naturally obtained from the test plant may play a major role in the antioxidant defense mechanism.

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**CONFLICTS OF INTEREST:** The authors declare no conflict of interest.

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