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# *In vitro* evaluation of anti-acetylcholinesterase and free radical scavenging potential of leaf extracts of some selected medicinal plants

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ABSTRACT

**Objective:** To evaluate the phytochemical present in various solvent extracts from leaves of *Ocimum sanctum* (L.), *Swertia chirayita* (L.), *Butea monosperma* (Lam.) and *Stevia rebaudiana* (Bert.) as well as antioxidant and anticholinergic activities employing different *in vitro* models. **Methods:** Total phenol content of diethyl ether, chloroform and methanolic extracts obtained from leaves of different medicinal plants was determined by Folin-Ciocalteau's spectrophotometric method. Moreover, antioxidant and anticholinergic studies were conducted by four different *in vitro* methods which included diphenyl picrylhydrazyl radical scavenging, 2,2-azinobis (3-ethylbezoline-6-sulphonic acid), reducing activity by ferrous reduced antioxidant power and anti-acetylcholinesterase assay, in order to ensure pharmacological potential of the plants. **Results:** The methanolic leaf extract of *Ocimum sanctum* showed the highest total phenol content which was (21.13±1.04) GAE/g DW and antioxidant activities compared to other plants with the $IC_{50}$ value of 40.43 μg/mL in diphenyl picrylhydrazyl radical scavenging assay and 53.5 μg/mL in 2,2-azinobis (3-ethylbezoline-6-sulphonic acid) assay as well as metal ion reduced by (78.22±0.38) TE/g DW in ferrous reduced antioxidant power assay. The inhibition percentage of the anti-acetylcholinesterase assay was (94.22±0.26)%. **Conclusions:** The results of our current study show that *Ocimum sanctum* leaf is the most significant source of phytochemicals that possesses antioxidant and anticholinergic properties. However, further investigation on isolation and characterization of active compound which is responsible for the pharmacological potential is needed.

## 1. Introduction

Alzheimer's disease (AD), at present is considered to be the most prominent age-related neurodegenerative health complication worldwide, based on its frequency of occurrence in the population. According to different works of literature, the report indicated that about 33% of individuals aged 85 and above are mostly affected by this disease[1]. Progression is irreversible in deterioration of cognitive abilities, which leads to complete dependence of individual which symbolizes as nature of AD[2,3]. In this disorder,

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inflammation and neuronal loss of some specific region of forebrain are diagnosed which is due to amyloid beta plaques growing into neurofibrillary tangles, which slowly damages memory and thinking skills[4]. Oxidative stress plays a vital role in the cause of this disorder, it had been found that free radicals do activate memory inadequacy in AD patients, which is evident in many studies[5,6]. At present, in the treatment for AD, acetylcholinesterase (AChE) inhibitors like donepezil, galantamine, rivastigmine, and tacrine are used and mostly these drugs could accomplish the case to reform the expression of dementia. However, due to the lack of selectivity of cholinesterase inhibiting drugs in the commercial market, AD patients suffer from other issues like nausea or vomiting[7].

Nature is the best combinable chemist and feasibly has been used to heal almost any medical issues faced by mankind[7]. Demand of herbal medicine treatment for the AD is increasing because of their potential activities against the AD. The folkloristic concepts of medicinal plants play a vital role in the cure of different medical issues. The medicinal plant and plant-related products are used in an increasing manner day by day[8]. Many noticeable incidents came around, which highlights that in Western society the reduction in usage of synthetic products had become a growing interest and allied to upgradation in the demand for natural remedies[9]. Tulsi [*Ocimum sanctum* (*O. sanctum*)], is a multifunctional herb employed in the indigenous system of medicine culture. The roots and seeds of *O. sanctum* possess multitudes of medicinal properties. It has the wide range of influence on the human body mainly as a cough alleviator, a sweat inducer and a moderator of indigestion and anorexia and also acts as memory enhancer[10]. *Butea monosperma* (*B. monosperma*) (Lam.) is widely used in folk medicine due to its anticonvulsive, antistress, antidiabetic and antiaging properties. Extracts of *B. monosperma* exhibit pharmacological activities like wound healing, antihyperglycemic, anti-inflammatory, hepatoprotective and antitumoral properties found *in vivo* and *in vitro* studies[11]. *Stevia rebaudiana* (*S. rebaudiana*) is a perennial shrub which is historically employed as a sweetener in South America. *Stevia* extracts consist of polyphenolic compounds which have been reported to exhibit strong antioxidant activity by reducing series of free radicals such as DPPH, hydroxyl radical, nitric oxide, superoxide anion and hydrogen peroxide[12]. *Swertia chirayita* (*S. chirayita*) has been reported to possess most important medicinal properties such as anti-inflammatory, hypoglycemic, hepatoprotective, antioxidant and antispasmodic *etc*. There are many isolated chemical compounds from *S. chirayita* and one of them is swertiamarin which shows biological activity of anticholinergic potential[13]. In this study, an attempt was made to investigate the diethyl ether, chloroform and methanolic extracts of *O. sanctum* (L.), *S. rebaudiana* (Bert.), *B. monosperma* (Lam.) and *S. chirayita* (L.) leaves for their potential antioxidant activity by using diphenyl picrylhydrazyl (DPPH) radical scavenging activity, ferrous reduced antioxidant power (FRAP), and 2,2-azinobis (3-ethylbezoline-6-sulphonic acid) (ABTS) along with anti-acetylcholinesterase (anti-AChE) activity assays. These different assays were the simpler methodologies to estimate the free radical scavenging potential. Further, these extracts can be used to reduce oxidative stresses which are responsible for many neurodegenerative disorders. Anti-AChE cholinergic study has been done to estimate plants extractability to inhibit AChE which is responsible for hydrolysis of acetylcholine (ACh), resulting in hindrance in nerve impulse transmission.

## 2. Materials and methods

### 2.1. Plant materials and preparation of extracts

Leaves of plants *B. monosperma*, *O. sanctum*, *S. rebaudiana* were collected from medicinal plant garden of Birla Institute of Technology, Mesra, Ranchi, Jharkhand and leaves of *S. chirayita* were attained from their natural habitat of Darjeeling, West Bengal. Plant materials were shade dried at room temperature and ground in a mortar. Twenty-five gram of each plant powder was extracted in solvents which were diethyl ether, chloroform or methanol by successive maceration (48 h). Subsequently, each solvent extract was analyzed by different methodologies. The solvent was removed and concentrated extracts were froze dried for further analysis[14].

### 2.2. Total phenol determination

The total phenolic content was determined by the Folin-Ciocalteau's spectrophotometric method[15–17]. One mL extract (1 mg/mL) was mixed with Folin-Ciocalteau's phenol reagent, 0.5 mL of 7% $Na_2CO_3$ solution was then added to the reaction mixture followed by the addition of 13 mL of deionized distilled water. The mixture was allowed to stand in the dark for 15 min at the temperature of 23 ℃. The absorbance was recorded at 750 nm. The total phenol content was estimated from the prognostication of the calibration curve which was made by gallic acid solution. The evaluation of the phenolics compounds was carried out in triplicates.

### 2.3. DPPH radical scavenging activity

DPPH scavenging activity was estimated using a modified methodology of Laghari *et al*[18]. The DPPH (0.1 mM) working solution was prepared using methanol to attain an absorbance of about (1.10±0.02) at 517 nm. One mL of sample was supplemented to 3 mL of the methanolic DPPH solution, then the mixture was allowed to stand for 90 min at 23 ℃. Furthermore, the antioxidant potential of Trolox as standard reference was assayed. The inhibition of DPPH radicals by the plant extracts was calculated as follows: DPPH inhibition (%) = [(A-B/A)] × 100 (Where A is the absorbance without extract and B is the absorbance with extract).

### 2.4. Total antioxidant capacity assay using ABTS radicals

An ABTS radical scavenging potential of different medicinal plants was estimated by an elaboration by Miller and Rice-Evans as well as Arnao *et al*. with minor modifications[19,20]. The ABTS

solution was processed by mixing 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution in ratio of 2:1. The mixture allowed for reacting for 16 h at room temperature in the dark. The solution was then mixed to 1 mL ABTS solution with 60 mL methanol to obtain an absorbance of (0.70±0.02) units at 734 nm using the spectrophotometer. A total of 225 μL of different extracts were allowed for reacting with 4 275 μL of ABTS for 2 h in a dark condition. Then the absorbance was observed at 734 nm using the spectrophotometer. The standard curve of Trolox was linear between 50 to 200 μg/mL.

### 2.5. Determination of FRAP assay

For measuring reducing power of extracts, FRAP assay was employed as reported by Szollosi and Varga, with few modifications[21]. The stock solutions consisted of 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_2O_2$), pH 3.6; and 10 mM TPTZ (2,4,6- tripyridyl-s-triazine) solution. Plant extracts (200 μL) were allowed for reacting with 5 000 μL of the FRAP solution for 30 min in the lightless condition at 37 ℃. The absorbance of the colored product (ferrous tripyridyltriazine complex), was observed at 593 nm. The standard curve of Trolox was linear between 20 to 100 μg/mL.

### 2.6. Anti-AChE assay

The enzyme inhibition for AChE (purified purchased from Sigma-Aldrich) and measurement of release of acetylcholine from synaptosomes on the administration of plant extract were evaluated according to the method previously reported by Ellman *et al*., and modified by Sancheti *et al*. and Lee *et al*[22-24]. The potentials of all four medicinal plants' extracts were evaluated against AChE which was determined using enzyme at a concentration of 0.3 U/mL and galanthamine as standard reference was assayed. All plant extracts were examined for their inhibitory activities at the 40 μg/mL of concentration.

### 2.7. Statistical analysis

All analyses were carried out at least in triplicates, and these values along with their standard deviations were elucidated. Data analysis was carried out using Graph Pad Prism version 5.0 software. Statistical comparisons were made with one-way analysis of variance (ANOVA) and $P$-value <0.05 was taken as significant difference.

## 3. Results

### 3.1. Total phenolics content

The comparative study of total phenol content on all leaf extracts in different solvents was conducted by using the Folin-Ciocalteau's spectrophotometric method. The total phenol content in the extracts was computed from the regression equation (y=0.011 2x+0.046 9, $R^2$=0.966 8) of the calibration curve. The total phenol content of plant methanol extracts showed parameters from (16.40±0.87) to (21.13±1.04) GAE/g DW. In this study, methanol extract of *O. sanctum* leaves showed the highest value in total phenol content [(21.13±1.04) GAE/g DW] followed by *S. chirayita* [(20.93±3.04) GAE/g DW], *B. monosperma* [(20.30±2.00) GAE/g DW], *S. rebaudiana* [(16.40±0.87) GAE/g DW] which showed the lowest content in methanolic extracts as shown in Figure 1. According to the observations in our study, the high contents of phenol in these extracts could illustrate their strong free radical scavenging capacity.

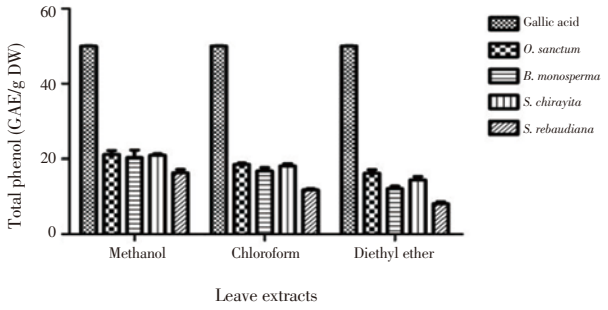


**Figure 1.** Total phenol content in leaf extracts of different medicinal plants. Gallic acid was taken as a control; GAE: Gallic acid equivalent, DW: Dry weight of the sample.

### 3.2. Results of DPPH radical scavenging activity

The outcome of DPPH scavenging potential for various leaf extracts of four plants is presented in Table 1. The twelve fractions of different plants showed various degrees of antioxidant activities. It could be concluded that from the results of Table 1, $IC_{50}$ values ranged from 40.43 μg/mL to 102.05 μg/mL. The highest antioxidant activities were determined in *O. sanctum* in its respective solvents, in which the methanol extracts had $IC_{50}$ value (40.43 μg/mL) followed by chloroform (52.25 μg/mL) and diethyl ether extracts (66.87 μg/mL). Moreover, among these plants, methanolic extract of *O. sanctum* leaves showed the highest DPPH scavenging ability followed by *S. chirayita* methanolic extract that may include the therapeutic potential of the extract against oxidative stress.

**Table 1**

Free radical scavenging activities, represented by $IC_{50}$ in various extracts of each plant tested by DPPH assay (μg/mL).

| Plant | Methanol | Chloroform | Diethyl ether |
|---|---|---|---|
| *O. sanctum* | 40.43 | 52.25 | 66.87 |
| *S. chirayita* | 52.87 | 65.92 | 76.05 |
| *S. rebaudiana* | 64.87 | 70.55 | 85.96 |
| *B. monosperma* | 80.06 | 92.85 | 102.05 |

### 3.3. Results of ABTS assay

For the $IC_{50}$ values in ABTS assay shown in Table 2, we found that the $IC_{50}$ varied from 53.5 μg/mL to 88.9 μg/mL. The extracts of leaves using methanol as the solvent were shown to be the strongest inhibitor which showed the $IC_{50}$ values in ABTS assay at its lowest concentration when compared to other solvents. In this study, *O. sanctum* methanolic extract showed the highest scavenging activity followed by *S. chirayita*, which attributes to the presence of phenol in higher concentration as compared to other plants.

**Table 2**

Free radical scavenging activities, represented by $IC_{50}$ in various extracts of each plant tested by ABTS assay (μg/mL).

| Plant | Methanol | Chloroform | Diethyl ether |
|---|---|---|---|
| *O. sanctum* | 53.5 | 61.2 | 69.9 |
| *S. chirayita* | 55.2 | 63.8 | 73.3 |
| *S. rebaudiana* | 68.2 | 75.6 | 83.8 |
| *B. monosperma* | 75.8 | 82.2 | 88.9 |

### 3.4. Results of FRAP assay

Amongst all extracts, *O. sanctum* methanolic extract [(78.22±0.38) TE/g DW] showed the highest reducing capability followed by its diethyl ether extract [(76.58±0.78) TE/g DW] and chloroform extract [(74.00±1.99) TE/g DW] (Figure 2).

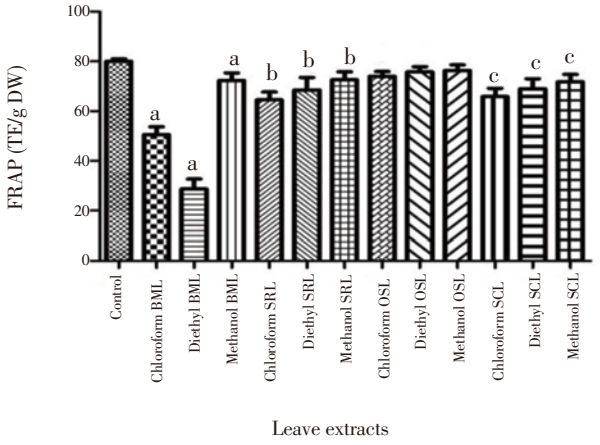


**Figure 2.** Activity of ferrous ion chelation by four plant extracts in various solvents.

BML-*B. monosperma* leaves, SRL-*S. rebaudiana* leaves, OSL-*O. sanctum* leaves, SCL-*S. chirayita* leaves. Data were expressed as mean±SD. [a]superscript in each value showed the significant difference ($P$<0.001), [b]superscript in each value showed the significant difference ($P$<0.01), [c]superscript in each value showed the significant difference ($P$<0.05).

### 3.5. Results of anti-AChE activity

In our study, the methanolic extract of *O. sanctum* showed the highest percentage of AChE inhibition (94.22±0.26)% followed by its diethyl ether and chloroform extracts when compared to galanthamine (98.23±0.34)% as shown in Figure 3.

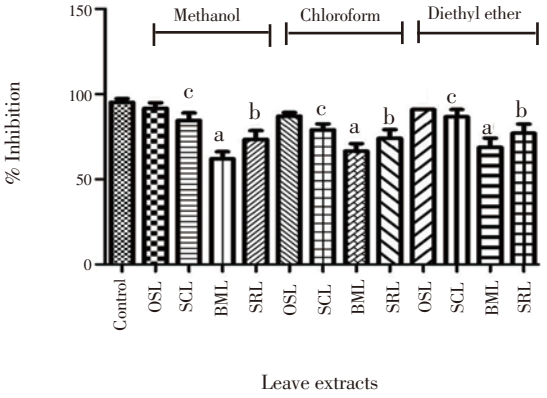


**Figure 3.** Inhibitory effect of leaf extracts of different medicinal plants prepared in different solvents on AChE activity (acetylcholinesterase of electric eel) *in vitro*.

Galanthamine was taken as a control. BML-*B. monosperma* leaves, SRL-*S. rebaudiana* leaves, OSL-*O. sanctum* leaves, SCL-*S. chirayita* leaves. Data were expressed as mean±SD. [a]superscript in each value showed the significant difference ($P$<0.001), [b]superscript in each value showed the significant difference ($P$<0.01), [c] superscript in each value showed the significant difference ($P$<0.05).

## 4. Discussion

Plants are well supplemented with multiple phytochemical constituents *i.e.* vitamins, terpenoids, phenolics, lignin, tannins, flavonoids, quinones, oils and resins and other metabolites which have high antioxidant activity. From past few years, researchers have shown great interest in the medicinal plant which is the source of many phytochemicals with great pharmacological activities[25,26]. The natural phenolics which are reported as reducing agents demonstrate strong antioxidant properties because these molecules have the potential to terminate the multiplication of free radicals chain reactions in the existence of hydroxyl groups[27]. In total phenolics determination assay, according to previous studies by Suriyavathana and Punithavanthi, it was found that the amount of phenolic compounds (14.55 w/w) in methanolic fraction was higher than others solvents' extracts in *O. sanctum* leaves[28]. In our study, methanol fraction was studied extensively because it was observed that maximum phenol content can possibly be found in polar solvents' extracts [*i.e* (87.32±1.32) mg GAE/g DE] reported by Lee *et al*[29].

In DPPH scavenging assay, the $IC_{50}$ value assists in the evaluation of herb concentration which is able to inhibit 50% of used DPPH. The study which was done by Rana *et al*. suggested that methanolic extract of *O. sanctum* leaves showed higher $IC_{50}$ value than other solvents' extracts[30]. In our study, we also found *O. sanctum* methanolic extracts exhibited great potential to scavenge free

radicals. Similarly, a study was done by Keshari *et al*., in which they compared the percentage of free radicals scavenging property of *O. sanctum* and vitamin C[31]. In their results, *O. sanctum* showed slightly higher scavenging percentage, indicating its greater scavenging activity than vitamin C. Property of ABTS free radical scavenging activity possessed by plant sample is helpful in forming more stable product by modifying free radicals in the influence of hydrogen which terminates oxidation process[32]. In the present study, we found that *O. sanctum* methanolic extracts had the lowest $IC_{50}$ value (53.5 μg/mL) as compared to diethyl ether and chloroform. Similar results were found by Basak *et al*., in which *O. sanctum*'s methanolic extract showed better ABTS scavenging activity than other extracts with different polarities[33]. Antioxidants electron donating competency is tested by FRAP assay. The process is comprised of increase in absorbance at 700 nm, indicating higher reducing power, which is due to the reduction of ferric ion ($Fe^{3+}$)[34]. Agarwal *et al*., found that *O. sanctum*'s ethyl acetate extract showed a better result than methanolic extract[35]. But, in our work, we observed that *O. sanctum* methanolic extract exhibited better reducing capability than diethyl ether and chloroform.

The nerve impulse transmission is hindered by AChE through hydrolysis of ACh in neurodegenerative disorder. So, inhibition of AChE is one of the foremost strategies of pharmacotherapy to inhibit this neuro dysfunctionality[36]. *O. sanctum* has shown better inhibition efficacy compared to *B. monosperma* leaf extract. *B. monosperma* crude extract has indicated minimum efficacy, but to the best of our knowledge, this is the first report which demonstrates the inhibition of *B. monosperma* leaf extract against *in vitro* AChE assay. So, this could be a novel therapeutic material for AD, however further studies are needed to gain insight into its medicinal phenomena. According to the study by Uddin *et al*., it was found that *B. monosperma* leaves showed anti-inflammatory, and the formation of thrombus and membrane stabilizing property, this result concludes that *B. monosperma* leaves had ability to reduce oxidative degradation of cellular components[37]. In the case of AChE inhibition of *O. sanctum* leaves methanolic extract, the study conducted by Singh *et al.* suggested that it showed the lowest inhibition amongst other *Ocimum* species[38]. But in our study, it showed the highest inhibition percentage. According to Sembulingam *et al*., it studied that the reduction of ACh content in the discrete areas of the brain in rats was caused due to noise stress, but after pretreatment of *O. sanctum* extract, stress values of cholinergic parameters had brought back to normal[39].

In conclusion, the present study revealed that *O. sanctum* leaves have significant antioxidant potential and anti-AChE activity. In the anti-AChE assay, the methanolic extract of *O. sanctum* showed the highest activity, in comparison to other plants' extracts in various solvents. *B. monosperma* leaves showed the least inhibition in all assays but still up to some extent it showed some inhibition in case of anti-AChE activity which is least studied. This *in vitro* antioxidant study indicated that *O. sanctum* is an important natural source of antioxidants and might be significant for preventing the oxidative stress and damage to our body cells. Our future study assures further investigation about isolation, characterization, and *in vitro* studies on animal models to appraise the potency of the active compounds present in *O. sanctum*.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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