Pharmacogn. Mag.
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ORIGINAL ARTICLE

Antioxidant and Antiacetylcholinestrase Studies of *In vitro* Regenerated and Transformed Hairy Roots of *Ocimum sanctum* (L.)

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Submitted: 19-Dec-2020 Revised: 02-Mar-2021 Accepted: 22-Apr-2021 Published: 15-Sep-2021

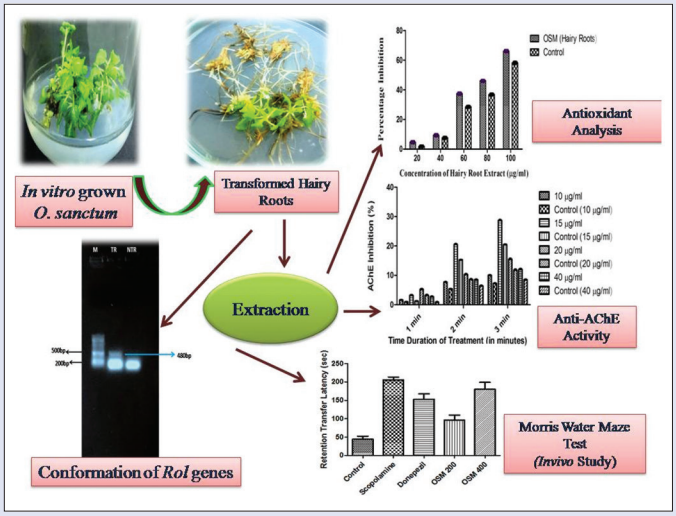
ABSTRACT

Background: *Ocimum sanctum* (L.) is a well-known extensively employed Indian medicinal shrub in traditional and modern therapeutics. Hairy roots exhibit similar or superior capability to inhibit oxidants responsible for many disorders. **Objectives:** *Agrobacterium*-mediated transformation protocol has developed for *O. sanctum* which influences T-DNA delivery. The antioxidant, antiacetylcholinesterase (AChE) potential, and *in vivo* study of hairy root extract of *O. sanctum* has been evaluated. **Materials and Methods:** Sterilized *O. sanctum* shoot tips had grown *in vitro* by employing Murashige and Skoog basal media, Benzyl amino purine (BAP) (0.5 mg/L), and other additives. *In vitro* grown leaf explants were infected by *Agrobacterium rhizogenes* ATCC 15834 for various time durations. Polymerase chain reaction (PCR) employed to confirm the genetic assimilation of *Agrobacterium*. The hairy root extract's antioxidant potential has been tested by diphenyl picric hydroxyl (DPPH) and ferrous ion reducing assay (ferrous reduced antioxidant power [FRAP]) along with anti-AChE potential. *In vivo* study was done employing behavioral and biochemical estimation. **Results:** For *in vitro* culturing of *O. sanctum*, administration of BAP (0.5 mg/L) shows growth rate of 32.7% ±0.19% in 7 days. The *Agrobacterium*-infected explants show the good yield of hairy root in 1 h of infection duration. The PCR results show the genomic integration of transgene *rol A* and *rol C*. The methanolic extracts of hairy roots showed potential antioxidant activities for DPPH-free radical (66.15% ±0.34%) and FRAP assay (226.35 ± 0.17TE/gDW of the sample). These extracts effectively inhibit AChE (28.710 ± 0.26 µg/mL at 3 min incubation duration). In *in vivo* study, the pretreatment of OSM (200 and 400 mg/kg) exhibited significant increase of spatial and long term after administration of scopolamine. **Conclusion:** The obtained results exhibited a simple protocol for micropropagation and transgenic roots production with competently targeting AChE and related disorders.

Key words: Acetosyringone, *Agrobacterium*, Alzheimer's, mercuric chloride, meropenem, micropropagation

SUMMARY

- *Ocimum sanctum* L. is known as one of the indigenous plants of India. It has numerous phytochemical constituents such as rosemarinic acid, ursolic acid, eugenol, apigenin, and beta-pinene. In this study, *O. sanctum* plantlets were grown by sterilizing them with $HgCl_2$. *Agrobacterium rhizogenes*-mediated transformation (ATCC 15834) had performed to procure hairy roots. The hairy root extracts were tested for FRAP reducing and DPPH scavenging activity. Along with anti-AChE inhibition, activity of hairy roots in different concentrations and time durations was tested.



Abbreviations used: DPPH: Diphenyl picric hydroxyl; FRAP: Ferrous reduced antioxidant power; $HgCl_2$: Mercuric chloride; BAP: Benzyl amino purine; $AdSO_4$: Adenine sulfate; MS: Murashige and Skoog; YEB: Yeast extract beef extract broth; PCR: polymerase chain reaction; TBE: Tris, boric acid, EDTA: EDTA: Ethylenediamine tetraacetic acid; EtBr: Ethidium bromide; DTNB: 5,5′-Dithiobis-(2-nitrobenzoic acid); ATCI: Acetylcholine iodide; ANOVA: One-way analysis of variance; BChE: Butyrylcholinesterase; AD: Alzheimer's disease; TPTZ—Fe^{2+}: 2,4,6-Tripyridyl-S-triazine-ferrous complex; TE: Trolox equivalent; MWM: Morris Water Maze; PSA: Passive Avoidance Paradigm; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; GSH: Reduced glutathione; MDA: Malondialdehyde; STL: Step transfer latency

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DOI: 10.4103/pm.pm_539_20

Access this article online
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INTRODUCTION

Since the Vedic period, *Ocimum sanctum* L. (well-known as *Ocimum tenuiflorum*, Tulsi) is manifested as a medicinal plant that is indigenous to India. This plant has been known for its healing power, endorsement of life's longevity, and maintenance of an individual's health. Due to these traits, it is classified as "Rasayana."[1] *O. sanctum* L. (Tulsi) is a shrub of 30–40 cm tall, erect, branched with simple opposite green or purple

Cite this article as: Toppo AJ, Chandra S. Antioxidant and antiacetylcholinestrase studies of *In vitro* regenerated and transformed hairy roots of *Ocimum sanctum* (L.). Phcog Mag 2021;17:S151-61.

leaves. The leaves of the plant consist of strong aroma. Its stems are hairy in texture. The length of the leaves is up to 5 cm long and has petiole and ovate. The characteristic feature of the flower consists of purplish color having elongate racemes, patterned in close whorls. It is bred mainly for religious and therapeutic purposes.[2] For the cure of different ailments such as common flu, digestive, and hepatic issues, the leaf extract of this flora has been extensively used.[3,4] Phytochemical constituents majorly found in *O. sanctum* are rosemarinic acid, ursolic acid, eugenol, apigenin, and beta-pinene.[5,6] It also possesses some nootropic competency against diazepam, scopolamine in mice models.[7]

A wide range of secondary metabolites that profusely found for pharmacological importance, results in the destruction or uprooting of the whole plant for the extraction of noble compounds. The production of the disease-free plant, plant genome transformation, and multiplication of rare genotypes has profusely depended on the *in vitro* techniques.[8] *In vitro* techniques such as plant tissue culture, cell suspension techniques exhibit excellent results. However, the development of hairy roots culture through varied species of *Agrobacterium rhizogenes* gave a promising perspective toward the production of bioactive components.[9,10] It is a Gram-negative soil-borne bacterium, induces exogenous Ri plasmid into the host plant cell. The formation of a branch-like neoplastic hairy root has been caused due to *rol* genes. The whole phenomenon of induction is due to stability in the integration of Ri T-DNA in the host plant genome. The hairy root possesses the ability to grow profusely in devoid of any phytohormones which is their unique characteristic.[4,11] One of the greatest advantages of the hairy root is that they exhibit similar or superior capability of secondary metabolites in the comparison of mother plants.[12,13]

As *O. sanctum* is a repository of numerous medicinal properties, the study aimed to germinate the plant by employing potential protocol in a shorter period. Along with, hairy roots induction in the same plant and investigation of methanolic extracts of hairy roots for their potential antioxidant activity using diphenyl picric hydroxyl (DPPH) radical scavenging activity, ferrous reduced antioxidant power (FRAP), and antiacetylcholinesterase (anti-AChE) activity assays. These various antioxidant assays were employed because they are simple and quick methodologies for the estimation of free radical scavenging capacity. Anti-AChE cholinergic study has been employed to investigate the capability of hairy roots to inhibit AChE which is the causative agent for neurodegenerative diseases such as Alzheimer's. However, to the best of our knowledge, there is no analytical report concerning the effect of hairy roots extract on AChE considering different parameters (i.e. time duration and concentrations). Further, these *in vitro* studies had validated using *in vivo* model. This *in vivo* model investigated to recognize the protective effect of *O. sanctum* hairy root extract on scopolamine-induced memory deficits in Swiss albino mice.

MATERIALS AND METHODS

Sterilization of explants and *in vitro* development of plantlets

O. sanctum (L.) (Accession No.-T0134) plants were procured from the medicinal garden of Birla Institute of Technology, Mesra, Ranchi, Jharkhand. The shoot tips were harvested from the field-grown plants. Explants were cleaned under running tap water for 10 min washed with bavistin for another 10 min, and cleansed thrice using distilled water. The final step of sterilization was followed by mercuric chloride ($HgCl_2$) treatment in varied concentrations (0.025%, 0.05%, and 0.1%) for 5 min and then thoroughly cleansed with sterilized distilled water. The sterilized shoot tips were excised up to 1.0 cm and inoculated onto 0.8% (w/v) agar solidified medium containing benzyl amino purine (BAP) (0.5, 1.0, and 1.5 mg/L), adenine sulfate ($AdSO_4$) (25, 50, and 75 mg/L), and citric acid (1.0, 2.0, and 3.0 mg/L) in Murashige and Skoog (MS) basal[14] (Himedia Mumbai, Maharashtra, India) medium with 30 g/L sucrose, pH 5.8 ± 0.2. The explants inoculated in agar solidified hormone-free MS basal medium employed as control. The culture established was achieved after 4 weeks of inoculation at 25°C ± 2°C and 16/8 h (light/dark) photoperiod. *In vitro* grown shoot cultures were maintained by eventual subculturing. Later, these explants were employed in transformation experiments.

Agroinfection and Induction of hairy roots

The transformation experiments are carried out by employing *A. rhizogenes* ATCC 15834. *Agrobacterium* strain was grown in Yeast extract Beef extract broth medium[4] for 24 h at 28°C ± 2°C with shaking (120 rpm) in the dark until absorbance between 0.4 and 0.8 at 600 nm were attained. The bacterial culture grown in liquid medium was centrifuged at 5000 g for 5 min. Then, bacterial pellets were re-suspended in 200 ml MS basal liquid medium supplemented with 75 μM acetosyringone.[15] The cultures were allowed to acclimatize for another 2–3 h at 28°C ± 2°C at 60 rpm.

The leaf explants were excised from the *in vitro* cultivated plantlets. The excised explants were cut into 1–2 cm length, wounded carefully using a sterile blade (Sterile Scalpel Blade No. 22 Himedia, Maharashtra, India). Then, explants were immersed in bacterial suspension for different infection time durations (1, 2, and 3 h). The explants were blotted mildly on sterile paper towels and then co-cultured on MS (full strength, ½, and 1/4) basal medium supplemented with meropenem (6.25 mg/L)[16] and B5 vitamin (1 ml/L)[17] for 48 h at 25°C ± 2°C in the light free zone. After that, explants were made bacteria free by washing them by sterile distilled water supplemented with 500 mg/L of cefotaxime (Alkem, India)[13] for 5 min. Then, explants were relocated to agar-solidified MS basal medium devoid of any hormone and incubated at 25°C ± 2°C under 16/8 h photoperiod conditions. After 5–6 days of incubation, the commencement of hairy roots has been observed. All experiments were repeated five times, each set of experiments consist of twenty explants. Some of the explants were incubated in MS basal medium without *Agrobacterium* infection to serve as the control. Later, profusely grown hairy roots were transferred to MS basal semi-solid medium for further growth at 25°C ± 2°C with an eventual subculturing process.

Molecular characterization of hairy root through the polymerase chain reaction

All materials used for RNA isolation were free from RNase. Freshly cut hairy roots were pulverized with a pestle in a mortar having liquid nitrogen. The total RNA was extracted according to the manufacturer's instructions employing a commercial RNA extraction solution (RNeasy Plant Mini Kit Qiagen, USA).

The cDNA synthesis was done using the protocol suggested by Kang *et al.*[18] To study the presence of Ri T-DNA in the hairy root lines, cDNA was further analyzed. Specific primers were used for amplifying *rol* A gene (403 bp) (forward primer: 5'-ACGGTGAGTGTGGTTGTAGG-3', reverse primer: 5'-GCCACGTGCGTATTAATCCC-3') and *rol* C gene (480 bp) (forward primer: 5'-TGTGACAAGCAGCGATGAGC-3', reverse primer: 5'-AAACTTGCACTCGCCATGCC-3').[18,19] The polymerase chain reaction (PCR) reaction was endured in a DNA thermal cycler (Himedia, Maharashtra, India) under the conditions as follows: hot start at 96°C, 5 min, 96°C 1 min (denaturation), 55°C 1 min (annealing), 72°C 1 min (amplification), and 72°C out for 35 cycles.

The agarose gel of 0.7% (Duchefa Biochemie, The Netherlands) was employed for fractionating amplified sequences in 0.5 × TBE (Tris 44.5 mM: Boric Acid 44.5 mM: EDTA 0.1M) (Sigma-Aldrich

Co., USA) buffer. The staining of the gel was performed using 0.5 µg/L ethidium bromide Sigma-Aldrich Co, USA, added during casting gel and after than electrophorized gel was observed under ultraviolet-transilluminator (A260).

Antioxidant and antiacetylcholinesterase analysis

The transformed hairy roots were shade dried at room temperature and grounded finely in a mortar. Two grams of plant powder was extracted in solvent (methanol) employing the maceration technique for 24 h. After that, extracts were analyzed by different methodologies.[20]

To determine total antioxidant potential of methanol extracts, FRAP assay was employed. In this, 200 µl of the extract and 3 ml FRAP reagent were mixed in test tubes and vortexes, respectively. All the test tubes containing reaction mixture were incubated carefully in a water bath for 30 min at 37°C, and the absorbance of the samples was recorded against blank at 593 nm. For these analyses, $FeSO_4.7H_2O$ was employed as a standard curve. The values evaluated were expressed as µM of ferrous equivalent Fe (II)/g of the sample.[21]

DPPH-free radical scavenging method was employed to test the free radical inhibition activity of extracts. Two milliliters of an extract of various concentrations (20–100 µl/mL) was taken and mixed with 2 mL of methanol and DPPH solution. In this, DPPH solution devoid of test sample was taken as control. The mixture was incubated at room temperature at light-free zone for 30 min. The free radical scavenging activity of the DPPH was analyzed by evaluating the absorbance at 517 nm until the reaction reached the steady state.[22] All the experiments were performed thrice. The DPPH-free radical scavenging activity was evaluated by following equation: % inhibition = (A1 − A0/A0) ×100

A1 and A0 are the absorbances of the test sample and control, respectively.

Inhibition of the anti-AChE activity was analyzed using the microplate reader by following Ellman *et al.* and Toppo *et al.* methods with few modifications.[23,24] In these analyses, mixture of 0.1M of sodium phosphate buffer (pH 8.0), an enzyme solution (20 µl was added to 20 µl of Ellman's reagent/DTNB [5,5′-Dithiobis-(2-nitrobenzoic acid)]) (10 mM). Then, the reaction was initiated by the addition of substrate (10 µl of acetylcholine iodide [ATCI]). The hydrolysis of the ATCI in the reaction can be observed by the formation of the colored product 5-thio-2-nitrobenzene anion. This product is formed by the reaction between DTNB and thiocholine, released due to hydrolysis of the concerned enzyme. All the experiments were performed thrice. The development of the colored product was evaluated at 412 nm employing a microplate reader.

Animals

Swiss Albino Mice (25–30 g) of either sex was procuring from Birla Institute of Technology, Department of Pharmaceutical Sciences and Technology, Mesra, Ranchi, Jharkhand. The animals were housed and maintained at 25±°C (12 h light-dark cycles) in the departmental animal house. The design of the study has been authorized by the Institutional Animal Ethics Committee (IAEC) (approval no. 1972/PH/BIT/33/18/IAEC). The care of the animals had been taken as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals Ministry of Environment and Government of India.

Study design

After the acclimatization, the animals were divided into five groups having five animals in each group.

- Group I: Control group. Animals were given normal saline (10 ml/kg)
- Group II: Scopolamine-treated group (diseases). Animals were given scopolamine (1 mg/kg)
- Group III and IV: OSM-treated group. Animals were administered OSM at the dose of 200 mg/kg and 400 mg/kg
- Group V: Donepezil-treated group. Animals were administered donepezil (5 mg/kg)

The animals were administered with respective treatments once daily for 7 days. At the end of the treatment schedule, except for Group I, all mice were administered with scopolamine intraperitoneally (1 mg/kg), for 60 min afterward, extract or standard drug (donepezil) was administration. The parameters for cognition were estimated after 30 min of scopolamine dosage.[25]

Behavioral study

Morris Water Maze

Spatial learning and memory of the tested animal were executed by employing a water maze test following protocol by Raghavendra *et al.*,[26] Tripathi *et al.*[27] with few modifications. In this experimentation, a circular tank of 90 cm (diameter) and 45 cm (height) was utilized containing water which was maintained at 25°C ± 2°C. The target platform having dimensions of 10 cm × 10 cm and 22 cm (height) was used. The platform was submerged up to 1–1.5 cm beneath the surface of the water. Different quadrant positions were marked to the tank-like North (N), East (E), South (S), and West (W). In the trial period, the animal was allowed to swim in the tank filled with water for 120s. Later, mice were conceded to stay on the platform for the 20s and then removed from the platform. In the probe trial, the animal was allowed to swim in the tank for 120s in the absence of a platform. Ethovision video tracking (model no. KL35V81S, Noldus) system was employed to record time for escape latency, target quadrant, and zone frequency.

Passive shock avoidance paradigm

This is experimentation setup employed for the evaluation of long-term memory in rodents. This is based on the negative reinforcements of memory.[25,28] It consists of one open compartment and one closed compartment (dark) box. The dark chamber contains a grid floor. In the acquisition phase, the mice were placed in an open compartment. Later, the time taken by mice to move in the dark compartment was recorded. Afterward, in the vicinity of the grid floor, an electric shock of 50V, 0.2 mA was provided to the mice for 3s. Then, after 24 h, mice were once again positioned at the open chamber, and the time duration of entrance in the dark chamber was recorded. The retention trial phase, in the dark chamber no electric foot was provided, and the cutoff was recorded as 300s.[27]

Biochemical estimation

After treatment duration, the animals were sacrificed by decapitation using diethyl ether as anesthesia. Mice brains were removed and homogenized in 10% of chilled 0.1M of PBS (pH 7.4) solution and centrifuged at 3000 rpm for 15 min. The supernatant was collected and transferred in sterilized tubes. The collected supernatant was again centrifuged at 12,000 rpm for 20 min. The supernatant was collected in the sterilized tube for the analysis of superoxide dismutase (SOD) activity. The brain homogenate solution is employed for the estimation of thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH).[29] All the experiments were performed in triplicates.

Estimation of superoxide dismutase activity

The production of toxic products in the body is mainly due to oxidative stress. To counteract these oxidative stress molecules, the body deploys antioxidant agents (like SOD) to scavenge these molecules and defend against cellular level deterioration. SOD level in brain homogenate was estimated by the method of Zhang *et al.*[30]

Estimation of thiobarbituric acid reactive substance

Malondialdehyde (MDA) is the common marker of lipid peroxidation. This affects cell integrity and injuries. MDA was estimated by the color reaction with TBA. Thus, it is known as TBARS level. Its level was estimated in brain homogenate by the method of Ameen and Shafi.[31]

Estimation of reduced glutathione level

During excessive reactive oxygen species generation production of glutathione, enzyme takes place. The level of GSH was estimated in the brain homogenate by the method of Ameen and Shafi.[31]

Estimation of brain acetylcholinesterase activity

The level of AChE activity in the brain was determined by Ellman *et al.*[23] methodology.

Statistical analysis

Tissue culture and transformation experiments' data presented (mean ± standard deviation [SD]) are the mean value of five replicates with 20 explants in each set. Antioxidant and anti-AChE data were carried out in triplicates, along with their mean inhibition percentage and SD values. All data analyses were carried out employing GraphPad Prism 5.0 software. Statistical comparisons were elucidated through one-way analysis of variance (ANOVA) with a significant difference of $P < 0.05$. All *in vivo* data presented as mean ± standard error of mean. Statistical comparisons were elucidated through one-way ANOVA followed by Dunnett's multiple comparison test with a significant difference of $P < 0.05$.

RESULTS AND DISCUSSION

Sterilization of explants and *in vitro* development of plantlets

The thriving plant tissue culture initiates with a suitable procedure for explants employed. In this investigation, various concentrations of $HgCl_2$ were used (0.025%, 0.05%, and 0.1%) for evaluation of sterilization of shoot tip for overcoming contamination of field-derived explants of *O. sanctum*. It has been observed that 26.4% ±0.40% explants of *O. sanctum* were contamination-free and healthy when treated with 0.1% of $HgCl_2$ for 10 min but 0.05% of $HgCl_2$ showed 32.7% ±0.19% of survival rate after 7 days of inoculation [Figure 1a and b]. A similar study was done by Mishra,[32] in which 0.1% of $HgCl_2$ shows the best result at 3–4 min by having 30% of shooting response. In many works of literature, 0.1% $HgCl_2$ shows aseptic culture survival and bud breaking of *O. citriodorum*,[33] but in our study, 0.05% was sufficient for the sterilization process.

For shoot development and multiplication of *O. sanctum*, various concentrations of BAP (0.5, 1.0, and 1.5 mg/L), $AdSO_4$ (25, 50, and 75 mg/L), and citric acid (1.0, 2.0, and 3.0 mg/L) were studied. The highest percentage has been recorded for the number of shoot bud breaking (48.9 ± 0.38) in MS basal media augmented with 0.5 mg/L of BAP with $AdSO_4$ (25 mg/L) and citric acid (1 mg/L) in 21 days of inoculation [Figure 2a and b]. A study by Susila *et al.*[34] on *Rauwolfia serpentina* Benth. found multiple shoot formation with a high concentration of BAP (2.5 mg/L) with a combination of NAA (0.1 mg/L). Similarly, a study by Jamal *et al.*,[35] in which *O. sanctum* shoot tip and nodal segment show the maximum number of shoots in the administration of 2.0 mg/L BAP with 0.5 mg/L NAA in media. In many works of literature, the reduced concentration of cytokinin (BAP) has been proved to be significant for bud breaking and increment in the proliferation of shoots.[36,37] $AdSO_4$ is also known as a precursor of adenine. During the DNA replication process, it is indirectly responsible for the rejuvenation of plant vigor. Therefore, *in vitro* grown shoots are supplemented with $AdSO_4$ in MS basal medium to rejuvenate it after subculturing.[38] A similar study by Singh *et al.*,[39] in which supplementation BAP in the combination of $AdSO_4$ shows a higher rate of multiplication. Citric acid is a well-known antioxidant in the medium that can reduce the lethal effect of phenolic compounds responsible for the browning of tissues and culture medium.[40] From the present study, it is evident that a combination of BAP, $AdSO_4$, and citric acid could easily induce shoot proliferation.

Agroinfection and induction of hairy roots

In the present work, transformation protocol through *A. rhizogenes* has been developed for *O. sanctum* as it is well-known source for many

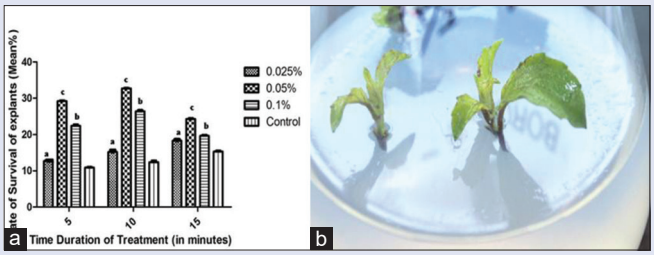

Figure 1: Effect of $HgCl_2$ treatment on micropragation initiation (a) Optimization of $HgCl_2$ treatment for micropropagation of *Ocimum sanctum*. Each treatment has five replicates with 20 explants in each set of experiment. Data represent mean percentage (±) standard deviation of these replicates. a: Superscript is each value shows the significant differences ($P < 0.001$), b: Superscript is each value shows the significant difference ($P < 0.01$), c: Superscript is each value shows the significant difference ($P < 0.05$). Control refers to the regeneration of explants untreated with $HgCl_2$. The data were retrieved after 7 days of culture in respective manner. (b) Inoculated shoot tips after treatment of 0.05% $HgCl_2$ after 7 days of inoculation. Reproduction size: (at column width)

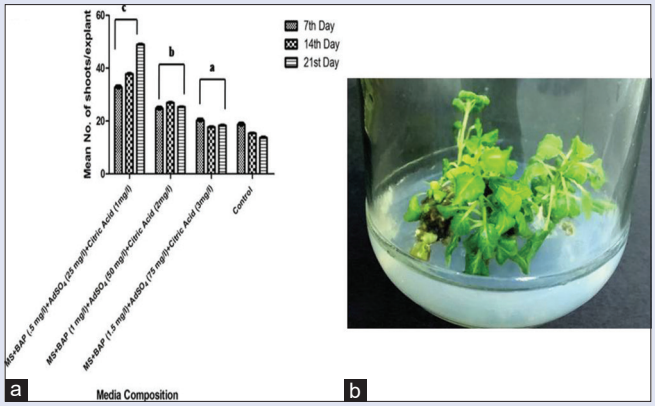

Figure 2: Effect of various concentration of phytohormones on micropropagation (a) optimization of different concentration of benzyl amino purine, $AdSO_4$, and Citric acid on shoot regeneration. Each treatment has five replicates with twenty explants in each set of experiment. Data represent mean percentage (±) standard deviation of these replicates. a: Superscript is each value shows the significant differences ($P < 0.001$), b: superscript is each value shows the significant difference ($P < 0.01$), c: Superscript is each value shows the significant difference ($P < 0.05$). Control refers to the regeneration of shoot tips without supplementation of hormones in the respective media. *The data were retrieved after 30 days of culture in respective manner. (b) Regeneration of shoots from the explants inoculated on Murashige and Skoog basal medium supplemented with benzyl amino purine (0.5 mg/L), dSO_4 (25 mg/L), and Citric acid (1 mg/L) in 21 days. Reproduction size: (at column width)

secondary metabolites. *A. rhizogenes* is an Agropine type strain which is mostly utilized for their powerful potential to induce hairy roots.[41] Initially, the explants have been inquisition with inflammation within 3–5 days of infection [Figure 3a]. In the next 5–6 days, those leaves infected with *Agrobacterium* for 1 h show origination of transformed roots [Figure 3b and c]. In many works of literature, root initiation almost took 10 days in the case of *Withania somnifera*[42] 3 weeks in *Ocimum bacilicum*[43] and 5 weeks in *Trachyspermum ammi.*[44] A similar study was done by Vyas and Mukhopadhyay,[4] in which they observed root initiation within 4–5 days of infection in leaf explants.

In this study, leaves show root initiation specifically from midrib and leaf blades in 1 h of infection duration. We observed that, from the blades and midrib portion of the leaves explants, the growth of single and multiple hairy roots was higher in number. In many works related to *Agrobacterium,* transformation observed that 2 h or 1 day of infection produce a good yield of hairy roots.[45,46] However, in our case, 1 h of infection duration initiates hairy root within 5–6 days (18.6 ± 0.51 mean number of the hairy roots in 7th day). The mean number of roots was higher in 1 h of infection (32.0 ± 1.53 in 21st day) as compared to 2 h (17.5 ± 1.42) and 3 h (8.75 ± 2.08) [Figure 4a].

The variation in the infection time depends on the type of explants and plant species. Hence, identification of optimal or exact infection time could be critical to enhance the transformation capability of particular plant species. It has been realized that the characteristic of relocation and unification of T-DNA calculated on the optical density of the *Agrobacterium* culture. Hence, we can say that this factor would be responsible for attaching bacterial cells to explants' wounded sites. Yang *et al.*[47] had even observations in their article's Figure 2b exhibits a higher rate of transformation in 60 min of an infection duration that other time durations (5, 30, and 120 min).

The features observed in the initial stages of hairy root obtained in this work were mostly unbranched and densely covered with hairy roots. In later stages, the hairy roots had a moderate root length of 7–9 cm.

The supplementation of 75 µM of acetosyringone in the coculture media exhibits a compelling effect on the transformation ability of *Agrobacterium* strain. The studies by Balasubramanian *et al.* and Dehdashti *et al.*[15,42] reported that the enhancement of hairy roots was more effective after supplementation of 75 µM acetosyringone in coculture media for leaf explants.

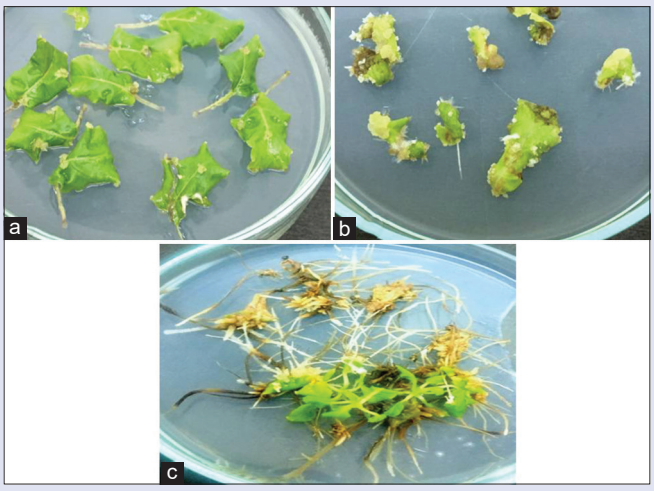

Figure 3: Hairy roots initiation and its growth (a) Initiation of hairy root in leaf explants of *Ocimum sanctum* (b) Emergence of hairy roots from transformed leaves (in 5–6 days) in 1/4 Murashige and Skoog basal media (c) Prolonged growth of hairy root after 30 days of infection. Reproduction size: (at column width)

It is noticeable in various studies that the biomass accumulation of hairy roots by *A. rhizogenes* significantly exhibits variations building upon on the nature of the media.[15] Biomass accumulation was higher (8.42 ± 0.52 g FW; 1.26 ± 0.09 g DW) in 1/4 MS basal medium in comparison to other the other media (MS and 1/2 MS) [Figure 4b]. In respect to the usage of media, in the study reported by Liu *et al.*,[48] in which adoption of 1/4 MS basal salts to obtain steady expression of the transgene in *Sedum alfredii* and *S. plumbizincicola*.

polymerase chain reaction-based confirmation of transformed root cultures

For determination of the genetic prominence of the transformed roots, PCR-based analysis was targeted toward *rol A* and *rol C* of *A. rhizogenes.* The difference in the integration site and copy numbers of Ri-TDNA could be the differentiation and regeneration potential of various root lines.[49] The confirmation of Ri plasmid T-DNA incorporation in the plant genome had detected by the presence of *rol A* (403 bp) and *rol C* (480 bp). Amplification of long fragments of *rol A* and *rol C* showed transgene integration into the plant genome. There were no amplification products obtained from the nontransformed plant [Figure 5a and b]. As *rol C* gene present on the plant-derived DNA and located on TL-DNA of Ri plasmid confirms the integration of the plant genome. The expression of the *rol* gene is important for increased biomass of transformed plants. It also causes increased branching, internodes shortening, and reduced apical dominance.[49] All hairy root lines selected based on their profuse growth in 1/4MS media devoid of any growth hormones.

Antioxidant and anti-acetylcholinesterase analysis

To evaluate the antioxidant capability of samples, FRAP and DPPH are the most accepted assays that have been being employing in several studies. The estimation of ferric ion reducing antioxidant potential (FRAP) samples can deduce by their capacity to oxidize colorless TPTZ-Fe $^{3+}$ to TPTZ-Fe^{2+} (2,4,6-Tripyridyl-S-triazine-ferrous complex) (blue colored samples). In the case of DPPH, the hydrogen donating ability of the compound attributes the scavenging capability of the extract.[50] The antioxidant activity of transformed roots enhances as the capability of the sample used for assay increased from 20 to 100 µg/mL in both the assays [Figure 6a and b]. In the case of *O. sanctum*, we have recorded a hike in 226.35 ± 0.17 FRAP TE/g dry weight of sample and 66.15% ±0.34% scavenging activities of the transformed plant in 100 µg/ml of sample. Furthermore, the extracts of ATCC 15834 hairy root have the potential to inhibit DPPH-free radicals in a concentration-dependent manner.

A similar work involving the evaluation of DPPH inhibiting capability of hairy roots were studied in *Bacopa monneri*[49] induced with MTCC 532. In the case of *Lactuca serriola* hairy roots infected with strain AR15834 correlated with control, a hike in total reducing power and antioxidant potential observed.[51] Matvieieva *et al.*[52] and Muthusamy and Shanmugam[53] recorded a higher amount of antioxidant activities in the hairy roots of *Artemisia vulgarise* and *Raphanus sativus*. Through various works of literature and observations, we can assume that the T-DNA insertion in the plant genome enhances its antioxidant potential. It intermeddles with the biosynthetic pathway responsible for the secondary metabolite production. Hence, the biologically active secondary metabolites of the plant upgrade its quality of secondary metabolite in Ri transformed plants.

AChE is known as predominant cholinesterase in the human brain. It hydrolyzes the ACh to choline and acetate results in hindering in the

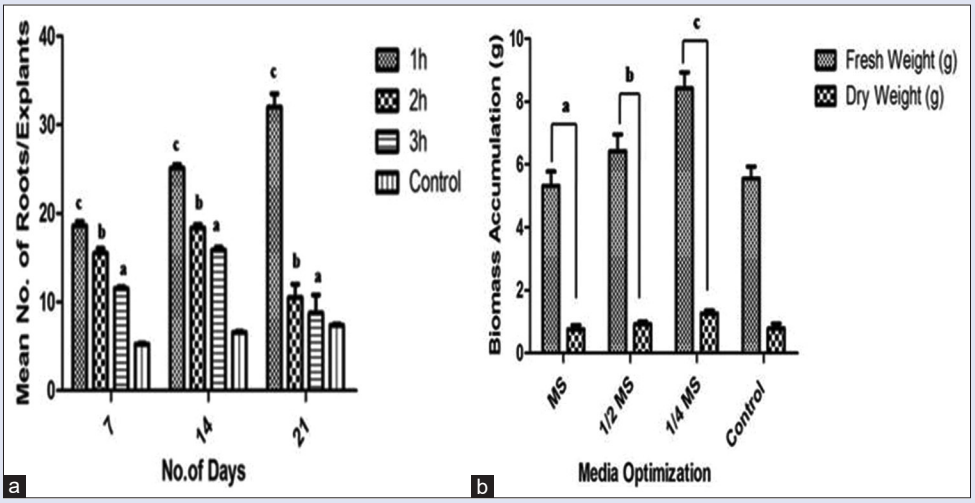

Figure 4: Hairy roots intiation in various infection time duration and its biomass accumulation (a) Optimization of different infection time duration on the transformation of explants through *Agrobacterium rhizogenes* ATCC 15834 (b) Optimization of media on biomass accumulation from hairy root cultures of *Ocimum sanctum* infected with ATCC 15834. Each treatment has five replicates with 20 explants in each set of experiment. Data represent mean percentage (±) standard deviation of these replicates. a: Superscript is each value shows the significant differences ($P < 0.001$), b: Superscript is each value shows the significant difference ($P < 0.01$), c: Superscript is each value shows the significant difference ($P < 0.05$). Control refers to the untransformed roots which has undergone same treatment but without infection through *Agrobacterium* shoot tips without supplementation of hormones in the respective media. *The data were retrieved after 30 days of culture in respective manner. Reproduction size: (at column width)

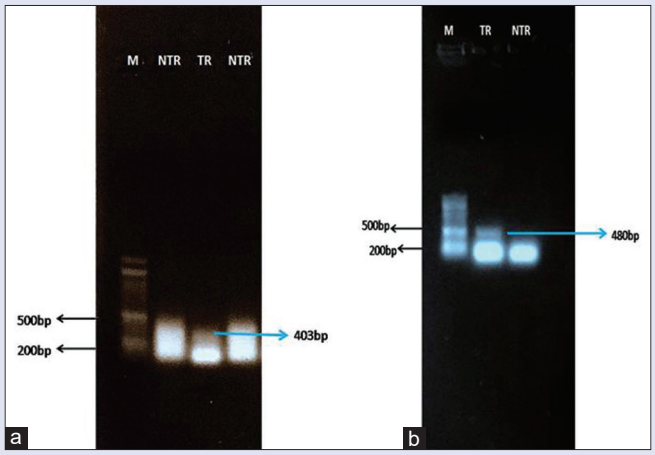

Figure 5: Polymerase chain reaction analysis of hairy roots of *Ocimum sanctum*. The fragments with length of *rol A gene* i.e. 403 bp (a) and *rol C gene* i.e. 480 bp (b) from Ri plasmids of ATCC 15834 (lane M: Marker, lane NTR: Nontransformed roots, lane TR: Transformed Roots) Reproduction size: (at column width)

effect of neurotransmitter at cholinergic synapses, causes neuronal loss also affects due to oxidative stress. Therefore, AChE is the key factor in negotiating the cholinergic failure in AD patients. Current pharmaceutical additives for AD are limited and show an area of need that is currently unmet. Suggested by many theories, oxidative damage due to free radical and oxidative stress captures a key role in the degeneration of brain cells. Hence, prevention and treatment for AD could go both hands in hand, when treatments entirely eradicate oxidative stress.[54] In this case, plants from the Lamiaceae family have been enumerating to acquire a wide array of biological conditioning and a wide variegation of phytochemicals.[55] Findings say that the alcoholic extracts of Lamiaceae species plants such as *Romarinus officials*, *Salvia offcinalis*, *Lavandula agustifolia,* and *Teucrium polium* reported AChE inhibitory ability in a dose-dependent manner.[56] In the present study, the evaluation has executed for different concentrations of hairy root extract for the inhibitory effect on the activity of AChE on different time durations. This study shows that a concentration of 15 µg/mL concentration of hairy roots extract exhibits maximum inhibition percentage (28.710 ± 0.26 µg/mL) in 3 min of time duration followed by 20 µg/mL (15.470 ± 0.35 µg/mL) and 10 µg/mL (10.0 ± 0.18 µg/mL) [Figure 6c]. In many works of literature, 0.25, 0.50, and 1 mg/mL showed inhibition when the reaction mixture incubated for 10 min. However, in the present study (methanol), extract of hairy roots from *O. sanctum* exhibited inhibition within 3 min of incubation. The previous reports say that alcoholic extract of leaf and aerial parts of *O. sanctum* consists of many flavonoids, alkaloids, and phenols. Some of them are ursolic acid, luteolin, apigenin-7 O-glucoronide, luteolin, orientin, gallic acid, rosmarinic acid, etc., which were responsible for many antioxidant activities. Oliveira *et al.*[57] studied on Huntington's mouse striatal cells showed a positive effect due to the luteolin derivative contains antioxidant potential. Although in our best of knowledge, current work shows the first report about the hairy roots' methanolic extract obtained through the leaf hairy roots of *O. sanctum*, exhibits the quick reactivity toward the inhibition of AChE. Similarly, a study by Kuźma *et al.*[58] observed the inhibitory action of hairy roots of *S. austriaca* toward AChE and BChE separated from human blood. The observed efficacy recommend that not only the extract have the capability to inhibit AChE but also the liberation of these extracts may positively regulate human biology under circumstances of excessive oxidative stress.

Behavioral study

Induction of scopolamine to cause memory impairment is extensively assessed in an *in vivo* study to estimate the antiamnesic effects of the drug. Scopolamine impairs learning and memory in mice as it is an antagonist of muscarinic acetylcholine.[59]

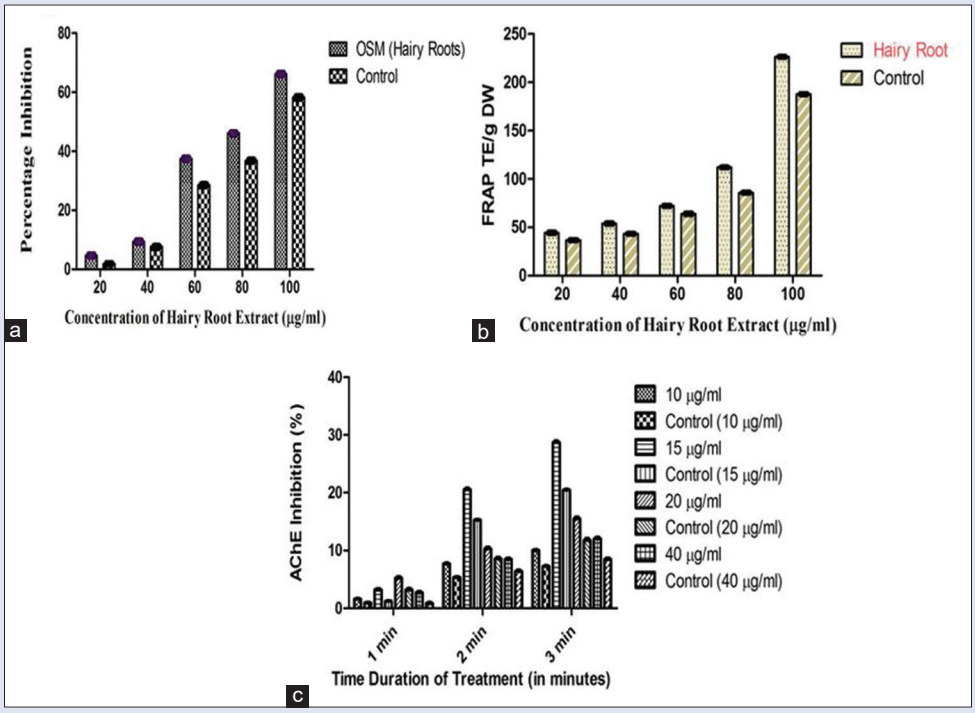

Figure 6: Antioxidant and Anti-cholinesterase potential of hairy roots extract. (a) Inhibition activity of diphenyl picric hydroxyl scavenging activity by hairy root extracts. (b) Activity of ferrous ion chelation of hairy roots. (c) Inhibition activity of AChE (acetylcholinesterase of electric eel) of hairy root.*Each experimentation has three replicates. Data represent mean percentage of inhibition (±) standard deviation with significance difference ($P < 0.05$) of these replicates. Control refers to untransformed plant's extracts. Reproduction size: (at column width)

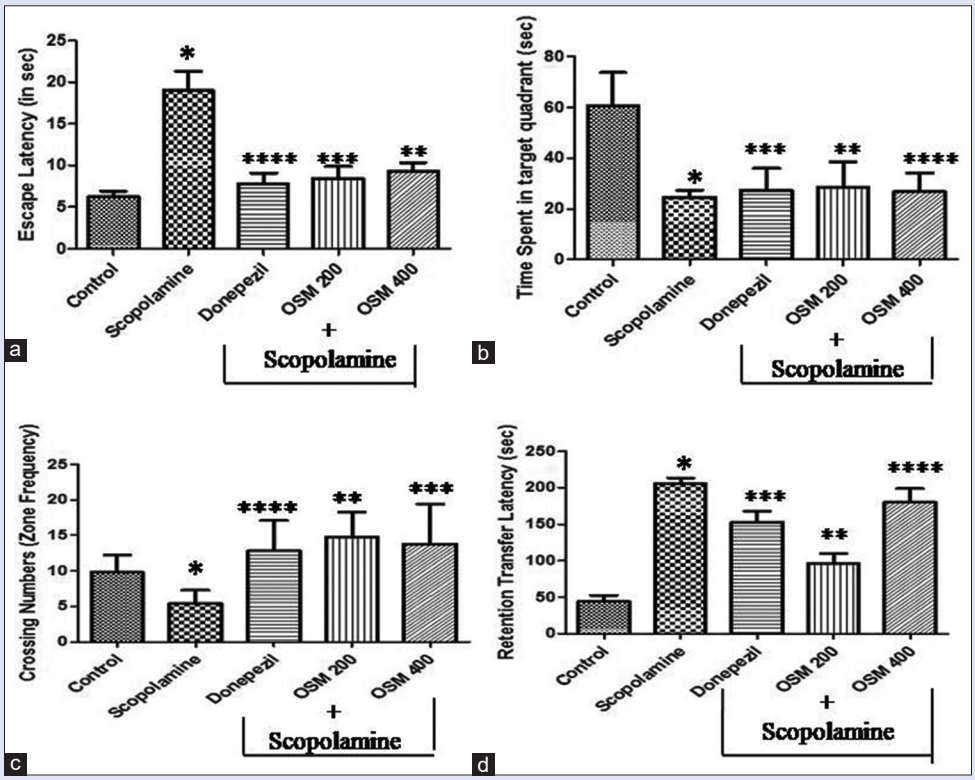

Figure 7: Effects of test compounds on escape latency, probe trial and retency latency training trials on Morris Water Maze test (a-c) and passive avoidance test (d). Data represented are expressed as mean ± standard error of mean, analyzed by one way analysis of variance Dunnette's multiple comparison test ($n = 5$). *$P < 0.05$ when scopolamine group compared to control. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ when to treated groups is compared with control. In case of probe trail (b) and passive avoidance test (d) same is follows but **$P < 0.001$, ***$P < 0.001$, $P < 0.0001$ is considered when treated group is compared with scopolamine induced group. Reproduction size: (at column width)

Morris Water Maze Test

To estimate spatial learning (short or long term), Morris water maze (MWM) test has been employed. Among all the groups, the scopolamine-induced animals show increased escape latency as compared to the control group ($P > 0.05$) [Figure 7a]. During the probe trial, the significant group induced with extract OSM (200 and 400 mg/kg) exhibited lower swimming times as compared to other groups [Figure 7b and c]. Mice pretreatment with *O. sanctum* hairy root extracts (methanol) exhibited swimming latencies toward the goal platform. This indicates their impaired spatial memory through the MWM. Animals present in the scopolamine-induced group shown compromised effects of spatial memory as they were not able to find the target quadrant. Similar results were also observed by Raghavendra *et al*.,[26] in which extracts of *O. sanctum* leaves successfully reserved the impact of neurotoxins.

Passive avoidance paradigm

The passive avoidance step transfer latency (STL) best was performed after 7 days of the study to estimate the effects of OSM, scopolamine, and donepezil in mice. The dosage of scopolamine (1 mg/kg, i.p.) exhibited a significant ($P < 0.05$) decrease in STL when compare to the control group which proves the impairment in mice's memory. In the case of donepezil (5 mg/kg) and OSM (200 and 400 mg/kg), treatment significantly ($P < 0.05$) shows the capability to revert the effects of deprivation caused by the introduction of scopolamine in mice's body system [Figure 7d]. In the case of the passive avoidance paradigm, the animal's memory was significantly increased escape latencies with OSM treatment. This indicates an improvement of cognition with contemplate to negative augmentation to electric shock. The passive avoidance paradigm works on anxiety, while the MWM works on spatial learning.[60]

In different works of literature, it was noted that scopolamine is responsible for causing the generation of free radicals, results in lipid peroxidation, detriment endogenous antioxidants levels, and neuronal death.[25,61]

Biochemical and acetylcholinesterase estimation

For biochemical analysis, antioxidant markers such as SOD, GSH, and lipid peroxidation (TBARS) levels estimation assays have enumerated in scopolamine-induced and pretreated drug-induced animal groups. Memory impairment in mice can be related to increased oxidative stress in neuronal cells, can be explored by an increased level of free radicals, and decreased level of endogenous antioxidant activity.[62] *O. sanctum* is rich in phytoconstituents and is known to posses' antioxidant activity.[24]

Estimation of thiobarbituric acid reactive substance

The effect of OSM on the TBARS level is shown in Figure 8a when the control was compared with the scopolamine-induced group; there is a significant increment of MDA. In the groups which are pretreated with hairy root extract (OSM 200 mg/kg), their MDA level decreased as compared to the scopolamine-induced group ($P < 0.05$).

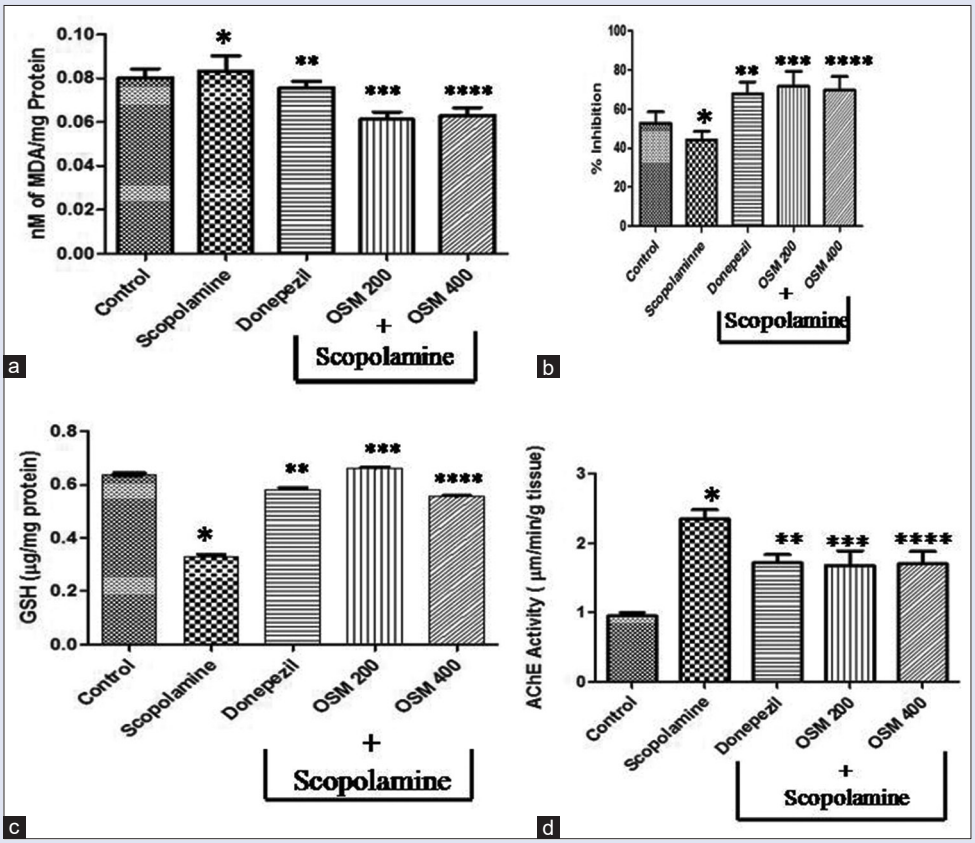

Figure 8: Effects of test compound on thiobarbituric acid reactive substances (a), superoxide dismutase (b), reduced glutathione (c) and acetylcholinesterase activity (d). Data are expressed as mean ± standard error of mean, analyzed by one way analysis of variance using Dunnett's Comparison Test ($n = 5$). *$P < 0.05$, $P < 0.0001$ when scopolamine group compared with control group. **$P < 0.01$, $P < 0.001$, $P < 0.0001$ when control group compared with donepezil induced group. ***$P < 0.05$, $P < 0.01$, $P < 0.0001$ when control group compared with OSM 200. ****$P < 0.01$, $P < 0.001$ when control group compared with OSM 400

Estimation of superoxide dismutase activity

The SOD is an antioxidant enzyme. The enzyme was compared among the control and the scopolamine-induced group there is a significant ($P < 0.0001$) decrease in SOD level. The groups were pretreated with OSM (200 and 400 mg/kg) exhibited increment of SOD activity ($P < 0.05$) as compared to the scopolamine-induced animal group [Figure 8b].

Estimation of reduced glutathione level

The level of GSH decreases significantly ($P < 0.05$) in the scopolamine-induced group as compared to the control. In case of animal groups which were pretreated with OSM (200 and 400 mg/kg) exhibited increment in the GSH group ($P < 0.0001$, $P < 0.001$) as compared with the scopolamine-induced group. These results show the promising antioxidant potential of OSM against memory impairment caused by scopolamine [Figure 8c].

Similar work is done by Kochan *et al*.[63] 2020, in which *P. quiquefolium* shows significant results by decreasing the level of intracellular oxidative stress (GSH) and activate the intracellular enzymatic defense.

Estimation of brain acetylcholinesterase activity

ACh is one of the known biomarkers of the cholinergic neutron system. The administration of scopolamine is responsible for the inducement of the β-amyloid precursor protein. This protein triggers the deposition of β-amyloid plaques.[64]

As the oxidative stress increases in the brain, AChE enzyme comes into play, which was estimated by Ellman's assay.[23] The AChE activity increases significantly ($P < 0.05$) in the scopolamine-induced group as compared with the control. Animal groups pretreated with OSM extracts (200 and 400 mg/kg) significantly ($P < 0.01$, $P < 0.001$) exhibited a decrease in AChE activity as compared to the scopolamine-induced animal group [Figure 8d]. Pretreatment with donepezil and OSM defined a significant declination of AChE activity in mice brain affected by scopolamine. In different works of literature, the extract of *O. sanctum* significantly responsible for the decrement of the level of AChE in the mice model.[65]

Hence, in the present study, *O. sanctum* hairy root extract (200 and 400 mg/kg) administered orally improved learning and memory of mice estimated by the behavioral models such as passive avoidance, MWM, and also biochemical models like AChE, SOD, GSH, and TBARS method well emulated with the scopolamine-induced group.

CONCLUSION

In the present study, the improvised and productive micropropagation procedure for field-grown *O. sanctum* has been achieved. The transformation through *Agrobacterium* was reported in many plants. However, in some cases, many of them are still recalcitrant. The present study introduces many effective agents to overcome the challenges, with the optimization of many aspects in media to enhance the efficiency of transformation. The hairy root derived from ATCC 15834 in *O. sanctum* exhibits higher antioxidant and anti-AChE potential. The *rol A* and *rol C* genes in genetically transformed hairy roots were verified using PCR. Apart from all these, this paper presents the first-ever data for AChE inhibitory activities employing hairy root's extract of *O. sanctum* at a concentration of 15 µg/mL achieves maximum inhibition within 3 min of incubation. In case of *in vivo* study, the pretreatment of OSM effectively enhance the cognition of animal's brain. Hence, we suggest that the present study could be competent for the development of antioxidants and therapeutic compounds at an industrial scale for significant health benefits by employing the *O. sanctum* hairy root culture system.

Acknowledgements

Authors would like to acknowledge Centre of Excellence TEQIP-II for Grant no.-NPIU/TEQIP II/FLN/31/158, Birla Institute of Technology, Mesra, Ranchi, Jharkhand and Rajiv Gandhi National Fellowship (SC/ST) Fellowship-UGC (RGNF-2014-15-ST-JHA-77633) for financial support. Authors would also like to acknowledge Dr. Sanjay Harke, Mahatama Gandhi Mission University, Institute of BioSciences and Technology, Maharashtra, India for his consistent guidance in the molecular studies of hairy root obtained from leaf plant of *O. sanctum*. Authors would also like to acknowledge Dr. Papiya Mitra Mazumder, Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, for her constant guidance in the *in vivo* study.

Financial support and sponsorship

This study was financially supported by Centre of Excellence (COE) TEQIP-II for Grant no.- NPIU/TEQIP II/FLN/31/158, Birla Institute of Technology, Mesra, Ranchi, Jharkhand and Rajiv Gandhi National Fellowship (SC/ST) Fellowship-UGC (RGNF-2014-15-ST-JHA-77633).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Navin M, Ajay L, Naseem S, Seema S, Surendra S, Isha N. Preliminary *ex-vivo* and an animal model evaluation of *Ocimum sanctum*'s essential oil extract for its antibacterial and anti- inflammatory properties. Oral Health Dent Manag 2013;12:174-9.
2. Pattanayak P, Behera P, Das D, Panda SK. *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview. Pharmacogn Rev 2010;4:95-105.
3. Simon JE, Morales MR, Phippen WB, Vieira RF, Hao Z. Basil: A source of aroma compounds and a popular culinary and ornamental herb. Perspectives on New Crops and New Uses 1999;16:499-505.
4. Vyas P, Mukhopadhyay K. Development of a rapid and high frequency *Agrobacterium rhizogenes* mediated transformation protocol for *Ocimum tenuiflorum*. Biologia 2014;69:765-70.
5. Ahmad A, Rasheed N, Gupta P, Singh S, Siripurapu KB, Ashraf GM, *et al*. Novel Ocimumoside A and B as anti-stress agents: Modulation of brain monoamines and antioxidant systems in chronic unpredictable stress model in rats. Phytomedicine 2012;19:639-47.
6. Venuprasad MP, Hemanth Kumar K, Khanum F. Neuroprotective effects of hydroalcoholic extract of *Ocimum sanctum* against H_2O_2 induced neuronal cell damage in SH-SY5Y cells via its antioxidative defence mechanism. Neurochem Res 2013;38:2190-200.
7. Giridharan VV, Thandavarayan RA, Konishi T. *Ocimum sanctum* Linn.(Holy Basil) to improve cognition. In: Diet and Nutrition in Dementia and Cognitive Decline. Academic Press; 2015. p. 1049-58.
8. Altpeter F, Springer NM, Bartley LE, Blechl AE, Brutnell TP, Citovsky V, *et al*. Advancing crop transformation in the era of genome editing. Plant Cell 2016;28:1510-20.
9. Hao G, Ji H, Li Y, Shi R, Wang J, Feng L, *et al*. Exogenous ABA and polyamines enhanced salvianolic acids contents in hairy root cultures of *Salvia miltiorrhiza* Bge. f. alba. Plant Omics 2012;5:446.
10. Sharan S, Sarin NB, Mukhopadhyay K. Elicitor-mediated enhanced accumulation of ursolic acid and eugenol in hairy root cultures of *Ocimum tenuiflorum* L. is age, dose and duration dependent. South Afr J Bot 2019;124:199-210.
11. Gangopadhyay M, Chakraborty D, Bhattacharyya S, Bhattacharya S. Regeneration of transformed plants from hairy roots of Plumbago indica. Plant Cell Tissue Org Cult (PCTOC) 2010;102:109-14.
12. Kim YJ, Weathers PJ, Wyslouzil BE. Growth of *Artemisia* annua hairy roots in liquid-and gas-phase reactors. Biotechnol Bioeng 2002;80:454-64.
13. Mottaki Z, Rezayian M, Niknam V, Ebrahimzadeh H, Mirmasoumi M. Using hairy roots for

production of secondary metabolites in *Artemisia*. Plant Biotechnol Rep 2019;13:263-71.

14. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plant 1962;15:473-97.
15. Balasubramanian M, Anbumegala M, Surendran R, Arun M, Shanmugam G. Elite hairy roots of *Raphanus sativus* (L.) as a source of antioxidants and flavonoids. 3 Biotech 2018;8:128.
16. Ogawa Y, Mii M. Meropenem and moxalactam: Novel β-lactam antibiotics for efficient Agrobacterium-mediated transformation. Plant Sci 2007;172:564-72.
17. Pileggi M, Pereira AA, Silva JD, Pileggi SA, Verma DP. An improved method for transformation of lettuce by *Agrobacterium tumefaciens* with a gene that confers freezing resistance. Braz Arch Biol Technol 2001;44:191-6.
18. Kang HJ, Anbazhagan VR, You XL, Moon HK, Yi JS, *et al*. Production of transgenic *Aralia elata* regenerated from *Agrobacterium rhizogenes*-mediated transformed roots. Plant Cell Tissue Org Cult 2006;85:187-96.
19. Piispanen R, Aronen T, Chen X, Saranpää P, Häggman H. Silver birch (*Betula pendula*) plants with aux and rol genes show consistent changes in morphology, xylem structure and chemistry. Tree Physiol 2003;23:721-33.
20. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 2009;14:2167-80.
21. Szőllősi R, Varga IS. Total antioxidant power in some species of Labiatae: Adaptation of FRAP method. Acta Biol Szeged 2002;46:125-7.
22. Laghari AH, Memon S, Nelofar A, Khan KM, Yasmin A. Determination of free phenolic acids and antioxidant activity of methanolic extracts obtained from fruits and leaves of *Chenopodium album*. Food Chem 2011;126:1850-5.
23. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
24. Toppo AJ, Chandra S, Jha D, Mazumder PM. *In vitro* evaluation of anti-acetylcholinesterase and free radical scavenging potential of leaf extracts of some selected medicinal plants. Asian Pac J Trop Biomed 2019;9:60.
25. Singh V, Kahol A, Singh IP, Saraf I, Shri R. Evaluation of anti-amnesic effect of extracts of selected Ocimum species using *in-vitro* and *in-vivo* models. J Ethnopharmacol 2016;193:490-9.
26. Raghavendra M, Maiti R, Kumar S, Acharya SB. Role of *Ocimum sanctum* in the experimental model of Alzheimer's disease in rats. Int J Green Pharm 2009;3:265-70.
27. Tripathi S, Kumari U, Mitra Mazumder P. Ameliorative effects of apple cider vinegar on neurological complications via regulation of oxidative stress markers. J Food Biochem 2020;44:e13504.
28. Jarvik ME, Kopp R. An improved one-trial passive avoidance learning situation. Psychol Rep 1967;21:221-4.
29. Al-Enazi MM. Protective effects of combined therapy of rutin with silymarin on experimentally-induced diabetic neuropathy in rats. Pharmacol Pharm 2014;2014.
30. Zhang J, Chen R, Yu Z, Xue L. Superoxide dismutase (SOD) and catalase (CAT) activity assay protocols for *Caenorhabditis elegans*. Bio Protocol 2017;7:2505-15.
31. Ameen N, Shafi S. Biochemical and *in-vivo* antioxidant parameters for evaluation of memory enhancing activity. Int J Pharm Chem Biol Sci 2016;6.
32. Mishra T. Protocol establishment for multiplication and regeneration of 'Holy Basil'(*Ocimum sanctum* Linn). An important medicinal plant with high religious value in India. J Med Plants Stud 2015;3:16-9.
33. Santhi K. *In vitro* micropropagation of *Ocimum citriodorum* and standardization of growth hormone. Ann Plant Soil Res 2019;21:386-9.
34. Susila T, Reddy GS, Jyothsna D. Standardization of protocol for *in vitro* propagation of an endangered medicinal plant *Rauwolfia serpentina* Benth. J Med *P* Res 2013;7:2150-3.
35. Jamal MA, Sharif IH, Shakil MM, Rahman AR, Banu NA, Islam MR, *et al*. *In vitro* regeneration of a common medicinal plant, *Ocimum sanctum* L. for mass propagation. Afr J Biotechnol 2016;15:1269-75.
36. Wang D, Li X, Cheng Z, Long C. *In vitro* preservation and micropropagation of Oreocharis mileense (WT Wang) M. Möller and A. Weber (Gesneriaceae) through shoot organogenesis. *In vitro* Cell Dev Biol Plant 2018;54:606-11.
37. Revathi J, Manokari M, Latha R, Priyadharshini S, Kher MM, Shekhawat MS. *In vitro* propagation, *in vitro* flowering, *ex vitro* root regeneration and foliar micro-morphological analysis of *Hedyotis biflora* (Linn.) Lam Vegetos 2019;32:609-19.
38. Khatik NI, Joshi R. An efficient protocol for *in vitro* shoot regeneration from different explants of *Murraya koenigii* (L.) Spreng J Phytol Res 2017;30:59-67.
39. Singh S, Banerjee M, Kumar M. An efficient protocol for plant regeneration of Phlogacanthus thyrsiflorus Nees: An important medicinal shrub. In: Applications of Biotechnology for Sustainable Development. Singapore: Springer; 2017. p. 15-20.
40. Rathore MS, Chikara J, Mastan SG, Rahman H, Anand KG, Shekhawat NS. Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR markers. Appl Biochem Biotechnol 2011;165:1356-65.
41. Zhou LG, Wang JJ, Yang CR. Progress on plant hairy root culture and its chemistry. Nat Prod Res Dev 1998;3:87-95.
42. Dehdashti SM, Acharjee S, Kianamiri S, Deka M. An efficient *Agrobacterium rhizogenes*-mediated transformation protocol of *Withania somnifera*. Plant Cell Tissue Org Cult 2017;128:55-65.
43. Tada H, Murakami Y, Omoto T, Shimomura K, Ishimaru K. Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. Phytochemistry 1996;42:431-4.
44. Vamenani R, Pakdin-Parizi A, Mortazavi M, Gholami Z. Establishment of hairy root cultures by *Agrobacterium rhizogenes* mediated transformation of *Trachyspermum ammi* L. for the efficient production of thymol. Biotechnol Appl Biochem 2020;67:389-95.
45. Marwani E, Pratiwi D, Wardhani K, Esyanti R, Marwani E, Pratiwi D, *et al*. Development of hairy root culture of *Andrographis paniculata* for *in vitro* adrographollide production. J Med Bioeng Vol 2015:446-50.
46. Renouard S, Corbin C, Drouet S, Medvedec B, Doussot J, Colas C, *et al*. Investigation of *Linum flavum* (L.) hairy root cultures for the production of anticancer aryltetralin lignans. Int J Mol Sci 2018;19:990-8.
47. Yang XF, Yu XQ, Zhou Z, Ma WJ, Tang GX. A high-efficiency Agrobacterium tumefaciens mediated transformation system using cotyledonary node as explants in soybean (*Glycine max* L.). Acta Physiol Plant 2016;38:60-70.
48. Liu H, Zhao H, Wu L, Xu W. A genetic transformation method for cadmium hyperaccumulator *Sedum plumbizincicola* and non-hyperaccumulating ecotype of *Sedum alfredii*. Front Plant Sci 2017;8:1047-57.
49. Largia MJ, Satish L, Johnsi R, Shilpha J, Ramesh M. Analysis of propagation of *Bacopa monnieri* (L.) from hairy roots, elicitation and Bacoside A contents of Ri transformed plants. World J Microbiol Biotechnol 2016;32:131.
50. Ebrahimzadeh MA, Nabavi SM, Nabavi SF. Correlation between the *in vitro* iron chelating activity and poly phenol and flavonoid contents of some medicinal plants. Pak J Biol Sci 2009;12:934-8.
51. El-Esawi MA, Elkelish A, Elansary HO, Ali HM, Elshikh M, Witczak J, *et al*. Genetic transformation and hairy root induction enhance the antioxidant potential of *Lactuca serriola* L. Oxid Med Cell Longev 2017;2017:1-8.
52. Matvieieva N, Drobot K, Duplij V, Ratushniak Y, Shakhovsky A, Kyrpa-Nesmiian T, *et al*. Flavonoid content and antioxidant activity of *Artemisia vulgaris* L. "hairy" roots. Prep Biochem Biotechnol 2019;49:82-7.
53. Muthusamy B, Shanmugam G. Analysis of flavonoid content, antioxidant, antimicrobial and antibiofilm activity of *in vitro* hairy root extract of radish (*Raphanus sativus* L.). Plant Cell Tissue Org Cult 2020;140:619-33.
54. Lee HP, Zhu X, Casadesus G, Castellani RJ, Nunomura A, Smith MA, *et al*. Antioxidant approaches for the treatment of Alzheimer's disease. Expert Rev Neurother 2010;10:1201-8.
55. Štefan MB, Rodríguez JV, Blažeković B, Kindl M, Vladimir-Knežević S. Total hydroxycinnamic acids assay: Prevalidation and application on Lamiaceae species. Food Analy Meth 2014;7:326-36.
56. Vladimir-Knežević S, Blažeković B, Kindl M, Vladić J, Lower-Nedza AD, Brantner AH. Acetylcholinesterase inhibitory, antioxidant and phytochemical properties of selected medicinal plants of the Lamiaceae family. Molecules 2014;19:767-82.
57. Oliveira AM, Cardoso SM, Ribeiro M, Seixas RS, Silva AM, Rego AC. Protective effects of 3-alkyl luteolin derivatives are mediated by Nrf2 transcriptional activity and decreased oxidative stress in Huntington's disease mouse striatal cells. Neurochem Int 2015;91:1-12.
58. Kuźma Ł, Wysokińska H, Sikora J, Olszewska P, Mikiciuk-Olasik E, Szymański P. Taxodione and extracts from *Salvia austriaca* roots as human cholinesterase inhibitors. Phytother Res 2016;30:234-42.
59. Terry AV Jr., Buccafusco JJ. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: Recent challenges and their implications for novel drug development. J Pharmacol Exp Ther 2003;306:821-7.
60. Brandeis R, Pittel Z, Lachman C, Heldman E, Luz S, Dachir S, *et al*. AF64A induced cholinotoxicity: Behavioral and biochemical correlates. In: Alzheimer's and Parkinson's Disease. Boston, MA: Springer; 1986. p. 469-77.
61. Kaur R, Singh V, Shri R. Anti-amnesic effects of Ganoderma species: A possible cholinergic

and antioxidant mechanism. Biomed Pharmacother 2017;92:1055-61.

62. Ghumatkar PJ, Patil SP, Jain PD, Tambe RM, Sathaye S. Nootropic, neuroprotective and neurotrophic effects of phloretin in scopolamine induced amnesia in mice. Pharmacol Biochem Behav 2015;135:182-91.
63. Kochan E, Nowak A, Zakłos-Szyda M, Szczuka D, Szymańska G, Motyl I. *Panax quinquefolium* L. Ginsenosides from hairy root cultures and their clones exert cytotoxic, genotoxic and pro-apoptotic activity towards human colon adenocarcinoma cell line caco-2. Molecules 2020;25:2262.
64. Bennett S, Grant MM, Aldred S. Oxidative stress in vascular dementia and Alzheimer's disease: A common pathology. J Alzheimers Dis 2009;17:245-57.
65. Giridharan VV, Thandavarayan RA, Mani V, Ashok Dundapa T, Watanabe K, Konishi T. *Ocimum sanctum* Linn. leaf extracts inhibit acetylcholinesterase and improve cognition in rats with experimentally induced dementia. J Med Food 2011;14:912-9.\