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Title: **Antioxidant and Anticancer Activity of Tannins Isolated from Callus Cultures of Achyranthes aspera L.**

Author (s): Madieha Ambreen[1], Safdar Ali Mirza[2], Zahida Bano[3], Wasiq Ikram[1,4], Muhammad Nazim[5], Syed Muhammad Waqas Gillani[6], Muhammad Talha Hassan[7], and Sajida Begum[2]

Affiliation (s):
[1]Minhaj University, Lahore, Pakistan
[2]Government College University, Lahore, Pakistan
[3]Heinrich Heine University, Düsseldorf, Germany
[4]Mohammed VI Polytechnic University, Ben Guerir, Morocco
[5]Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, China
[6]Pakistan Tobacco Board, Ministry of National Food Security and Research, Pakistan
[7]Superior University, Lahore, Pakistan

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Antioxidant and Anticancer Activity of Tannins Isolated from Callus Cultures of *Achyranthes aspera* L.

Madieha Ambreen[1*], Safdar Ali Mirza[2], Zahida Bano[3], Wasiq Ikram[1,4], Muhammad Nazim[5], Syed Muhammad Waqas Gillani[6], Muhammad Talha Hassan[7], and Sajida Begum[2]

[1]School of Botany, Minhaj University, Lahore, Pakistan
[2]Botany Department, Government College University, Lahore, Pakistan
[3]Heinrich Heine University, Düsseldorf, Germany
[4]Chair of Global Critical Zone Science, Mohammed VI Polytechnic University, Ben Guerir, Morocco

[5]Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, China
[6]Pakistan Tobacco Board, Ministry of National Food Security and Research, Pakistan
[7]Superior University, Lahore

ABSTRACT

In the current study, the impact of oxidative stress induced by free radicals generated during metabolic processes was investigated. It was found to be linked to a variety of diseases due to their detrimental effects on nucleic acids and proteins. The primary focus remained on investigating the free radical-scavenging properties and potential anticancer activities of tannins found in callus cultures of *A. aspera*. It was observed that the induction of callus formation was notably successful when using leaf and root explants, resulting in a callus index as high as 160, as compared to stem explants in the presence of auxins. The extraction yield of callus tannins was the most abundant in chloroform extracts, although the overall antioxidant activity was comparable among chloroform, methanol, and petroleum ether callus extracts. Among these extracts, the chloroform extract from stem callus cultures grown on the MSDN medium showed the highest total antioxidant activity. Notably, tannins extracted from *A. aspera* leaf callus extracts displayed significant anticancer effects against the Jurket cell line. These effects were evaluated through measures such as cell viability and colony formation. The anticancer activity was notably higher in callus culture extracts as compared to the control. In summary, the results showed that in vitro biomass production can be a valuable approach for

*Corresponding Author: madiha.ambreen@yahoo.com

augmenting the production of bioactive compounds, such as tannins, from selected medicinal plants including *A. aspera*. Tannins extracted from *A. aspera* hold promise for their potential role in the development of new medicines.

Keywords: anticancer activity, antioxidant activity, biomass production, callus culture, cell viability, jurket cell line, medicinal plant, oxidative stress, scavenging activity, tannins content

1. INTRODUCTION

In recent years, the exploration of natural compounds with potential therapeutic benefits has gained considerable momentum, driven by the pursuit of novel and effective treatments with fewer side effects [1, 2]. Plant possess numerous bioactive constituents and have been considered for new drug molecules. Indeed, numerous studies on medicinal herbs have confirmed their antioxidant and anticancer potential [3, 4]. Among the diverse array of bioactive compounds, tannins have emerged as compounds of significant interest, demonstrating notable antioxidant and anticancer properties [5]. Hence, this research is aimed to determine the anticancer and antioxidant activity of tannins isolated from the extracts of *Achyranthes aspera.*

Achyranthes aspera is a medicinal plant that has been extensively utilized in traditional medicine systems because of its diverse pharmacological properties [6]. In particular, callus cultures derived from different parts of *A. aspera* remain promising for the sustainable production of bioactive compounds. Callus cultures serve as reservoirs of secondary metabolites, offering a sterile and controlled environment for the isolation of pharmacologically active components [7]. The focus on tannins in this study is motivated by their well-documented antioxidant effects. Tannins are polyphenolic compounds known for their ability to neutralize free radicals, such as RNS and ROS. These free radicals, originating from both endogenous metabolic processes and exogenous sources, play a pivotal role in modifying various biomolecules such as proteins, lipids, and nucleic acids, thereby disrupting redox reactions and contributing to heightened oxidative stress. The latter has been implicated in a wide spectrum of health disorders including neurodegenerative conditions, respiratory ailments, diabetes, and in several forms of cancer (lung, colon, breast, bladder, and prostate cancers) [2, 7]. Moreover, the

anticancer potential of tannins, particularly in selectively inducing apoptosis in cancer cells while sparing normal cells, has garnered significant attention [8]. The intricate mechanisms behind this selective action, such as acetylation and methylation processes initiated through direct binding with proteins, make tannins promising candidates for cancer therapeutics [9]. Therefore, this study specifically focuses on isolating tannins from callus cultures of *A. aspera*, aiming to explore and harness their antioxidant and anticancer properties. The findings not only contribute to expand the understanding of the medicinal potential of *A. aspera* but also provide insights into sustainable approaches to obtain bioactive compounds with therapeutic relevance.

2. MATERIALS AND METHODS

2.1. Procurement and Identification of Seeds

Seeds of *A. aspera* (Voucher No. GC. Herb. Bot. 3492) were procured from the local market, identified by a taxonomist of GC University, Lahore, and assigned voucher specimens after comparing them with herbarium specimens in Sultan Ahmed Herbarium, Botany Department, GCU, Lahore. The research was performed at the Plant Biotechnology Laboratory of GCU, Lahore.

2.2. Seedling Development

For *in vitro* seed germination, seeds were surface sterilized with 5% sodium hypochlorite solution under aseptic conditions and shifted to growth room on petri plate on wet cotton pads at 27ºC for the development of seedlings [10].

2.3. Inoculation

The leaf, stem, and root explants were placed in glass jars with sterilized MS media and sealed with cling film [11]. The whole experiment was performed under aseptic conditions and culture jars were shifted to culture chamber for callus induction at 26±°C temperature with 16 hours of dark and light periods at light intensity of 2000-3000 lux m^{-2} s^{-1}.

2.4. Callus Biomass Production

Callus biomass production was achieved under various combinations of plant growth regulators (PGRs) and shifted to petri plates to determine

their fresh weight. These callus masses were kept in an oven at 40°C for one week by wrapping them in Whatman filter paper to measure their dry weight. The following formula was used to compute callus biomass:

Callus biomass = W1-W2/W1,

2.5. Maceration of Callus

The fine powder of callus was dissolved in petroleum ether, methanol, chloroform, and aqueous solvents in small, pre-weighed, glass jars. The jars were shaken twice or three times a day. The mixture in glass jars was filtered after a week and the percentage yield was calculated by weighing the filtrate which was stored for further anticancer and antioxidant research.

2.6. Antioxidant Assay

The phosphomolybdate antioxidant method was carried out according to [12].

2.6.1. Preparation of Solutions. To prepare a solution of 18M sulfuric acid, 3.33 ml of acid was carefully measured and added to a 100 ml volumetric flask. Subsequently, distilled water was added to bring the total volume to 100 ml. For the sodium phosphate solution, 0.487 g of sodium phosphate was dissolved in water within a flask and the volume was adjusted to the mark. Similarly, for the ammonium molybdate solution, 0.48 g of ammonium molybdate was dissolved in water within a volumetric flask and the volume was increased to 100 ml.

2.6.2. Preparation of Plant Extracts. To prepare plant extracts, 0.08g of tannins was isolated from both seedling tissues and callus culture extracts. Subsequently, each extract was dissolved in a mixture of polar and non-polar solvents and the final volume was adjusted to 8 ml to create a working solution with a concentration measured in mg/ml.

2.6.3. Preparation of Phosphomolybdate Reagent. To prepare the phosphomolybdate reagent, a mixture was created by combining 100 ml of 4 mm ammonium molybdate, 100 ml of 20 mm sodium phosphate, and 100 ml of 0.6 mm sulphuric acid. The resultant phosphomolybdate reagent was then utilized in further analyses.

In a vial, 300 microliters of each extract including seedling tissues, callus culture extracts, and a standard solution were mixed with 3 ml of

phosphomolybdate reagent. Following an incubation period of 90 minutes at 95°C, absorbance was measured at 765 nm against a blank using the standard solution of ascorbic acid. The antioxidant activity of *A. aspera* was quantified in terms of g/ml of AAE (ascorbic acid equivalents) based on the ascorbic acid standard curve. The outcomes were represented as TAA (total antioxidant activity) in g/ml.

2.7. Anticancer Activity

2.7.1. Cell Viability Assay. Cells were plated in a 96-well plate. After 24 hrs, the cells were treated with plant extracts at concentrations of 10 and 20 µg/ml. Subsequently, MTT assay was employed involving the addition of MTT solution at a concentration of 5 mg/ml to the wells. The yellow-colored MTT salt underwent a conversion to an insoluble, purple-colored solution. Cell viability was determined using a plate reader at 490 nm, as per the methodology of Orellana and Kasinski [13].

2.7.2. Morphology. Cells were cultured in a 6-well plate and subjected to treatment with plant extracts at concentrations of 10 and 20 µg/ml, along with a control group. The assessment of cellular morphology was conducted using bright-field microscopy, following fixation and staining with 0.5% crystal violet.

2.7.3. Colony Formation Assay. For the colony formation assay, the impact of plant extracts on cell survival was examined through the observation of colony formation. After 24 hrs, cells were seeded in a 9-well plate and allowed to grow for 15 days until visible colonies were formed. The colonies were then fixed and stained using 0.5% crystal violet. Subsequently, images were captured and the colony density was quantified.

2.8. Statistical Analysis

The results were presented as mean ± SEM for a sample size of n=3. Statistical analysis included the application of Duncan's test at a significance level of $p < 0.05$. Further, one-way ANOVA was employed to assess the means.

3. RESULTS

3.1. Callus Induction

Seedling was grown from *A. aspera* seed in an aseptic condition, followed by callus induction from leaf, stem, and root on MS medium with different concentrations of plant growth regulators (PGRs). Callus induction was achieved by placing leaf explants on MS media supplemented with PGRs, that is, MSNB medium (2.0mg/l+2.0 mg/l NAA and BAP), resulting in the development of large, light green, and granular callus cultures. The stem explant in MSDI medium (2.0 mg/l+4.0 mg/l 2,4-D and IBA) also resulted in green and granular callus cultures. Utilizing root explants on MSNB medium (2.0 mg/l+2.0mg/l NAA and BAP) yielded small-sized, green, and compact calluses (Figure 2). The production of callus biomass from different explants on MS medium with various combinations of PGRs is detailed below in Table 1.

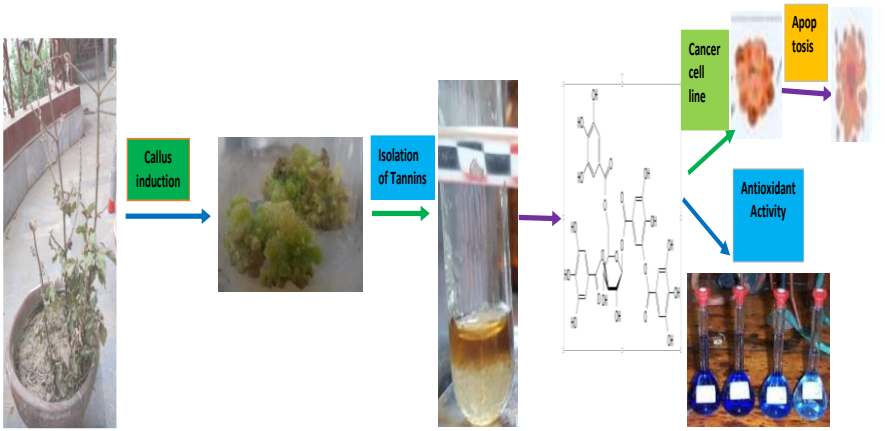


Figure 1. Schematic Representation of Callus Formation, Isolation of Tannins from *A. aspera*, and Assessment of Antioxidant Activity

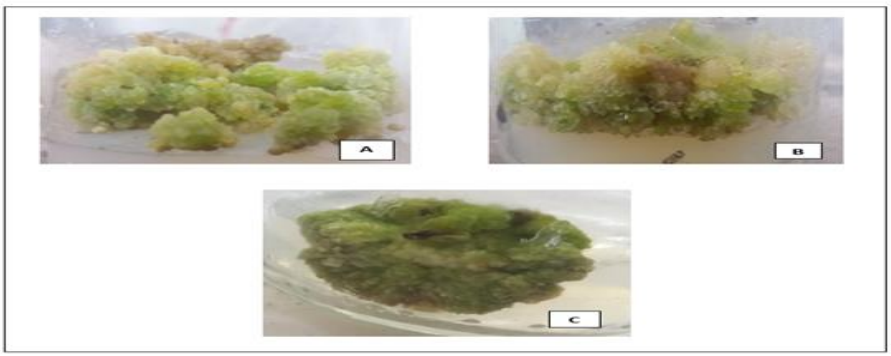


Figure 2. Callus Biomass Production from Various Tissues of *A. aspera* as Explant. (A) Leaf Explant Employed on MS Medium with NAA + BAP

(2.0 mg/l+2.0mg/l) (MSNB). (B) Stem Explant Grown on MS Medium with 2,4-D+IBA (4.0mg/l+2.0mg/l) (MSDI). (C) Root Explant Cultivated on MS Medium with NAA + BAP (2.0 mg/l+2.0mg/l) (MSNB).

Table 1. Callus Biomass Production on MS Medium with Various Combinations of PGRs from Different Explants

MS medium + PGRs (mg/L)	Abbreviations	Explant	Callus induction (%)	Callus induction duration (Days)	Callus index	Callus weight (g)	Callus morphology
2,4-D+BAP 1.5+0.5	MSDB	Leaf	40	19±0.65	70	0.17±0.00	Light green and granular
NAA+BAP 2.0+2.0	MSNB	Leaf	20	16±0.36	112.5	0.67±0.007	
NAA+ 2,4-D 2.0+4.0	MSDN	Leaf	40	13±0.34	160	1.21±0.02	
NAA+IAA 6.0+4.0	MSDIA	Stem	60	25±0.40	70	0.34±0.00	
2,4-D+NAA 6.0+8.0	MSDN	Stem	60	17±0.18	70	0.32±0.00	Greenish brown and granular
2,4-D+IBA 4.0+2.0	MSDIB	Stem	20	15±0.27	100	0.55±0.00	Green and granular
2,4-D+NAA 4.0+6.0	MSDN	Root	20	19±0.49	160	0.73±0.00	Light green and compact
NAA+BAP 2.0+2.0	MSNB	Root	20	16±0.60	70	0.16±0.01	Brown and granular
2,4-D+IBA 4.0+2.0	MSDIB	Root	20	17±0.58	60	0.35±0.00	Brown and granular

3.2. Estimation of Tannins

For quantitative analysis, tannins were extracted from the callus culture of leaf, stem, and root using distilled water, methanol, chloroform, and petroleum ether. Figure 1 illustrates the processes of callus induction, the isolation of tannins from *A. aspera*, and the assessment of antioxidant activity. The composition of culture medium is given in Table 2.

Table 2. Composition and Abbreviation of PGRs

Culture type	Hormonal Combination	Abbreviation
	Control	C
Leaf	2,4-D 0.5 mg/l + 1.5 mg/l BAP	MSDB
	2,4-D, 2.0 mg/l+4.0 mg/l NAA	MSDN
	NAA, 2.0 mg/l+ 2.0 mg/l BAP	MSNB

Culture type	Hormonal Combination	Abbreviation
	Control	C
	2,4-D 4.0 mg/l+6.0 mg/l IAA	MSDIA
Stem	2,4-D, 6.0 mg/l + 8.0 mg/l NAA	MSDN
	2,4D, 2.0 mg/l+ 4.0 mg/l IBA	MSDIB
	2,4-D, 4.0 mg/l +6.0 mg/l NAA	MSDN
Root	2,4 D, 2.0 mg/l+ 2,0 mg/l BAP	MSNB
	2,4-D, 2.0 mg/l+ 4.0 mg/l IBA	MSDIB

3.2.1.Leaf. In the petroleum ether extract of *A. aspera*, leaf seedling tissue exhibited the highest tannin content across all cultures and solvents, measuring 12.72±0.08 mg/g. Following closely, tannins in the chloroform extract of leaf callus culture induced by MSDN were recorded at 12.41±0.10 mg/g. In contrast, the callus induction medium of the leaf containing MSDB in aqueous extract displayed the lowest tannin content, measuring 7.4±0.03 mg/g (Figure 3).

3.2.2. Stem. Among all solvents, the methanol extract of stem callus culture from medium MSDI exhibited the highest tannin content. This was followed by the chloroform extract of tannin content from stem seedling tissue in all solvents, measuring 10.78±0.06 mg/g. Conversely, aqueous extract on the MSDI medium displayed minimum tannin content (Figure 4).

3.2.3. Root. The highest tannin content was observed in the petroleum ether extract of root callus culture containing MSNB medium, measuring 10.22±0.02 mg/g. Subsequently, tannins in the methanol extract from the seedling root tissue (control) were recorded at 9.96±0.03 mg/g. Conversely, the root callus biomass production of MSNB medium showed the minimum amount of tannins, measuring 3.00±0.02 mg/g (Figure 5). More details about the percentage yield of callus cultures and plant tissues are given in Table S1.

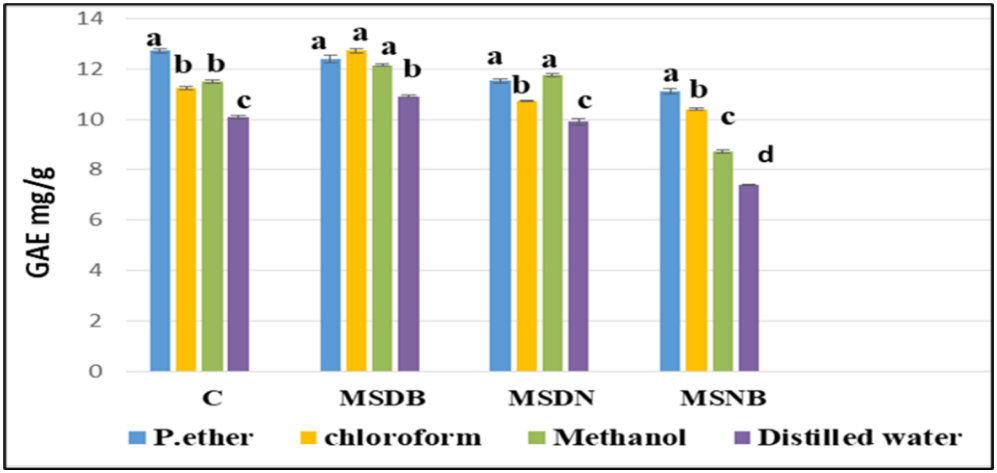


Figure 3. Measurement of Tannin Concentration from Callus Culture and Leaf Tissue Extracts of *A. aspera* Expressed as GAE mg/g. *A. aspera* Leaf Callus Explants Grown on Various Concentrations of PGRs Containing MSDB, MSDN, MSNB, and C (Control) Represent Leaf Tissue. The Application of Duncan's Novel Multiple Range Test with a Significance Level of $p < 0.05$ Resulted in Distinct Letters (a-d) Indicating Significance.

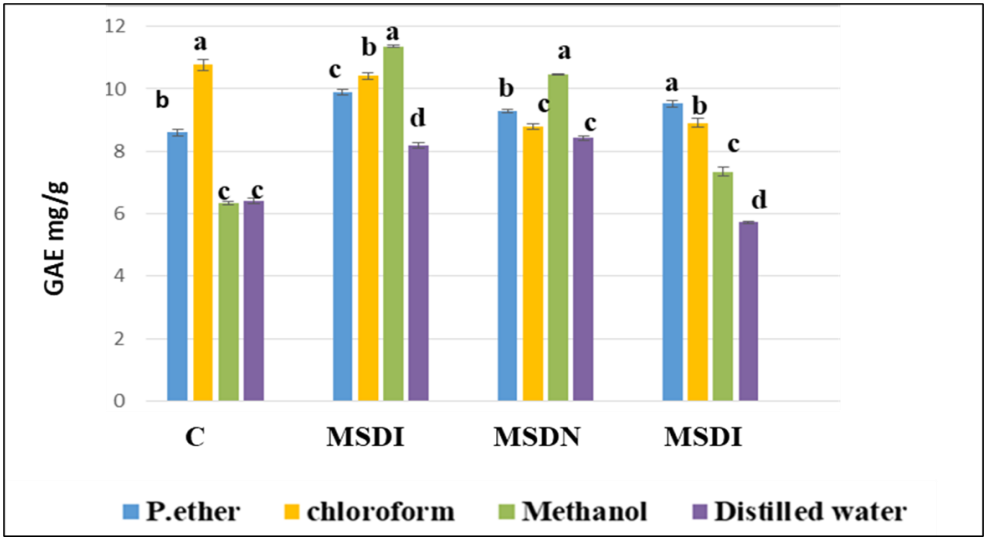


Figure 4. Tannin Concentration in *A. aspera* Stem Tissue and Callus Culture Expressed as GAE mg/g. *A. aspera* Leaf Callus Explants Grown on Various Concentrations of PGRs Containing MSDI, MSDN, and

MSDI, with Stem Tissue Used as Control (C). The Application of Duncan's Novel Multiple Range Test with A Significance Level of $p < 0.05$ Resulted in Distinct Letters (a-d) Indicating Significance

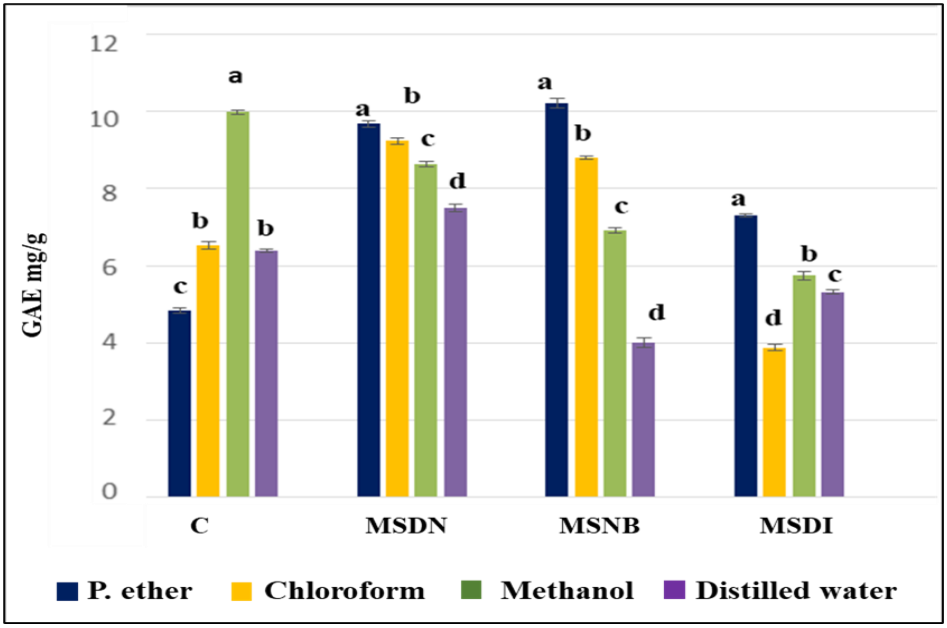


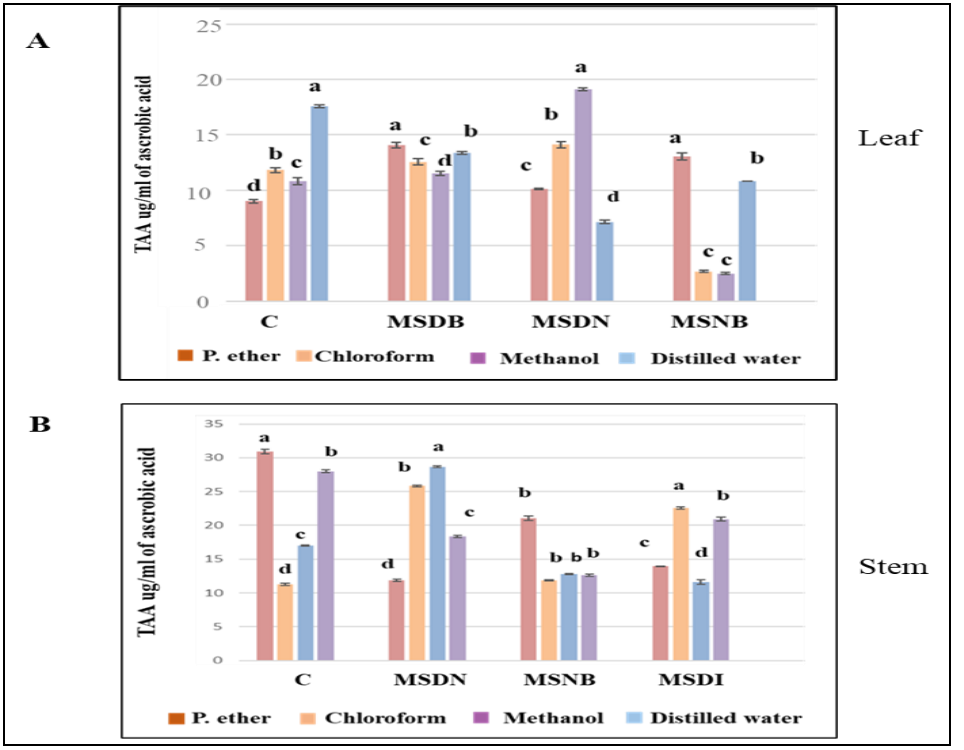
Figure 5. Measurement of Tannin Concentration from the Root Tissue and Callus Culture of *A. aspera***.** Tannin Concentration in *A. aspera* Root Tissue and Callus Culture Extracts is Expressed as GAE mg/g. *A. aspera* Root Callus Culture on MSDN, MSNB, and MSDI is Compared to Root Tissue C (Control). The Application of Duncan's Novel Multiple Range Test with a Significance Level of $p < 0.05$ Resulted in Distinct Letters (a-d) Indicating Significance

3.3. Assessment of Tannins' Antioxidant Activity

The assessment of tannins' antioxidant efficacy encompassed various plant parts of *A. aspera* including its leaf, stem, and root seedling tissues and their callus culture extracts. Among these, the callus culture extract from leaves demonstrated the highest antioxidant potential, surpassing seedling leaf tissue extracts. Notably, the methanolic extract from callus induction in MSDN exhibited the overall peak antioxidant activity at a concentration of 20 g/ml. In the case of seedling leaf tissue, the aqueous extract exhibited an antioxidant activity of 18 g/ml, whereas chloroform

and methanolic extracts from leaf callus culture in medium MSNB displayed minimum antioxidant potential at 4 g/ml (Figure 6A).

Moving to stem extracts, callus culture manifested superior antioxidant activity as compared to *A. aspera* seedling stem tissue. Specifically, stem callus culture with MSDN in chloroform extract displayed the highest antioxidant potential at 25 g/ml. Conversely, the petroleum ether extract in the same medium exhibited a lower antioxidant potential of 19 g/ml than the chloroform extract. Stem callus culture of MSDI in petroleum ether extract demonstrated minimum antioxidant activity at 7 g/ml (Figure 6B). In the context of root extracts, seedling root tissue extracts exhibited the highest total antioxidant activity, particularly the petroleum ether extract showing an antioxidant potential of 32 g/ml. On the other hand, the root callus culture of MSDI in chloroform extract displayed the lowest antioxidant potential at 11 g/ml (Figure 6C).



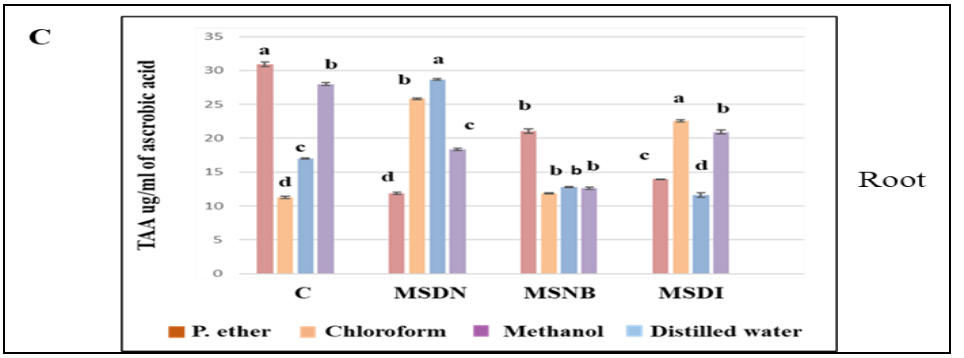


Figure 6. Antioxidant Capacity of Tannins Extracted from the Seedlings of Leaf, Stem, and Root Tissues and Callus Cultures of *A. aspera*. The Mean SEM Values were Determined from Three Replicates (n=3). Separate Alphabets (a-d) on the Bars Indicate Significant Difference at $p \leq 0.05$. The Treatments Included (A) Leaf Explants Cultured on MSDB (0.5 mg/l 2,4-D+1.5 mg/l BAP), MSDN (2.0 mg/l +4.0 mg/l), and MSNB (2.0 mg/l NAA+2.0 mg/l BAP) Medium, with Leaf Tissue Used as Control (C). (B) Stem Explant Cultured on MSDI (2,4-D 4.0 mg/l + 6.0 mg/l IAA), MSDN (6.0 mg/l 2,4-D + 8.0 mg/l NAA), and MSDI (2.0 mg/l 2,4-D + 4.0 mg/l IBA) Medium, with Stem Tissue used as Control (C). (C) Root Explant Cultured on MSDN (4.0 mg/l 2,4 D+6.0 mg/l NAA), MSNB (2.0 mg/l 2,4-D+ 2.0 mg/l BAP), and MSDI (2.0 mg/l 2,4-D+ 4.0 mg/l IBA) Medium, with Root Tissue Used as Control (C).

Table 3. Correlation Coefficient Between Tannin Content and Antioxidant Activity of the Leaf of Selected Plant.

Tannin content				Antioxidant activity			
Petroleum ether	Chloroform	Methanol	Distilled water	Petroleum ether	Chloroform	Methanol	Distilled water
1							
-0.30506	1						
0.422991	0.126125	1					
*0.712569	-0.36496	**0.852206	1				
0.056768	-0.47598	*0.651982	*0.713511	1			
-0.01517	-0.65127	0.436503	0.591929	**0.964765	1		
0.225311	-0.82467	-0.6064	-0.10302	-0.09898	0.133768	1	
0.415278	0.490759	0.162872	0.288577	-0.1719	-0.82359	0.366553	1

Tannins extracted from leaf callus cultures were evaluated for their potential anticancer properties against the HJC 106 (Human Jurket Cell Line). The results indicated a notable reduction in cancer cell proliferation with increasing concentrations of tannins (Figure 7).

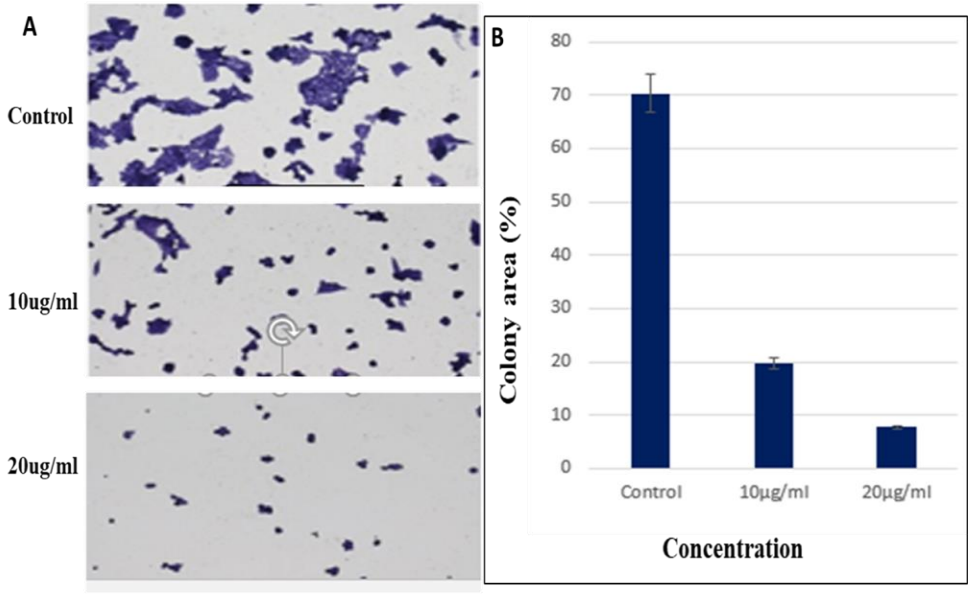


Figure 7. Anticancer Effect of Tannins from Leaf Callus Culture Extracts against HJC 106 (Human Jurket Cell Line). **(A)** Colony Formation of HJC106 after Treatment of Leaf Callus Culture Extracts. (**B**) Graph Represents the Percentage of the Colony Area of HJC106 in a Concentration Dependent Treatment of Leaf Callus Culture Extracts and Control

4. DISCUSSION

This research focused on investigating the anticancer and antioxidant properties of tannins derived from callus biomass production of *Achyranthes aspera* L. plant species. The primary objective was to deepen the understanding of the medicinal potential inherent in *A. aspera*, while exploring sustainable methods for obtaining bioactive compounds of therapeutic significance. The anticipated results hold the potential to expand the current knowledge and contribute to advancements in medicinal plant research, potentially leading to sustainable approaches for acquiring valuable bioactive compounds.

In this study, seedling leaf, stem, root, and callus cultures grown on MS medium were subjected to treatments with different combinations and concentrations of various plant growth regulators (PGRs) including IBA, BAP, NAA, and 2,4-D. Across all selected hormone concentrations and combinations, *A. aspera* leaf, stem, and root explants exhibited a callogenic response. The shape, texture, and color of the callus changed due to the combined effects of hormones, with auxin and cytokinin influencing callus establishment [14]. MSDN (NAA and 2,4-D) resulted in the formation of large-sized, light green, and compact calluses from leaf and root explants, respectively. The interaction of 2,4-D with cytokinin led to the formation of callus, indicating DNA synthesis and cell division (Table 1). Similar results were reported in a study employing *A. aspera* leaf explants from MSDN [15].

The methanol extract of leaf callus culture from the medium MSDI yielded the highest percentage production of tannins (Figure 4), followed by seedling leaf tissue in the methanol extract of the selected plant, among all callus and plant seedling tissue extracts. Previous phytochemical studies revealed the presence of various beneficial substances including tannins in seedling tissues and callus cultures. Tannins were found to be present in all callus culture extracts, with their amount varying depending on callus concentration and combinations (Figure 2, 3, 4). *A. aspera* was found to contain condensed tannins, consistent with the findings of previous studies [16]. Petroleum ether extract showed the highest amount of tannins in seedling leaf tissue, followed by tannins in the petroleum ether extract of leaf callus culture of MSDB (2.4-D and BAP) of *A. aspera* (Figure 6A).

Polyphenolic compounds such as tannins have antioxidant capabilities due to their scavenging activity [17–19]. Tannin content and antioxidant activity of leaves showed a positive correlation ($r = 0.8526602$ for methanol extract, $r = 0.964765$ for petroleum ether extract) (Table 3). There was established a direct link between tannin concentration and antioxidant potential. The antioxidant potential of the root correlated significantly with the quantity of tannins in aqueous and chloroform extracts, with $r = 0.881742$ and $r = 0.877695$, respectively. The anticancer potential of extracts also showed a strong correlation with phytochemical compounds inlcuding tannins, which are considered as effective candidates for antioxidant and anticancer agents [20–23]. Tannins

demonstrated concentration-dependent reduction against the human Jurket cell line in the MTT assay. The callus culture extract of tannins from the leaf of the plant reduced cell viability by 19.7% and 7.7% at 10 µg/ml and 20 µg/ml DMSO concentrations respectively, inhibiting the Jurket cell line by 81.3% and 3.3%, respectively (Figure 7).

4.1. Conclusion

The study concludes by establishing a callus culture procedure for the medicinal plant *A. aspera*, offering a novel approach for extracting essential phytochemical components such as tannins from *in vitro* cultures, rather than employing traditional planting methods. Tannins from callus cultures, possessing antioxidant and anticancer properties, may be explored for creating pharmacological profiles targeting disorders caused by oxidative stress. Pre-clinical evidence suggests that bioactive chemicals from *A. aspera* callus culture or biomass may find applications in clinical settings.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVALIABILITY STATEMENT

Data availability is not applicable as no new data was created.

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SUPPLEMENTARY FIGURES AND TABLES

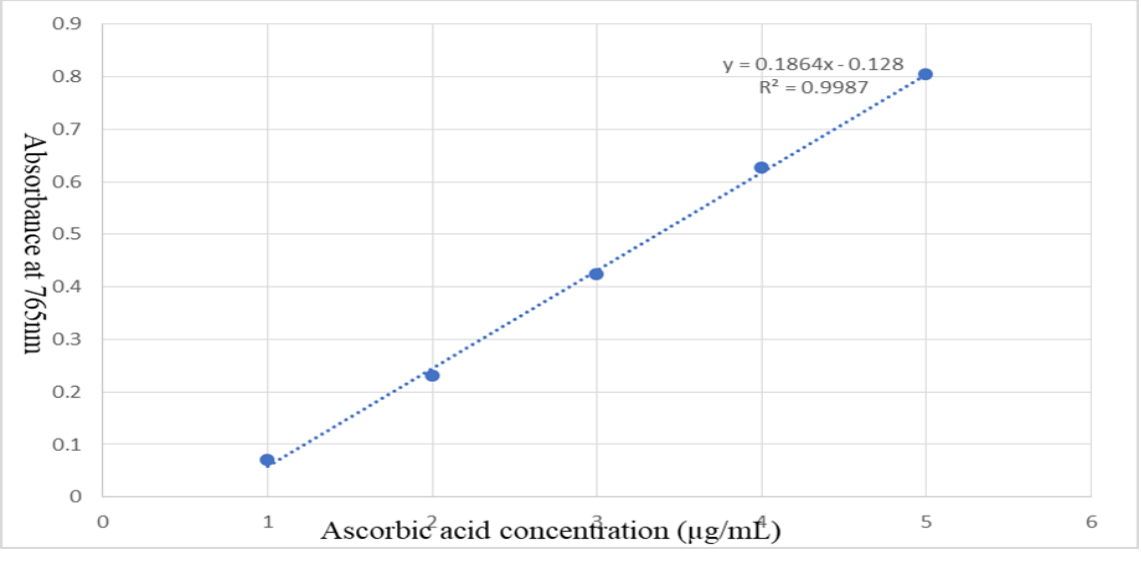

Figure S1. Linear Regression Analysis of Ascorbic Acid and 765 nm Absorption. *A. aspera* Seedling Tissues and Callus Culture Extracts have Antioxidant Activity

Table S1. The Percentage Yield of Callus Cultures and Seedling Plant Tissue

Solvent	Seedling Leaf Tissue Extract (%age Yield)	Leaf callus culture extracts (%age Yield)			Seedling Stem Tissue Extract (%age Yield)	Stem callus culture extracts (%age Yield)			Seedling Root Tissue Extract (%age Yield)	Root callus culture extracts (%age Yield)		
		MSDB	MSDN	MSN		MSDIA	MSDN	MSDIB		MSDN	MSNB	MSDIB
Petroleum ether	7±0.21	6.9±0.16	11±0.35	16±0.11	7.9±0.42	13±0.1	4±0.8	9±0.11	3±0.22	4.8±0.26	6.2±0.11	8.2±0.27
Chloroform	3.5± 0.14	7.6± 0.12	8.9±0.26	2.5±0.20	13±0.27	24.4±0.1	20.1±0.3	6.6±0.13	2.8±0.19	3.9±0.12	9.9±0.33	4.6±0.21

Solvent	Seedling Leaf Tissue Extract (%age Yield)	Leaf callus culture extracts (%age Yield)			Seedling Stem Tissue Extract (%age Yield)	Stem callus culture extracts (%age Yield)			Seedling Root Tissue Extract (%age Yield)	Root callus culture extracts (%age Yield)		
		MSDB	MSDN	MSN		MSDIA	MSDN	MSDIB		MSDN	MSNB	MSDIB
Methanol	27±0.10	31.2±0.1	22.5±0.5	3.5±0.29	6.5±0.11	3.2±0.1	28±0.49	15±0.22	19.7±0.13	8.5±0.10	13.5±0.23	10.3±0.14
Distilled water	5.2±0.24	4.3±0.25	9.1±0.16	4.4±0.19	3±0.15	6.5±0.3	13±0.34	17±0.29	3.3±0.14	11±013	5.5±0.14	3.2±0.26

Leaf callus culture medium code: MSDB (2,4-D 0.5 mg/l + 1.5 mg/l BAP) MSDN (2,4-D, 2.0 mg/l+4.0 mg/l NAA) MSNB (NAA, 2.0 mg/l+ 2.0 mg/l BAP).

Stem callus culture medium code: MSDIA (2,4-D 4.0 mg/l+6.0 mg/l IAA), MSDN (2,4-D, 6.0 mg/l + 8.0 mg/l NAA) MSDIB (2,4D, 2.0 mg/l+ 4.0 mg/l IBA).

Root callus culture medium code: MSDN (2,4-D, 4.0 mg/l +6.0 mg/l NAA) MSNB (2,4 D, 2.0 mg/l+ 2,0 mg/l BAP) MSDIB (2,4-D, 2.0 mg/l+ 4.0 mg/l IBA).

Table S2. Correlation Coefficient between Tannin Content and the Antioxidant Activity in the Stem Extract of *A. aspera.*

Tannins content				Antioxidant activity			
Petroleum ether	Chloroform	Methanol	Distilled water	Petroleum ether	Chloroform	Methanol	Distilled water
1							
0.568257	1						
-0.52011	0.38736	1					
-0.18657	0.187205	0.577482	1				
-0.67403	0.048609	*0.680994	-0.1635	1			
0.360044	-0.14231	-0.36926	0.54454	-0.89669	1		

Tannins content				Antioxidant activity			
Petroleum ether	Chloroform	Methanol	Distilled water	Petroleum ether	Chloroform	Methanol	Distilled water
0.302478	*0.666396	0.490696	**0.835059	-0.27343	0.471257	1	
-0.93642	-0.42327	0.66239	0.519381	0.531549	-0.12246	0.036682	1

Table S3. Correlation Coefficient between Tannin Content and the Antioxidant Activity in the Root Extract of *A. aspera*

Tannins content				Antioxidant activity			
Petroleum ether	Chloroform	Methanol	Distilled water	Petroleum ether	Chloroform	Methanol	Distilled water
1							
*0.685493	1						
*0.621423	*0.621963	1					
*0.633955	**0.728634	**0.88343	1				
-0.34667	0.4372	-0.2562	-0.13138	1			
0.602404	0.855532	0.847265	**0.8817	-0.32784	1		
0.252867	0.320783	**0.72652	*0.61233	-0.3683	**0.877695	1	
0.808327	0.490759	0.162872	0.288577	-0.1719	0.029828	-0.32975	1