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सं सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2023/Tech – 551 दिनांक/Date:27th July 2023

पादप प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen given by you for authentication is identified as ***Cynanchum tunicatum*** (Retz.) Alston - **APOCYNACEAE.**

अभिनिर्धारित प्रतिरूप को संबंधित कॉलेज/विभाग/संस्थान के पादपालय में परिरक्षण हेतु वापस किया जाता है।/ The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

27/07/2023

डॉ. एस. एस. हमीद /Dr. S. S. HAMEED
वैज्ञानिक 'ई' एवं कार्यालय प्रभारी/
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सेवा में / To

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Appendix L2

(Item No 5 of Check List) Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	In vitro seed culture optimization of Cynanchum tunicatum (Retz.) Ali ton using Response surface Methodology	Medicinal plants - International Journal of Phytomedicine and related Industries	Vol. 15(4), Dec 2023, 651-665	Scopus
2	Exploring the therapeutic potential of Cynanchum tunicatum (Retz.) Ali ton - assessment of phytochemicals and biological activities	Journal of King Saud University - Science	2024	Scopus

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : D Jaji

Supervisor : 07/05/2024

A. Vijayan 7/5/24

Checked By: 7/5/2024

HoD/Dean of Respective School

Medicinal Plants
Vol. 15 (4), December 2023, 654-665

doi : 10.5958/0975-6892.2023.00066.7

Research Article

In vitro seed culture optimization of *Cynanchum tunicatum* (Retz.) alston using Response Surface Methodology

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Received: May 25, 2023; Accepted: October 16, 2023

ABSTRACT

In vitro seed culture is the most successful method of preserving rare, threatened, endangered, and vulnerable medicinal plants. *Cynanchum tunicatum* (Retz.) Alston is a climbing shrub native to India and Sri Lanka. The plant seeds are used in traditional medicine to treat fever, skin diseases and infections. The plant seed is commercially important, and due to the over-exploitation of the medicinal plant, it may become vulnerable and the seed was collected from Sirumalai forest, Dindigul, Tamil Nadu, India. Producing ample plantlets through the seed culture technique is a boon and overcoming the exploitation of medicinal plants to optimize the experimental factors using Response Surface Methodology (RSM) 2FI Model. Since the significance of abiotic factors plays an importance role in plant tissue culture. This method is an effective statistical technique for analyzing various factors with a number of experimental trials. Hence, this experiment was conducted to optimize the influencing factors such as pH, photoperiod and sucrose concentration on Murashige and Skoog (MS) basal medium. The factors were optimized under various experimental conditions, the maximum percentage (96%) of seed germination was obtained at pH 5.8, photoperiod 16/8 hours with 3% of sucrose on MS basal medium.

Keywords: Dog strangling vine, pH, photoperiod, RSM and sucrose concentration

INTRODUCTION

Plants have been used as a source of traditional medicine since ancient times. Medicinal plants are an alternative role to therapeutic medicine (Swaminathan *et al*., 2020). Plant tissue culture methods are one of the best ways to preserve rare plants with adequate maintenance. The production of phytochemicals through plant tissue culture methods depends on using precise growing media and regulatory substances (Rezaldi *et al*., 2022).

Scientists in the field of biotechnology may find that tissue culture techniques can be used to assist farmers in accelerating crop propagation. These methods have the advantage that the plants are generated genetically like their mothers (Fadillah *et al*., 2022). In recent years, tissue culture has become increasingly popular for short- or medium-term conservation (slow development) and cryogenic conservation (long-term conservation) of vulnerable plant species. They proved to be more effective than traditional conservation measures in protecting plant

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species with resistant seeds or dormant seeds (Singh *et al*., 2022).

Conventional breeding methods blended with *in vitro* culture method serve as an excellent tool for crop improvement (Ebbie *et al*., 2022). In addition, cryopreservation preserves economically important plants and endangered species for an extended period without losing their viability (Abubakar *et al*., 2022). The *in vitro* seed culture method is simple and economically feasible for commercial applications. Seed culture is the most efficient approach for overcoming seed dormancy and low germination percentage (viability) having plant species. The culture medium used for this method significantly impact seed germination (Al-Ahmad *et al*., 2020). Therefore, selecting an appropriate medium is crucial factor for germinating all recalcitrant and low-viability seeds.

Seed germination is a simple way to save medicinal plants due to overexploitation by residents and climate change. Several factors can determine conductivity, such as temperature, salt stress, pH, sucrose concentration and medium composition (Belmehdi *et al*., 2018). The germination rate rises linearly with the correct photoperiod. Nevertheless, the germination rate falls dramatically without the proper photoperiod (Fallahi *et al*., 2015).

Response surface methodology (RSM) was used for experimental design, creating models, accessing the relative significance of numerous independent variables, and finding the optimal conditions for the desired outcome (Li *et al*., 2022). The surface response is obtained by fitting the collected data into a polynomial mathematical model by assessing the effects of each element and interaction. RSM process variables (independent variables) are simultaneously optimized to get the desired response variables (dependent variables). Although RSM has various advantages, its optimum condition depends on its mathematical design, while its validity depends on its statistical analysis (Mansor *et al*., 2022). It is commonly used to optimize bioactive compound production conditions and has already been utilized in the modelling and optimization of Plant Tissue Culture (PTC).

There are roughly 2,980 species in 315 genera in the Apocynaceae family. Besides, more than 300 compounds were identified from the *Cynanchum* species. Including steroids, alkaloids, terpenes, flavonoids, polysaccharides, and steroidal glycosides (Shan *et al*., 2006). *Cynanchum atrati* has high therapeutic properties, and it has C21 steroid glycosides such as fever, lymphangitis, vasoconstrictive syncope, relieving drenching, treating abscesses, detoxifying and other diseases (Zhang *et al*., 2022). *C. paniculatum* is mainly composed of alkaloids, steroids, and carbohydrates which have revealed anti-nociceptive, sedative, anti-inflammatory, anti-tumor, anti-viral, and neuroprotective activities (Ji *et al*., 2022). Furthermore, it is traditionally used for treating cancer, snake bites, stomach ulcers, hypercholesterolemia, constipation and liver disease (Sridharan *et al*., 2022).

Tubers of *C. taiwanianum* possess antioxidant, anti-cancer, anti-inflammatory, and anti-platelet aggregation activities (Tseng *et al*., 2022). A pharmacology study showed that *Cynanchum komarovii* has anti-bacterial, analgesic, anti-inflammatory, and anti-tumor, relieving asthma and cough (Wang *et al*., 2022). Interestingly, during the field survey at Dindigul, Tamilnadu district found that this species has efficient to heal maggot-infected wounds for domestic animals (Shivamanjunatha *et al*., 2019). The comments from indigenous people about this plant species hold significant value for antiviral activity. This species should have a lot of potential medicinal value to be analyzed.

To the best of our knowledge, no publication or literature has worked on the effect of abiotic factors on seed germination in *Cynanchum tunicatum*. Therefore, the effect of photoperiod, sucrose concentration, and pH on *C. tunicatum* seed germination was investigated in this study. The objective of our study is to mainly focus on the importance of abiotic factors with various parameters like pH, photoperiod, sucrose concentration and optimization with the help of Design-Expert software. Due to the potential medicinal values present in the various species of *Cynanchum* genus, we have chosen the species *Cynanchum tunicatum* to evaluate the medicinal use, to optimize the factors influencing growth parameters and this is the first report.

MATERIALS AND METHODS

Seed collection

Mature seeds (10 pods) were collected from Sirumalai forest, Dindigul, Tamil Nadu (N:10º16’45.1; E: 77 º59’55.1).

Healthy seeds were carefully collected to avoid any injury to the seeds. The plant is maintained in the herbal garden, at Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India.

Sterilization

Sterile solid Murashige and Skoog medium (MS) containing sucrose with various concentrations ranging from 2.3% to 3.8% and 0.8% agar were adjusted to pH ranging from 5.1 to 6.6 before sterilization (121 °C, 15 psi, for 15 minutes). Healthy pods were collected and washed thoroughly under running tap water for 25 minutes. Further, the sterilization process continues in the laminar airflow chamber. In order to avoid microbe interactions such as bacteria and fungi, the seeds were sterilized with 1% sodium hypochlorite (NaOCl) solution for 15-30 minutes. Then, the seeds were treated with sterile distilled water five times and mercuric chloride ($HgCl_2$) (0.1-0.3 %) for 2 to 3 minutes and then rinsed with distilled water.

Inoculation and incubation

Sterilized seeds were inoculated and incubated in controlled culture conditions. The different photoperiods with white fluorescent light were maintained from 9 hours of light and 15 hours of darkness (9/15) to 24 hours of light and zero hours of darkness (24/0), with an incubation temperature of 24 ± 2ºC.

Optimization through RSM

The analytical procedure of RSM was followed by (Nosratimovafagh *et al*., 2022) used by Design-Expert software version 13.0.8.0. Using numerical and graphical optimization methods were used to analyze the growth of seed culture-independent variables. The percentage of seed germination was calculated using a traditional graphical method. The experimental design was constructed employing a 3-variable, optimal design with 5 centre points. The three independent variables of abiotic factors were the pH (A), photoperiod (B), sucrose concentration (C) the details of the constituents of each factor and their upper and lower limits were determined from the graphical representation of the analysis of mean values from each level for a particular factor (Table 1).

Table 1: Factors used in Response Surface Methodology

S.No.	Parameter	Range
A	pH	5.1 - 6.6
B	Photoperiod	(9/15) - (24/0)
C	Sucrose Concentration	2.3% - 3.8%

Statistical analysis

The statistical effect of each factor was determined with the analysis of variance (ANOVA) method using Design-Expert (Version 12) at a confidence coefficient level of $\alpha = 0.05$. The model fit accuracy was assessed based on the model validity (lack of fit) and the explained variation (R^2). To validate the quality of the Design of Experiments (DoE) models, experiments were conducted under the predicted optimal process conditions, and compared to the predicted model outcomes (Nosratimovafagh *et al*., 2022).

RESULTS AND DISCUSSION

Optimization of *in vitro* seed culture

Response surface methodology was applied to optimize various abiotic factors in the seed culture of *Cynanchum tunicatum*. Five replicates were used for each variation of all the parameters. In our study, three independent parameters were investigated pH, photoperiod, and sucrose concentration on MS basal medium was used for the Two-Factor interaction model (2FI model). This method was effective in finding the most important factors with a few trials (Dron *et al*., 2002).

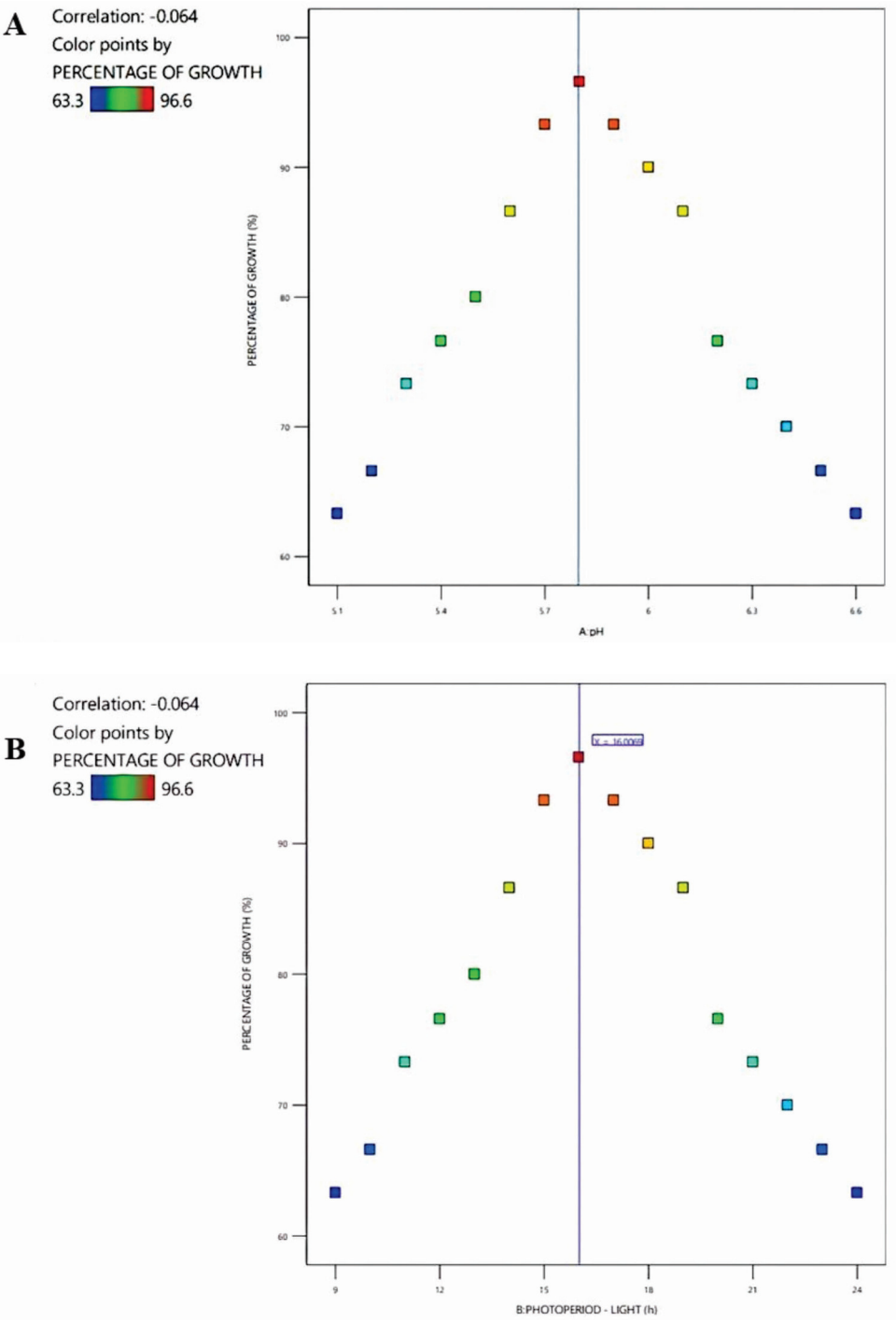
Effect of pH on seed germination percentage

Shoot culture, seed culture, and other tissue culture prospects for the production of explants could all be affected by pH changes (George *et al*., 2008). Although cadmium (Cd) toxicity was shown to be affected by the medium pH levels, pH 7 on growth and dry biomass was found to be minimal in terms of adverse effects of Cd toxicity (Ur *et al*., 2021). The medium of plant tissue culture has a pH of 5.6 to 5.8. The germination percentage responds based on the pH and if it was suitable for the growth, maximum output was obtained. Ramìrez *et al*. (2001) studied the optimization of individual parameters and proved that the highest astaxanthin production was obtained at 19.7°C temperature; 11.25 g L^{-1} carbon

concentration; 6.0 pH; 5% inoculum and 0.5 g L^{-1} nitrogen concentration. pH showed a significant effect on the production of melanin (Inamdar *et al*., 2014). Similarly, maximum seed germination occurs in *C. tunicatum* at pH levels ranging from 5.6 to 5.8 (Figure 1A).

Effect of photoperiod – seed germination percentage

In recent years, light-emitting diode (LED) technology has advanced rapidly, and LED lights have emerged as a valuable technology for increasing productivity in



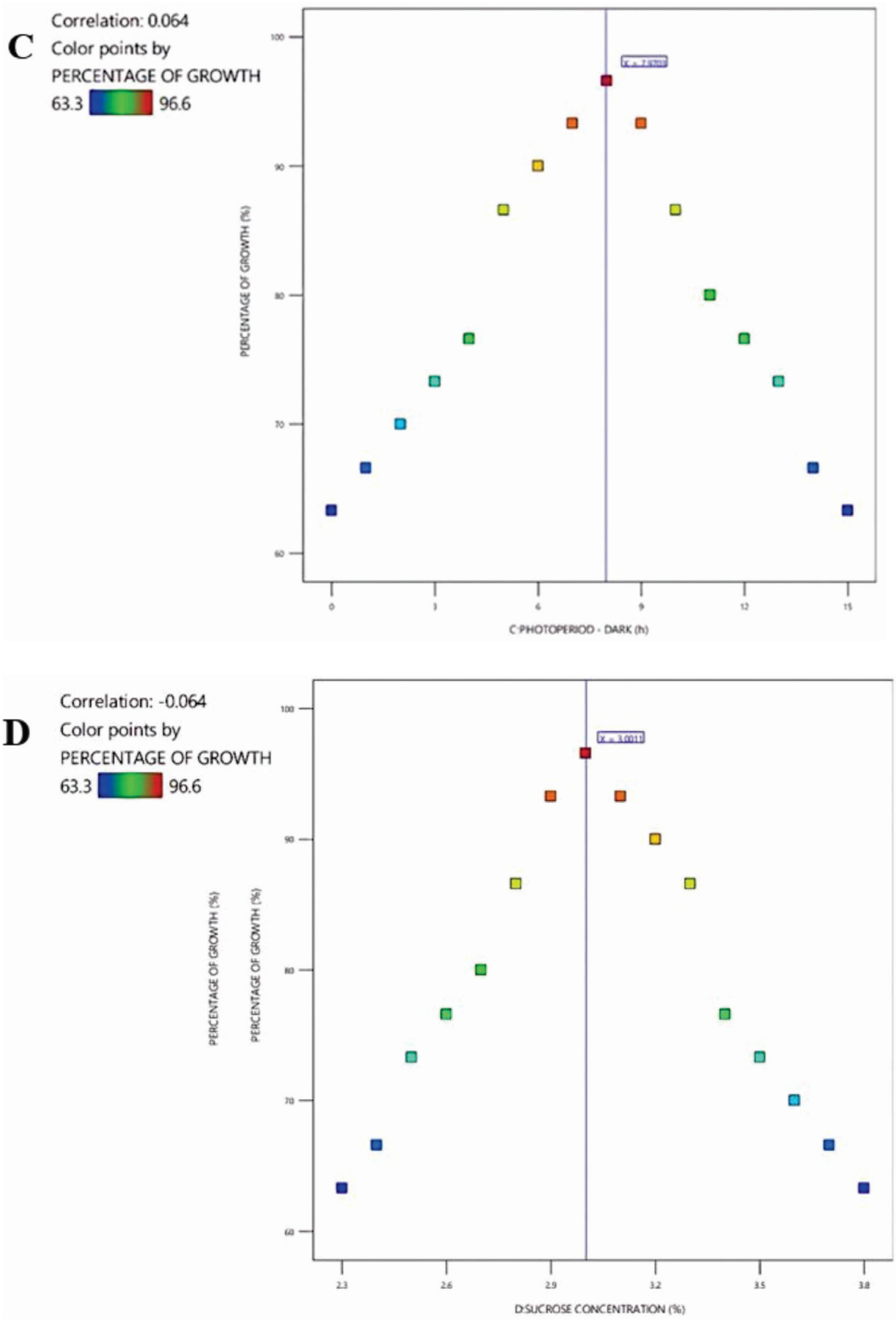


Figure 1: (A) Percentage growth of pH (B) Percentage growth of photoperiod (Light) (C) Percentage growth of photoperiod (Dark) (D) Percentage growth of sucrose concentration

commercial applications with controlled environments (Batista *et al*., 2018). The potential of LED technology used to produce secondary metabolites, this technique helps to understand biosynthesis pathways for the production of bioactive compounds which has been commercially important (Batista *et al*., 2018). Night interruption lights showed the most effective growth percentage and increase the yield (Flores *et al*., 2021).

The optimum photoperiod has given the best flowering and increases plant growth in *Cannabis sativa* (Moher *et al*., 2021). It was apparent that light quality and intensity were simple factors, however they were complex factors that interact with other factors such as sucrose levels to influence shoot development and growth as a non-linear, multifactorial, and complex process despite their apparent simplicity (Hesami *et al*., 2021b). Light and dark photoperiod cycle can cause a change in cellular content. *Isochrysis galbana* has high biomass in 18/6 photoperiod conditions in produces a lipid content. It becomes a suitable candidate for biodiesel production (Che *et al*., 2019). *C. tunicatum* exhibits maximum seed germination in 16 hours of light (Figure 1B).

Effect of photoperiod – seed germination percentage

The Reduction of the dark period affects the ability of plant regeneration. There was no positive evidence for increasing the germination rate in *Triticum timopheevii* (Miroshnichenko *et al*., 2021). Light intensity at low levels hampers photosynthetic efficiency, but high levels can cause damage to certain components of the photosynthetic apparatus and limit pigment synthesis (Cioc *et al.,* 2018). Each *in vitro* developmental stage requires specific light and dark conditions for their growth (Batista *et al*., 2018). Complete darkness may affect the germination percentage in both *in vivo* and *in vitro* plants. The effect of a dark period can change the germination percentage in *in-vitro* culture. Similarly, in *C. tunicatum,* a continuous dark period affected the germination percentage and showed a maximum response at the 8-hour dark period (Figure 1C).

Effect of sucrose concentration on seed germination percentage

In plant tissue culture, sucrose was used as a predominant carbon source. It was affiliated with foliar expansion, higher growth and gain of biomass, and more significant plant growth of *Vernonia condensata* (Fortini *et al*., 2021). Sucrose concentration in shoot cultures varied not only by species but also by genotype and cultivars. *In vitro*, Kolhari plants showed a good result in the optimal concentration of 3% sucrose, allowing it to regenerate efficiently. In response to the increased amount of sugar in the medium, the germination rate decreased proportionally (Cosic *et al*., 2021). Even though sucrose often stimulates photo-mixotrophic metabolic responses, light still plays a key role in the success of *in vitro* culture (Miler *et al*., 2019).

Batista *et al*. (2018) reported that sucrose was the most important for *in vitro* morphogenic and developmental processes, but the light intensity and spectrum were also important for plant growth and development. However, 3% sucrose was optimal for maximizing shoot length in *in vitro* culture. Similarly in the micropropagation of Cannabis, 3% sucrose was used generally for shoot growth and development (Hesami *et al*., 2021a). For instance, half-strength of MS medium supplemented with 3% sucrose showed a higher amount of seed germination percentage in *Eustoma grandiflorum* (Roni *et al*., 2018). Our study aimed to determine the sucrose concentrations ranging from 2.3 to 3.8 percent, in *C. tunicatum.* 3% sucrose concentration showed a good germination percentage in MS media (Figure 1D).

Results illustrated that *in vitro* seed cultures were optimized with various abiotic factors such as pH, photoperiod and sucrose concentration (Figure 2).

Statistical representation of ANOVA

Analysis of variance, the lack-of-fit test, and three R^2 statistics for pH, photoperiod (light and dark) and sucrose concentration were analysed. The seed culture of *C. tunicatum* showed a maximum result under proper abiotic conditions. The adjusted R^2 value and predicted R^2 value ranged from 0.8737 to 0.8905. The effect of the total model was highly significant ($p < 0.0001$), indicating that improper abiotic factors affected growth. ANOVA revealed the significant terms, with p-values < 0.0001, indicating good agreement among these values. The overall effect of the model was highly significant ($p < 0.0001$), indicating significant factor effects on pH, photoperiod and sucrose concentration (Table 2).

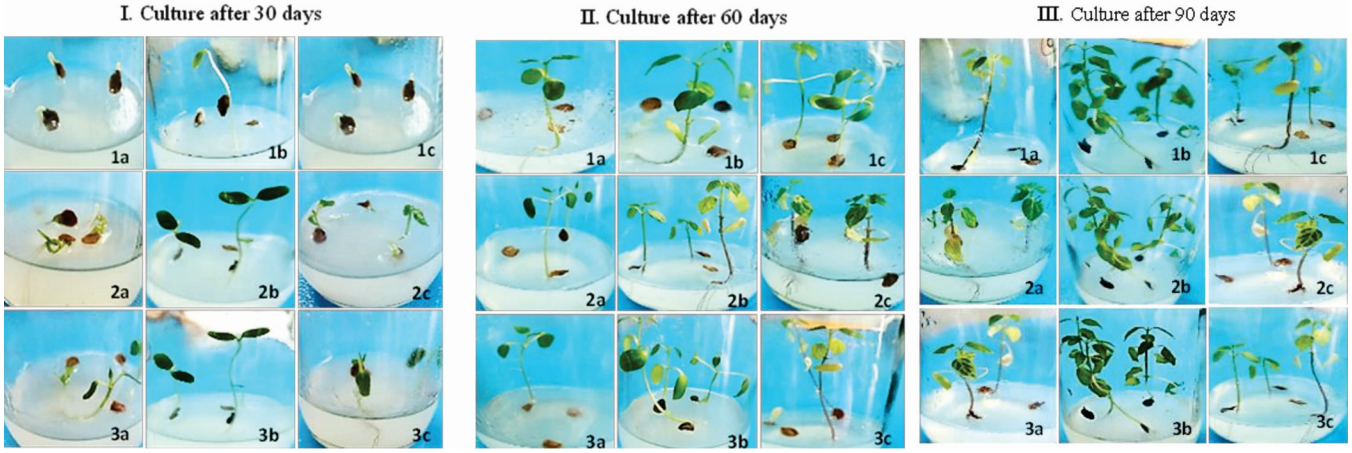


Figure 2: Effect of basal medium on *in vitro* seed culture with various abiotic factors (pH, photoperiod and sucrose concentration) of *Cynanchum tunicatum*. (1a) MS media with pH 5.1; (1b) MS media with pH 5.8; (1c) MS media with pH 6.6; (2a) MS media with photoperiod 9/15hrs (L/D); (2b) MS media with photoperiod 16/8hrs (L/D); (2c) MS media with photoperiod 24/0hrs (L/D); (3a) MS media with sucrose concentration is 2.3%; (3b) MS media with sucrose concentration is 3%; (3c) MS media with sucrose concentration is 3.8%

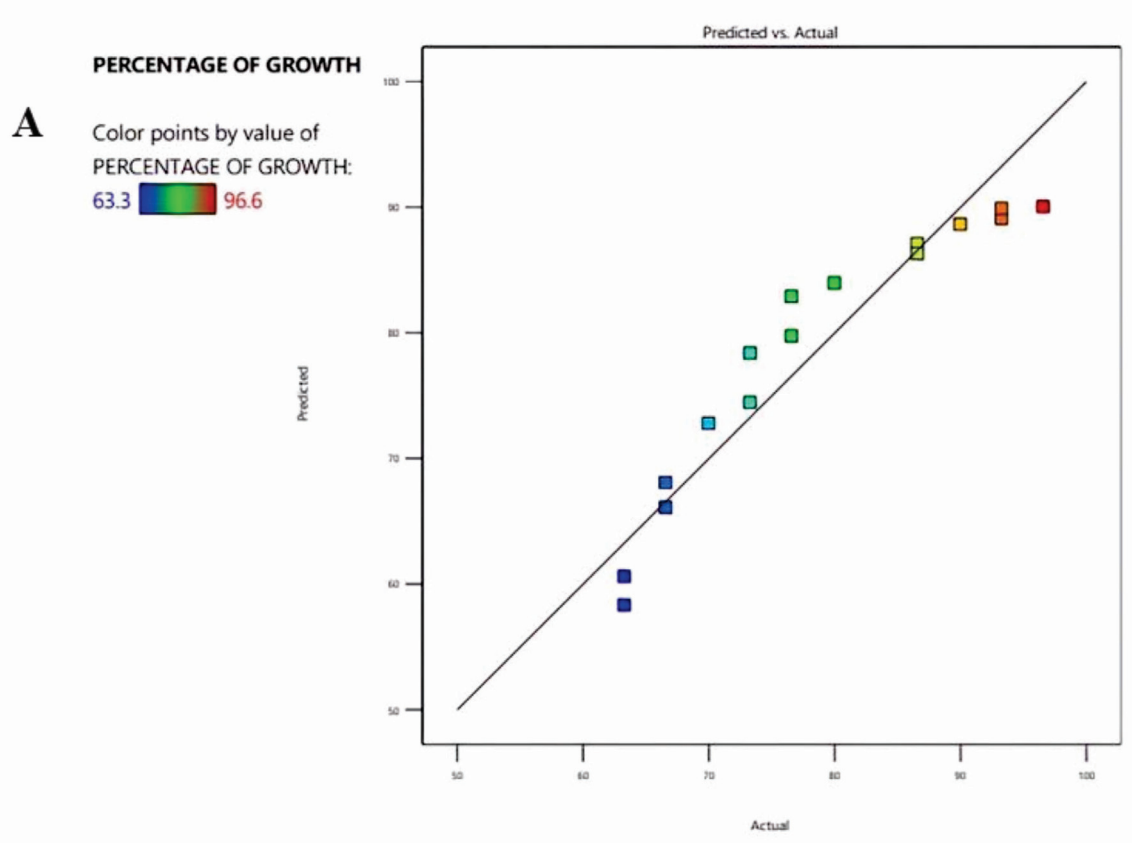
Table 2: Percentage of growth in *in-vitro* seed culture of *Cynanchum tunicatum*

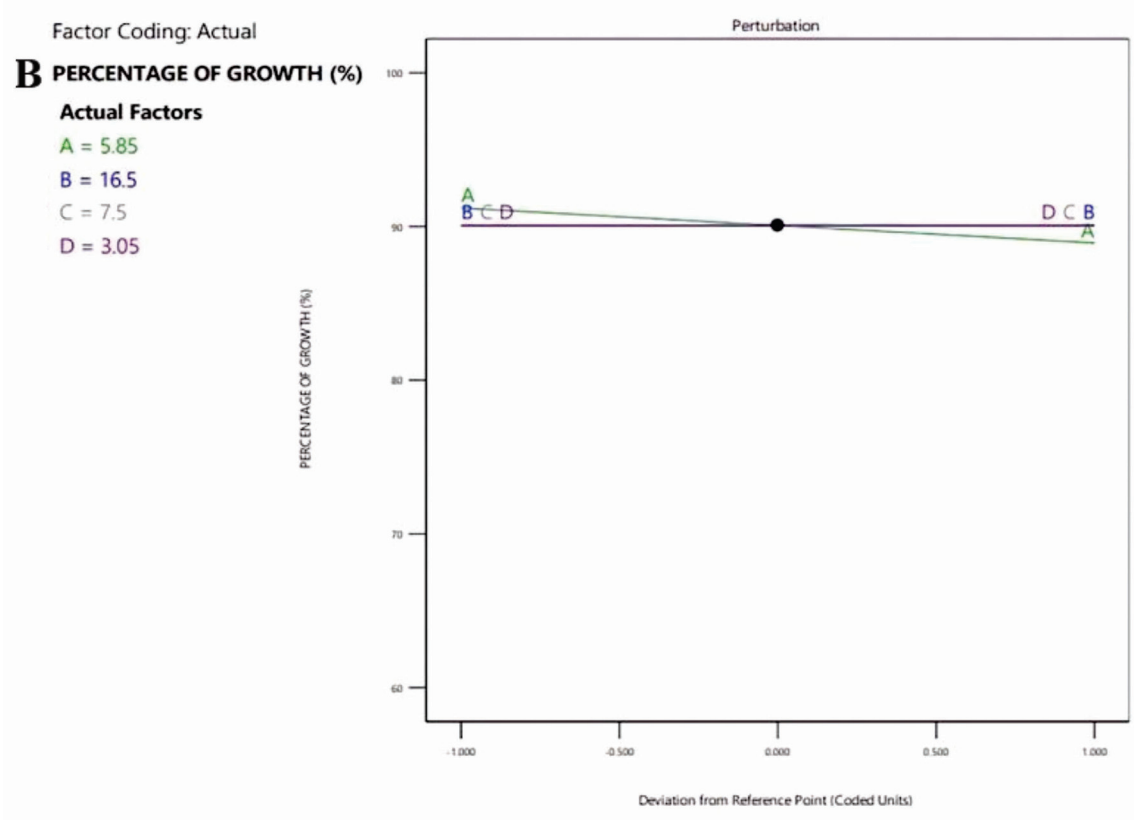
Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1701.10	2	850.55	52.86	< 0.0001	Significant
A-pH	7.80	1	7.80	0.4848	0.4985	
B-Photoperiod Light	0.0000	0				
C-Photoperiod Dark	0.0000	0				
D-Sucrose Concentration	0.0000	0				
AB	1693.29	1	1693.29	105.24	< 0.0001	Significant
AC	0.0000	0				
AD	0.0000	0				
BC	0.0000	0				
BD	0.0000	0				
CD	0.0000	0				
Residual	209.16	13	16.09			
Cor Total	1910.26	15				

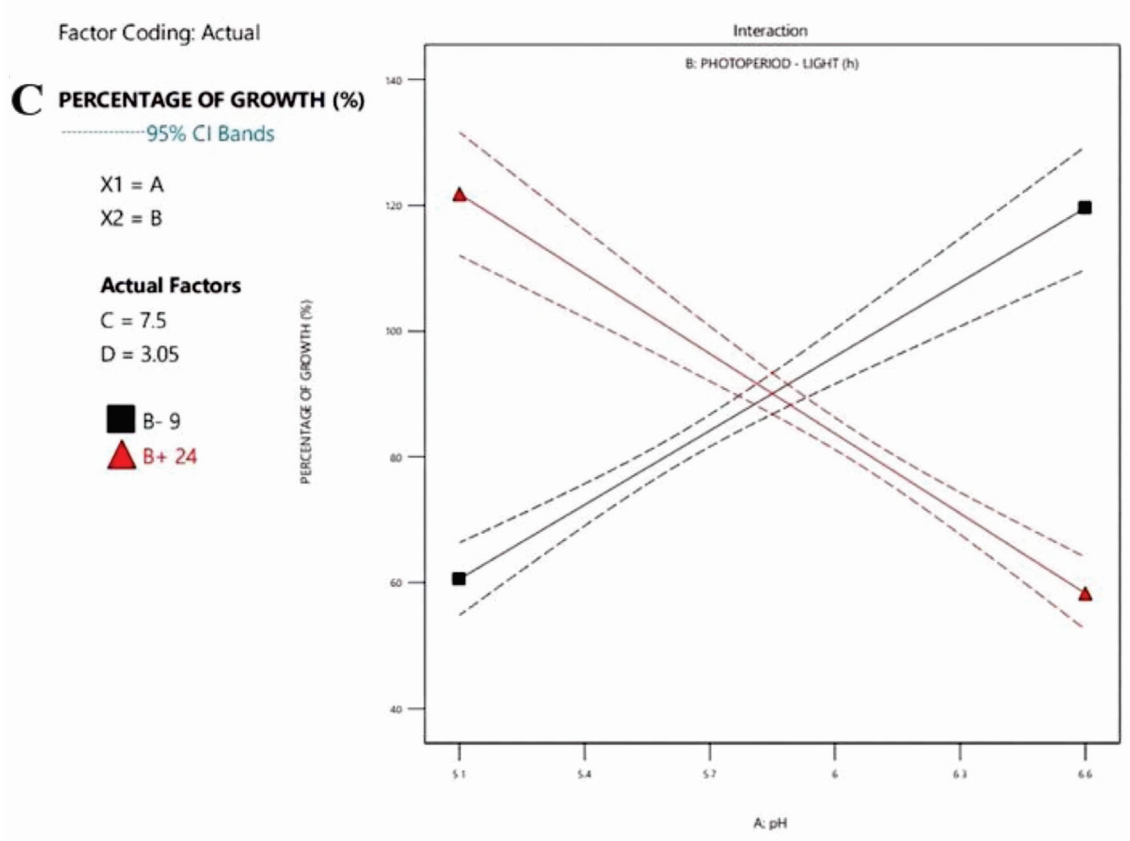
Growth percentage in plots

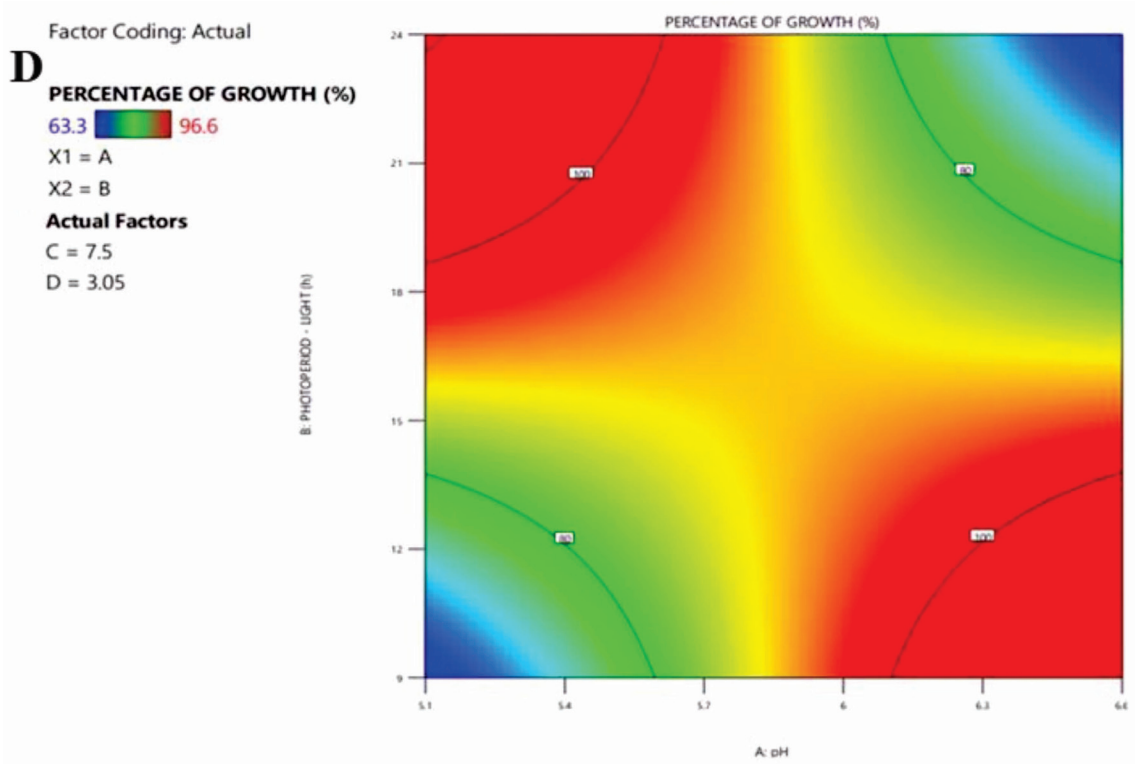
The percentage of growth was plotted in Predicted vs Actual (Figure 3A). The perturbation plot shows that other factors were aliased, the growth percentage was plotted by changing only pH (coded as A) over its range. By default, Design-Expert used all factors midpoints (coded 0) as the reference point. A factor with a steep slope or curvature represents that the response was sensitive to that factor. A relatively flat line showed that the factor was insensitive to change. The perturbation plot displayed the percentage of growth (Figure 3B).

An interaction occurred when percentage growth (response) varied with the combined effect of pH, photoperiod, and sucrose concentration (factors). Other interaction cannot be analyzed as the model was aliased (Figure 3C). The contour plot was a two-dimensional (2D) representation of the response (% growth) plotted against a set of two numerical factors (pH, photoperiod–light, dark and sucrose concentration). Aliased terms were not included


A
PERCENTAGE OF GROWTH
Color points by value of
PERCENTAGE OF GROWTH:
63.3
96.6
Predicted vs. Actual
Predicted
Actual
50
60
70
80
90
100


Factor Coding: Actual
B PERCENTAGE OF GROWTH (%)
Actual Factors
A = 5.85
B = 16.5
C = 7.5
D = 3.05
Perturbation
PERCENTAGE OF GROWTH (%)
A
B C D
D C B
A
Deviation from Reference Point (Coded Units)
-1.000
-0.500
0.000
0.500
1.000


Factor Coding: Actual
C
PERCENTAGE OF GROWTH (%)
95% CI Bands
X1 = A
X2 = B
Actual Factors
C = 7.5
D = 3.05
B- 9
B+ 24
Interaction
B: PHOTOPERIOD - LIGHT (h)
PERCENTAGE OF GROWTH (%)
140
120
100
80
60
40
5.1
5.4
5.7
6
6.3
6.6
A: pH


Factor Coding: Actual
D
PERCENTAGE OF GROWTH (%)
63.3
96.6
X1 = A
X2 = B
Actual Factors
C = 7.5
D = 3.05
PERCENTAGE OF GROWTH (%)
B: PHOTOPERIOD - LIGHT (h)
24
21
18
15
12
9
5.1
5.4
5.7
6
6.3
6.6
A: pH

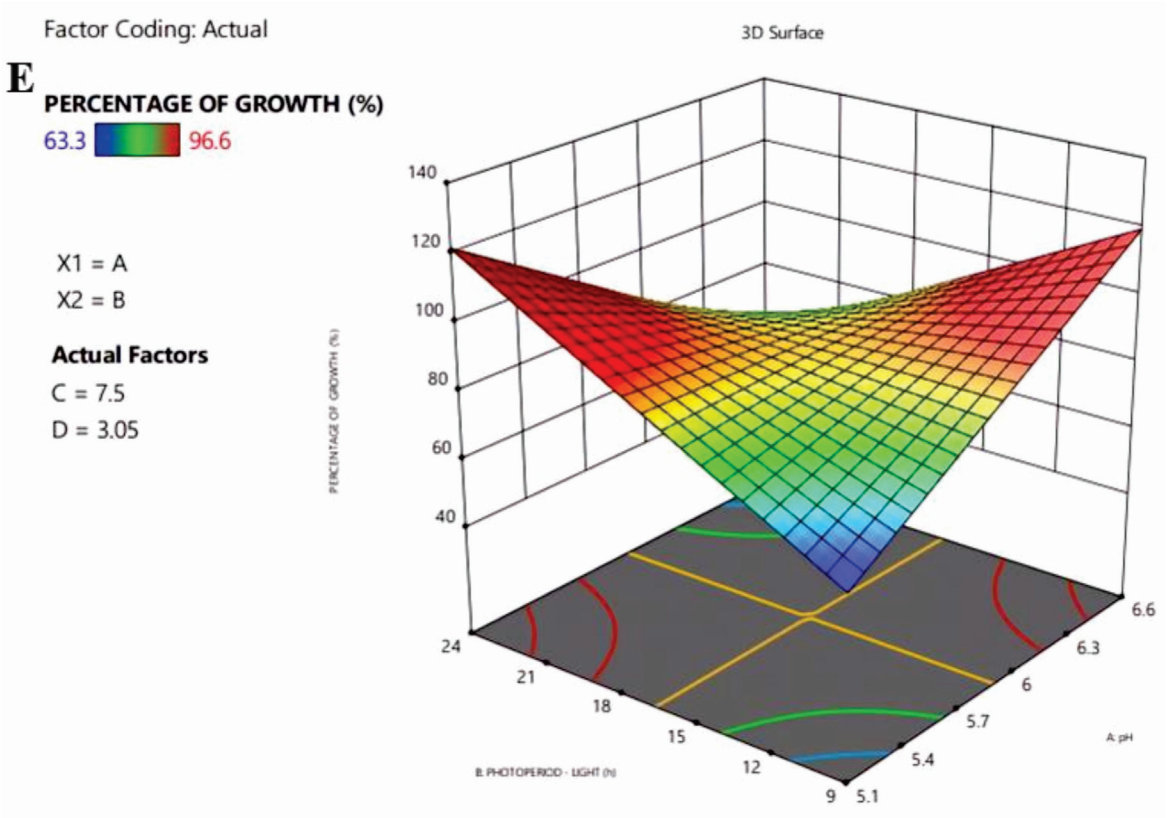


Figure 3: Optimization of *in-vitro* seed culture of *Cynanchum tunicatum.* A) Predicted vs Actual plot B) Perturbation C) Interaction D) Percentage of Growth E) Three-Dimensional surface

in this plot (Figure 3D). The three-dimensional response surface plot showed the photoperiod and percentage of growth for seed germination of *C. tunicatum*. Response surface plotted provide an overview of growth percentage in a three-dimensional view (Figure 3E).

CONCLUSION

This research work can be of a great use in medicinal plant cultivation, as it will enable us to understand the optimum conditions required for the successful germination of *Cynanchum tunicatum*. These results may guide the growers in setting up the best conditions for maximum germination and seedling establishment. It also sheds light on the significance of abiotic elements including pH, photoperiod, and sucrose content in determining the success of *C. tunicatum* germination. However, RSM is extensive to demonstrate the growth response by estimating predetermined inputs for germination is effective for various factors influencing growth parameters. The correlation coefficient (R^2) of the proposed model was 0.8905, indicating that the empirical model confirmed the experiment results. The findings of this study can also be utilized to create plans for protecting medicinal plant species that are in jeopardy of extinction.

ACKNOWLEDGEMENT

Our sincere thanks to the Department of Botany and Biotechnology for providing moral support to complete the project. We also thank CNR Rao laboratory, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India.

Conflict of interest

The authors declare that they have no competing interests.

Authors contribution

All authors are equally contributed.

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Journal of King Saud University - Science 36 (2024) 103238

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Full Length Article

Exploring the therapeutic potential of *Cynanchum tunicatum* (Retz.) Alston-assessment of phytochemicals and biological activities

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ARTICLE INFO

Keywords:
Cynanchum tunicatum
Antioxidant activity
Anti-inflammatory activity
And phytochemical analysis

ABSTRACT

Cynanchum tunicatum (Retz.) Alston is native to the Asian region and is distributed in the tropical areas of India and Sri Lanka. The aim of this study is to explore the phytochemical composition, antioxidant potential, and anti-inflammatory properties of *C. tunicatum*. Metabolic profiling was carried out using phytochemical screening to detect and quantify the secondary metabolites. To evaluate the potential secondary metabolites using the standard methods, Fourier transform infrared (FTIR), High-Performance Thin Layer Chromatography (HPTLC) and Gas Chromatography–Mass Spectrometry (GC–MS) from organic extracts of *C. tunicatum* and its biological activities. FTIR investigated peaks that represent alkane and aromatic compounds. GC–MS revealed the presence of 22 constituents such as 1-Hexacosene (0.145 %), l-(+)-Ascorbic acid 2,6-dihexadecanoate (8.129 %), Campesterol (5.243 %), Beta −Amyrin (10.614 %), Lupeol (13.061 %), Octadecanoic acid (0.751 %) are the major active compounds present. HPTLC fingerprinting confirms the bioactive compounds such as colchicine, strychnine, coumarin etc. which are represented with corresponding R_f values. Among all the extracts of *C. tunicatum* methanolic extract showed highest antioxidant activities. In 2,2-diphenylpicrylhydrazyl method exhibit the IC_{50} of (38.91 μg/mL), Ferric reducing antioxidant power assay (1.6 μg/mL), total antioxidant assay (IC_{50} = 32.91 μg/mL) and IC_{50} of anti-inflammatory activity (42.31 μg/mL) respectively. These findings enrich the knowledge of the species *Cynanchum tunicatum* for the possible application as a source of bioactive compounds in drug discovery.

1. Introduction

Medicinal plants are employed in the treatment of a wide array of diseases and serve as abundant sources of secondary metabolites. Folklore medicine has been used from ancient period in several countries (Sridharan et al., 2022). In rural communities, traditional medicine practitioners were used for many diseases (Archana & Bose, 2022). The secondary metabolites significantly shapes the biological and pharmacological efficacy of the product, consequently posing substantial challenges to achieving reproducibility in preclinical investigations and clinical trials (Alcazar Magana et al., 2020).

Cynanchum tunicatum belongs to the family Apocynaceae, which comprises approximately 300 species, including some types of swallowworts. Most of these species are climbers or twiners. In Chinese medicine, different varieties of *Cynanchum* species has their essential phytochemical constituents are prescribed to treat fever, cough, pneumonia and asthma (Bailly et al., 2023). Recent pharmacological studies have demonstrated that *Cynanchum* plants possess significant pharmacological effects, such as anti-oxidation, immune regulation, anti-inflammatory, and anti-tumor. A literature survey shows that approximately 450 compounds have been isolated from various *Cynanchum* species (Han et al., 2018). *Cynanchum* species harbor a myriad of therapeutic compounds; however, so far, researchers have not explored the biological activity of the specific plant species *C. tunicatum*.

A number of compounds such as β-sitosterol, conduritol F, geniposide, wilfoside was identified in *C. wilfordii* and *C. auriculatum*. Literature survey evidences that the most important lacuna of bioactive compounds present in the *C. tunicatum*. The primary objective of this study is to explore the bioactive compounds and their biological properties. Fingerprinting of *C. tunicatum* was conducted using Gas Chromatography-Mass Spectrometry (GC–MS) and Fourier Transform Infrared (FTIR) spectroscopy. Additionally, High-Performance Thin

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https://doi.org/10.1016/j.jksus.2024.103238
Received 5 January 2024; Received in revised form 3 May 2024; Accepted 5 May 2024
Available online 7 May 2024

Layer Chromatography (HPTLC) was employed as an effective technique for metabolite profiling.

2. Materials and methods

2.1. Plant material

The plant *C. tunicatum* was collected from Sirumalai forest, Dindigul, Tamil Nadu (N:10°16′45.1; E: 77 °59′55.1). The plant is maintaining in Herbal Garden, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore. The Plant was authenticated by Botanical Survey of India, Southern Region, Coimbatore (BSI/SRC/5/23/2023/Tech-551).

2.2. Solvents, chemicals, and extraction

Hexane, chloroform, ethyl acetate, methanol, toluene, acetone, formic acid, Mayer's reagent Wagner's reagent, Drangendorff's reagent, Molisch's reagent, Fehling reagent, Barfoed reagent, Bendict's reagent, Borntrager's reagent, sulphuric acid, ammonium solution, copper sulphate, sodium nitroprusside, ninhydrin, gallic acid, atropine, rutin, gallic acid and linalool, diclofenac sodium, DPPH reagent, Ascorbic acid, FRAP reagent, sulfuric acid, sodium phosphate, ammonium molybdate, Bovine serum albumin were used for further analysis. Atropine, Rutin, Ascorbic acid, Linalool from Sigma-Aldrich and other reagents were purchased from Sisco Research Laboratories.

Fresh and healthy plants were rinsed, shade-dried, and then pulverized. The extracts were obtained through sequential extraction of phytochemicals, transitioning from non-polar to polar solvents, employing hexane, chloroform, ethyl acetate, and methanol. All the crude extracts were dried using rotary vacuum evaporator (Roteva) and dissolved with respective solvents at mg/mL concentrations for further analysis.

2.3. Qualitative and quantitative phytochemical screening

The preliminary qualitative phytochemicals were analysed in all four extracts of *C. tunicatum* to determine the secondary metabolites (Harborne, 1998; Raaman, 2006). The Quantitative analysis of Alkaloids, Flavonoids, Phenols and Terpenoids were estimated by the following methods.

2.3.1. Total alkaloid content of C. tunicatum

The estimation of alkaloids was determined by bromocresol green method (Ajanal et al., 2012) for all four extracts of *C. tunicatum*. The extracts were dissolved in 2 N HCl (pH 2) and subsequently filtered. The resulting 10 mL solution was transferred to a separatory funnel and subjected to three washes with 50 mL of chloroform each. Following this, 5 mL of bromocresol green solution and 5 mL of phosphate buffer were added. Shake the mixture and extract yellow-coloured complex with 1-, 2-, 3- and 4-mL of chloroform in a separating funnel and collect the extract in 10 mL volumetric flask make up with chloroform. Atropine is used as a standard with different concentrations (0.2, 0.4, 0.6, 0.8 and 1 µg/ mL). The absorbance was measured using a spectrophotometer at 470 nm.

2.3.2. Total flavonoid content of C. tunicatum

The aluminium chloride method (Slinkard & Singleton, 1977) was used for flavonoid estimation using various extracts of *C. tunicatum*. Take 0.5 mL of all four extracts and mixed with 0.1 mL of 10 % aluminium chloride, 0.1 mL of potassium acetate and 2.8 mL of distilled water. It incubates at room temperature for 30 min. Rutin was used as a standard with various concentrations (0.2, 0.4, 0.6, 0.8, 1 µg/mL). The absorbance of the reaction mixture was measured at 415 nm.

2.3.3. Total Phenol content of C. tunicatum

Total phenolics were determined by Folin Ciocalteau (Slinkard & Singleton, 1977), using various extracts of *C. tunicatum*. One mL of extract was added with 1.0 mL of Folin Ciocalteau reagent followed by the addition of 3.0 mL of 2 % sodium carbonate and the mixture kept for 2 hrs incubation in dark condition. Gallic acid used as a standard. The blue colour indicates the presence of phenols and the absorbance was measured at 760 nm.

2.3.4. Determination of total terpenoid content of Cynanchum tunicatum

The total terpenoid content was determined by colorimetry assay (Indumathi et al., 2014) using various extracts of *C. tunicatum*. One mL of plant extract was mixed with 150 µL of vanillin-glacial acetic acid solution (5 %) followed by the addition of 500 µL of perchloric acid. The mixture was kept under water bath for 45 mins at 60℃. Glacial acetic acid (2.25 mL) was added to the mixture and observed reddish pink to blue color. The absorbance was measured at 548 nm. Linalool is used as a standard.

2.4. Determination of antioxidant activity

2.4.1. DPPH assay of C. tunicatum

The DPPH free radical scavenging activity was assessed according to the method described by Hatano et al. (1988). All four extracts of *C. tunicatum* at various concentrations (20, 40, 60, 80, 100 µg/mL) were combined with 2.7 mL of a 0.1 mM methanolic solution of DPPH. The resulting mixture was vigorously shaken and allowed to incubate in the dark for 30 min at room temperature. Subsequently, the absorbance was measured at 517 nm. A control sample, devoid of any extract but containing DPPH, was also measured. The percentage inhibition and IC_{50} value were then calculated. The absorbance of positive control is a pure compound. Hence it is showed higher inhibition percentage than plant crude extracts.

2.4.2. FRAP − assay of C. tunicatum

The capacity to reduce ferric ions was evaluated using a modified protocol based on the method outlined by Benzie & Strain, (1996). A portion of each extract, spanning various concentrations (20, 40, 60, 80, 100 µg/mL), was combined with 3 mL of FRAP reagent composed of 10 parts of 300 mM sodium acetate buffer (pH 3.6), 1 part of 10 mM 2,4,6-Tripyridyl-S-Triazine solution, and 1 part of 20 mM FeCl3 6H2O solution. The reaction mixture was then incubated in a water bath at 37℃ for 30 min. Gallic acid served as the standard reference. Subsequently, the absorbance was recorded at 593 nm, and the FRAP value was computed.

2.4.3. Total antioxidant activity of C. tunicatum

The total antioxidant capacity of *C. tunicatum* was assessed using the phosphomolybdenum method as described by (Prieto et al., 1999). A 0.5 mL aliquot of each of the four extracts, prepared at various concentrations (20, 40, 60, 80, 100 µg/mL), was combined with 4.5 mL of phosphomolybdenum reagent, consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixtures were then incubated in a water bath at 95℃ for 90 min. Ascorbic acid served as the standard reference. Subsequently, the absorbance was measured at 695 nm, and the inhibition percentage and IC_{50} value were determined.

2.5. Evaluation of anti-inflammatory activity

The anti-inflammatory activity of all four extracts of *C. tunicatum* was assessed using the bovine serum albumin denaturation assay, as described by Chandra et al., (2012). A reaction mixture of 5 mL was prepared, comprising 0.2 mL of bovine serum albumin, 2.8 mL of phosphate-buffered saline (pH 6.4), and 2 mL of various concentrations (20, 40, 60, 80, 100 µg/mL) of plant extracts. The mixtures were then

incubated at (37 ± 2) ℃ in a Biochemical Oxygen Demand (BOD) incubator for 15 min, followed by heating at 70℃ for 5 min. Subsequently, the absorbance was measured at 660 nm, with diclofenac sodium serving as the standard reference.

Percentage inhibition (%) = [Sample absorbance/(Control absorbance − 1)] × 100

2.6. FTIR (Fourier transform infrared) analysis

FTIR (Shimadzu Miracle 10) equipped with a temperature-stabilized detector was used for the analysis of functional groups present in phytochemicals in the range of 400–4000 cm^{-1} with a resolution of 16 cm^{-1}. The characteristic peaks and their functional groups were detected and recorded for further structure elucidated using FTIR.

2.7. GC–MS analysis

Mass spectrometry data were utilized in conjunction with GC–MS/MS analysis to identify compounds present within the extract. Interpretation of mass spectra was facilitated using the comprehensive database provided by the National Institute of Standards and Technology (NIST), which encompasses over 62,000 patterns. Unknown components were matched against known compounds stored within the NIST library. The identification process involved determination of the name, molecular weight, and molecular formula of the extract samples (Valdez et al., 2018).

2.8. HPTLC fingerprinting analysis

HPTLC profiling is used for the separation, identification, determination, and validation of phytoconstituents such as alkaloids, flavonoids, phenols and terpenoids from methanolic extracts of *C. tunicatum*. Aliquot of the test solution (100 mg / mL) and the standard solution were loaded as 5 mm band length in a silica gel 60F254TLC plate using a Hamilton syringe and LINOMAT 5 instrument (CAMAG, Muttenz, Switzerland). The samples were loaded in TLC plate with respective mobile phases and the images were captured in white light and UV light at 254 nm and 366 nm of developed and derivatized plates.

2.9. Statistical analysis

The experimental results were expressed as mean ± standard deviation. Statistical significance was evaluated by one-way analysis of variance (ANOVA). Values of $p \leq 0.05$ were significant using Tukey's test.

3. Results and discussion

3.1. Phytochemical analysis

The preliminary qualitative analysis was performed in four different extracts of *C. tunicatum*. The secondary metabolites are rich in ethyl acetate and methanolic extracts such as alkaloids, carbohydrates and glycosides, phenol and terpenoids, quinine, and phytosteroids (Table 1). Similar results were obtained in genus *Cynanchum* such as steroids, saponins, alkaloids, flavonoids, phenols and terpenoids (Han et al., 2018). According to Shrivastava et al., analyzed the terpenoids, flavonoids, tannins, saponins, steroids, carbohydrates, and alkaloids in methanolic fruit extract of *Trichosanthes dioica* (Shrivastava et al., 2021). In *Ricinus communis,* Rahman et al. studied the plant secondary metabolites in seed extract such as terpenoids, flavonoids, coumarin, steroids, and reducing sugar. These secondary metabolites are exclusively significant for the plant defence mechanism (Rahman et al., 2022).

Table 1
Preliminary qualitative phytochemical screening of *Cynanchum tunicatum.*

S. No	Tests	Hexane	Chloroform	Ethyl acetate	Methanol
1	**Alkaloids**				
A	Mayer's Test	−	−	++	++
B	Wagner's Test	++	−	+	+
C	Drangendorff's Test	+	−	−	+
2	Flavonoids	+	−	++	++
3	**Carbohydrates and glycosides**				
A	Molisch's Test	++	++	+	+
B	Fehling Test	−	+	++	+
C	Barfoed Test	+	+	++	++
D	Bendict's Test	−	−	++	++
E	Borntrager's Test	+	+	+	++
4	Saponin Test	−	−	+	+
5	**Oils and fats test**				
A	Spot Test	−	−	−	+
6	**Phenolic and terpenoid test**				
A	Ferric chloride Test	−	−	+	++
B	Gelatin Test	−	−	−	++
C	Lead Acetate Test	++	−	−	++
D	Alkaline Reagent Test	−	−	++	++
E	Magnesium and Hydrochloric Acid Test	+	−	−	++
F	Phlobaterpenoids	−	−	−	−
7	Quinone	++	−	++	+
8	Glycosides	++	−	++	++
9	Cardiac glycosides	+	−	−	−
10	Terpenoid	++	−	+	+
11	Coumarins	−	−	−	+
12	Steroids	−	−	+	+
13	Phytosterols	−	++	++	++
14	Protein	+	−	++	+

−: Absent, +: Present, ++: Strongly Present.

3.1.1. Estimation of total alkaloid content of Cynanchum tunicatum

Alkaloids are specific remedy for many diseases particularly in mammals because of their general toxicity, deterrence capability, adaptogenic activities and anti-inflammatory activity which help to alleviate pain endurance against stress and resistance to diseases (Zhang & Hu, 2020). In *C. tunicatum* the alkaloid contents were quantified in all the extract. Interestingly, the maximum alkaloid content was found in methanolic extract (1.37 ± 0.03 µg/mL) followed by ethyl acetate extract (0.78 ± 0.05 µg/mL), hexane (0.67 ± 0.03 µg/mL), chloroform (0.53 ± 0.04 µg/mL) of *C. tunicatum* (Fig. 1(e)). Atropine is used as a reference standard (Fig. 1(a)). Nagalakshmi et al. also studied that the maximum alkaloid content (5.62 mg/g) in methanolic extract of *Tinospora cordifolia* was observed (Nagalakshmi et al., 2023).

3.1.2. Total flavonoid content of C. tunicatum

The total flavonoid content was determined using aluminium chloride method of various solvents. The yellow colour represented the presence of flavonoid. The maximum flavonoid content was observed in methanolic extracts (4.23 ± 0.05 µg/mL) followed by ethyl acetate (2.37 ± 0.01 µg/mL), chloroform (1.28 ± 0.02 µg/mL) and hexane (0.104 ± 0.01 µg/mL) (Fig. 1(e)). Rutin is used for a reference standard (Fig. 1 (b)). Interestingly, Garg & Garg, analysed flavonoid content in methanolic leaf extract of *Ocimum sanctum,* obtained the maximum amount (4.75 mg /100 mg) and it is correlated with antioxidant activity (Garg & Garg, 2019).

3.1.3. Total Phenol content of C. tunicatum

The Phenolic content of hexane, chloroform, ethyl acetate and methanolic extracts of *C. tunicatum* showed 1.33 ± 0.01 µg/mL, 1.35 ± 0.007 µg/mL, 2.27 ± 0.063 µg/mL and 2.32 ± 0.037 µg/mL respectively, in which methanolic extract showed the maximum amounts of phenols (Fig. 1 (e)). Gallic acid is used as a reference standard (Fig. 1(c)).

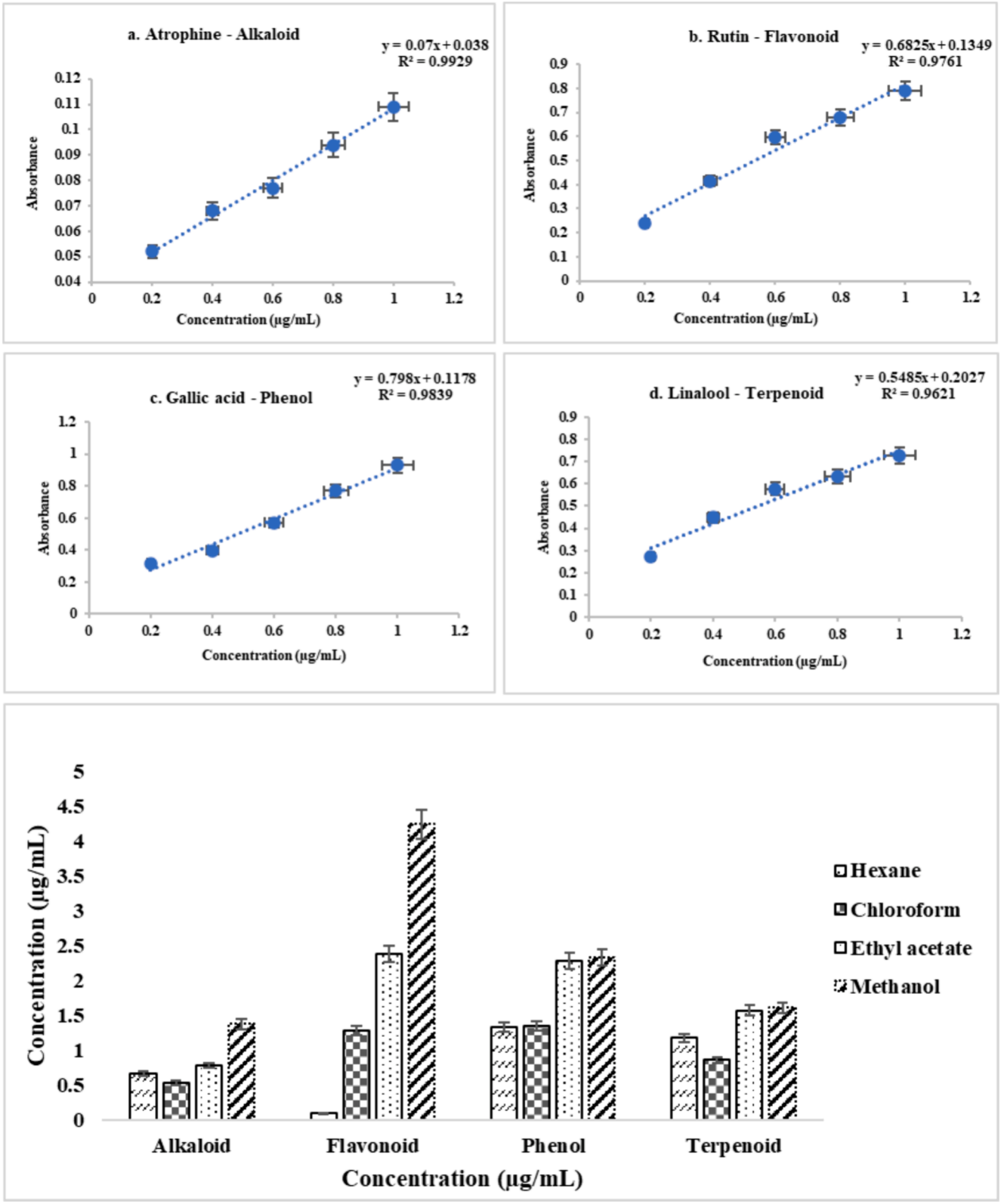

Fig. 1. a) Calibration curve of standard atropine for the quantification of total alkaloid content b) Calibration curve of standard rutin for the quantification of total flavonoid content c) Calibration curve of standard gallic acid for the quantification of total phenol content d) Calibration curve of standard linalool for the quantification of total terpenoid content e) Graphical representation of quantitative secondary metabolite screening of *Cynanchum tunicatum*.

Similarly, the previous researchers also analyzed the flavonoid content in various medicinal plants. Arya et al. studied the methanolic leaf extract of *Cichorium intybus* containing maximum phenolic content (302 ± 0.251 mg/mL) (Arya et al., 2022). Ahmed et al. investigated the methanolic extract of *Cannabis sativa* encompassing the maximum phenolic content (36.42 ± 1.905 μg/mL) (Abdel-Azeem et al., 2020).

3.1.4. Total terpenoid content of C. tunicatum

Terpenoid contents were analysed using the folin–ciocalteu method using various solvents of *C. tunicatum*. The maximum content of terpenoid was observed in methanol (1.60 ± 0.02 μg/mL) followed by ethyl acetate (1.57 ± 0.14 μg/mL), hexane (1.18 ± 0.02 μg/mL) and chloroform (0.86 ± 0.02 μg/mL) (Fig. 1 (e)). Linalool is used as a standard (Fig. 1 (d)). (Indumathi et al., 2014) analysed the highest terpenoid content in the methanolic leaf extract of *Enicostemma litorrale*. Terpenoids are significantly helpful for therapeutic properties like anticancer, antimicrobial and antioxidant activity (Roaa, 2020).

Preliminary phytochemical investigation of *C. tunicatum* facilitates the identification of phytochemical constituents and is quantified in the ranked as flavonoids > phenols > terpenoids > alkaloid. The differences in the values were statistically significant ($p \leq 0.05$). Based on the previous literature (Wang et al., 2021), *Cynanchum* species contains 232 compounds including alkaloids, terpenoids, C_{21} steroids, flavonoids, acetophenones and these phytochemical compounds are abundantly present in *C. tunicatum*. In future, based on the secondary metabolites biological activities will be carried out to confirm the medicinal properties of the *C. tunicatum*.

3.2. Antioxidant assays

Reactive oxygen species (ROS) damage the cells through pollution, radiations, and other environmental factors. These damages were protected through antioxidants which are the molecules that can neutralize the ROS by donating the electrons and stabilizes hazardous free radicals. The mechanisms of antioxidant activity either directly or indirectly by inhibiting the free radical damage, thereby preventing the nucleic acids, proteins, lipids, and other molecules. Plant derived bioactive compounds protect the cells from free radical damage by the free radical scavenging activity. In this present study, bioactive compounds obtained from *Cynanchum tunicatum* extracts showed more efficient antioxidant potential was analysed by DPPH, FRAP, and TAA.

3.2.1. DPPH assay of C. tunicatum

Free radicals cause damage to the plant cells, whereas antioxidants play a crucial role in protecting the cells. Natural antioxidants such as phenols and flavonoids have the potential to provide resistance against free radical-induced oxidative stress (Karale et al., 2022). The antioxidant activity was anlayzed for all four crude extracts of *C. tunicatum* using DPPH assay. The maximum inhibition percentage was obtained in the methanolic extract (IC_{50} = 38.91 μg/mL) followed by ethyl acetate (IC_{50} = 52.26 μg/mL) (Table 2). Ascorbic acid was used as a reference standard and its IC_{50} value is 53.101 μg/mL. This result confirmed the methanolic extract of *C. tunicatum* have potential for antioxidant activity. Still there is no previous antioxidant study were found on *C. tunicatum*. Akgül et al. reported that Ethyl acetate extract showed maximum inhibition (68.721 ± 1.694) of free radical scavenging activity in *Euphorbia eriophora* (Akgül et al., 2022).

3.2.2. FRAP assay of C. tunicatum

The determination of antioxidant activity, based on their ferric-reducing power, involves assessing the ability to reduce Fe_3 + to Fe_2+ (Ene-Obong et al., 2018). The antioxidant activity of all four crude extracts of *C. tunicatum* by using ferrous reducing assay. The maximum reduction was observed at a concentration of 100 μg/mL in methanolic extract (1.6 ± 0.11) followed by ethyl acetate (1.2 ± 0.007), chloroform (1.09 ± 0.009) and hexane (0.98 ± 0.007) (Table 3). The ferric-reducing assay of methanolic extract shows higher antioxidant capacity compared to gallic acid. According to Noreen et al. reported that the ethanolic extract of *Coronopus didymus* (aerial parts) observed the highest Optical Density (OD) value is 0.304 at the concentration of 50 μg/mL (Noreen et al., 2017).

3.2.3. Total antioxidant activity of C. tunicatum

The total antioxidant activity is based on the phosphomolybdate reagent which reduces Molybdenum VI to V with maximum absorption and it forms a green phosphate/Mo (V) complex. All four extracts of *C. tunicatum* with various concentrations were analysed and the maximum activity was observed at 100 μg/mL in methanol extract (IC_{50} = 32.91 μg/mL) followed by ethyl acetate (IC_{50} = 47.11 μg/mL), chloroform (IC_{50} = 86 μg/mL) and hexane (IC_{50} = 82.11 μg/mL) (Table 4). Hence the *C. tunicatum* is a potential candidate of antioxidant property. Ascorbic acid was used as a standard (IC_{50} = 53.38 μg/mL). In the previous research evidenced that the ethanolic leaf extract of *Limonia acidissima* showed maximum antioxidant activity of 5.055 μL (Parvez & Sarker, 2021). Bayliak *et al.*, studied that highest reducing ability of

Table 3
Evaluation of Ferric Reducing Antioxidant Power (FRAP) of *Cynanchum tunicatum*.

Concentration (μg/mL)	FRAP value			
	Hexane	Chloroform	Ethyl acetate	Methanol
20	0.02 ± 0.0005 ^{e}	0.24 ± 0.009 ^{e}	0.11 ± 0.005 ^{e}	0.29 ± 0.01 ^{e}
40	0.16 ± 0.01 ^{d}	0.43 ± 0.01 ^{d}	0.40 ± 0.01 ^{d}	0.45 ± 0.009 ^{d}
60	0.44 ± 0.01^{c}	0.59 ± 0.01^{c}	0.63 ± 0.02^{c}	0.89 ± 0.03^{c}
80	0.65 ± 0.004^{b}	0.84 ± 0.001^{b}	1.008 ± 0.009^{b}	1.20 ± 0.01^{b}
100	0.98 ± 0.007 ^{a}	1.09 ± 0.009 ^{a}	1.20 ± 0.007 ^{a}	1.60 ± 0.11 ^{a}

All values were expressed in mean ± standard deviations of triplicate measures. Each analysis was statistically significant ($p \leq 0.05$), 'a' expressed best results and 'e' showed poor results.

Table 2
Evaluation of Radical Scavenging activity of *Cynanchum tunicatum* by using DPPH method.

Concentration (μg/mL)	Percent scavenging of stable DPPH free radical (%)				
	Hexane	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
20	8.67 ± 0.003 ^{e}	26.4 ± 0.01 ^{e}	29.3 ± 0.004 ^{e}	40.3 ± 0.007 ^{e}	22.76 ± 0.004 ^{e}
40	20.7 ± 0.003 ^{d}	36.4 ± 0.006 ^{d}	39.4 ± 0.009 ^{d}	52.09 ± 0.004 ^{d}	40.4 ± 0.004 ^{d}
60	35.01 ± 0.002^{c}	48.4 ± 0.005^{c}	55.6 ± 0.002^{c}	57.9 ± 0.01^{c}	56.05 ± 0.007^{c}
80	54.1 ± 0.008^{b}	58.3 ± 0.005^{b}	68.1 ± 0.005^{b}	72.5 ± 0.004^{b}	71.39 ± 0.006^{b}
100	69.1 ± 0.004^{a}	70.8 ± 0.01 ^{a}	84.08 ± 0.01 ^{a}	85.9 ± 0.005 ^{a}	86.82 ± 0.002 ^{a}
IC_{50}	76.15	63.405	52.26	38.91	53.101

All values were expressed in mean ± standard deviations of triplicate measures. Each analysis was statistically significant ($p \leq 0.05$), 'a' expressed best results and 'e' showed poor results.

Table 4
Evaluation of Total antioxidant activity of *Cynanchum tunicatum* by phosphomolybdenum method.

Concentration (µg/mL)	Percent scavenging of TAA (%)				
	Hexane	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
20	13.6 ± 0.03 [e]	15.4 ± 0.07 [e]	29.9 ± 0.01 [e]	44.2 ± 0.005 [e]	27.3 ± 0.02 [e]
40	21.1 ± 0.023 [d]	17.5 ± 0.047 [d]	48.0 ± 0.008 [d]	54.1 ± 0.008 [d]	44 ± 0.02 [d]
60	30.0 ± 0.003[c]	31.2 ± 0.027[c]	60.3 ± 0.007[c]	62.4 ± 0.007[c]	53.1 ± 0.01[c]
80	57.0 ± 0.013[b]	45.1 ± 0.057[b]	70.3 ± 0.009[b]	75.1 ± 0.002[b]	68.1 ± 0.02[b]
100	58.6 ± 0.023 [a]	62.1 ± 0.017 [a]	81.8 ± 0.006 [a]	86.3 ± 0.003 [a]	83.6 ± 0.002 [a]

All values were expressed in mean ± standard deviations of triplicate measures. Each analysis was statistically significant ($p \leq 0.05$), 'a' expressed best results and 'e' showed poor results.

Rhodiola rosea aqueous extract was 3.66 ± 0.24 mg/mL (Bayliak et al., 2016).

3.3. Evaluation of anti-inflammatory activity assay

Protein is denatured by harmful pathogens or free radicals and inhibiting this process can be a potential mechanism for anti-inflammatory activity (Yesmin et al., 2020). Result displayed in Table 5 show IC_{50} inhibition of albumin denaturation of all the extracts of *C. tunicatum* at concentration between 42.31 to 68.44 µg/mL in the following order of methanol > ethyl acetate > hexane > chloroform. The standard diclofenac sodium an anti-inflammatory drug showed IC_{50} value at 60.14 µg/mL. According to *Phoenix dactylifera* (Jihl − a variety of date seed) showed a high inhibition (IC_{50} = 90.34 µg/mL) in scavenging nitric oxide free radicals and possessed the highest anti-denaturation effect (Hmidani et al., 2020). Similarly, Saleem et al. analysed BSA assay in *Moringa oleifera* showed maximum inhibition percentage of protein denaturation with IC_{50} value of butanol > n-hexane > ethyl acetate > piroxicam > diclofenac sodium > methanolic > aqueous (Saleem et al., 2020). In this present study, the methanolic extract contains alkaloids, flavonoids, phenols and terpenoids are the main factors influenced the anti-inflammatory activity.

3.4. FTIR analysis of C. tunicatum

FTIR spectrum of whole plant of *C. tunicatum* from hexane, chloroform, ethyl acetate and methanol crude extracts were analysed to identify the functional groups. The information in Fig. 2 showed peak values along with their functional group of various extracts, respectively. The main peak observed in FTIR spectra of hexane extract at 2870.08 cm^{-1} (medium), 2924.09 cm^{-1} (strong), 2954.95 cm^{-1} (narrow sharp strong), 1728.22 cm^{-1} (weak), 1465.90 cm^{-1} (strong), 1381.03 cm^{-1} (medium), 1242.16 cm^{-1} (weak), 1172.72 cm^{-1} (weak), 1041.56 cm^{-1} (weak), 887.26 cm^{-1} (medium), 725.23 cm^{-1} (strong), 817.82 cm^{-1} (weak), 563.21 cm^{-1} (weak). Infrared spectrum of chemical constituents of chloroform exhibited peaks at 3325.28 cm^{-1} (broad strong), 2947.23 cm^{-1} (medium), 2831.50 cm^{-1} (sharp medium), 1666.50 cm^{-1} (very weak), 1404.18 cm^{-1} (weak), 1026.13 cm^{-1} (strong) and 686.66 cm^{-1} (medium). The peak of ethyl acetate represented at 3633.89 cm^{-1} (weak), 2985.81 cm^{-1} (sharp medium), 2900.94 cm^{-1} (very weak), 1735.93 cm^{-1} (sharp narrow strong), 1442.75 cm^{-1} (medium), 1373.32 cm^{-1} (sharp medium), 1234.44 cm^{-1} (sharp strong), 1095.57 cm^{-1} (medium), 1041.56 cm^{-1} (sharp narrow strong), 786.96 cm^{-1} (broad medium), 848.68 cm^{-1} (sharp medium), 632.65 cm^{-1} (medium), 609.51 cm^{-1} (medium). The FTIR spectra of methanol extract showed peaks at 3317.56 cm^{-1} (broad medium), 2831.50 cm^{-1} (sharp medium), 2939.52 cm^{-1} (medium), 2522.89 cm^{-1} (weak), 2229.71 cm^{-1} (very weak), 2044.54 cm^{-1} (weak), 1666.50 cm^{-1} (weak), 1450.47 cm^{-1} (medium), 1111.00 cm^{-1} (medium), 1018.41 cm^{-1} (narrow strong), 671.23 cm^{-1} (broad medium), 601.79 cm^{-1} (medium), 524.64 cm^{-1} (medium).

The spectra of *C. tunicatum* with the frequency range from 2831.50 to 2985.81 represented the presence of alkane group (C–H stretch). The peak at 3325.28 and 3633.89 corresponds to the presence of alcohol (O–H stretch) (Vahur et al., 2016). The peak at 2522.89 was observed as carboxylic acid (O–H stretch). The peak at 2229.71 showed the presence of nitriles (C≡N stretch). The peak at 2044.54 signified the presence of isothiocyanate (N = C = S stretch). The peak at 1666.50 and 1728.22 to 1735.93 unveiled the presence of aromatic compounds (C–H bending). The peak at 1450.47 and 1465.90 displayed alkane (O–H bending) (Reignier et al., 2021). The peak at 1404.18 and 1442.75 showed carboxylic acid (O–H bending). The peak at 1381.03 and 1373.32 exhibited alcohol (O–H bending). The peak ranges from 1172.72 to 1242.16 represented by amine (C–H stretch). The peak ranges from 1018.41 to 1041.56 showed a sulfoxide group (S = O stretch). The medium peak at 887.26 exhibited alkene (C = C bending). The peak ranges from 563.21 to 848.68 showed a halo compound (C-Cl stretch). The medium peak at 524.64 contained a halo compound (C-I stretch) (Papakosta et al., 2020). The FTIR spectrum conforms to the presence of alkaloids, flavonoids, phenols, terpenoids, carboxylic acid, alkane, aromatic alkenes in *C. tunicatum*.

3.5. GC–MS analysis

The GC–MS analyses of the methanolic extract of the whole plant of C. *tunicatum* provided the separation of 22 components, from 11 different groups were well identified using their mass spectra and retention index data. The major compounds are 1-Hexacosene (0.145 %), 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2 (0.180 %), 1,3-Dioxolane, 4-methyl-2-pentadecyl (0.382 %), 7-Dehydrodiosgenin (0.155 %), Methyl Palmitoleate (0.119 %), Palmitic acid (0.988 %), Methyl (Z)-5,11,14,17-eicosatetraenoate (0.13 %), Octadecanoic acid (0.751 %), Myristic acid (0.141 %), Methyl linoleate (0.147 %), l-(+)-Ascorbic acid 2,6-dihexadecanoate (8.129 %), Campesterol (5.243 %), Phenol, 2,6-dimethoxy-4-(2-propenyl)- (0.108 %), 4.alpha.,14-Dimethyl-5. alpha-

Table 5
Evaluation of Anti-inflammatory activity of *Cynanchum tunicatum* by protein denaturation assay.

Concentration (µg/mL)	Percent inhibition of anti-inflammatory (%)				
	Hexane	Chloroform	Ethyl acetate	Methanol	Diclofenac sodium
20	24.8 ± 0.002[e]	2.51 ± 0.0005[e]	13.22 ± 0.0005[e]	49.2 ± 0.002[e]	39.3 ± 0.003[e]
40	118.009 ± 0.002[d]	29.5 ± 0.003[d]	28.1 ± 0.003[d]	71.01 ± 0.002[d]	122.09 ± 0.01[d]
60	194.6 ± 0.001[c]	62.8 ± 0.002[c]	50.7 ± 0.002[c]	102.8 ± 0.001[c]	221.7 ± 0.007[c]
80	268.08 ± 0.003[b]	97.4 ± 0.001[b]	71.4 ± 0.001[b]	147.8 ± 0.004[b]	301.1 ± 0.004[b]
100	310.7 ± 0.002 [a]	130.8 ± 0.002 [a]	85.7 ± 0.002[a]	171.01 ± 0.001[a]	374.5 ± 0.009[a]
IC_{50}	59.23	68.44	57.03	42.31	60.14

All values were expressed in mean ± standard deviations of triplicate measures. Each analysis was statistically significant ($p \leq 0.05$), 'a' expressed best results and 'e' showed poor results.

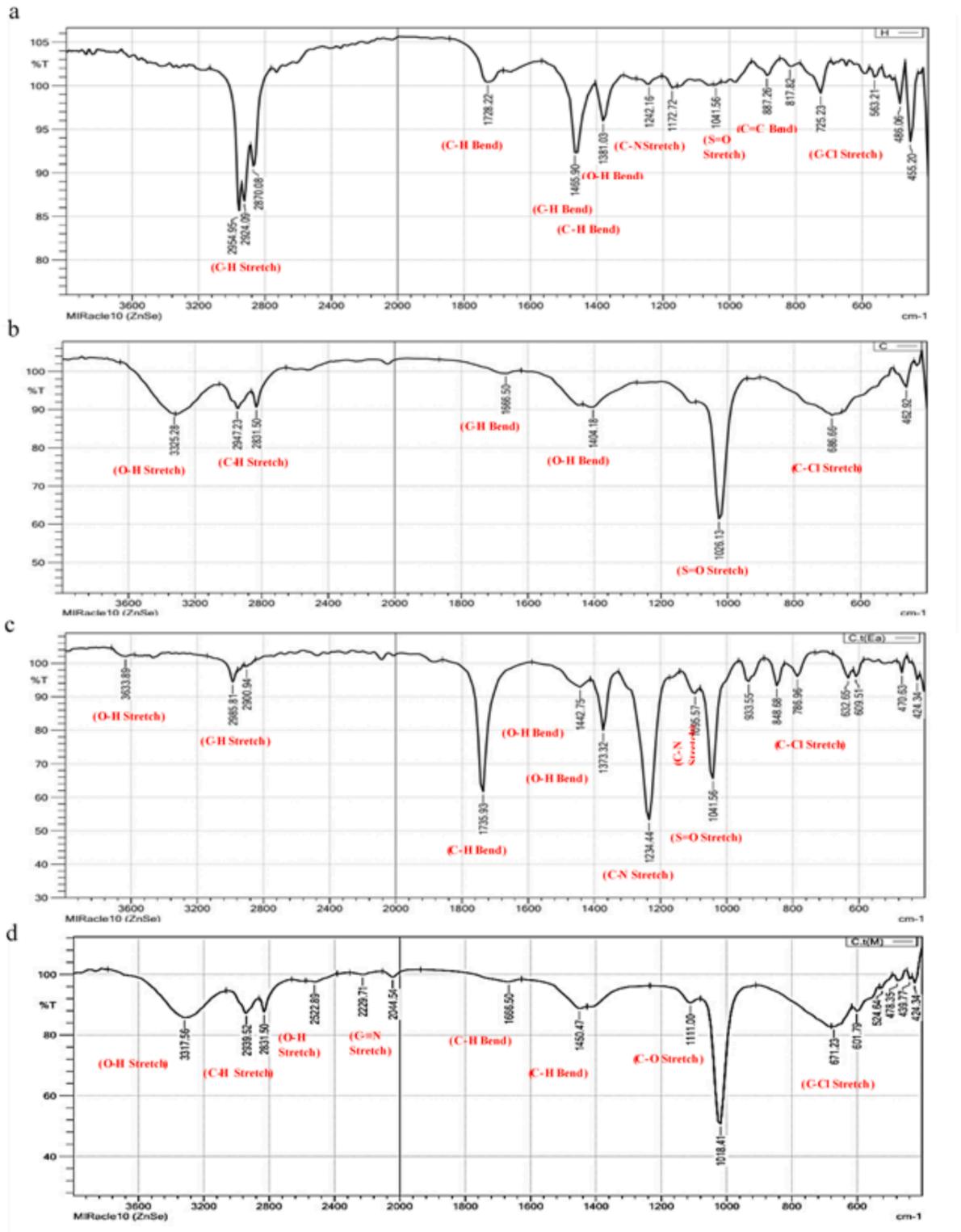

Fig. 2. Infrared spectra with Fourier transform in the fingerprint region of **a)** Hexane extract, b) Chloroform extract, c) Ethyl acetate extract and d) Methanol extract of *Cynanchum tunicatum.*

ergosta-8 (0.430 %), Pregn-5-en-20-one, 12-(acetyloxy)-3,8,14 (0.136 %), Cholesta-22,24-dien-5-ol, 4,4-dimethyl- (3.482 %), Lupeol (13.061 %), Megastigmatrienone (0.116 %), Lupenone (0.306 %), Beta −Amyrin (10.614 %), 17-(1,5-Dimethyl-3-phenylthiohex-4-enyl) (0.593 %) and Phytol (1.455 %). In Table 6 represents the compound name, molecular formula, retention time, peak area, functional group and reported medicinal applications for methanol extract of *C. tunicatum*. The structure of these metabolites was represented in Fig. 3. GC–MS analysis of *C. acutum* latex observed the chemical constituents such as lupeol, hexadecanoic acid, neophytadiene, octadecanoic acid and phytol were found with percentages of 15.36 %, 10.72 %, 9.15 %, 8.78 %, and 6.51 %, respectively (Soliman et al., 2022).

The GC–MS analysis of *Aristolochia tagala* leaf extracts revealed the presence of 42 compounds across various solvents such as petroleum

Table 6
Distribution of the identified metabolites through GC–MS in methanolic extracts of *Cynanchum tunicatum*.

Group	Compound name	Molecular formula	Retention time (min)	Peak Area (%)	Medical applications
Alkene	1-Hexacosene	$C_{26}H_{52}$	34.306	0.145	Antibacterial (Rani et al., 2019)
Cholestanoid	17-(1,5- Dimethylhexyl)-10,13-dimethyl-2	$C_{27}H_{46}O$	38.780	0.18	Antioxidant activity (Nyalo et al., 2023)
Dioxolanes	1,3-Dioxolane, 4-methyl-2-pentadecyl	$C_{21}H_{42}O_2$	3.104	0.382	Antimicrobial activity (Küçük et al., 2011)
Diosgenin	7-Dehydrodiosgenin	$C_{27}H_{40}O_3$	37.654	0.155	Antioxidant, Anti-inflammatory, Anticancer (Semwal et al., 2022)
Fatty acid	Methyl Palmitoleate	$C_{17}H_{32}O_2$	14.929	0.119	–
	Palmitic acid	$C_{16}H_{32}O_2$	25.998	0.988	Anticancer, Cardiovascular disease (Mancini et al., 2015)
	Methyl (Z)- 5,11,14,17- eicosatetraenoate	$C_{21}H_{34}O_2$	17.383	0.130	–
	Octadecanoic acid	$C_{18}H_{36}O_2$	18.714	0.751	Hypocholesterolemic activity (Selvaraju et al., 2021)
	Myristic acid	$C_{14}H_{28}O_2$	12.490	0.141	Larvicidal and Repellent activity (Chen et al., 2019)
	Methyl linoleate	$C_{19}H_{34}O_2$	17.266	0.147	Antioxidant activity (Davey et al., 2000)
L-Ascorbic acid	1-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	15.336	8.129	Antioxidant activity (Babouongolo et al., 2021)
Phytosterol	17-(1,5-Dimethyl-3-phenylthiohex-4-enyl) −4,4,10,13,14-pentamethyl 2,3,4,5,6,7,10,11,12-	$C_{36}H_{54}OS$	47.067	0.593	Anticancer (Suttiarporn et al., 2015)
	Campesterol	$C_{28}H_{48}O$	41.252	5.243	Anti-inflammatory and Cytotoxic activity (Bagewadi et al., 2019)
Phenol	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	$C_{11}H_{14}O_3$	11.909	0.108	Antioxidant activity (Molan et al., 2012)
Steroid	14-Dimethyl-5 alpha-ergosta-8	$C_{30}H_{50}O$	42.870	0.430	Anti-inflammatory, Anticancer (Rasheed & Qasim, 2013)
	Pregn-5-en-20-one,12-(acetyloxy)-3,8,14	$C_{23}H_{34}O_6$	38.302	0.136	–
Terpenoid	Cholesta-22,24-dien-5-ol,4,4-dimethyl-	$C_{29}H_{48}O$	41.877	3.482	–
	Lupeol	$C_{30}H_{50}O$	46.326	13.061	Anti-inflammatory (Tsai et al., 2016)
	Megastigmatrienone	$C_{13}H_{18}O$	11.065	0.116	Antioxidant activity (Kyslychenko et al., 2010)
	Lupenone	$C_{30}H_{48}O$	45.271	0.306	Anti-inflammatory (Xu et al., 2020)
	Beta-Amyrin	$C_{30}H_{50}O$	44.767	10.614	Antibacterial activity, Anti-inflammatory activity (Dash et al., 2023)
	Phytol	$C_{20}H_{40}O$	17.572	1.455	Anti-inflammatory, Antimicrobial activity (Bagewadi et al., 2019)

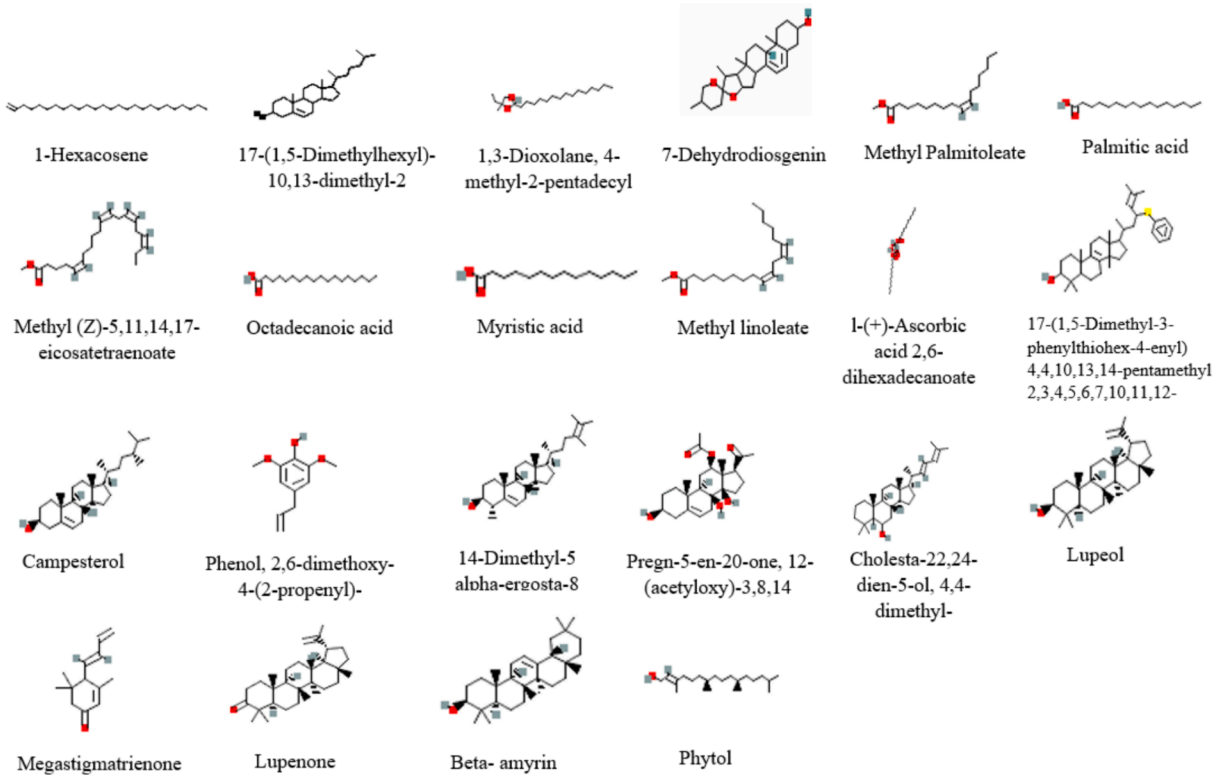

Fig. 3. Structure of the bioactive compounds obtained in the methanolic extract of *Cynanchum tunicatum* through GC–MS analysis.

ether, chloroform, ethyl acetate, methanol, and hydro-alcoholic (Mariyammal et al., 2023). Olivia *et al.*, utilized GC–MS analysis to identify twenty-three bioactive compounds in the hydromethanolic fraction of *Hibiscus asper* leaves such as 9,12,15-octadecatrien-1-ol, n-Hexadecanoic acid, octadecatrienol acid, methyl palmitate and phytol were significant phytoconstituents (Olivia et al., 2021). Lupeol and phytosterols possess various properties like antifungal, anti-inflammatory, antibacterial, anti-tumor, antioxidant and anti-ulcerative effects which plays their multifunctional biological roles (Ito et al., 2017). The GC–MS analyses showed preliminary insights into the bioactive components and evidenced the pharmaceutical applications for further research and drug discovery.

3.6. HPTLC profiling

In *C. tunicatum* the secondary metabolites were separated on HPTLC plates using various mobile phases such as Ethyl acetate: Methanol: Water (20:3:2), Ethyl acetate: Methanol: Formic acid: Water (20:3:1:2), Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2) and Toluene: Acetone: formic acid (4.5:4.5:1) for alkaloid, flavonoid, phenols and terpenoids respectively.

HPTLC Fingerprinting of alkaloid evidenced eight spots with their corresponding ascending order of R_f value of 0.032, 0.224, 0.302, 0.474, 0.597, 0.669,0.776, 0.976 in 2.5 µL of methanolic extract. Spot 1, 2, 3, and 5 were identified as strychnine (Anti-inflammatory, anticancer, hepatoprotective, antioxidant, cardio protective, antidepressant, antidote for snakebite), colchicine (decreases inflammatory cytokines) (Senguttuvan & Subramaniam, 2016). While in flavonoid showed eight bands in 2.5 µL of methanolic extract with corresponding R_f values were 0.048, 0.082, 0.310, 0.456, 0.684, 0.813, 0.890 and 0.966. Whereas in 5 µL of extracts, 6 bands were noted, and its corresponding R_f values were 0.076, 0.460, 0.703, 0.834, 0.900, 0.971.

Phenol showed three bands with respective R_f values were 0.031, 0.258, 0.813 in 2.5 µL of methanol extracts and eight bands formed with corresponding R_f values were 0.032, 0.090, 0.227, 0.448, 0.489, 0.785, 0.850, 0.961 in 5 µL of extract. In terpenoid, nine bands have appeared with the corresponding R_f values were 0.045, 0.113, 0.173, 0.192, 0.285, 0.398, 0.463, 0.553 and 0.629 in 2.5 µL of methanol extracts while in 5 µL of extract, ten bands with R_f values were 0.050, 0.118, 0.176, 0.285, 0.344, 0.402, 0.469, 0.561, 0.635 and 0.745 were obtained. The alkaloids, flavonoids, phenols and terpenoids were identified and quantified by comparing them with standards like colchicine, quercetin, gallic acid and oleanolic acid, respectively (Fig. 4). These bioactive components were reported strong antioxidant, antimicrobial, anti-inflammatory, anticancer, cardioprotective, anti-cardiovascular disease, antihemostatic, and chemopreventive activities (Deepika & Maurya, 2022). The metabolic screening showed that the primary constituents of the *C. tunicatum* extract comprised alkaloids, flavonoids, phenolic and terpenoids compounds.

4. Conclusion

In conclusion, the lacuna of requisite research in phytoconstituents of *Cynanchum tunicatum* influenced the spectrum of secondary metabolites, such as alkaloids, flavonoids, terpenoids and phenolic compounds were identified. In GCMS analysis methanolic extract of *C. tunicatum* showed 22 compounds were used for various therapeutic applications such as hypocholesterolemia, cancer, cardiovascular diseases, antiulcerative, antimicrobial, larvicidal and repellent activity. The phytochemicals identified using HPTLC fingerprinting were greatly beneficial for anti-inflammatory, anticancer, hepatoprotective, antioxidant, cardio protective, antidepressant, antidote for snakebite, and decreases inflammatory cytokines. Methanol extract of *C. tunicatum* evidenced potential in antioxidant and anti-inflammatory activities than the other

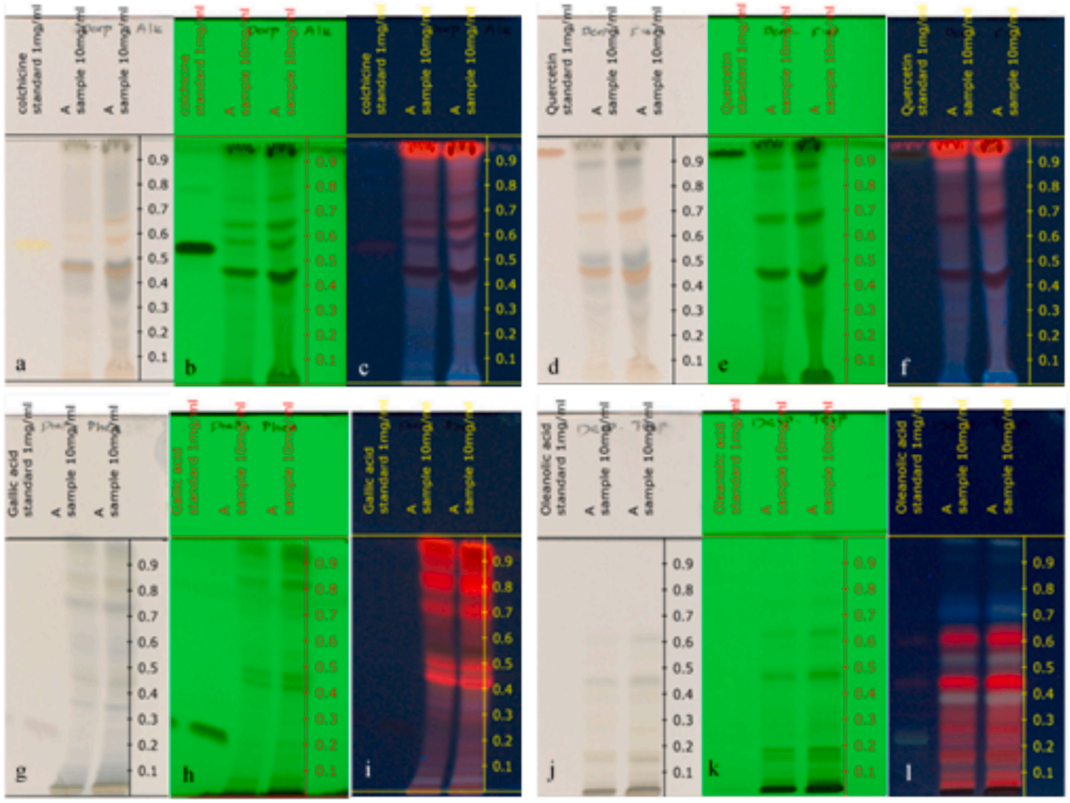

Fig. 4. TLC Profiles of *Cynanchum tunicatum.* a) Alkaloid at visible light b) Alkaloid at 366 nm c) Alkaloid at 254 nm d) Flavonoid at visible light e) Flavonoid at 366 nm f) Flavonoid at 254 nm g) Phenol at visible light h) Phenol at 366 nm i) Phenol at 254 nm j) Terpenoid at visible light k) Terpenoid at 366 nm l) Terpenoid at 254 nm.

extracts. Overall, the findings of this study of *C. tunicatum* as the major source of bioactive compounds with promising pharmacological activities suggested for further drug discovery from these identified compounds.

CRediT authorship contribution statement

Deepika Krishnamoorthy: Writing – original draft, Methodology, Investigation. **Amutha Swaminathan:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Amal Mohamed AlGarawi:** Writing – review & editing, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lavanya Nallasamy:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Girija Sangari Murugavelu:** Writing – review & editing, Formal analysis. **Swarna Lakshmi Selvaraj:** Writing – review & editing, Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

I would like to thank the Department of Botany and CNR Rao Laboratory and Advanced Research Laboratory, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu, India. The authors extend their appreciation to the Researchers supporting project number (RSPD2024R931) King Saud University, Riyadh, Saudi Arabia.

Funding

This work was supported by the Researchers supporting project number (RSPD2024R931) King Saud University, Riyadh, Saudi Arabia.

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