

Establishment of suspension cultures and their antioxidative activity in relation to biomass and phenolics content of *Artemisia amygdalina* D., a critically endangered endemic medicinal plant of Kashmir Himalaya

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ABSTRACT

For increment in the fabrication of total phenolic content and elevated antioxidative activities suspension cultures of *Artemisia amygdalina* D. were developed. The *in vitro* suspension cultures were established on MS medium (Murashige and Skoog's 1962) augmented with plant hormone 6-Benzylaminopurine (BAP) in different concentrations (2.5-15 μ M). The two experimental culture systems were established. In one of the system BAP was used singly while as in other system BAP was used along with 2.5 μ M Naphthaleneacetic acid (NAA). Further cultures were investigated for their kinetic patterns of growth, total content of phenolics and viz-a-viz antioxidative activities over a time of 45 days within the interim of 5 days. In our study it was analyzed that 10 μ M/l BAP and 2.5 μ M/l NAA resulted in highest dry biomass formation on 40th day of culture (11.7 g/l). Besides this on 40th day, highest profile for total content of phenolics (8.8 mg GAE/g DW) and high DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity (72.2 %) were recorded in suspension cultures augmented with 10 μ M/l BAP. The results of our study reveal significant correlation between DPPH free radical scavenging activity and total content of phenolics within suspension cultures of *A. amygdalina*.

Keywords: Antioxidative activities, 6-Benzylaminopurine, Dry biomass, Naphthalene acetic acid, Phenolic content, Suspension cultures

I. INTRODUCTION

Northwest Himalaya is known for rich repository of medicinal plant wealth. Among the treasure of these medicinal plants, one of the important medicinal plant is *Artemisia amygdalina* D. It is an endemic medicinal plant of Kashmir valley belonging to the family Asteraceae and grows in subalpine region of Kashmir Himalaya and North-West Frontier Province of Pakistan. The plant extract is used locally for the treatment of epilepsy, piles, nervous disorders, cough, cold, fever, and pain [1]. The women folk of the valley use it for amenorrhoea and dysmenorrhoea. As a consequence of overharvest and deforestation, this plant is considered as the critically endangered endemic species of Kashmir valley

[2]. Plant bioactive metabolites are distinctive sources of pharmaceuticals viz-a-viz industrial material either as a raw material or final product. Among these bioactive metabolites plant phenolics constitutes the large group of natural antioxidants. Phenolics along with flavonoids possess diverse biological activities including antioxidant, antiageing and protection from cardiovascular diseases [3]. Recently it has been put forth that phenolics plays an imperative role in treatment of Alzheimer's and Parkinson's diseases [4]. Nowadays phenolics are thought to be more effective cancer prevention agents as compared to vitamin E and vitamin C and henceforth phenolics can play a significant role in scavenging of free radicals under *in vivo* conditions [5]. *In vitro* cultures are suitable alternatives for the production of bioactive compounds whose production is obscure by traditional methods or financially not attainable [6, 7] (Rafi et al. 2016; Karioti et al. 2010). In view of the above discussion procedures ought to be embraced to monetarily upgrade the production of phenolic compounds by utilizing tissue culture systems. Our study focuses on the investigation of correlation between accretion of biomass, total content of phenolics and antioxidative activities within suspension culture systems of *A. amygdalina*.

II.METHODOLOGY

2.1 Collection and selection of plant material

The whole plants of *A. amygdalina* were gathered from Gurez valley, Kashmir (34.6333°N 74.8333°E). They were identified at Centre for Biodiversity and Taxonomy, University of Kashmir.

2.2 Establishment of suspension cultures

For the establishment of suspension culture, proliferated callus (35-day old) was transferred to 100 ml Erlenmeyer flasks containing MS basal medium supplemented with BAP (1.0 μ M - 15.0 μ M) either singly or in combination of NAA (2.5 μ M). The cultures were placed in rotatory shaker (25 °C, 120 rpm) for the development of suspension culture. The data recording and analysis of the growth kinetics was assessed at the interval of 5 days for period of 45 days.

2.3 Analytical method

For the analysis of biomass accretion the samples were retrieved from the medium and weighed for fresh weight (FW) analysis. Furthermore these samples were dried in oven (60°C, 24 h) for dry weight (DW) exploration. The extraction of the samples was done as per methodology adopted by [8] with slight modification. The dried samples were ground in a course grinder and powdered (100 mg) with 10 ml of 99 % (v/v) methanol. After that samples were sonicated four times with resting phase of 20 minutes and further subjected to centrifugation for 20 minutes at 7000 rpm. Subsequently supernatant was collected and stored at -20°C or analyzed immediately.

The determination of phenolics was done by utilizing Folin Ciocalteu's reagent as per the methodology of Malick and Singh, [9]. The absorbance of the samples was taken at 765 nm and calibrated against gallic acid.

For analysis of antioxidative activity DPPH free radicals were used [2].

$$\text{Inhibition\% of free radicals} = (A_c - A_s) / A_c \times 100$$

whereas 'Ac' is the absorbance of sample without any antioxidative substance and 'As' is absorbance of the sample along with standard antioxidant or plant extract.

2.4 Statistical analysis

Completely randomized design (CRD) was setup for carrying out the experiments and statistic analysis was carried out by means of one way ANOVA. Moreover, Duncan's multiple range test was used for the determination of significant differences ($P < 0.05$) for multiple comparisons.

III. RESULTS AND DISCUSSION

3.1 Effect of plant growth regulators on growth kinetics and biomass accumulation.

BAP when utilized alone at the concentration of 10 µM resulted in the formation of 118 g/l fresh biomass (FB) and 9.4 g/l dry biomass (DB) on 40th day of suspension culture. On the other hand amongst the various combinations and concentrations of plant growth regulators tested the highest production of FB (152g/l) and DB (11.7g/l) was recorded on the 40th day of suspension culture at 10 µM BAP and 5 µM NAA. Thus it is put forth that 2.5 µM NAA along with BAP positively effects the biomass formation. This has also been published in previous studies which ascribe the synergetic acquaintances of cytokinins and auxins on frequency of callus development for various medicinal plants [8, 10]. In our experimental line the utmost production of FB and DB were assessed on 40th day of culture at concentrations 2.5 µM, 5 µM, 7.5 µM and 10 µM of BAP either alone or in combination with NAA (5 µM/l). On further increasing the concentration of BAP it was found that frequency of biomass development decreased significantly and moreover on 35th day of suspension culture maximum biomass formation was analyzed.

During the investigation of kinetics patterns of growth for the accumulation of fresh and dry biomass in reaction to tested plant growth regulators a lag phase of 5 days was observed. Highest profiles of biomass were recorded on 40th and 35th days of culture depending upon the combinations and concentrations of plant growth regulators used.

Starting with the inoculum of 10 g/l more than double in FB with values 22.1 g/l, 29.3 g/l, 37.5 g/l and 44.3 g/l was analyzed on 10th day of culture whereas maximum FB with the values of 90 g/l, 99 g/l, 107 g/l and 118 g/l in response to 2.5 μ M, 5 μ M, 7.5 μ M and 10 μ M BAP respectively (Table 1). However on the other hand, at higher concentrations of BAP more than two fold increment in FB with values 33.2 g/l and 31.2 g/l was observed 15th day of culture and maximum accumulation was recorded 87.9 g/l and 85.3 g/l on 35th day of culture in reaction to 12.5 μ M and 15 μ M of BAP respectively (Table 1). Moreover two fold increment in DB was found to be fluctuating and not austere ally allied to particular day or days. The maximum values of DB 7.9 g/l, 8.5 g/l, 8.9 g/l and 9.4 g/l on 40th day of culture in reaction to concentrations 2.5 μ M, 5 μ M, 7.5 μ M and 10 μ M of BAP, followed by 8.3 g/l and 8.0 g/l on 35th day of culture in response to 12.5 μ M and 15 μ M of BAP respectively (Table 1).

The accumulation of biomass was also analyzed in response to different concentration of BAP along with 2.5 μ M NAA. The maximum values of FB 128 g/l, 134 g/l, 142g/l and 152 g/l and DB 9.4g/l, 10.2 g/l, 11.1 g/l and 11.7 g/l were assessed on 40th day of culture in reaction to 2.5 μ M, 5 μ M, 7.5 μ M and 10 μ M BAP (along with 2.5 μ M NAA) correspondingly (Table 2). In addition to this, maximum levels of FB 116 g/l and 105.7 g/l viz-a-viz DB 8.2 g/l and 7.4 g/l were recorded on 35th day of culture in reaction to 12.5 μ M and 15 μ M BAP (along with 2.5 μ M NAA) (Table 2). This indicates that BAP at higher concentrations either alone or along with NAA significantly decrease rate of recurrence of biomass development and viz-a-viz reduced log phase of growth curve in suspension cultures of *A. amygdalina*.

3.2 Antioxidative activity and its correlation with accumulation of biomass and total content of phenolics

Suspension cultures of *A. amygdalina* were assessed for free radical scavenging activity for a time of 45 days with an interim of 5 days. A significant positive correlation was analyzed between dry biomass accumulation, antioxidant activity at lower concentrations of BAP (2.5-10) μ M. The cultures developed on MS media augmented with 2.5 μ M, 5 μ M, 7.5 μ M and 10 μ M revealed highest values for dry biomass accumulation (7.9 g/l, 8.5 g/l, 8.9 g/l and 9.4 g/l correspondingly), total content of phenolics (6.3, 7.3, 8.2 and 8.8) mg GAE/g DW, and antioxidant activity (65.8 %, 67.2 %, 70.1 % and 72.2 %) on 40th day (stationary phase of growth kinetics) of culture (Fig 1 a-d). Notably antioxidant activity at higher concentrations of BAP (12.5 μ M and 15 μ M) was analyzed to be dependent on biomass and independent to total phenolics content (Fig 1 e, f). Thus our results revealed that at lower concentrations of BAP phenolics plays a vital role in antioxidative activities. Further at higher concentrations a fluctuating patterns of biomass accumulation, total phenolics and antioxidant activity

was observed. Maximum antioxidant activity 62.5% and 61% viz-a-viz maximum biomass accumulation 8.3 g/l and 8.0 g/l was assessed on 35th day (stationary phase of growth kinetics), Moreover maximum total phenolic content 6.0 mg GAE/g DW and 5.7 mg GAE/g DW were recorded on 30th day of culture (log phase) in response to 12.5 μ M and 15 μ M of BAP respectively (Fig 1 e, f). This result could be associated with the conjecture that at higher concentration of plant growth regulators other antioxidative compounds besides phenolics are synthesized.

Antioxidative activity in case of lower concentrations BAP and along with NAA was found to be dependent on total phenolic content almost in all the culture systems. Suspension cultures in reaction to concentrations of BAP (2.5 μ M, 5 μ M and 7.5 μ M) and 2.5 μ M IAA showed high values of total phenolic content (6.2, 6.7 and 7.2) mg GAE/g DW and antioxidant activity (63%, 65.5% and 69.2%) on 30th day (log phase) of culture (Fig 2 a-c). In addition to this cultures at 10 μ M BAP concentration along with 2.5 μ M IAA showed raised values of phenolic content (7.7mg GAE/g DW) and antioxidant activity (70.1%) on 35th day (log phase) of culture (Fig 2d). Moreover at higher concentrations of BAP (12.5 μ M and 15 μ M) along with 2.5 μ M NAA significant correlation was analyzed between maximum dry biomass accumulation (8.2 g/l and 7.4 g/l) and maximum antioxidant activity (60.9% and 58.2%) on 35th day of culture (Fig 2 e, f) signifying the involvement of other antioxidative compounds besides phenolics within the stationary phase of growth kinetics in suspension cultures. Our results are in line with several reports which put forth significant correlation of antioxidative activity and phenolic content within many plants [11, 12,13, 14]. Noteworthy to mention that in various in vitro culture systems of different medicinal herbs significant correlation between antioxidant activities and phenolic content has also been analyzed [8,15,16].

IV.FIGURES AND TABLES

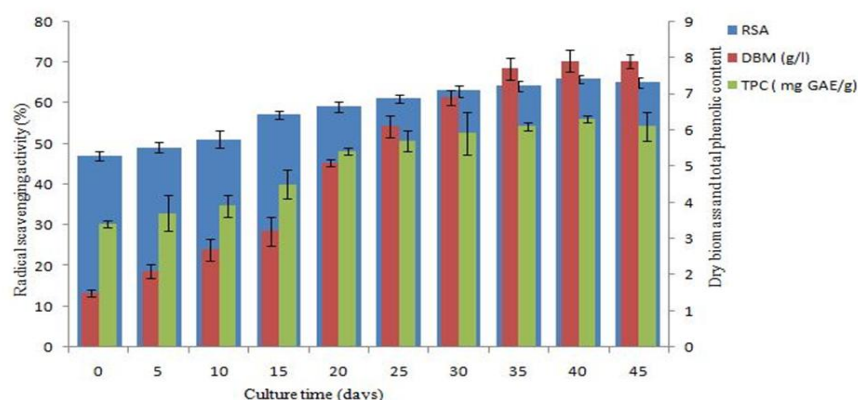


Fig 1a. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass(g/l) within callus in response to 2.5 μ M BAP.

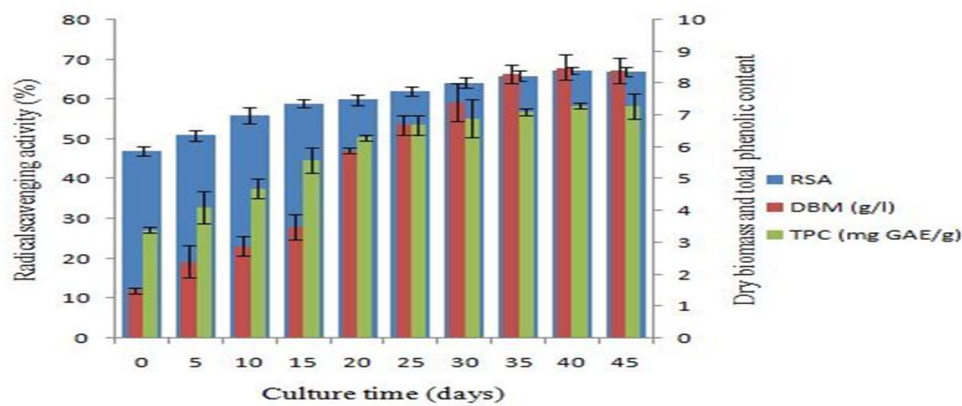


Fig 1b. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 5.0 μM BAP.

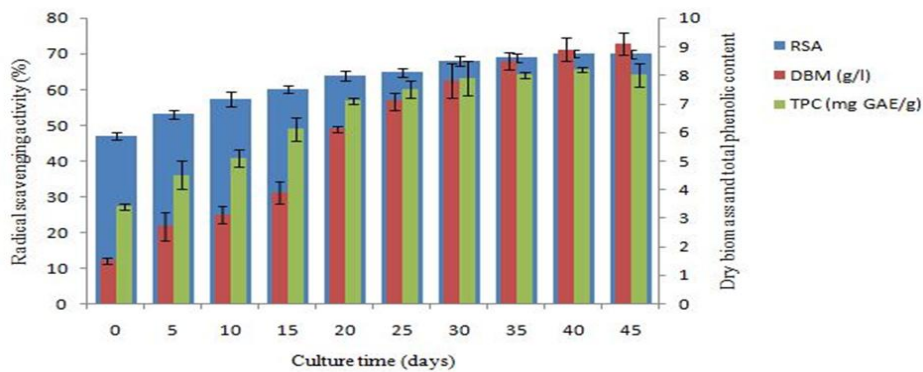


Fig 1c. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 7.5 μM BAP.

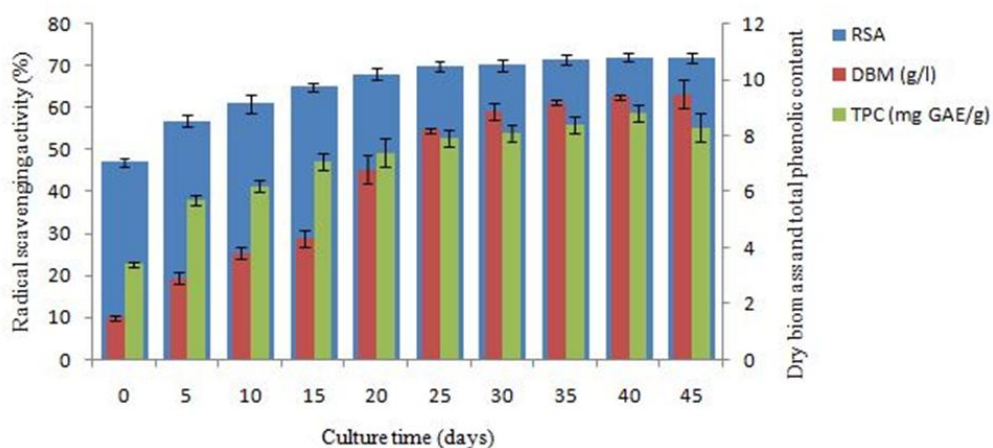


Fig 1d. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 10.0 μM BAP.

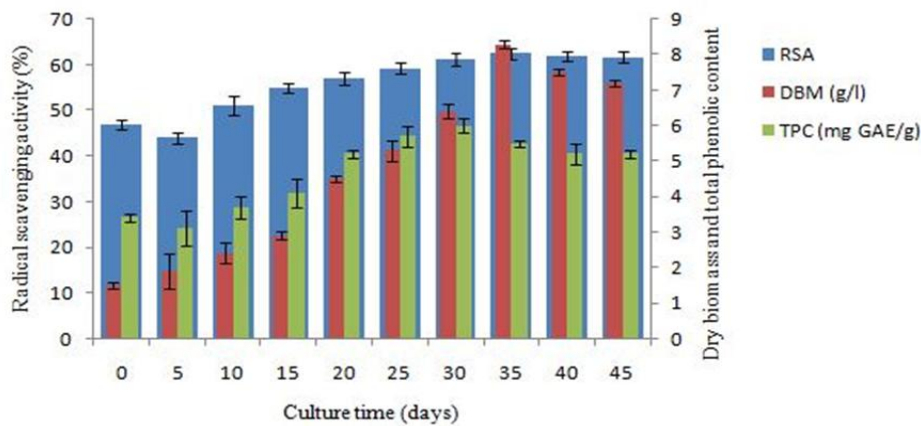


Fig 1e. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 12.5 µM BAP.

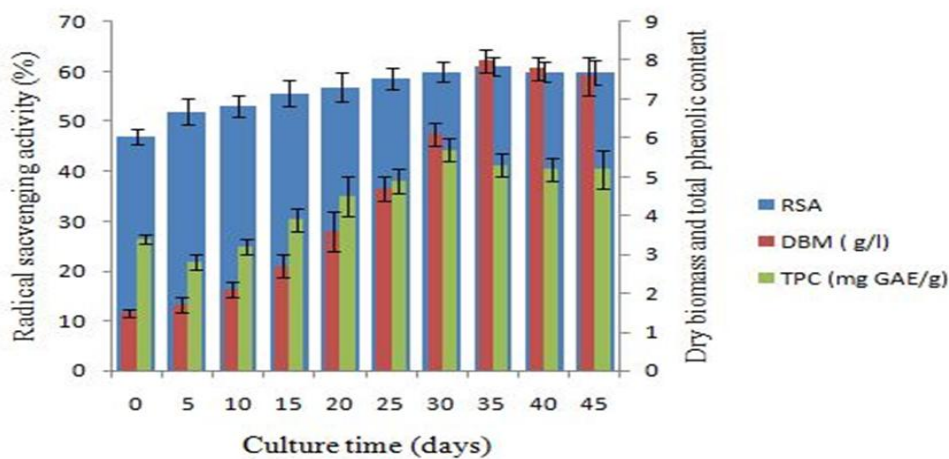


Fig 1f. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 15.0 µM BAP.

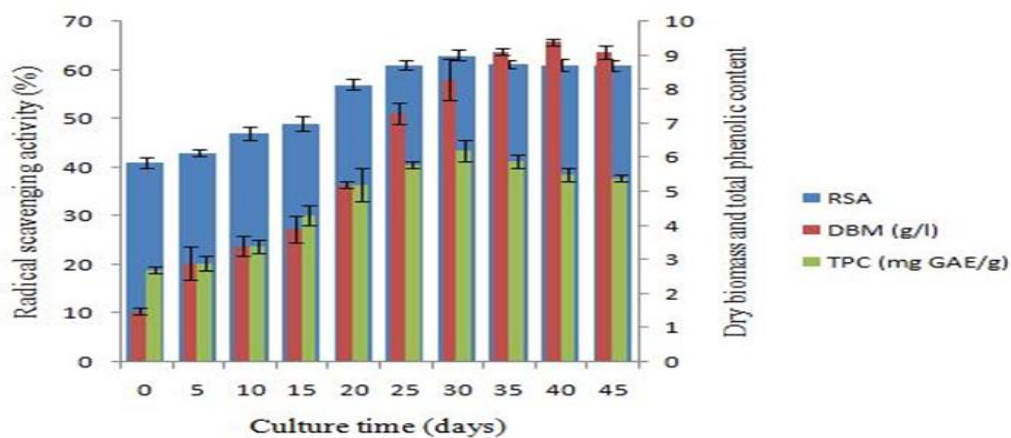


Fig 2a. Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 2.5 µM BAP + 2.5 µM NAA.

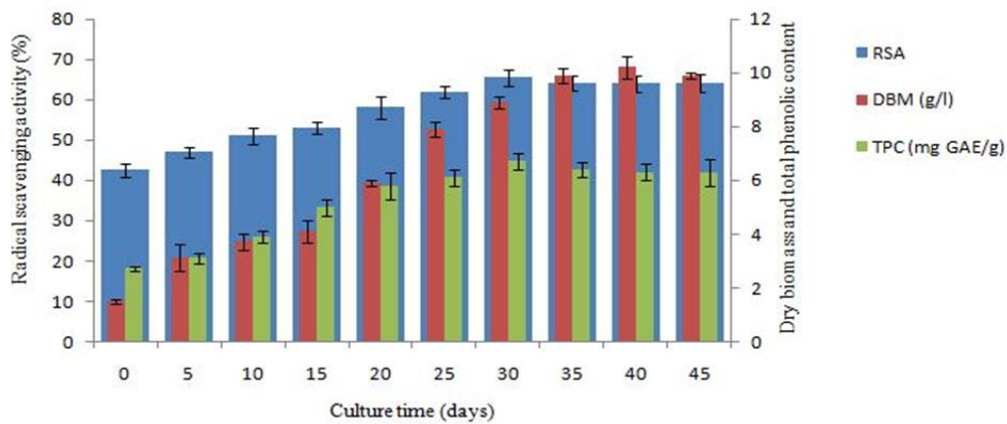


Fig 2b Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 5.0 μM BAP + 2.5 μM NAA.

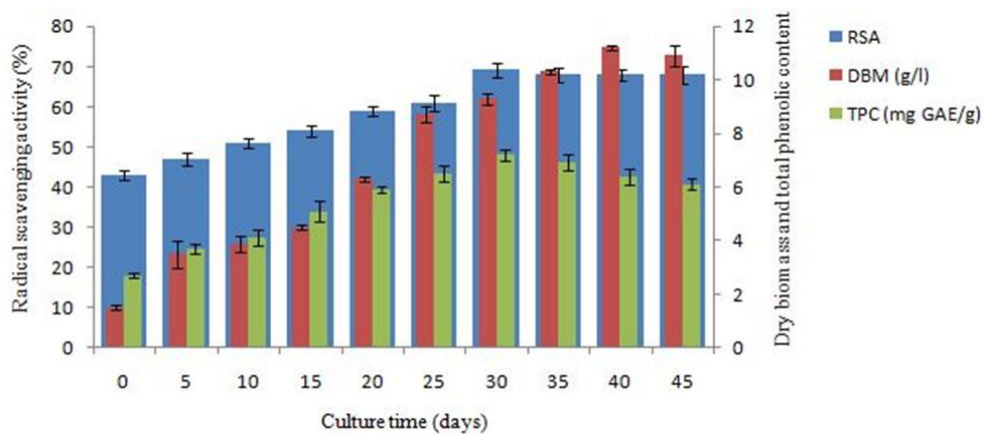


Fig 2c Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 7.5 μM BAP + 2.5 μM NAA.

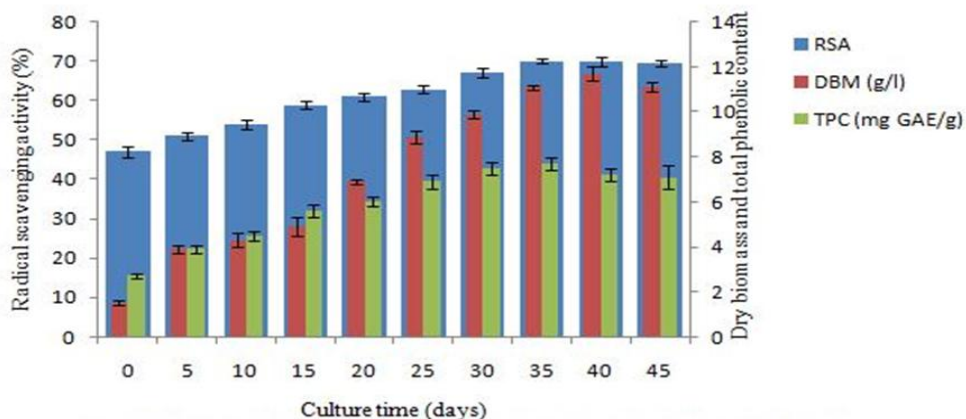


Fig 2d Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 10.0 μM BAP + 2.5 μM NAA.

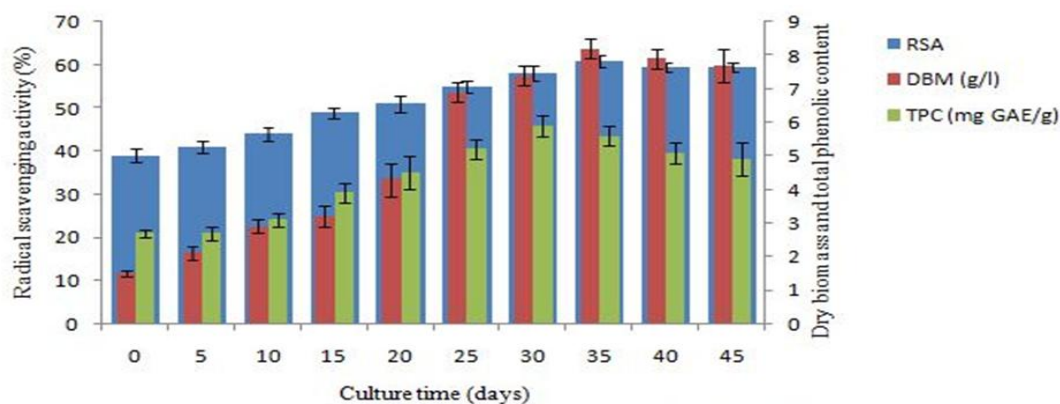


Fig 2e Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 12.5 μM BAP + 2.5 μM NAA.

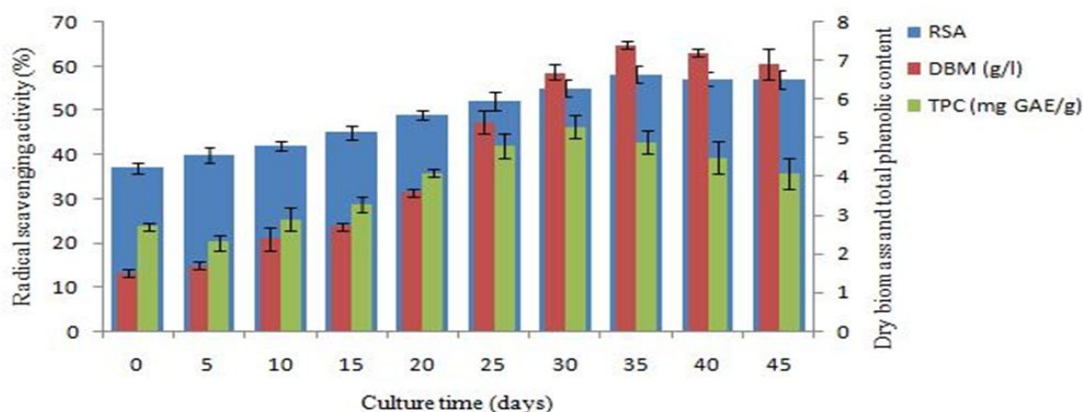


Fig 2f Antioxidant activity (%) total phenolic content (mg GAE/g DW) and dry biomass (g/l) within callus in response to 15.0 μM BAP + 2.5 μM NAA.

Table 1. Fresh biomass and Dry biomass of *Artemisia amygdalina* on MS media augmented with different concentrations of BAP

Cultured days	2.5 μM BAP		5.0 μM BAP		7.5 μM BAP		10 μM BAP		12.5 μM BAP		15 μM BAP	
	FM	DM	FM	DM	FM	DM	FM	DM	FM	DM	FM	DM
0	10.0 ± 1.2	1.5 ± 0.2	10.0 ± 1.4	1.5 ± 0.3	10.0 ± 0.5	1.5 ± 0.3	10.0 ± 1.4	1.5 ± 0.3	10.0 ± 0.1	1.5 ± 0.1	10.0 ± 1.0	1.5 ± 0.4
5	14.2 ± 0.3	2.1 ± 0.5	17.8 ± 1.3	2.4 ± 0.2	19.6 ± 1.4	2.7 ± 0.5	21.7 ± 1.5	2.9 ± 0.1	15.3 ± 0.6	1.9 ± 0.4	14.1 ± 1.0	1.7 ± 0.3
10	22.1 ± 1.4	2.7 ± 0.5	29.3 ± 1.6	2.9 ± 0.5	37.5 ± 1.2	3.1 ± 0.5	44.3 ± 1.5	3.8 ± 0.5	18.3 ± 0.5	2.4 ± 0.5	17.6 ± 1.4	2.1 ± 0.7

	0.4	.2	0.3	.1	.5							
15	39.7± 1.3	3.2± 0.5	42.5 ±1.5	3.5±0 .2	59.2± 1.3	3.9± 0.5	61.5± 1.7	4.3±0 .0	33.2±1 .3	2.9±0 .4	31.2± 1.3	2.7±0.5
20	52.8± 1.7	5.1± 0.3	49.7 ±1.7	5.9±0 .2	68.4± 1.0	6.1± 0.1	75.9± 1.3	6.8±0 .1	48.7±1 .1	4.5±0 .7	41.5± 1.1	3.6±0.4
25	63.5± 1.5	6.1± 0.7	69.9 ±1.4	6.7±0 .2	89.3± 1.0	7.1± 0.1	95.4± 1.5	8.2±0 .1	58.2±1 .1	5.3±0 .3	49.7± 1.1	4.7±0.3
30	72.5± 1.3	6.9± 0.2	82.1 ±1.3	7.4±0 .2	95.4± 1.7	7.8± 0.5	106.3 ±14	8.9±0 .4	79.5±1 .4	6.4±0 .3	67.2± 1.3	6.1±0.7
35	83.7± 1.7	7.7± 0.3	90.6 ±1.2	8.3±0 .2	101.4 ±1.5	8.5± 0.7	115.2 ±1.4	9.2±0 .1	87.9±1 .3	8.3±0 .6	85.3± 1.4	8.0±0.3
40	90±1. 4	7.9± 0.7	99±1 .2	8.5±0 .2	107±1 .1	8.9± 0.4	118±1 .0	9.4±0 .3	84.7±1 .2	7.5±0 .4	83.9± 1.5	7.6±0.3
45	88±1. 5	7.9± 0.4	97.7 ±1.5	8.4±0 .2	106.4 ±1.1	9.1± 0.3	116.3 ±1.0	9.2±0 .2	82.7±1 .0	7.2±0 .3	82.9± 1.4	7.1±0.7

Data scored for 45 days with interval of 5 days of suspension culture and represented as mean±SD. Statistical analysis was done by ANOVA using Duncan's multiple range test (SPSS17.0). The values with are provided with superscript different superscript in the columns are statically significant at P<0.005.

BAP=6-Benzylaminopurine.

Table 2. Fresh biomass and Dry biomass of *Artemisia amygdalina* on MS media augmented with different concentrations of BAP along with 2.5 µM NAA

Cultu e days	2.5 µM BAP + 2.5 µM NAA		5.0 µM BAP+ 2.5 µM NAA		7.5 µM BAP+ 2.5 µM NAA		10 µM BAP + 2.5 µM NAA		12.5 µM BAP + 2.5 NAA		15 µM BAP+ 2.5 µM NAA	
	FM	DM	FM	DM	FM	DM	FM	DM	FM	DM	FM	DM
0	10.0±1.1	1.5±0	10.0±	1.5±0	10.0±	1.5±0	10.0±	1.5±0	10.0±	1.5±	10.0±	1.5±
		.1	1.3	.1	1.3	.1	1.2	.3	1.7	0.5	1.2	0.4
5	17.8±1.0	2.9±0	19.5±	3.1±0	23.3±	3.5±0	27.2±	3.9±0	16.9±	2.1±	16.4±	1.7±

	.7	1.3	.4	1.1	.7	1.7	.3	1.1	0.1	1.4	0.1	
10	42.5±1.2	3.4±0	45.7±	3.7±0	49.8±	3.9±0	56.2±	4.3±0	38.7±	2.9±	34.7±	2.4±
	.4	1.3	.2	1.4	.3	1.3	.4	1.1	0.6	1.1	0.5	
15	56.7±0.9	3.9±0	58.3±	4.1±0	64.9±	4.5±0	72.3±	4.9±0	49.5±	3.2±	42.9±	2.7±
	.5	1.3	.6	1.5	.9	1.1	.3	1.5	0.4	1.0	0.1	
20	70.8±1.7	5.2±0	76.5±	5.9±0	83.5±	6.3±0	89.7±	6.9±0	64.5±	4.3±	57.4±	3.6±
	.1	1.3	.1	1.1	.7	1.0	.2	1.0	0.1	1.6	0.6	
25	97.6±1.9	7.3±0	101.2	7.9±0	110.4	8.7±0	114.9	8.9±0	87.5±	6.9±	79.5±	5.4±
	.1	±1.3	.2	±10	.4	±1.1	.3	1.1	0.5	1.7	0.5	
30	108.4±1.	8.3±0	113.7	8.9±0	124.9	9.3±0	129.7	9.9±0	105.2	7.4±	89.9±	6.7±
	1	.4	±1.3	.5	±1.7	.1	±1.1	.1	±1.6	0.6	1.0	0.4
35	117.5	9.1±0	124.3	9.9±0	131.9	10.3±	143.2	11.1±	116.0	8.2±	105.7	7.4±
	±1.7	.8	±1.3	.4	±1.1	0.1	±1.5	0.3	±1.7	0.6	±1.1	0.4
40	128±1.1	9.4±0	134.0	10.2±	142.0	11.1±	152.0	11.7±	114.3	8.1±	104.6	7.1±
	.9	±1.3	0.1	±1.6	0.5	±1.7	0.3	±1.9	0.7	±1.6	0.6	
45	126.8±1.	9.1±0	133.6	9.9±0	140.8	10.7±	150.7	11.1±	113.2	7.8±	103.6	6.7±
	5	.1	±1.3	.2	±1.1	0.1	±1.0	0.3	±1.1	0.4	±1.8	0.5

Data scored for 45 days with interval of 5 days of suspension culture and represented as mean±SD. Statistical analysis was done by ANOVA using Duncan's multiple range test (SPSS17.0). The values with are provided with superscript different superscript in the columns are statically significant at P<0.005.

BAP=6-Benzylaminopurine, NAA= Naphthalene acetic acid.

V.CONCLUSION

The present study showed that phenolics production was positively regulated in suspension cultures of *Artemisia amygdalina* in response to BAP. A highly significant correlation between DPPH free radical scavenging activity and total content of phenolics. The results suggest the exploitation of suspension cultures of *Artemisia amygdalina* by treatment with other PGRs and elicitors to enhance total phenolic content and antioxidant activity.

VI.ACKNOWLEDGMENTS

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