Optimization of plant growth hormone concentrations on callus induction and comparative phytochemical profiling in leaves and callus of *Rosmarinus officinalis* L.

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Abstract

Optimizing the culture medium for efficient callus induction in Rosmarinus officinalis (rosemary) using various plant growth regulator (PGR) combinations and production of effective secondary metabolites are evaluated in the present study. Young leaves of Rosmarinus officinalis were selected as explants for callus induction. The results showed that equal combinations of growth hormones (BAP and 2,4-D) initiated callus induction in Rosmarinus officinalis. Highest callus biomass (0.3741±0.1768 gm) was procured from the leaf explants refined on MS medium embellished with 0.2 mg/l of BAP and 0.2mg/l of 2,4-D. A 100% callus induction rate was inoculated from leaf explants cultured on MS medium supplemented with equal concentrations of plant growth regulators (PGR) BAP and 2,4-D at 0.5 mg/L, 0.8 mg/L and 1.6 mg/L respectively.

The proposed medium compositions can be effectively used to obtain callus proliferation in Rosmarinus officinalis L. A comparative phytochemical analysis proved the detection of bioactive compounds like phenol and tannin among leaves and in vitro cultured callus of Rosmarinus officinalis (rosemary). The low concentration of tannins in the callus of Rosmarinus officinalis presents the potential for large-scale callus propagation and its subsequent use as a raw material for herbal tea production.

Keywords: Callus, Phytochemical, Phenol, Tannin.

Introduction

The native of Mediterranean area, volatile and aromatic evergreen plant belonging to family Lamiaceae, Rosemary (*Rosmarinus officinalis* L.) are distributed widely. The leaves of rosemary contain a variety of secondary metabolites such as flavonoids, volatile oils, tannins, triterpenoid acid, phenolic acids and phenolic diterpenoid bitter substances[42]. This traditional remedy is used to treat a number of illnesses such as headache, rheumatism, epilepsy, dysmenorrhea, nervous agitation, memory enhancement and physical and mental exhaustion[34,37]. It has various medicinal properties such as astringent, anti-inflammatory, expectorant, carminative, antirheumatic, analgesic, antimicrobial and hypotensive properties[3,20]. Because of its health benefits, the rosemary plant is used as a culinary condiment and to make body perfumes using essential oil extracted using hot extraction techniques[1].

The chosen plant cultivars are utilized in the form of whole dried herbs or powders, while both fresh and dried leaves are commonly employed in preparing teas and liquid extracts[33,39]. The aromatic oil can be extracted from the entire plant and the primary reasons for the growing popularity of rosemary plants are their anti-oxidant and health-promoting qualities[15].

The utilization of rosemary extract in therapeutic purpose as a natural antioxidant was first reported in 1955[44]. The active ingredients in rosemary plants, such as phenolic diterpenes prevent lipid oxidation and exhibit antioxidant properties[16]. The rosemary extract contains three different kinds of phenolic diterpenes namely carnosic acid, carnosol and rosmanol compounds which are used in making Ayurvedic products. The main phenolic diterpene found in the rosemary extract is mainly carnosic acid[7,16]. The derived/synthesized rosmarinic acid can increase shelf-life of perishable materials due to its acidic nature[30,44]. The antimicrobial constituents present in the leaf tea decoction contribute to its effectiveness in fighting infections and promoting wound repair.

In industrial sector especially in "Aromatherapy", this plant and its variants are extensively utilized in the perfume industry for their distinctive aromatic properties. Ensuring the conservation of the plant species and optimizing large-scale extraction[32] processes are vital for sustainable industrial exploitation. Plant tissue culture method is widely accepted in producing callus, multiple shoots and roots from medicinal plants[17,27]. The cells, tissue or organ culture in plants have become a powerful tool for biomass production and synthesising variety of secondary metabolites[25]. This study aims to optimize the culture medium for callus induction in *Rosmarinus officinalis*, followed by phytochemical screening to quantify phenolic and tannin content and to assess antioxidant activity in both dried leaves and *in vitro* cultured callus of rosemary.

Material and Methods

Collection of explant: Young leaves of *Rosmarinus officinalis* (L.) was collected as explants. Two-month-old plants of *Rosmarinus officinalis* (L.) was procured from

Kerala Agricultural University, Thrissur, for use in the present study and maintained in the Botanical Garden of Department of Botany, St. Joseph's College for Women, Alappuzha, Kerala.

Medium preparation: Murashige and Skoog media containing 3% sucrose were used to inoculate young leaf explants[10,11]. The medium was supplemented with specific amounts of hormones, BAP and 2,4-D (concentrations such as 0.2 mg/l, 0.5 mg/l, 0.8 mg/l and 1.6 mg/l) and pH was adjusted to a range of 5.6–5.8[41,43]. Medium was solidified using 0.8% agar.

Surface Sterilization of explants: The leaf explants were initially cleansed in tap water for one minute, followed by a 5-minute treatment with the detergent cleansol. After this, the explants were rinsed with tap water four times to ensure complete removal of detergent residues. Subsequently, they were exposed to 2 drops of 70% ethanol for 30 seconds for preliminary sterilization. The explants were then rinsed four times with tap water. The last stage of the sterilisation procedure involved treatment with 0.1% (w/v) sodium hypochlorite for three to five minutes followed by four to five rinses with sterile distilled water to remove any traces of sodium hypochlorite[6,38].

Incubation of culture bottles: Culture bottles were incubated under controlled temperature of 22°C and 60 to 70 % relative humidity[4]. The photoperiod of 16hr and 2000-3000 lux light intensity was provided using white fluorescent tubes.

Preparation of Extracts: One gram each of callus tissue and leaves of *Rosmarinus officinalis* were soaked in 100 milliliters of distilled water and incubated in a water bath at 50°C for 24 hours. After being filtered via Whatmann filter paper, the resultant aqueous extracts were refrigerated in dark bottles for further examination.

Test for Tannin: Approximately 0.5 ml of the aqueous extract was mixed with a few drops of a 0.1% ferric chloride solution. The formation of a brownish-green or blue-black coloration indicated the presence of tannins.

Test for Phlobatannin: Aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid. The presence of phlobatannins was demonstrated by the production of a crimson precipitate following boiling.

Test for Flavonoids: The aqueous plant extract was mixed with five millilitres of diluted ammonia solution followed by the addition of concentrated sulphuric acid (H_2SO_4). The onset of a yellow tint suggested the presence of flavonoids.

Test for Terpenoids: 5ml of the extract were mixed with 2 mL of chloroform, followed by the careful addition of 3 mL of concentrated sulfuric acid (H_2SO_4) along the side of the test tube. The appearance of a reddish-brown coloration at the interface indicated the presence of terpenoids.

Test for Essential Oils: 2ml of the extract was mixed with 0.1 ml of 2 M sodium hydroxide, followed by the addition of a small quantity of 2 M hydrochloric acid. The formation of a white precipitate indicated the presence of essential oils.

Test for Phenols: Two millilitres of ferric chloride ($FeCl_3$) solution were added to the reaction mixture containing two millilitres of the extract. Phenols were present when a deep bluish-green colouration developed.

Estimation of Total Phenolic Content[3]: The Folin-Ciocaltaeu test was used to determine the dry extracts' total phenolic content. One millilitre of Folin Ciocalteu's phenol reagent (1 mg/ml) was combined with one ml of the sample. After five minutes, the mixture was mixed well with 10 ml of a 7% sodium carbonate solution and 13 ml of deionized distilled water. The absorbance was measured at 760 nm after the mixture was left in the dark for 90 minutes at 23^0C. The calibration curve, which was created by making a solution of gallic acid, was extrapolated to calculate the total phenolic concentration. Measurements of the phenolics were made for statistical value. Gallic acid equivalents (GAE) milligrams per gram of dried material was the unit of measurement used to express the TPC.

Estimation of Total Tannin Content[35]: The "Folin-Ciocalteu" method was used to determine the tannins. A volumetric flask (10 ml) containing 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu phenol reagent and 1 ml of 35% sodium carbonate solution was filled with approximately 0.1 ml of the sample extract. The flask was then diluted to 10 ml with distilled water. After giving the mixture a good shake, it was allowed to sit at room temperature for half an hour. Tannic acid reference standard solutions (20, 40, 60, 80 and 100 µg/ml) were made using the previously mentioned technique. An ultraviolet visible spectrophotometer was used to measure the absorbance of the test and standard solutions at 700 nm in relation to the blank. Three separate measurements were made in order to estimate the tannin content. The amount of tannin was stated in of mg of tannic acid equivalents/ g of dried sample.

Statistical Analysis: The data were statistically analyzed using Microsoft Excel (Microsoft Corporation, USA) to determine the mean and standard deviation values for callus induction and phytochemical analysis.

Results and Discussion

Callus Induction: Plant tissue culture is a well-established technique for producing a large number of genetically identical plantlets. Plant growth hormones are important factors that regulate metabolite synthesis, differentiation and cell growth. The right concentration of the medium is another element that controls callus development and metabolite synthesis in apical bud of plants[28]. Secondary metabolite synthesis from medicinal plants depends on proper growing conditions for the particular plant species[14]. One of the most popular *in vitro* methods for obtaining

secondary metabolites is callus culture as per the reports of Jain et al[24].

The addition of specific growth hormones induces the production of secondary metabolites that exhibit antibacterial, antioxidant and neuroprotective activities in plants[8]. The current work used MS medium supplemented with different amounts of "BAP and 2,4-D" to induce callus from the leaf explants. Young leaves, or explants, were sterilized using 70% ethanol and sodium hypochlorite solution[40]. They were then inoculated on MS medium that was supplemented with varying amounts of "growth regulators". The growth chamber was then kept at a controlled temperature and light level.

Our study demonstrated that leaf explants of *Rosmarinus officinalis* exhibited effective callus induction when grown on Murashige and Skoog (MS) medium supplemented with different amounts of 2,4-D and BAP (0.2 mg/L, 0.5 mg/L, 0.8 mg/L and 1.6 mg/L). The highest callus initiation rate (100%) was observed on MS medium supplemented with 2,4-D and BAP at 0.2 mg/L, 0.5 mg/L and 0.8 mg/L concentrations. The callus was pale yellow in colour and the dry weight ranged from 0.0001-0.1241 gm (Table 1). Highest biomass was obtained (0.3741 ± 0.1768 g) on MS medium containing 0.2 mg/L BAP and 0.2 mg/L 2,4-D. In conclusion, among the tested concentrations, the combination of 0.2 mg/L 2,4 -D and 0.2 mg/L BAP was most effective for promoting callus growth in *R. officinalis* (Table 1 and 2).

According to Coskun et al[9] and El-Zefzafy et al[13], the highest rate of callus induction in *R. officinalis* L. was obtained with growth hormone concentration ranging from 0.5 to 1.5 mg/l. Al-Masoody et al[2] reported the callus formation from leaf explants of *R. officinalis* on MS medium supplemented with "BAP and NAA" and found that 1.5 mg/l BAP and 1.5 mg/l NAA produced the largest volume of callus when cultured under 16 hours of light and 8 hours of darkness[5].

In an *in vitro* study, Al-Saeedi et al[5] reported the effects of growth regulators and light conditions on callus of rosemary. The combination of 2,4-D (1.0 mg/l) and BAP (0.5 mg/l) produced the largest callus (3.60g) in the medium.

Preliminary phytochemical Screening: Preliminary phytochemical study conducted on both leaves and callus of *R.officinalis* confirmed the presence of secondary metabolites. Tannin, terpenoids, phenol and flavonoids were detected in all samples, although the initial analysis of the aqueous extracts of the chosen samples did not reveal any phlobatannin or essential oil content (Table 3). The detection of phytocompounds in *Rosmarinus officinalis* leaves and callus confirmed its medicinal properties, particularly its antioxidant potential, which can be attributed to the presence of phenolic compounds and flavonoids[9,21]. Both the leaf and callus extracts tested positive for tannins, terpenoids, flavonoids and phenols when appropriate reagents were added to the aqueous extracts.

Table 1
Effect of plant growth regulators on callus fresh weight, callus dry weight and callus colour

Treatments (mg/L) Control	Callus fresh weight (g)	Callus dry weight (g)	Callus colour
2,4-D0.5 + BAP 0.5	0.0056±0.0038	0.0001± 0.0038	Pale Yellow
2,4-D0.2 + BAP0.2	0.3741 ± 0.1768	0.1241 ± 0.1768	Pale Yellow
2,4-D0.8 + BAP0.8	0.0314 ±0.0195	0.0038 ±0.0195	Pale Yellow
2,4-D1.6 + BAP1.6	0.3394 ±0.1978	0.0595±0.1978	Pale Yellow

Mean±SD

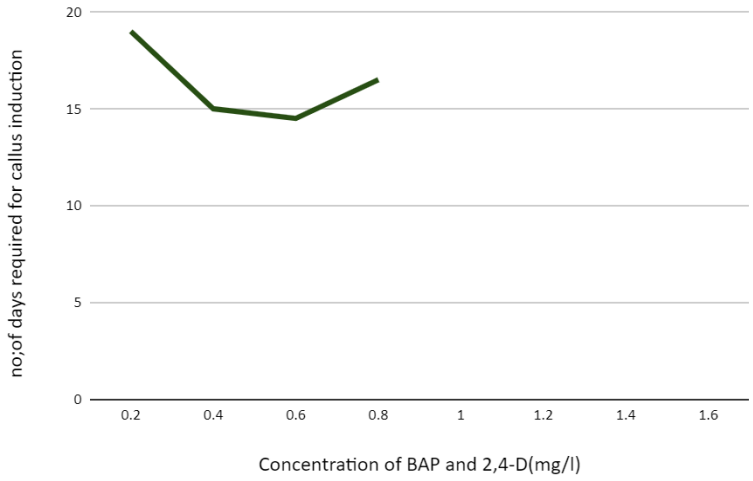

Fig. 1: Graph showing number of days of callus induction in *R. rosmarinus*

Table 2
Effect of plant growth regulators on percentage and duration of *R. rosmarinus* callus formation

Treatments	Days to callus induction	Percentage of callus induction
2,4-D(0.5mg/l) + BAP (0.5mg/l)	19±2.02	100
2,4-D(0.2mg/l) + BAP(0.2mg/l)	15±2.02	100
2,4-D(0.8mg/l) + BAP(0.8mg/l)	14.5 ±2.02	100
2,4-D(1.6mg/l)+ BAP (1.6mg/l)	16.5±2.02	80

Mean± SD

Table 3
Test showing preliminary detection of Secondary metabolites

S.N.	Test	*Rosmarinus officinalis* (Leaves)	*Rosmarinus officinalis* (Callus)
1.	Tannin	+	+
2	Phlobatannin	-	-
3	Terpenoids	+	+
4	Flavonoids	+	+
5	Essential Oil	-	-
6	Phenol	+	+

+: Present. -: Absent

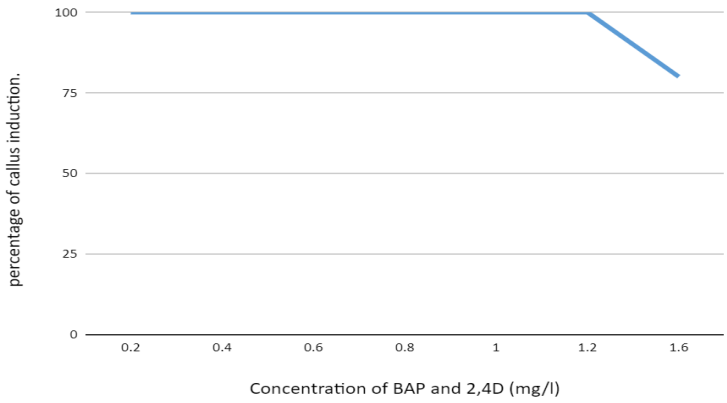

Fig. 2: Graph showing percentage of callus induction in *R. rosmarinus*

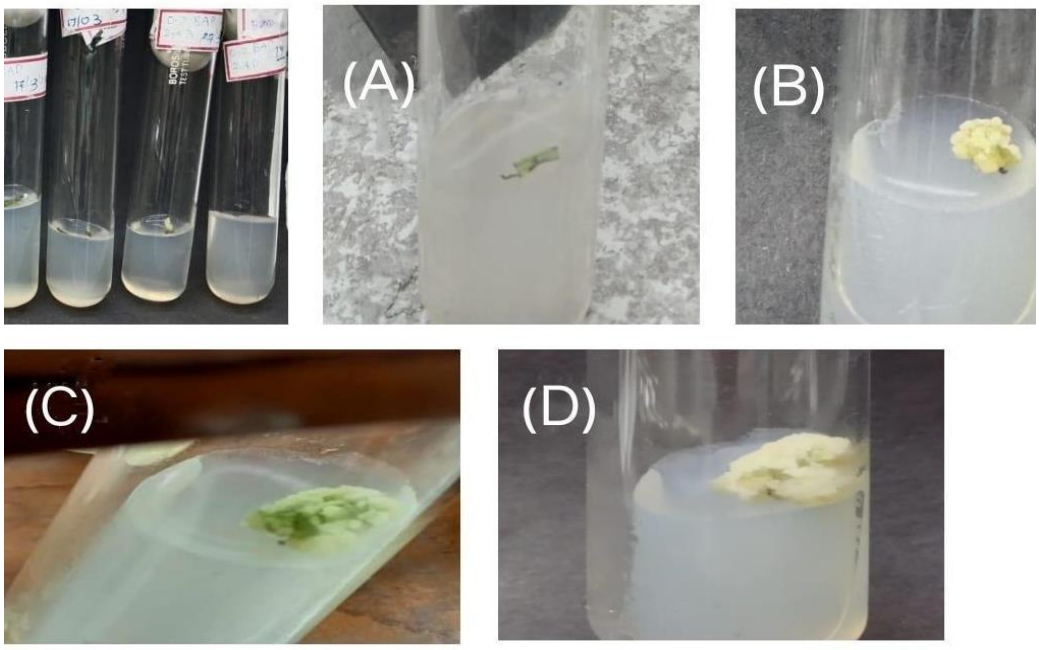

Fig. 3: Inoculated leaf explants of *R. rosmarinus* (0.2mg/l BAP and 0.2mg/l 2.4-D) and Callus formation after A- 6 days, B- 12 days, C- 36 days and D-48 days

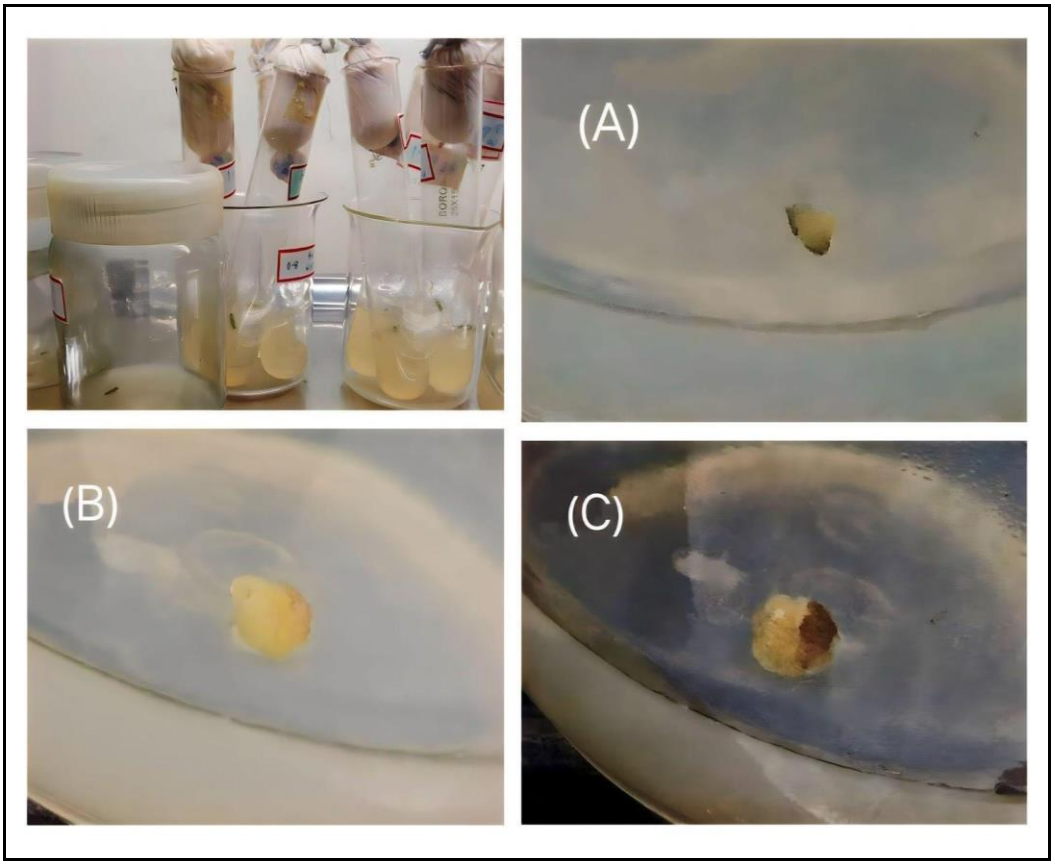

Fig. 4: Inoculated leaf explants of *R. officinalis* (0.8mg/l BAP and 0.8mg/l 2.4-D) and Callus formation after A-8 days, B-15 days and C-20 days

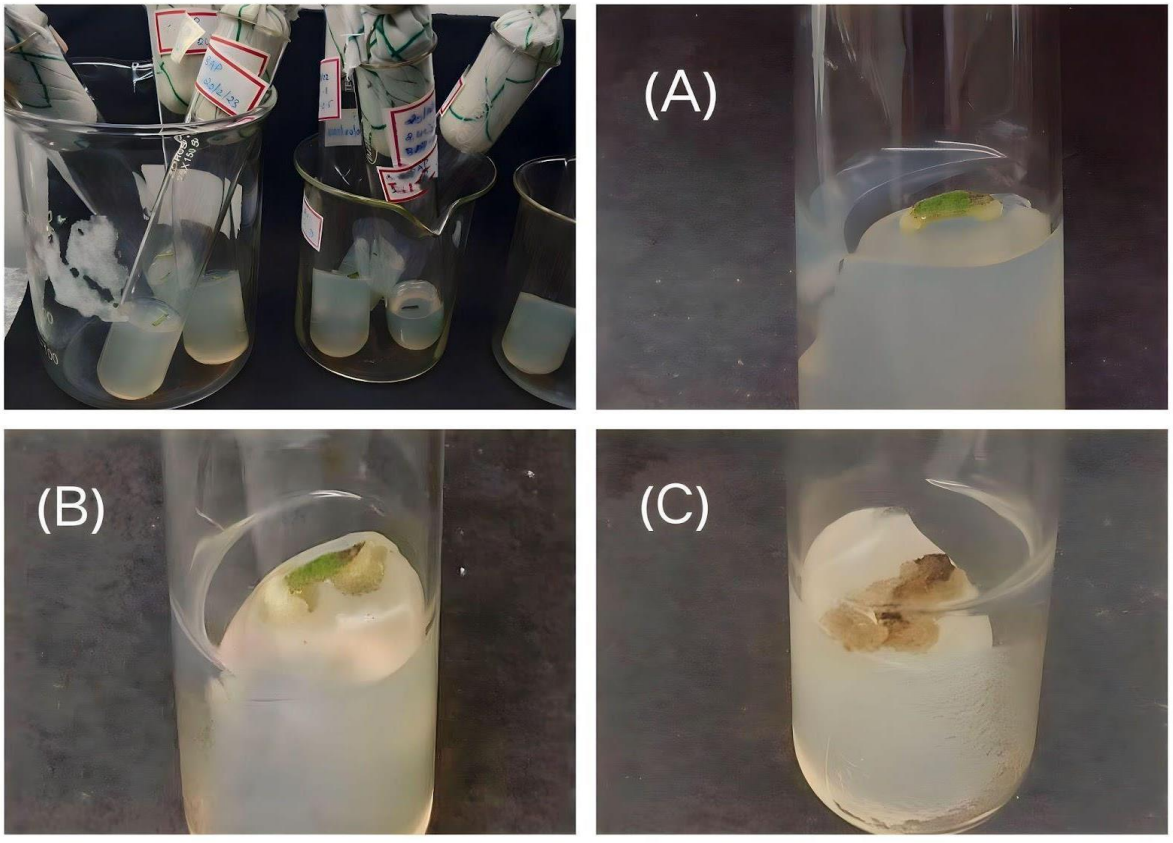

Fig. 5: Inoculated leaf explants of *R. officinalis* (1.6mg/l BAP and 1.6mg/l 2.4-D) and Callus formation after (A) 6 days (B) 28 days (C) 48 days

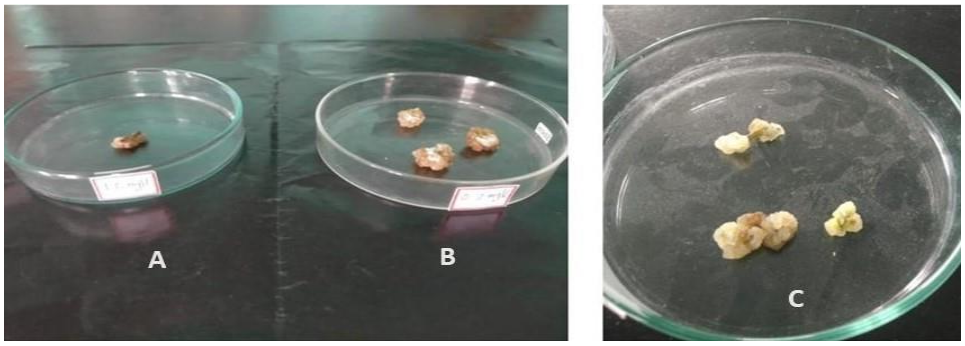

Fig. 6: Harvested callus of *R. officinalis* cultured on MS medium components with concentrations variation of 24-D and BAP (A- 0.2mg/l BAP and 0.2mg/l 2.4-D; B- 0.8mg/l BAP and 0.8mg/l2.4-D; C-1.6mg/l BAP and 1.6mg/l 2.4-D)

Table 4
Estimation of Phenol in *Rosmarinus officinalis* L. Leaves

S.N.	Test Sample	Absorbance of leaves (nm)	Absorbance of callus (nm)
1.	2 ml	0.187±0.05	0.02±0.01
2.	4 ml	0.238±0.72	0.09±0.04
3.	6 ml	0.302±0.24	0.14±0.39
4.	8 ml	0.352±0.04	0.23±0.21

Mean ± SD

Table 5
Estimation of Tannin in *Rosmarinus officinalis* L.

S.N.	Test Sample	Absorbance of leaves (nm)	Absorbance of callus (nm)
1.	2 ml	0.16±0.03	0.04±0.07
2.	4 ml	0.29±0.05	0.11±0.05
3.	6 ml	0.41±0.23	0.23±0.12
4.	8 ml	0.56±0.22	0.31±0.24

Mean ± SD

The majority of antioxidant activities in plants and plant derivatives depends on phytochemicals, the most significant class of phenolics. Table 4 and 5 showed the estimation of phenol and tannin content in leaves and callus of Rosemary plant. The amount of tannin and phenol content varied in both Rosemary leaves and callus in a concentration dependent manner. It was observed that the tannin content ranged from 0.16±0.03 nm to 0.56±0.22nm in Rosemary leaves.

Callus showed reduction in tannin content ranging from 0.04±0.07nm to 0.31±0.24 nm respectively (Table 5, Fig. 7). Phytochemical analysis revealed that the callus tissue of *Rosmarinus officinalis L.* contained lower levels of tannins than the leaves of normally grown plants. Gautheret[17] and Germ et al[18] reported that by controlling the conditions during plant growth, the amounts of bioactive compounds in St. John's wort herb can be changed. *In vitro* culturing on the callus from *R. officinalis* showed a significant reduction in tannin content which supported the reviewed reports. The large scale production of Rosemary leaves through micro-propagation was considered as an ideal method for industrial purposes[29]. Phenol content was compared which indicated the presence of phytocompound ranging from 0.16±0.03 nm to 0.56±0.22nm (*R. officinalis* leaves) and 0.04±0.07 nm to 0.31±0.24 nm (*R. officinalis* -callus) respectively (Fig. 7). The halophytes are used as a herbal tea due the presence of tannin and phenols[36]. These phytocompound had antioxidant, bioactive compounds and nutritive potential. The investigated plants offer a potential supply of bioactive chemicals, natural antioxidants and vital nutrients that can be used for a variety of home and commercial purposes.

Natural phenolic compounds have been reported to exhibit anticancer properties and numerous medicinal plants synthesize substantial quantities of these compounds[22]. The wide range of biological activities displayed by phenolics-including anti-inflammatory, antimutagenic and anticarcinogenic effects-highlights their pharmacological significance. Phytocompounds, particularly phenolic compounds, can directly influence metabolic processes and thereby regulate the cell cycle and apoptosis[26]. The present study highlights the optimal levels of phenols and tannins in the callus of *Rosmarinus officinalis L.* compared to the leaves of *R. officinalis* (Fig. 7). Furthermore, herbal tea formulations containing adequate concentrations of such phytochemicals have been reported to enhance antioxidant potential[20,23].

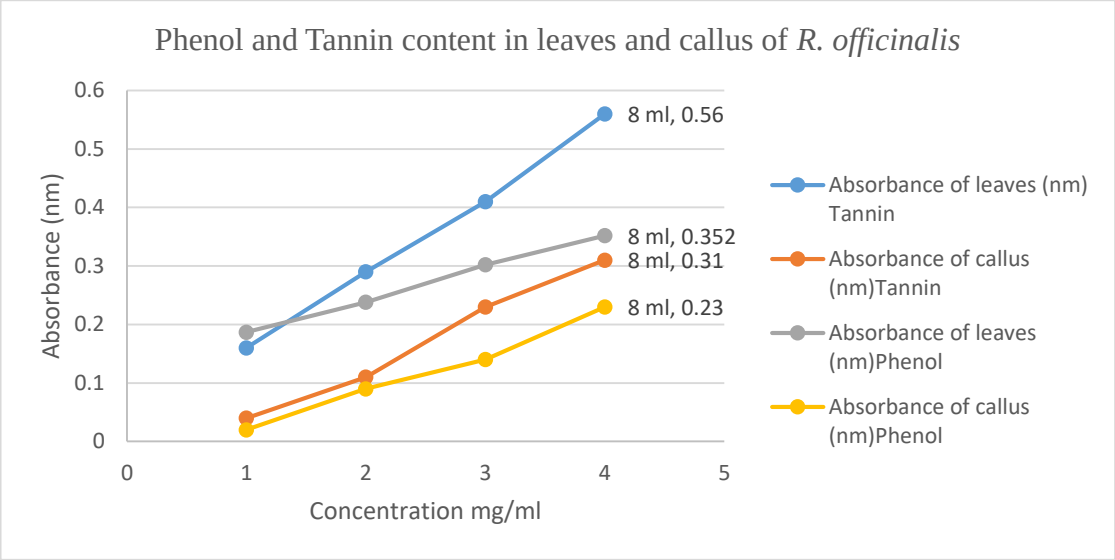

Fig. 7: Comparison of Phenol and Tannin content in Rosemary Leaves and Callus.

The callus induced from Rosemary plant showed similar phytoconstituents compared to normal leaves of *R. officinalis* plant. The callus from *Gymnema sylvestre* was used for the preparation of tea bags and showed antioxidant and phenol content[19]. Through somatic embryogenesis, new embryos can be produced with more primary and secondary metabolites in Rosemary as per reports of Perry et al[34]. Cell suspension technique is also used in production of secondary metabolites in medicinal plants, which was reported by Shekhawat et al[40] where *Arnebia hispidissima* produced alkannin content via this culture technique. Reports from different data sources like Pubmed, Science Direct showed that phytocompounds present in plants can be utilized for preparing Ayurvedic infusions[45]. Herbal tea bags with phytoconstituents can be supplemented for promoting health and also efficient brain functioning.

Conclusion

This study presents a standardized protocol for "callus induction" from indirect plant regeneration from *Rosmarinus officinalis* (Rosemary) leaves, offering a valuable tool for future research on enhanced production and potential somaclonal variation to support the genetic improvement of this medicinal herb. By establishing an optimized protocol for callus initiation, media composition and hormonal concentration, this work provides a framework for generating a reliable source of pharmacologically active compounds through callus culture. Cultivating *R. officinalis* and utilizing it as a herbal tea component holds potential for industrial application and sustainable research practices. The preliminary screening and secondary metabolite estimation in callus and leaves of *Rosmarinus officinalis* revealed that there is a strong correlation between flavonoid and phenolic content. The detection of these compounds proved the antioxidant properties among the callus and leaves of traditional herb. The observed results align with previous reports indicating that higher phenolic content contributes to the reduction of oxidative stress. The low tannin concentration in *R. officinalis* callus suggests its suitability for large-scale propagation, providing an abundant raw material source for herbal tea production.

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