In vitro Propagation and Variation of Antioxidant and Docking Properties of *Bacopa monnieri* (L.) Wettst. a Threatened Medicinal Plant

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Abstract

Bacopa monnieri (L.) Wettst Ayurveda herb has anti-cancer, anti-ulcer, antispasmodic, anti-asthmatic, neuro-tonic, immuno-modulator, adaptive, tranquillizing, memory and learning improving, cerebral activator and therapeutic value. It is the most well-known for its nootropic properties. Through the antioxidant and molecular docking stimulation of several shoots, Bacopa monnieri(L.) Wettst is analyzed. In this investigation, methanol extracts exhibited the strongest MeoH free radical scavenging capabilities, with the antioxidant activity increasing noticeably and regenerated in vitro in the current study. The molecular docking study evaluated the binding affinities of various compounds with four protein targets. The docking scores (in kcal/mol) indicate the strength of interaction where a lower (more negative) value suggests stronger binding affinity. Leaf explants were used. Explants that had been surface-sterilized were aseptically cultivated on MS media that included varying amounts of plant growth regulators.

The leaf responded most favorably to shoot regeneration and subsequent plant development on MS. After six weeks of culture, the leaf explants produced an average of 12.94 ± 2.81 shoots/explants in this combination. The optimal medium for root induction was determined to be MS half-strength supplemented with 0.5 mg/L IBA. Thus, MeoH has great potential as a natural remedy for a number of inflammatory, oxidative and infectious disorders. For additional research, screening of its active components is required. The in vitro grown rooted plantlets were placed in plastic pots to garden in a 50:50 soil vermiculite mixture. After that, the acclimatized plants were successfully moved into the soil.

Keywords: *Bacopa monnieri*, *in vitro* regeneration, leaf explants.

Introduction

Medicinal plant contains substance synthesis for useful drug authorizing to Sofowora[23] and Sofowora[24]. WHO has developed this description of medicinal plants. Plants that have therapeutic potentials or have positive pharmacological effects on animals are referred[6]. Herbal plants have a significant role to cure illness[12]. They are crucial in helping rural residents receive primary healthcare facilities. They are key raw materials and therapeutic agents used in the production of contemporary medicine[7,20]. Because of their complexity and large number of secondary metabolites, medicinal plants have a wide variety of chemical and pharmacological components. Medicinal plants are an important portion of the Asian countries. They play a significant role in primary health care services to rural people[20]. They aid as therapeutic agents and important raw materials for the manufacture of modern medicine[7].

Medicinal plants are characterized by diverse chemical and pharmacological constituents owing to their complexity and abundance of secondary metabolites. The utilization of medicinal plants to cure a variety of illnesses and the creation of indigenous medicines have significant economic benefits[5]. The majority of people, particularly those living in rural areas, are still compelled to use traditional medicines for their everyday illnesses because of a lack of modern medical facilities, poverty, ignorance and limited communication options. The majority of these individuals come from the lowest echelon of the medicinal plant trade[15].

In places where using plants is still very important, a wealth of information about how to use them to treat various disorders should have developed[8]. Wetlands and muddy beaches are home to the perennial creeping herb *Bacopa monnieri* (L.) Pennell. Common names include "Water Hyssop" and Brahmi (Centella asiatica and other herbs are also known by the Ayurveda term Brahmi). Herbaceous plants called *Bacopa monnieri* (L.) Wettst are typically found in temperate climates. In addition to Florida and other southern States of the United States, where it can be cultivated in moist circumstances around a pond or bog garden, it is often found in marshy places in China, Taiwan, India, Nepal and Sri Lanka. This species has commercial value as a decorative plant for aquatic gardens, potted plant and medicinal plant.

Propagation is often realized through cuttings. *Bacopa monnieri*(L.) Wettst[13] possesses antioxidant qualities. It has been shown to lessen blood-stream fat oxidation, a risk factor for cardiovascular disorders. For generations, people with epilepsy have used it to improve their memory and lessen anxiety brought on by stress. It is classified as a nootropic, which is a medication that improves cognitive function. This herb has also long been used in India to consecrate newborns in the hope that it may unlock their

intelligence. Recent studies suggest Bacopa may improve intellectual activity[21,25]. This plant is also known as 'Thyme-leafed gratiola' and 'Moneywort'. Synonyms include *Herpestris monnieria, Moniera euneifolia, Lysimachia monnieri* and *Bacopa monnieri*(L.) Wettst. *Bacopa monnieri*(L.) Wettst is a very important medicinal plant. It is a neuro tonic, immune madulator, adaptogen, tranquilizing, memory and learning enhancing, cerebral activator, anti-cancer, anti-ulcer, antispasmodic, anti-asthmatic Ayurveda herb.

In India, an estimated 100,000 tons of material are harvested annually for commercial use from the wild[3]. The plant is in danger of going extinct as a result of this. Large amounts of plant material are needed for drug extraction since, despite the plant's abundance in wetlands, its drug content is quite low (0.2%). The plant contains saponin[19]. To overcome all these problems, *in vitro* technique is the tool for mass production and conservation of this plant only. We should develop a standard protocol for plant production via indirect organogenesis using leaf explant and we should standardize a protocol for rooting and hardening.

Material and Methods

Collection of plants: *Bacopa monnieri* (L.) Wettst were collected from the Sitheri Hills of Dharmapuri(Dt) growing in wet, damp and marshy places. Bacopa monnieri (L.) Wettst grows in marshy areas throughout India.

Superoxide Radical Scavenging Assay: Superoxide scavenging activity of the extracts was determined based on the ability of the extracts to inhibit formazan production by bleaching the superoxide radicals generated by nitroblue tetrazolium salt with riboflavin and light. The extracts (0.1 mL) were added to the reaction mixture [0.1 mg NBT, 12 mM EDTA and 20 μg riboflavin in 50 mM sodium phosphate buffer (pH 7.6)] and illuminated by light. After 90 seconds the absorbance was measured at 590 nm[17].

Hydroxyl Scavenging: At 562 nm, the absorbance of the hydroxyl scavenging was detected. Ascorbic acid served as a positive control and the following formula was used to determine the percentage of inhibition:

$$\text{Scavenging activity} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100$$

where A_0 is absorbance of control, A_1 is absorbance of sample and A_2 is absorbance.

Docking studies: In our investigation, molecular docking was employed to simulate the binding interactions between proteins and potential antiviral compounds derived from the leaf extract. This approach sought to validate the findings from network pharmacology studies. Using the Discovery Studio Client platform, we identified promising compounds that could effectively interact with key proteins involved in the lifecycle of the yellow fever virus. Relevant protein structures were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/) and were prepared by removing non-essential molecules and adding necessary modifications, such as hydrogen atoms and charges. Ligands were also optimized for interaction studies.

Preparation of MS Stock Medium: The same composition of MS basal medium as used by Khan et al[14] was used for the study, except for the vitamins. B5 vitamins were used[18] for convenience throughout the study.

Culture Techniques

Sterilization of Glasswares: Following glassware were used such as tissue culture tubes, conical flasks, beakers, pipettes, standard flasks, auto-clavable cap bottles, Petri dishes and measuring cylinders. Microorganisms can enter the culture medium through any of these sources and therefore need to be sterilized. Sterilization of glassware involves the following procedure. The glassware was first soaked in sulfuric acid for four hours and then washed thoroughly with a jet of tap water. They were again soaked in detergent and washed again under the jet of running tap water to remove traces of the detergent. Finally, they were washed in distilled water and dried at high temperature. They were then autoclaved at 15 psi (120°C) for 20 min[26].

Surface sterilization of the explants: Without taking plants out of their natural home, explants were gathered in the field. Explants included nodes, internodes, leaves and stems. These might also have contaminated origins. To get rid of soil particles and other tiny, unnecessary particles, the explants were first cleaned for 30 minutes under running tap water. For ten minutes, the explants were surface sterilized using a 5% teepol solution (detergent). After repeatedly washing the explants in sterile distilled water, the teepol detergent was eliminated. After that, the explants were placed in 70% ethanol and left for two to three minutes. Sterilized distilled water was used to wash the samples three more times. After that, the explants were surface-sterilized for one to two minutes using 0.1% $HgCl_2$. A laminar airflow chamber was used to perform the complete surface sterilization procedure. For a variety of investigations, surface-sterilized explants were inoculated in MS medium with varying hormone doses[9].

Preparation of MS Medium: Different ingredients were added to 500mL of double-distilled water in a 1L of standard flask. Following chelation with Na_2EDTA, iron was added[28]. Sucrose was added to get the concentration down to 3% (30g/L) and 1L using d.Dis.H_2O- for Hormone preparation. Each aliquot received a sufficient amount of hormones. The pH was lowered to 5.7 using either 0.1N HCl or 0.1N NaOH. To solidify the media, bacteriological-grade (0.8%) agar was utilized. Culture tube was filled with 10 mg/mL of the melting media, sealed with cotton and then bundled in bundles of ten culture tubes, each of which was assumed to be a duplicate of a particular concentration or combination. The culture tubes with the media were autoclaved for 15 mints at 121°C and 15 psi.

0.1 HCl and 0.1N NaOH were used to raise the pH from 5.6 to 5.8 using a single electrode electronic pH meter. pH should be maintained at its optimal level because it affects ion uptake. 0.8% agar was added and the medium was heated to 60°C to dissolve it. The medium was sterilized using the autoclaving method, which employs water vapors under high pressure. The medium was autoclaved at 15 psi and 121°C for 20 mints. After solidification, the autoclaved material was used to inoculate the transplants.

Inoculation Procedure and Sterilization of the transfer area: There is a considerable risk that the nutrient media will get contaminated when the explant is placed in the culture medium. As a result, aseptic inoculation is required. The aseptic insertion of the explants into the growth medium involves three steps. The Atlantis LAF chamber was used for the inoculation. The floor of the laminar airflow chamber was cleaned and sterilized using spirit. There were forceps, a knife holder with a sterile blade and flame-sterilized sterile Petri dishes inside the room. The equipment and the entire chamber were subjected to UV radiation for around 30 mins.

Explant Initiated and Transfer and Cultivation and Hardening: Every possible precaution was taken when the explants were being transferred. They were wiped with spirit and properly cleaned with detergent. In the presence of a spirit lamp, the surface-sterilized explants were sliced to the appropriate size and inoculated on previously sterilized media. The culture tubes were then sealed with a non-absorbent cotton plug wrapped in gauze cloth. After the 15^{th} day, a subculture was made on the same medium for each treatment. The cultures were kept at 20±2°C with a photoperiod of 16 hours in white light and 8 hours in dark each day using fluorescent light (2000–3000 LUX).

After being taken out of the culture tubes, the rooted plants were cleaned under running water. They were moved to vermiculite-filled, sterile plastic cups. Since the plants require 95–100% humidity, they were covered with plastic bags that were perforated or holed. After 15 days, the plantlets in the plastic pots were moved to the soil and left in the shade for about 30 days.

Statistical Analysis: All the experiments were set up in a completely randomized design with three replications per treatment and the assays were performed in triplicate to verify the reproducibility of the results. Significant differences among the treatments were determined by Analysis of Variance (ANOVA) followed by Duncan multiple range tests at a 5% probability level by using SAS computer package (SAS Institute Inc., NC, USA).

Results and Discussion

Antioxidant effect on *Bacopa monnieri* Superoxide (O_2^-) Radical Scavenging Assay: Plant extracts inhibited the formation of formazan by scavenging the O_2^- radicals generated by NBT-riboflavin-light complex and the scavenging capacity of the extracts was compared with the positive control, ascorbic acid (Figure 1). The superoxide radical scavenging activity of *Bacopa monnieri* leaf extracts showed high reduction of superoxide radicals related to the high scavenging activity conducted by particular samples (Figure 1).

Superoxide radicals are highly harmful to the cell organelles and superoxide radicals act as the precursor for the formation of ROS. In addition, the larger amount of O_2^- in the cells enhances the dismutation reaction that results in H_2O_2 production further leading to the oxidative stress. Therefore, efficient scavenging of O_2^- is inevitable for the antioxidants.

Hydroxyl Radical Scavenging Activity: Higher reduction of hydroxyl radical scavenging activity is connected with the high scavenging activity performed by particular sample. In the present study, the hydroxyl radical scavenging activity in the range of MeOH 9.44- 61.26 % was chloroform extract 5.5 – 48.23% scavenging activity and compared to 15.23 - 69.23µg/mL (Figure 2). The ability of methanol extract was found to be of higher activity. The hydroxyl radical can induce oxidative damage to DNA, lipids and proteins.

Docking for selected molecules: The interaction of 1V40 with 2-(1,3-benzothiazol-2-ylsulfanyl) ethanol has an affinity of -9.3 kcal/mol, involving two hydrogen bonds and six hydrophobic interactions. (1,3-benzothiazol-2-ylsulfanyl) ethanol showed similar binding to1V40 at residues ASPC:497, SERB:300; ARGB:2014, LEUC;257, LYSB;250 (Figures 3-4).

Table 1
Effect of different concentrations of BAP and NAA on callus induction from Leaf explants of *Bacopa monnieri*(L.) *Wettst*

Hormonal concentrations (mg/l)	% of Callus Induction (Mean±S.D)	Callus Colour
BAP+NAA		
0.5+0.3	25±1	Dark green
1.0+0.3	45±3	Dark green
1.5+0.3	70±4	Dark green
2.0+0.3	80±5	Dark green
2.5+0.3	85±5	Dark green
3.0+0.3	95± 5	Dark green

A compound or ligand that has the same residue interaction activity as the native ligand will have a lower binding energy value so that the compound shows a stronger bond with the target protein, 1V40 vs 2-(1,3-benzothiazol-2-ylsulfanyl) ethanol. These docking results suggest that 2-(1,3-benzothiazol-2-ylsulfanyl) ethanol may act as an inhibitor for 1V40.

***In vitro* propagation of explants:** In order to induce callus, leaf explants were inoculated on MS medium enhanced with different concentrations of BAP (0.5–3.0 mg/L) along with 0.3 mg/L NAA. BAP (0.5 mg/L) and NAA (0.3 mg/L), a 25% callus was first seen. The highest percentage (95%) of dark green callus was seen when MS medium was supplemented with BAP (3.0 mg/L) and NAA (0.3 mg/L) when the BAP concentration was raised by 0.5 mg/L but the NAA concentration remained constant (Table 1; Figure 1).

The well-developed dark green callus was transferred into MS medium enclosing BAP (2.0-7.0mg/L) and NAA (0.05mg/L) for multiple shoot production. Initially, 10 shoots were formed and gradually the number of multiple shoots were observed when MS medium was supplemented with 6.0 mg/L BAP and 0.05mg/L NAA (Table 2).

The shoot lets were transferred to the rooting medium consisting of MS basel salts + IBA (0.5-2.5mg/L) +15% sucrose +0.8 Agar. The best frequency of roots was observed on MS medium containing 0.5mg/L IBA. (Table 3). After being carefully cleaned with tap water and tipped in MS basal medium for an hour, the well-developed plantlets were taken out of the agar medium. They were then moved to sterile vermiculite and irrigated with half-strength MS basal liquid medium.

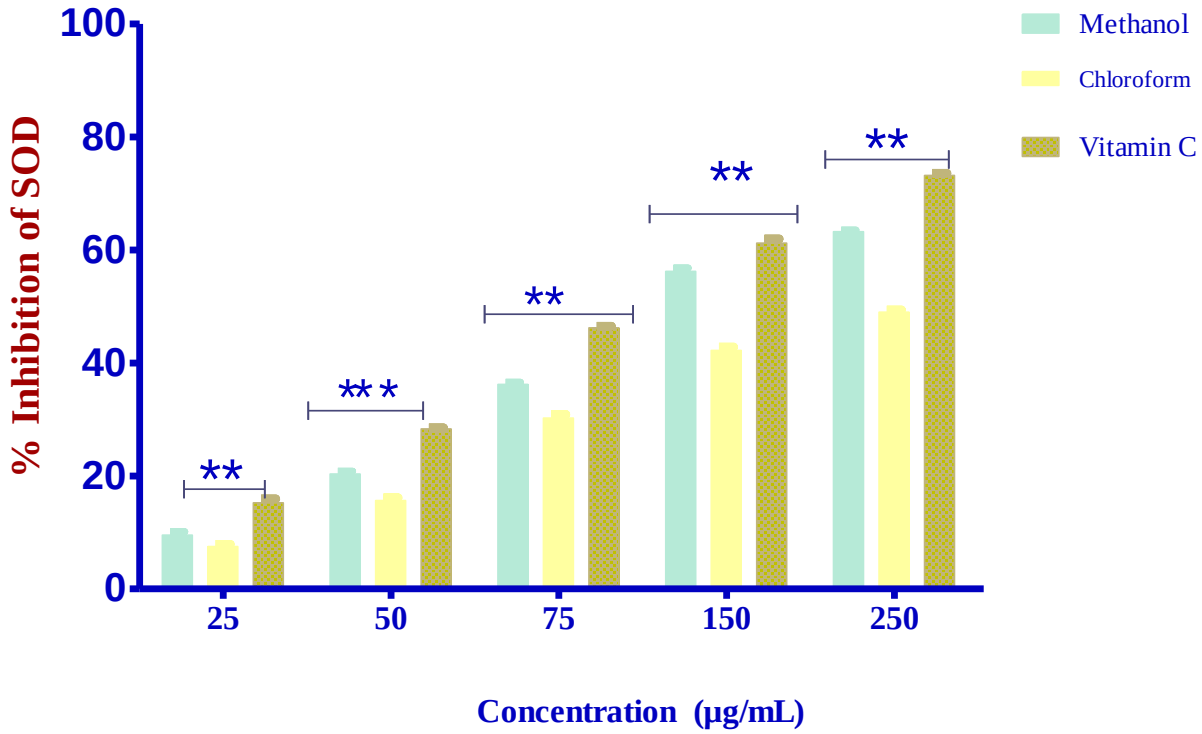

Figure 1: Antioxidant activity of the *Bacopa monnieri* Methanol and chloroform measured in the SOD assay at different concentrations (25, 50, 75, 150 and 250 µg/mL). The values expressed as mean ± SD values, analyzed by one-way analysis of Variance (ANOVA). Followed by Dunnett's multiple comparison tests (P<0.05; P<0.001; P <0.0001). Asterisk (*) (**) (***) indicates significance differences among treatments compared in control groups

Table 2
Effect of BAP in combination with NAA on organogenesis from Callus of *Bacopa monnieri*(L.) *Wettst*

Hormonal concentrations (mg/l)		No. of tubes responded/Total no. tubes inoculated.	Percentage of multiple shoot proliferation.	No. of shoots/explants. (Mean±SD)
BAP	NAA			
2.0	0.05	7/20	35%	2.71±1.49
3.0	0.05	10/20	50%	5.20±1.93
4.0	0.05	12/20	60%	6.81±1.83
5.0	0.05	15/20	75%	8.57±3.03
6.0	0.05	18/20	90%	12.94±2.81
7.0	0.05	11/20	55%	6.90±2.73

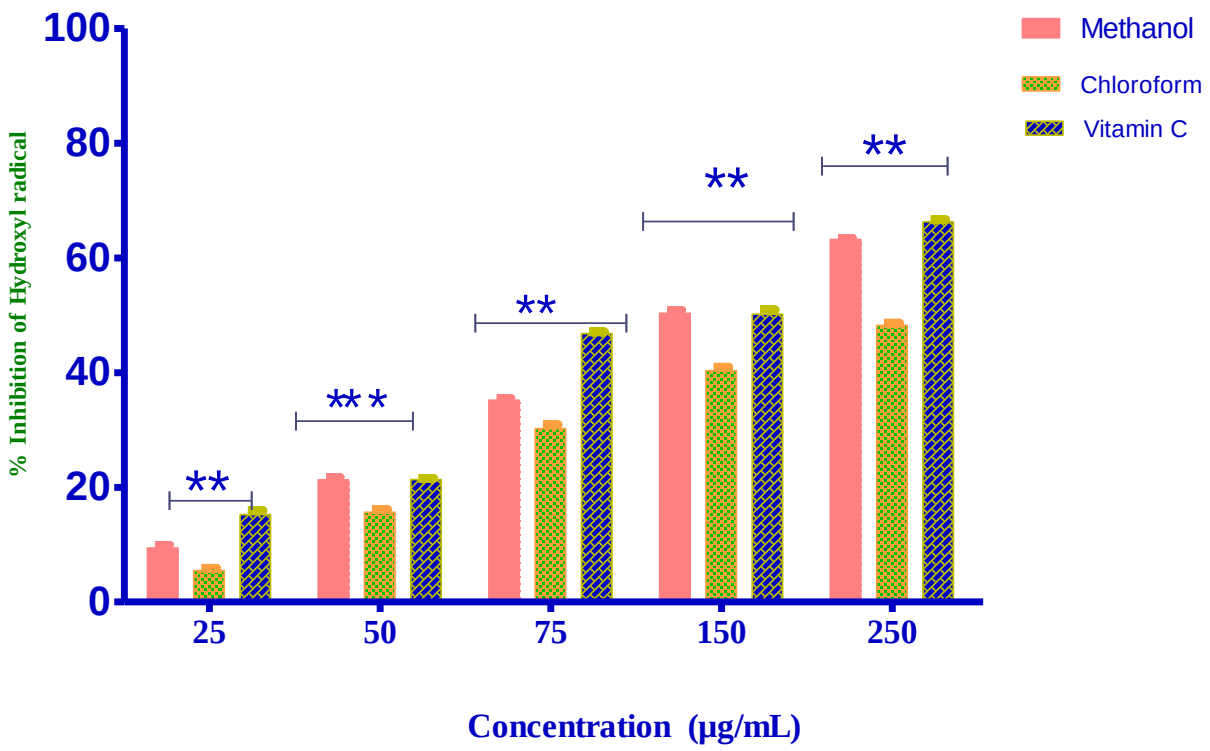

Figure 2: Antioxidant activity of the *Bacopa monnieri* Methanol and chloroform measured in the hydroxyl radical assay at different concentrations (25, 50, 75, 150 and 250 µg/mL). The values represent the percentage of radical scavenging. The values expressed as mean ± SD values, analyzed by one-way analysis of Variance (ANOVA) followed by Dunnett's multiple comparison tests (P<0.05; P<0.001; P <0.0001). Asterisk (*) (**) (***) indicates significance differences among treatments compared in control groups

1V40 vs 6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one

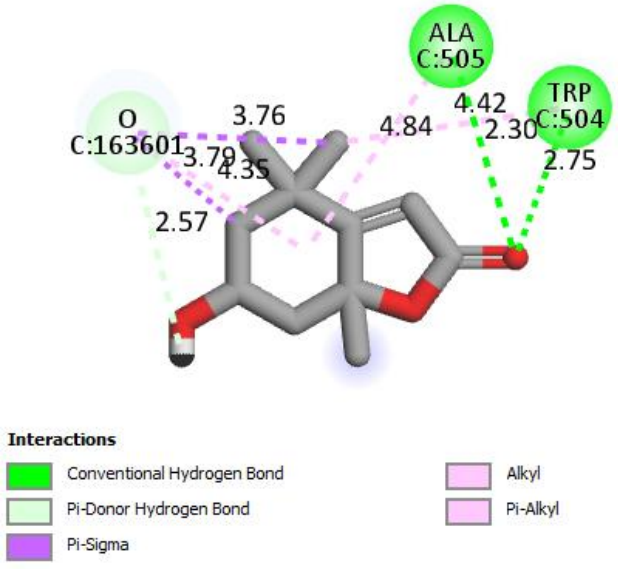

Figure 3: Docking complex and amino acid interactions of target proteins First Inhibitor Complex Structure of Human Hematopoietic Prostaglandin D Synthase (PDBID: 1V40) with 6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one (CID:14334) with binding energy of -7.2 kcal/mol.

Finally, the plantlets were placed in plastic cups filled with sterile vermiculite and an equal amount of garden and farmyard soil. The survival rate was 85% after 15 days. 6% of the plants survived after an additional fifteen days of hardening. (Figures 5-6). The optimal concentrations for callus development from the leaf explant in this investigation were 3.0 mg/L BAP and 0.3 mg/L NAA as in table 3. The best hormone for callus development in Pedalium murex was found to be 0.3 mg/L 2,4-D alone[22].

1V40 vs 2-(1,3-benzothiazol-2-ylsulfanyl) ethanol

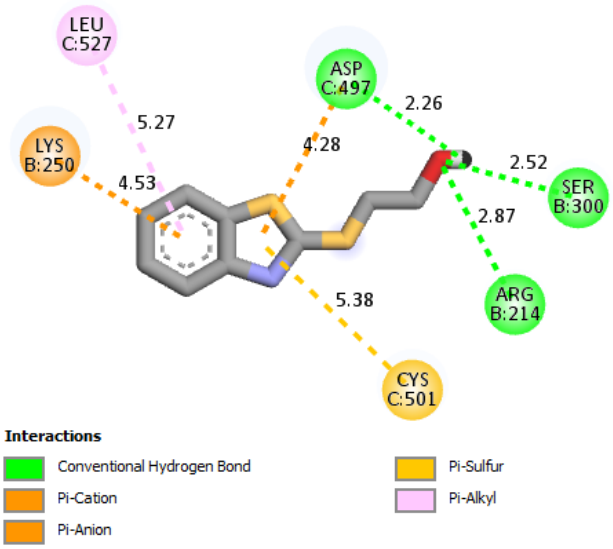

Figure 4: Docking complex and amino acid interactions of target proteins First Inhibitor Complex Structure of Human Hematopoietic Prostaglandin D Synthase (PDBID: 1V40) with -(1,3-benzothiazol-2-ylsulfanyl) ethanol (CID:20790) with binding energy of -5.6 kcal/mol.

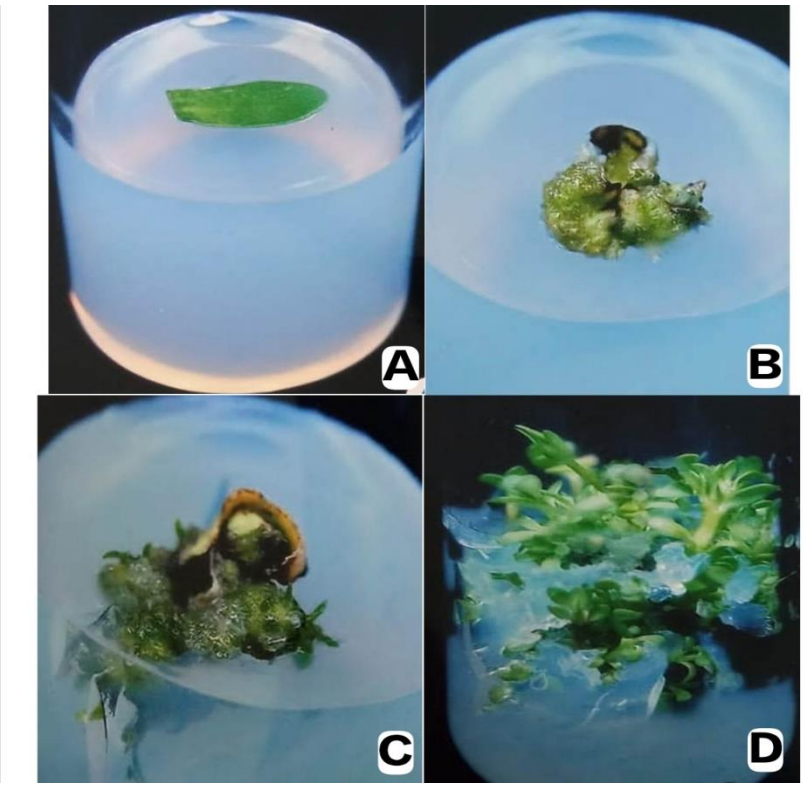

Figure 5: *In vitro* Propagation of *Bacopa monnieri (L.) Wettst using* (A) Leaf explant (B) Initiation of callus, (C) Initiation of multiple Shoots from Callus, (D) Well developed Multiple Shoots.

Table 3
Effect of IBA on root formation from the regenerated shoots of Leaf explants of *Bacopa monnieri*(L.) *Wettst*

Hormonal concentrations (mg/l)	Percentage of Root induction	No. of Roots / Shoot (Mean±SD)
0.5	71.0	7.2±2.14
1.0	58.0	7.7±1.88
1.5	48.0	2.7±1.33
2.0	32.0	2.0±0.81
2.5	-	Callus

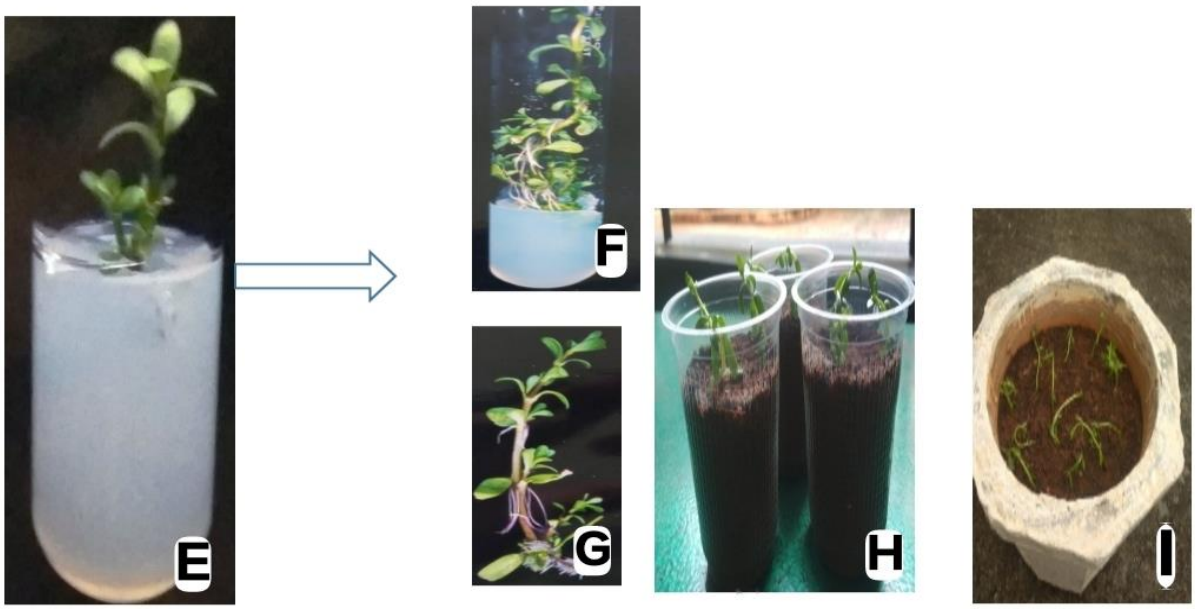

Figure 6: Shoot let transferred for Root induction (E) Rooting of the *in vitro* plant (F and G) Acclimatization of plants. (H) Hardening of the *in vitro* plants transferred to Plastic cups (I) *in vitro* plants transferred to the soil (field).

The optimal concentration for multiple shoot induction in the medicinal plant *Solanum varium* was found to be 8.0 mg/L 2ip+1.0 mg/L IAA. The maximum rate of multiple shoot development was achieved by cultivating the callus on MS medium supplemented with 6.0 mg/L BAP and 0.05 mg/L NAA[16]. It was found that when the callus was sub-cultured 3.0 mg/L BAP and 0.3 mg/L NAA, adventitious shoots were produced in *Acmella calva* L. Vinothkumar et al[27] found that the regenerated shoots were moved to the rooting media that contained varying amounts of IBA. The IBA concentration for root induction was found to be 1.5 mg/L. Amini et al[4] observed a similar observation in their early experiments.

Conclusion

In the current work related to *Bacopa monnieri* (L.) Wettst leaf, methanol extract fraction of this species exhibited possible antioxidant and enzyme inhibitory properties. The presence of flavonoids and phenolic substances was demonstrated by the phytochemical analysis of this extract. 0.5 mg/L IBA had the best rooting potential. The rooted plant has a 60% survival rate after being toughened appropriately. This medicinal plant can be commercially propagated and conserved.

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