Advances in Bioresearch
Adv. Biores., Vol 14 (2) March 2023: 103-111
©2023 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.14.2.103111

Advances in Bioresearch

ORIGINAL ARTICLE

Antihypertensive and Antioxidant Activities of *Oldenlandia diffusa* (Willd.) Roxb. Essential Oil from Assam, India

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ABSTRACT
Oldenlandia diffusa (Syn. Hedyotis diffusa Willd.) belongs to the class of Magnoliopsida and family Rubiaceae, and is a wild edible herb widely found in Assam, North East India. In this communication, Angiotensin Converting Enzyme (ACE) inhibitory activity and antioxidant (DPPH, hydroxyl and reductive ability) properties of the essential oil extracted from Oldenlandia diffusa (Willd.) Roxb. were described. Essential oil was extracted using hydrodistillation method. The amount of essential oil collected was 0.44% (v/v). DPPH, Hydroxyl radical scavenging and reductive ability of the essential oil were found to be 26.88±0.319 µg/mL (IC_{50}), 2.89±0.04 µg/mL (IC_{50}) and 0.74±0.008 respectively at maximum concentration of 100 µg/mL in comparison to Butylated Hydroxytoluene (0.99±0.011). The essential oil has good ACE inhibitory activity and IC_{50} was found to be 22.87±0.286 µg/mL. All these data suggested that essential oil of Oldenlandia diffusa has a good potential to combat hypertension along with antioxidant property. Further Oldenlandia diffusa can be used as potential herbal candidate for cardiovascular research in natural product chemistry.
***Key words:** Oldenlandia diffusa; Essential Oil; antihypertensive; antioxidant*

Received 12.12.2022 Revised 10.01.2023 Accepted 27.02.2023

How to cite this article:
Bardwi Narzary, Kashyap J. Tamuli, Manobjyoti Bordoloi, Dipsikha Bora, Antihypertensive and Antioxidant Activities of *Oldenlandia diffusa* (Willd.) Roxb. Essential Oil from Assam, India . Adv. Biores. Vol 14 [1] March 2023; 01-09

INTRODUCTION

The main cause of death worldwide is due to cardiovascular diseases (CVDs), a non communicable and chronic ailment such as coronary heart, cerebrovascular, peripheral arterial, rheumatic heart and congenital heart diseases in addition to deep vein thrombosis and pulmonary embolism. Both developed as well as developing nations acknowledge hypertension or elevated blood pressure as a risk factor for CVDs. According to WHO, in 2019, approximately 17.9 million deaths were recorded due to CVDs which corresponded to 32% of total deaths globally. Heart attack and stroke accounted for 85% of these fatalities [28]. It has been predicted that more than 1.56 billion individuals would have hypertension globally by 2025 due to the rising incidence of the condition, which is the primary reason of premature mortality [11]. Through the renin-angiotensin and kinin-kallikrein systems, angiotensin-I converting enzyme (ACE) is vital for mediating blood pressure. ACE facilitates the process by which angiotensin I is transformed into the strong vasoconstrictor angiotensin II and deactivates the vasodilator bradykinin [15]. As a result of ACE's twin effects, blood pressure rises and subsequently hypertension develops [10]. Therefore, it is thought that inhibiting ACE activity is a viable treatment strategy for engaging with hypertension issues [6].

In aerobic metabolism, oxidation is a crucial activity, especially in vertebrates and humans, but it also promotes the generation of free radicals [19]. These unstable free radicals can disrupt biomolecules when they are overly abundant or when cellular defences are vulnerable owing to a lack of anti-oxidative molecules. Consequently, oxidative stress generates free radicals which would harm proteins, lipids, and nucleic acids (DNA or RNA) and in turn leading to cell death and tissue damage. Furthermore, a study suggests oxidative stress is a factor in a number of human disorders, such as cancer, cardiovascular conditions, stroke, and hypertension [23]. Even though the human body possesses a defensive mechanism

against free radicals, it is not particularly good at averting their impact from occurring entirely. Therefore, foods loaded in anti-oxidants can be employed to support and shield the human body from these oxidative effects [12, 14].

Moreover, Angiotension II interferes with a number of cellular functions at high blood pressure by encouraging the development of intracellular reactive oxygen species (ROS), which raises oxidative stress [21]. Because ACE inhibitors prevent the production of angiotensin II, they have also been demonstrated to improve the anti-oxidative defense mechanism in both people and animals, in addition to lowering blood pressure. Functional food items with several bioactivities are receiving more attention attributable to the undesirable side effects of chemically produced ACE inhibitors. Medicinal plants have been used for the cure of different ailments including cancer and hypertension for thousands of years. Plant-derived substances, such as tannins, phenylpropanoids, flavonoids, terpenoids, xanthones, proanthocyanidins, fatty acids, alkaloids, peptides/amino acids and oligosaccharides were found to have in vitro ACE inhibitory activity [17]. Large numbers of plants having both antioxidative and ACE inhibitory properties are available in nature but they are not fully phytochemically investigated. It should be of particular interest to explore the antioxidative and ACE inhibitory potential of the medicinal plant extracts and essential oils, for isolation and characterization of the active principles so that better, safer and cost effective drugs can be developed for controlling the CVD, hypertension and oxidative stress**.**

One such example is *Oldenlandia diffusa* (Syn. *Hedyotis diffusa* Willd.). It belongs to the class of Magnoliopsida and family Rubiaceae, and is an annual herbaceous plant widely found in East and Southeast Asia, e.g. China, Indonesia, Malaysia and Japan. *O. diffusa* has immense medicinal value with antitumour, anti-inflammatory, immunomodulatory and other pharmacological effects, and it has been used for the treatment of tonsillitis, boils, hepatitis, appendicitis, dysentery, urethritis and snake bites for years [24, 5]. Essential oil forms an important part of the immune system of the plant. It is volatile in nature and is composed of numerous compounds having health benefits. From time immemorial, these oils are used as an alternative therapy for various common diseases. Study on essential oil composition of *O. diffusa* has been conducted but their biological activity has never been reported [29, 9].

The unique climatic and topographical conditions in Assam, India result in an area with a high biodiversity. It's fascinating to consider that the indigenous population of this area relies heavily on locally accessible flora for basic health care and food. *O. diffusa* herb is prepared traditionally as a cuisine in form of curry or fried item alone or with potato in Assam by various ethnic groups. The herb has been used as herbal medicine for a long time to treat various common diseases such as stomach trouble, jaundice, arthritis etc. Therefore, in extension of research by our team in quest of bioactive components from natural sources [16, 20], in this manuscript we have described the extraction of the essential oil from *O. diffusa* collected from paddy field areas in Jorhat, Assam. The essential oil was then subjected to antioxidant and ACE inhibitory assay. To our belief this study will be the first of its kind that describes the bio-activity of essential oils extracted from *Oldenlandia diffusa.* It holds a huge potential for developing drug molecules and this study will open further research opportunities in the field of herbal drug discovery.

MATERIAL AND METHODS

Collection of plant material

Fresh *Oldenlandia diffusa* (Willd.) Roxb. herbs were collected in February 2019 from paddy field area from Sahpuria gaon, Jorhat, Assam, India. Identification of the plant was done by Prof M Bordoloi and confirmed at BSI, Howrah (Voucher specimen No NPC/273).

Chemicals

Anhydrous sodium sulphate, DPPH, Methanol, Ascorbic acid, EDTA, $FeCl_3$, H_2O_2, Deoxyribose, Phosphate buffer, NaOH, BHA, TCA, TBA, Potassium ferricyanide, BHT, HCl, HHL, Borate buffer, NaCl, ACE, Hippuric acid, Captopril. All analytical grade chemicals were procured from Sigma-Aldrich. Water from Milli-Q system (Merck Millipore Corporation, Merck KGaA, Darmstadt, Germany) was used for preparation of stock solutions.

Extraction of essential oil

For extracting the essential oil, the method described by Saikia *et al.,* [20] was used with minor alterations. The collected plant material of *O. diffusa* (aerial parts) weighing 383 gm were washed and cut into small pieces. The essential oil was obtained from the herbs with Clevenger-type apparatus. The plant materials were put in a round bottom flask with 1L distilled water and heated at 100**°C** using a heating mantle. The condenser of Clevenger-type apparatus was connected with Buchi Recirculating Chiller F-305, circulating the mixer of ethylene glycol and water (1:1) at -10°C led to cool down the volatile components and get collected in the tube. The whole procedure was repeated 3 more times. The essential

oil was then carefully collected at the end of experiment in glass bottles**.** Anhydrous sodium sulphate was used to dry the moisture content of the essential oil. The oil was kept in sealed containers in the refrigerator at 4 **°C** until it was used for chemical analysis and biological activities. The yield of essential oil was calculated by using the following formula:

$$\text{Yield (\%)} = \text{Dry weight of crude oil (g)/Raw material taken (g)} \times 100\% \quad (1)$$

Phytochemical screening

Oldenlandia diffusa essential oil was qualitatively analysed for the presence of several phytochemicals utilizing a number of previously established phytochemical techniques [7].

Antioxidant activity of the essential oil

Numerous *in vitro* techniques were used to examine the *Oldenlandia diffusa* essential oil's antioxidant properties.

Free radical scavenging activity:

The approach of Nath *et al.,* [16] was customized to determine the amount of DPPH that could scavenge free radicals. Concisely, the 3mL whole reaction mixture, which comprised 2.8mL of test solution at different essential oil concentrations (1, 5, 25, 50, 75, 100, 150, 200, 350, 500 µg/mL) and 0.2mL of DPPH (100 µM), was formulated in triplicates in methanol and maintained at 37°C for 30 minutes. A negative test solution with varying doses was made by substituting 0.2 mL of methanol for DPPH. At 517 nm in relation to the blank, the DPPH reduction in end product was estimated in a UV-Vis spectrophotometer. As a reference standard, ascorbic acid was employed and the level of inhibited DPPH radical was determined using the method below by correlating the test results to those which were not administered with essential oil.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100 \quad (2)$$

Hydroxyl radical scavenging assay

The customized protocol of Bordoloi *et al.,* [4] was used to determine the hydroxyl radical scavenging capability. In order to conduct the experiment, the following ingredients pre-prepared with Milli Q deionized water were added in the orderly fashion: 1.0 mL essential oil (various concentrations), 0.1 mL each of EDTA (100 µM), H_2O_2 (1 mM) and ascorbic acid (100µM), 0.33 mL phosphate buffer (20 mM, pH-7.4), 0.01 mL $FeCl_3$ (100 µM) and 0.36 mL deoxyribose (2.8 mM). Then it was blended by vigorous shaking prior to being kept in dark at 37˚C for 1 hr. Later, 0.5% TBA (added in 0.025 M NaOH together with 0.025% BHA) and 10% TCA, 2mL each, was appended to it. The pink chromogen was formed by heating the solution for an extended 15 mins at 95°C in a water bath. This color formation was assessed at 532 nm. Standard employed for the experiment was BHT. Inhibition of deoxyribose cleavage expressed as a percentage represents the essential oil's capacity to scavenge hydroxyl radicals which are equated by

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100 \quad (3)$$

Reductive ability

According to a slightly tweaked version of Bordoloi *et al.,* [4] research, the capacity of the test samples' antioxidant components to produce coloured complexes with potassium ferricyanide, TCA, and $FeCl_3$ was used to evaluate their reducing power. A concoction was made and incubated for 20 minutes at 50°C comprising 0.5 mL of potassium ferricyanide (1%), 0.2M phosphate buffer (pH 6.6), and varying doses (5, 25, 50, 75 and 100 µg/mL) of essential oil. The subsequent step was centrifuging the concoction along with 10% TCA (0.5 mL) in it for 5 minutes at 5000 rpm. 100µL $FeCl_3$ (0.1%) and 1 mL water were added after 1 mL of supernatant was removed. After being held at 37°C for 10 minutes, the optical density of the reaction mixture was assessed at 700nm in relation to a suitable blank solution. BHT was employed as reference standard.

Angiotensin Converting Enzyme (ACE) Inhibition activity

The technique reported by Gogoi *et al.,* [8] was modified to execute the Angiotensin I-Converting Enzyme inhibitory assay of *Oldenlandia diffusa* essential oil. 200µL of 5 mM hippuryl-L-histidyl-L-leucine (HHL) was pre-incubated for 3 mins at 37 °C with a 80µL of *O. diffusa* essential oil at various quantities (10, 50, 100, 150, 200 and 250 µg/mL), both of which were made in borate buffer (100mM) along with sodium chloride (300mM) at a pH of 8.3. 20µl of 0.1 U/mL ACE prepared in sodium borate buffer was introduced, and the reaction was started and was allowed to sit for 30 min at 37 °C. By adding 250µL of HCl (0.1 M), the enzyme process was halted. Following that, 1.7 mL of ethyl acetate was appended and vortex-mixed

for 15 s to extract the generated hippuric acid (HA). An eppendorf tube was taken to transfer the top level (1mL), where it was then dispersed for 15 minutes at 90 °C. After 1 mL of distilled water was utilized to suspend the generated HA, an ultraviolet/visible spectrophotometer (U-3900, Hitachi) was used to detect the absorbance at 228 nm. As a standard, Captopril was employed. ACE inhibition percentage was estimated as following

$$\% \text{ ACE Inhibitory activity} = [(B - A)/ B] \times 100 \quad (4)$$

Wherein A represents HA absorbance while an ACE inhibitor was administered, and B represents HA absorbance while an ACE inhibitor was not administered. The sample concentration (µg/mL) that inhibits ACE activity by 50% (IC_{50}) was obtained from the graph plotting sample concentration vs ACE inhibitory activity.

Statistical analysis

All experiments were conducted in triplicates and the calculated parameters were reported as mean ±SD. The IC_{50} values were calculated using MS excel 2007. Here, $P<0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

Yield of the *Oldenlandia diffusa* essential oil

The yield of the isolated essential oil from *Oldenlandia diffusa* aerial parts was found to be 0.44% (v/w). The color of the essential was light yellow. Jian *et al.,* 2016 found that the oil yield of *Hedyotis diffusa* Willd. harvested in China was 0.1 (v/w) and hexadecanoic acid (48.89%), pentadecanoic acid (6.11%), D-limonene (5.74%), and fatty acids made up the majority of the constituents. According to Wong and Tan, 1995, *H. diffusa* Willd., which was collected in Malaysia, yielded 0.02% w/w of pale yellow oil, with the phenolic chemicals p-vinylphenol (22.2%) and p-vinylguaiacol (18.6%) and the terpenoid linalool (13.6%) being the most significant ones. The reported constituents of these studies are different from one another of same herb found in China and Malaysia. The variation in the qualitative and the quantitative framework of the constituents might be possibly due to the geographical location, climatic factors and edaphic factors. Owing to these reason, essential oils' chemical makeup and biological activity differ from one country to another [3].

Phytochemical screening

In the essential oil of *O. diffusa*, preliminary phytochemical analysis showed the presence of different types of physiologically important secondary metabolites such as steroids, flavonoids, terpenoids, etc as exhibited in Table 1. Plants that contain phytochemical substances serve as natural antioxidants that augment the body's requirements. Among the numerous beneficial health effects of eating fruits and vegetables are their high nutritional content and therapeutic characteristics [1]. Plants typically comprise phenolic and flavonoid compounds, which are acknowledged to have a range of biological effects. Antioxidant, cytotoxic, antimutagenic, antineoplastic, antimicrobial, anti-platelet, antihypertensive, antiviral, anti-inflammatory, antihepatotoxic, and antiulcerogenic activities are only a few of these advantageous traits. Numerous of these biological processes are linked to antioxidant and free radical action [18]. Awoyinka *et al.,* [2] accounted another class of phytochemicals with anti-inflammatory properties includes steroids and glycosides. In order to exert their antibacterial effects, terpenoids made up of monoterpenes, hemiterpenes, diterpenes, sesquiterpenes, triterpenes, tetraterpenes, and polyterpenes permeate through cell membranes and target intracellular components [25].

Table 1. Phytochemicals found in *Oldenlandia diffusa* essential oil.

Phytochemicals	Aerial part
Flavonoids	**++**
Saponin	**-**
Steroids	**+**
Reducing sugars	**-**
Tannins	**-**
Alkaloids-wagners reagents	**+**
Alkaloids-Draghandroff-reagents	**+**
Terpenoids	**+++**
Key: + = present, - = absent	

Antioxidant assay of the *Oldenlandia diffusa* essential oil

It is theorized that antioxidants' ability to donate hydrogen is what causes them to affect DPPH radical scavenging activity [22]. Consequently, a highly reactive radical generated from oxygen, the hydroxyl radical is found in all living things and numerous biomolecules, including nucleic acids, lipids, and

proteins are subject to oxidative injury as a result of it. Moreover, the ability of plants to donate electrons is described as their reductive ability. Hence, the potential of *Oldenlandia diffusa* essential oil to scavenge DPPH and Hydroxyl radicals and for electron donation was assayed. Promising antioxidant activity was observed in the essential oil of *O. diffusa* for DPPH and Hydroxyl radical scavenging assay, and Reductive ability in dose dependent manner. The data are listed in Table 2. The IC_{50} values for DPPH radical scavenging activity is 26.88±0.319 µg/mL and hydroxyl scavenging activity is 2.89±0.04 µg/mL respectively and are comparable to their respective standards. The dose dependent activity of essential oil in DPPH and Hydroxyl scavenging assay is represented graphically by % inhibition vs concentration in Fig.1 and 2 respectively. The absorbance for reductive ability is 0.74±0.008, at maximum concentration of 100 µg/mL in comparison to BHT (0.99±0.011) showed good reducing power (Table 2). It was seen that the absorbance of the essential oil amplified with the rise of their concentrations (Fig. 3) directly correlating to increase in reducing power. The presence of major phytochemical compounds may be attributed for the notable antioxidant test results performed for the first time in essential oil of *Oldenlandia diffusa* found in Assam. A few antioxidant studies on *H. diffusa* extracts showed strong activity of them. According to Yang *et al.,* [30] studied, using DPPH assay, the 80% alcohol extract (0.1-4.5 mg/mL) had the best activity when aqueous, methanolic, and 80% acetonic extracts of *H. diffusa* were examined for antioxidant activity. By measuring the peroxide value, Yu *et al.,* 2002 examined petroleum benzine, ether, chloroform, acetonic, alcoholic, and aqueous extracts from *H. diffusa* for the antioxidant effects and discovered that the 0.12% acetone extract had the strongest antioxidant impact. Another research by Lu *et al.,* [13]mentioned flavonoids and iridoids, two of *H. diffusa's* constituents, may be the source of the plant's antioxidant effects. Phenolics are potent chain-breaking antioxidants and efficient electron donors that can speed up the transformation of H_2O_2 to H_2O. Because of their potential to squelch free radicals and the direct contribution that their hydroxyl groups may make to antioxidant activity, phenols are significant plant components [26]. Consequently, terpenoids and vitamin E are anticipated to aid in the inhibition of DPPH free radicals. The total antioxidant effect of an essential oil, however, might be difficult to assign to a single or small group of active ingredients. Wang *et al.,* 2008 illustrated the potential of a synergistic effect is increased by the relevance that both minor and major components should have in the action of oil.

Table 2. Antioxidant activity of *Oldenlandia diffusa* Essential oil.

Name of Samples	**IC_{50} (µg/mL)±Std Dev**		**Absorbance (at 700nm) ±Std Dev**
	DPPH radical scavenging activity	**Hydroxyl radical scavenging activity**	**Reductive ability at 100 µg/mL**
Oldenlandia diffusa Essential oil	26.88±0.319	2.89±0.04	0.74±0.008
Ascorbic acid	1.58±0.033	ND	ND
BHT	ND	0.76±0.008	0.99±0.011
DPPH- 2, 2-diphenyl-1-picrylhydrazyl; BHT- Butylated Hydroxytoluene; IC_{50}-Concentration at which the sample can scavenge 50% of the radical; ND, Not done. **Values are expressed as mean ±SD (n = 3).**			

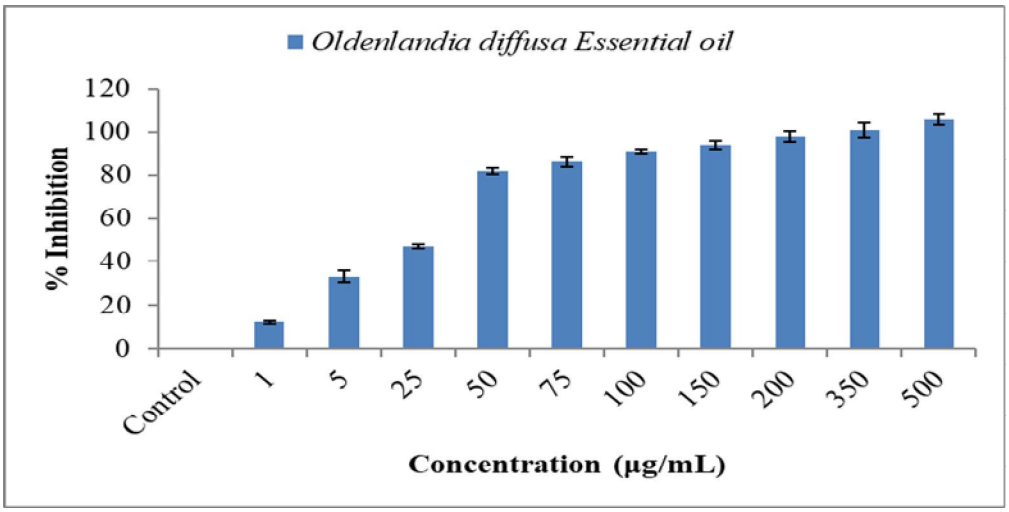

Fig. 1. DPPH Radical Scavenging Activity of *Oldenlandia diffusa* Essential oil

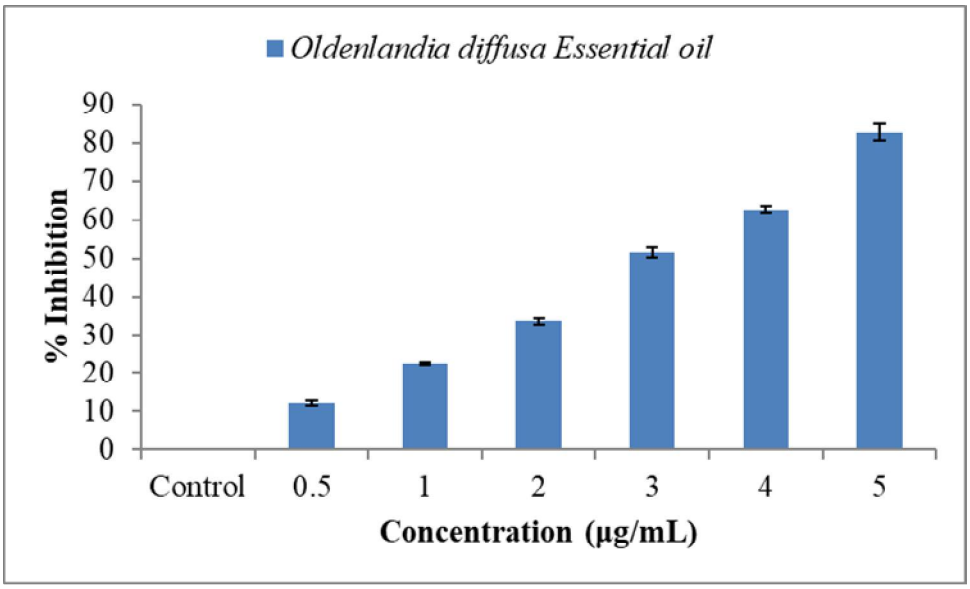

Fig. 2. Hydroxyl Radical Scavenging Activity of *Oldenlandia diffusa* Essential oil

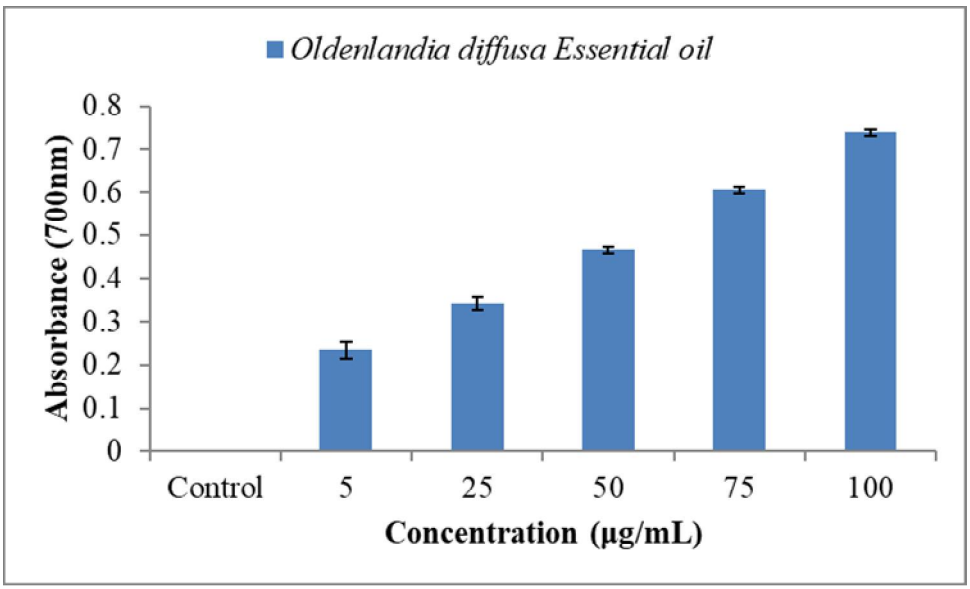

Fig. 3. Reductive Ability of *Oldenlandia diffusa* Essential oil

Inhibition of ACE by *Oldenlandia diffusa* essential oil

The essential oil isolated from *Oldenlandia diffusa* from Assam demonstrated good ACE inhibitory activity which was performed for the first time is shown in Table 3. IC_{50} value of the essential oil and captopril is found to be 22.87±0.286 µg/mL and 8.2±0.033 µg/mL respectively which was calculated from the dose response graph of % ACE inhibition with respect to concentration. As shown in Table 4 and Fig. 4, *O. diffusa* oil inhibited ACE in dose dependent manner with inhibition % of 44.67, 91.24, 92.85, 95.04, 96.50 and 99.12 corresponding to dosages of 10, 50, 100, 150, 200, 250 µg/mL, respectively. These noteworthy results of ACE inhibitory assay urged the employment of *O. diffusa* oil as ACE inhibitor to treat hypertension. *Thymus algeriensis* essential oil with an IC_{50} of 150 µg/mL demonstrated interesting ACE inhibitory activity [32]. Lacaille-Dubois *et al.,* 2001 accounted at 0.33 mg/mL, the flavonoids isovitexin and vitexin, which were isolated from diverse plants, respectively reduced the ACE activity by 45% and 20%. As mentioned by Chen *et al.,* 1992, the occurrence of substances that compete with the substrate for an enzyme's active site to produce chelate complexes, which in turn restrict ACE function, is thought to be the cause of essential oils' inhibitory properties.

Table 3. Angiotensin Converting Enzyme (ACE) Inhibitory activity of *Oldenlandia diffusa* Essential oil.

Name of Samples	IC_{50} (µg/mL)±Std Dev
	ACE Inhibitory activity
Oldenlandia diffusa Essential oil	22.87±0.286
Captopril	8.2±0.033
ACE- Angiotensin-I-converting enzyme; IC_{50}-Concentration of the sample that inhibits 50% activity of ACE; **Values are expressed as mean ±SD (n = 3).**	

Table 4. Percentage Inhibition of Angiotensin Converting Enzyme (ACE) due to *Oldenlandia diffusa* Essential oil at different concentrations.

Concentration (µg/ml)	% ACE Inhibition ±Std Dev
Control	0±0.00
10	44.67±0.442
50	91.24±0.837
100	92.85±0.697
150	95.04±0.280
200	96.50±0.448
250	99.12±0.442

Values are expressed as mean ±SD (n = 3).

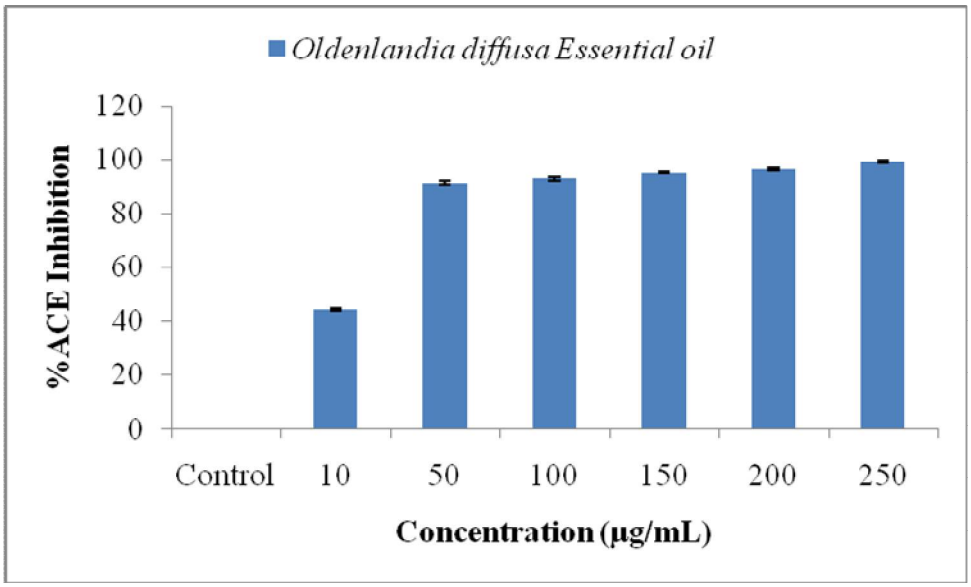

Fig. 4. Angiotensin Converting Enzyme (ACE) Inhibitory activity of *Oldenlandia diffusa* essential oil at different concentrations

CONCLUSION

This is the first report reciting the antioxidant and ACE inhibitory potential of the essential oil of *Oldenlandia diffusa* from Assam, India. Preliminary phytochemical analysis of light yellow colored *O. diffusa* essential oil revealed the presence of a wide variety of physiologically important secondary metabolites such as phenolic acids, flavonoids, terpenoids. Notably, our results illustrate a significant potential of *O. diffusa* essential oil of antioxidant activity and inhibitory effect on ACE showing twin bioactivities in dose dependent fashion. This indicates the probability of *O. diffusa* essential oil to be utilized as safe alternative to treat hypertension. This study will offer new viewpoint to treat CVDs and diseases associated with free radicals and to formulate an herbal drug.

ACKNOWLEDGEMENTS:

BN thanked Ministry of Tribal Affairs (MoTA), India for National Fellowship & Scholarship for Higher Education of Scheduled Tribe Students (NFST scheme) (201718-NFST-ASS-00448). The authors are also thankful to the Director, CSIR-NEIST for providing research facilities for part of the work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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