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Evaluation of Phytochemical & Antimitotic Potential of Annona Reticulata Extracts by Onion Root Model †

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Abstract: This study investigated photochemical investigation and extraction methods with polar and non polar solvent which could be used to compare and see the effectiveness of each on the chromosomes. The FTIR spectrum shows the presence of acetogenins in the extracts. Cytotoxic effects were studied using Allium cepa root tip cells. Eight onion bulbs were subjected to the treatment groups which were made using water, methanol, Ethyl acetate, acetone and hexane extracts of A. reticulata. Three different concentrations from each extraction solvent were used. The A. cepa root tip cells were subjected for 24 h to three different leaf extractions after left to grow in water. Concentrations of 0.1, 1, 10 and 50 mg/L were used. All studied concentrations of A. reticulata methanol extract showed significant ($p < 0.05$) drop in the mean mitotic index when compared with control Overall decrease in MI was contributed to by all the stages of mitosis.

Keywords: Annona reticulate; antimitotic potential; *A. cepa* root tip; Phytochemical Analysis; acetogenins

1. Introduction

Annona reticulata Linn. (Bullock's heart) is a versatile tree and its fruits are edible. Parts of *A. reticulata* are used as source of medicine and also for industrial products. It possesses several medicinal properties such as Antioxidant and antibacterial activities (Prasad GJ, et al., 2015), Anti-hyperglycemic activity (Soumya PR et al., 2013) Analgesic and Anti-inflammatory activities (Ndiaye M. et al., 2006 and Machindra J.C., et al., 2012), anthelmintic (Nirmal S.A., et al., 2012), Antipyretic activity (Prasad GJ, Amruta SW, 2015), anticancer (Chavan SS et al., 2014), wound healing (Ngbolua KN et al., 2013) and Antiproliferative effects (Suresh HM, Shivakumar B, Shivakumar SI 2012). It is widely distributed with phytochemicals like tannins, alkaloids, phenols, glycosides, flavonoids and steroids (J Tradit, 2015). In order to initiate the search for drugs from plants, the antimitotic activity of the extracts were tested by Allium cepa assay (Levan, 1949). The Allium cepa root meristem assay is considered widely as a practical and reliable system for the screening of environmental mutagens and carcinogens (Fiskc - jo, et al., 1985; Stich et al., 1975).

Allium cepa species (common onion) is ideal for use in bioassays (Fonrose, X., et al., 2007, Iwasaki, S. 1993). It has also been widely used for detection of cytostatic, cytotoxic and mutagenic properties of different compounds, including anticancer drugs of plant origin (Mangale, S.M et al., 2012).

The present study was carried out to evaluate the cytotoxic and antimitotic potential of annona reticulata by standard assay method using Allium cepa root meristem model (Mondal, D.; Mondal, T. 2012). The effect was compared with control.

2. Material and Methods

2.1. Collecting the Material (Leaves)

The leaves of A. reticulata and Allium sativum (bulbs) were collected from regions of Karjat Dist-Raigad, Maharashtra, India in October 2019. Plant materials were authenticated at "The Blatter Herbarium" -St. Xavier's College, Mumbai.

2.2. Sample Preparation

Annona reticulata leaves were dried and powdered using mechanical blender and 50g *Annona reticulata* powder was macerated with 95% ethanol for 5 days. Using a rotary evaporator ethanol was evaporated and the sludge was redissolved in acetone. The solution was filterated by using a Buchner funnel with silica gel 60 on a filter paper. F1 and F2 fractions were obtained by using the solvents water, ethanol to leach the solid crude extract. Then ethanol, ethyl acetate, acetone and hexane were used consecutively, combined and evaporated using a rotary evaporator to obtain fraction F1, F2, F3 and F4 (Luna et al., 2006).

2.3. Identification of Acetogenin by Using Kedde Reagent

Thin layer chromatography was carried out on silica gel 60 as stationary phase and chloroform-methanol (9:1) as mobile phase. Kedde's reagent is used for visualization of Acetogenins on the TLC plate. Kedde's reagent was prepared with equal volumes of 2% (*w*/*v*) solution of 3,5-dinitrobenzoic acid in ethanol and 5.7% (*w*/*v*) solution of KOH in ethanol. After spaying Keddes reagent it shows pinkish purple color spot for the presence of acetogenin (Vinothini R and Lali Growther 2016) (Table 1).

Table 1. Fractions of solvents with color and Kedde test.

Sample	Eluent	Color	Kedde Test
F0	Aqueous fraction	Dark reddish pink	Positive
F1	Ethanol	Faint reddish pink	Positive
F2	Acetone	Faint reddish pink	Positive
F3	Ethyl acetate	Faint reddish pink	Positive
F4	Hexane	Green	Negative

2.4. Phytochemical Investigation of the A. Reticulata Leaves Extracts and Fractions

All the plant extracts and fractions were tested for the analysis of phytochemicals by following testes.

Qualitative Phytochemical Tests

The different qualitative tests was performed for its chemical composition. *Annona reticulata* leaves (ARL) aqueous extract (ARL-Aq), ethanol extract (ARL-EOH), acetone extract (ARL-Ac), ethyl acetate extract (ARL-EA) and hexane extract (ARL-Hx) were analysed for the presence of various phytoconstituents by following standard phytochemical tests (Table 2).

Table 2. Preliminary Phytochemical Analysis of the leaves of *Annona reticulata L.*

Sr. No.	Tests	Different Solvent Extracts of Leaves (SL)				
		ARL-Aq	ARL-EOH	ARL-Ac	ARL-EtAc	ARL-Hx
1.	Carbohydrates	-	-	-	-	-
2.	Starch	-	-	-	+++	++
3.	Reducing sugar	-	+++	+++	+++	-
4.	Amino acids	-	-	-	-	-
5.	Proteins	-	-	-	-	-

6.	Acid	-	-	-	-	-
7.	Quinones	-	+	-	-	-
8.	Coumarins	-	+++	++	++	-
9.	Gums and mucilages	-	-	-	-	-
10	Steroids	-	++	++	+++	+
11	Tannins	-	+++	+++	+++	+
12.	Phlobatannins	-	-	-	-	-
13.	Phenols	-	+++	+++	+++	-
14.	Cardiac glycosides	-	-	-	-	+
15.	Alkaloids	++	-	-	++	+
16.	Anthraquinones	-	-	-	-	-
17.	Betacyanins	++	+	+	+++	+++
18.	Emodols	++	+++	+++	+++	-
19.	Saponins	+	+	-	-	-
20.	Volatile oils	-	++	+	+++	-
21.	Flavanoids	++	+++	+++	+	-
22.	Terpenoids	-	-	-	-	+
23.	Resins	-	-	-	-	-
24.	Fixed oils and fats	-	-	-	-	-

(+) Low levels; (++) Moderate levels; (+++) High levels.

2.5. Characterization of the Leaf Extract

FT-IR Spectroscopy

FT-IR spectra were recorded with a FT-IR Shimadzu FT-IR 8400S spectrometer. The technique used was KBr pelleting techniques. The FT-IR spectra were recorded in the middle infrared (4000cm^{-1} to 400cm^{-1}) (Figures 1–3).

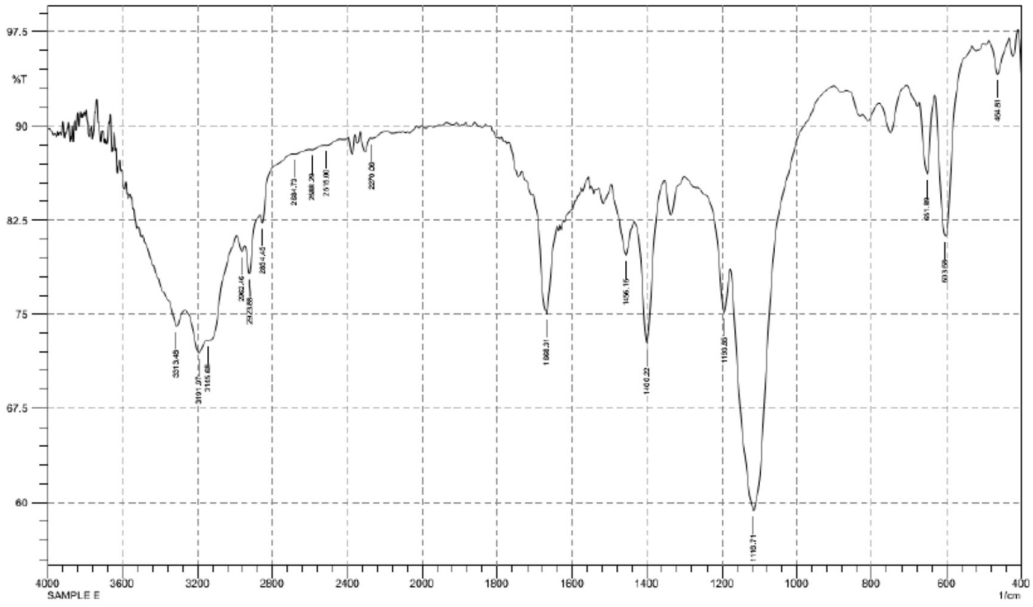

Figure 1. FT-IR spectrum of Ethyl acetate extract.

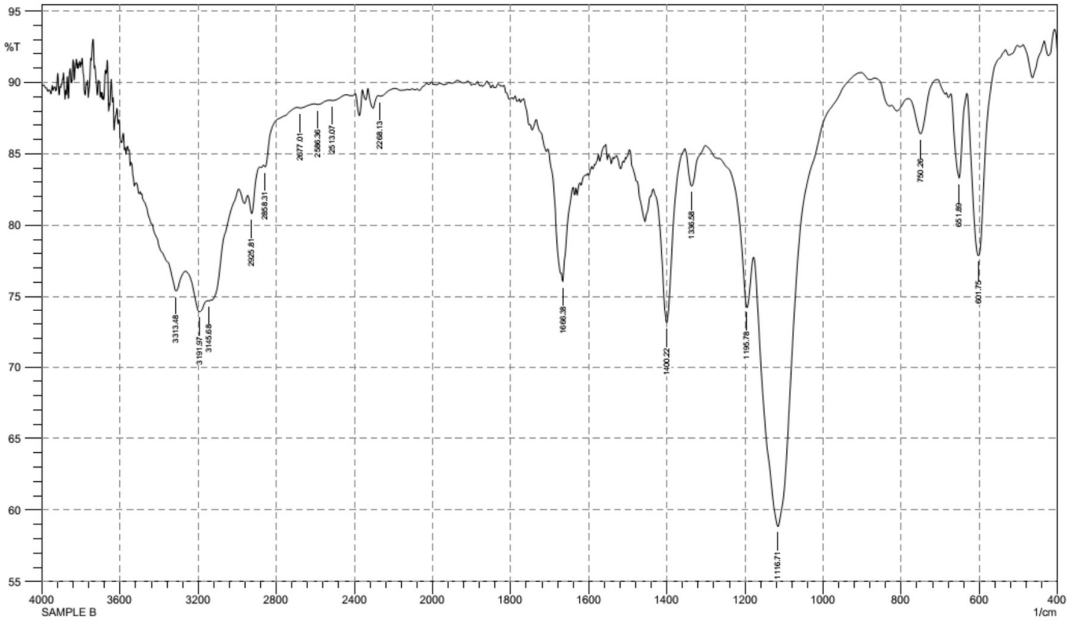

Figure 2. FT-IR spectrum of Acetone extract.

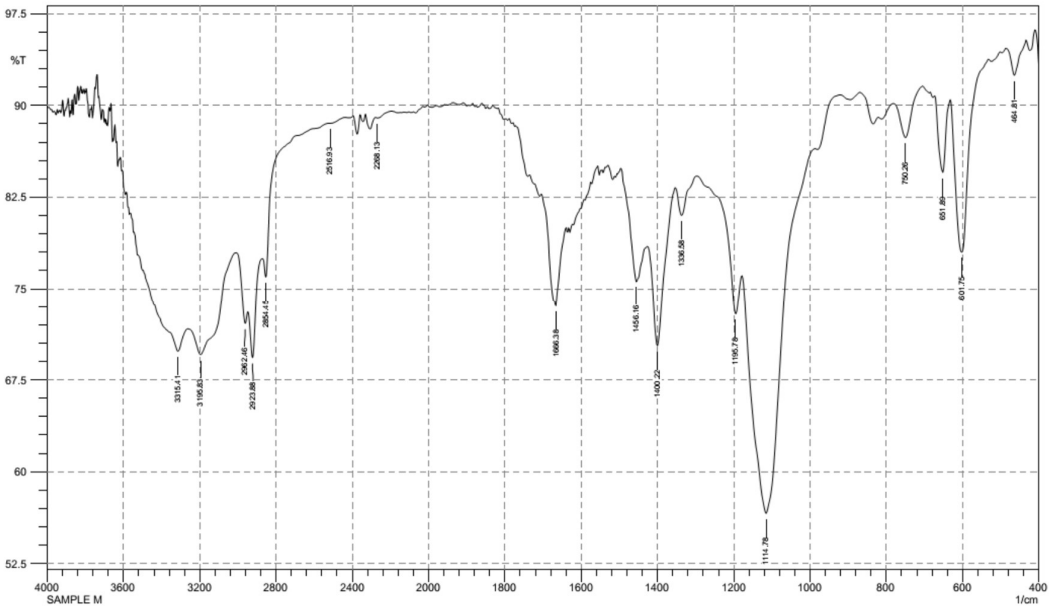

Figure 3. FT-IR spectrum of Ethanol extract.

2.6. Allium Cepa Root Cap Cells Preparation

Onion bulbs were collected from a local market. Onion outer scales were removed with the dry bottom plates of the bulbs without destroying the root primordia. A series of eight bulbs were placed in distilled water for 48 h to germinate for each separate extract with certain concentration. These glass beakers were covered with a black plastic to keep in dark (Akinboro et al., 2011) and placed in a room at room temperature. Newly grown roots were 1-2 cm in length, then the onion roots were treated with the particular leaf extracts before for 24 h for the recovery again dip into distilled water for another 24 h.

This makes exposure of root tips to distilled water as negative control. Treatment is when after exposure to negative control root tips are put in extracts for 24 h and recovery is when after this treatment, the root tips are put back into distilled water for another 24 h.

After 24 h under each exposure, 5 root tips were cut from each bulb (8 bulbs for each treatment method which makes it up to 40 root tips for each exposure), and returned for next step. The root tips were fixed in 3:1 (*v*/*v*) ethanol: glacial acetic acid and stored overnight at 4°C. They were placed in 70% (*v*/*v*) aqueous alcohol the next day and refrigerated until used. Five slides on average were made for each bulb for each treatment using root tips that were hydrolyzed in 1N HCl for 3 min. Stained root tips were squashed in acetocarmine stain. Each slide was viewed (Figure S4) at 100x using a compound microscope to determine the % MI (Rehana Bibi, et al., 2019)

2.7. Analysis of Cytotoxicity and Genotoxicity

(i) The mitotic index (MI) was calculated per 400 cells by using the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage (Balog, 1982; Sehgal etal., 2006; Al-Ahmadi, 2013).(Table 8)
(ii) Cytogenic effects were evaluated (Akinsemolu et al., 2015) (Tables 3–6).

Mitotic index % = (Total number of dividing cells /Total number of cells examined) ×

100

Table 3. Cytogenetic analysis of *A. cepa* root tip cells exposed to different concentrations of extract for Prophase.

Extract	Concentration in mg/mL	Prophase (Mean SE±)	Metaphase (Mean SE±)	Anaphase (Mean SE±)	Telophase (Mean SE±)
Control	0.1	63.33 ± 5.17	32 ± 3.51	23 ± 1.73	41 ± 2.00
Standard colchicine	0.1	147 ± 8.00	110 ± 4.16	48 ± 2.08	51 ± 2.00
ARL-Aq	0.1	96 ± 1.53	53 ± 3.21	36 ± 2.52	28 ± 2.65
ARL-EOH	0.1	73 ± 3.92	52 ± 2.65	41 ± 3.06	27 ± 1.53
ARL-Ac	0.1	147 ± 8.89	93 ± 3.51	24 ± 2.31	61 ± 3.46
ARL-EtAc	0.1	94 ± 2.31	73 ± 2.52	36 ± 2.65	30 ± 1.00
ARL-Hx	0.1	107 ± 7.21	58 ± 2.52	32 ± 2.31	44 ± 2.52
ANOVA	-	7494.67	5488.67	670.92	1564
p value	-	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Table 4. Cytogenetic analysis of *A. cepa* root tip cells exposed to different concentrations of extract for Metaphase.

Extract	Concentration in mg/mL	Prophase (Mean SE±)	Metaphase (Mean SE±)	Anaphase (Mean SE±)	Telophase (Mean SE±)
Control	1	132 ± 3.06	58 ± 2.89	53 ± 3.00	42 ± 2.08
Standard colchicine	1	127 ± 6.03	83 ± 2.65	53 ± 2.89	62 ± 2.52
ARL-Aq	1	141 ± 2.65	61 ± 1.73	54 ± 3.06	47 ± 2.65
ARL-EOH	1	111 ± 10.06	68 ± 4.51	38 ± 2.52	37 ± 6.08
ARL-Ac	1	118 ± 1.73	76 ± 3.21	53 ± 4.93	38 ± 1.53
ARL-EtAc	1	116 ± 2.52	67 ± 2.31	48 ± 2.00	52 ± 3.51
ARL-Hx	1	138 ± 2.89	73 ± 3.61	43 ± 1.53	59 ± 2.00
ANOVA	-	1828.92	1046.92	800	1466.92
p value	-	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Table 5. Cytogenetic analysis of *A. cepa* root tip cells exposed to different concentrations of extract for Anaphase.

Extract	Concentration in mg/mL	Prophase (Mean SE±)	Metaphase (Mean SE±)	Anaphase (Mean SE±)	Telophase (Mean SE±)

Control	10	55.33 ± 0.88	29 ± 1.53	21 ± 2.08	27 ± 2.65
Standard colchicine	10	110 ± 8.08	83 ± 2.65	50 ± 2.08	61 ± 3.61
ARL-Aq	10	58 ± 1.73	29 ± 2.31	26 ± 2.65	24 ± 1.15
ARL-EOH	10	51 ± 5.99	18 ± 4.51	17 ± 2.65	21 ± 2.52
ARL-Ac	10	127 ± 3.00	71 ± 4.36	52 ± 3.21	45 ± 1.15
ARL-EtAc	10	73 ± 2.52	42 ± 3.61	22 ± 1.53	36 ± 2.65
ARL-Hx	10	111 ± 3.51	73 ± 5.20	31 ± 1.73	44 ± 3.06
ANOVA	-	10,424.67	7472.25	1879.67	2515
p value	-	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Table 6. Cytogenetic analysis of *A. cepa* root tip cells exposed to different concentrations of extract for Telophase.

Extract	Concentration in mg/mL	Prophase (Mean SE±)	Metaphase (Mean SE±)	Anaphase (Mean SE±)	Telophase (Mean SE±)
Control	100	128 ± 9.02	57 ± 0.58	56 ± 2.08	35 ± 1.00
Standard colchicine	100	131 ± 5.03	84 ± 3.61	42 ± 2.08	63 ± 4.51
ARL-Aq	100	119 ± 4.91	67 ± 2.65	45 ± 3.00	38 ± 2.00
ARL-EOH	100	63 ± 5.66	29 ± 1.53	21 ± 4.16	26 ± 1.73
ARL-Ac	100	131 ± 3.51	75 ± 3.00	57 ± 4.00	40 ± 2.00
ARL-EtAc	100	111 ± 2.00	68 ± 2.89	38 ± 2.08	37 ± 4.58
ARL-Hx	100	107 ± 6.66	73 ± 5.20	58 ± 3.46	43 ± 3.21
ANOVA	-	6843.67	3896	1976.00	1372.25
p value	-	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

3. Results

3.1. Isolation of Acetogenin by Column Chromatography

Positive fraction of acetogenins with F1, F2, F3 and F0 was sent to an open column chromatography with silica gel 60 as the stationary phase. Ethanol, acetone, ethyl acetate and hexane solvents were used as eluents. All F1, F2, F3 and F0 fraction showed the positive reaction with kedde's reagent which indicated by the formation of pink to reddish color. Other fraction of solvents will not produce pink to reddish color formation after addition of kedde's reagent and the color disappears within a minute (Table 1). This indicates that polar fraction alone contain acetogenin compound. This again further confirmed by rechromatographed on TLC with silca gel 60 as stationary phase and chloroform-methanol (9:1) as mobile phase.

3.2. FT-IR Spectroscopy

The physicochemical properties of the leaf extract were studied by FT-IR spectroscopy. The infra red spectrum of Ethyl acetate crude revealed absorption bands as shown in (Figure 1).

–OH stretching absorption band found at 3313.48 and 3191.97 and 3145.68 cm^{-1} and at 2962.46 and 2923.88 and 2854.45 cm^{-1} the aliphatic C-H stretching observed. In the infrared spectrum Peak at 3450 and 2850 cm^{-1} shows presence of The OH stretching and the aliphatic -CH stretching band are aligned and appear as a broad band. Similarly, the primary amino group $–NH_2$ peak observed at 1116.71 and 1193.85 cm^{-1}. The acetyl amine peak represents at 1668.31 cm^{-1}. The spectrum shows presence of δ-CH spectrum, the γ-CH at 1400.22 cm^{-1} which is the standard spectrum of can be seen in the spectral standard wavelength 2923.88 cm^{-1}.

The FT-IR spectrum for extract of Acetone (Figure 2) showed the CH-OH major band peak at 1116.71 and 1195.78 cm^{-1}. The spectrum also shows δ-CH spectrum, the γ-CH at 1400.22 cm^{-1} which is the standard spectrum of can be seen in the spectral standard wavelength 2925.81 cm^{-1}. The peak of acetyl amine group was observed at 1666.38 cm^{-1}.

For the FT-IR spectrum of Methanol extract, the primary amino group NH_2 shows the band at 1114.78 cm^{-1}. The spectrum of δ-CH spectrum observed at 1400.22 cm^{-1} which is the standard spectrum, the γ-CH can be seen in the spectral standard wavelength 2923.88 cm^{-1}, CH-OH spectral

can be seen at 1195.78 cm^{-1}. Thus the spectral Peak is formed at the region of CH, CH-OH, -NH_2 and –OH with the bands 750.26, 651.89 and 464.81 cm^{-1} have been decreased respectively.

4. Discussion

Analyses of variance of the mitotic indices indicated significant differences ($p < 0.05$) between concentrations of *Annona reticulata* aqueous extract (ARL-Aq) as far as inhibition effect and MI was concerned (Table 7). Overall decrease in Mitotic index was contributed to by all the stages of mitosis. MI was above 75.75% in control groups which decreased to 34.25% with 10 mg/mL ARL-Aq extract exposure. The recovery rate declined as the concentration of the extract increased.

Table 7. Cytogenetic analysis of *A. cepa* root tip cells exposed to different concentrations of extract with mitotic index.

Extract	Concentration in mg/mL	Total No. of Cells	Cells in Interphase	Cell in Division	Mitotic Index %
Control	0.1	400	241	159	39.75
	1	400	115	285	71.25
	10	400	268	132	33
	100	400	124	276	69
Standard colchicine	0.1	400	44	356	89
	1	400	75	325	81.25
	10	400	96	304	76
	100	400	80	320	80
ARL-Aq	0.1	400	187	213	53.25
	1	400	97	303	75.75
	10	400	263	137	34.25
	100	400	131	269	67.25
ARL-EOH	0.1	400	207	193	48.25
	1	400	146	254	63.5
	10	400	293	107	26.75
	100	400	261	139	34.75
ARL-Ac	0.1	400	75	325	81.25
	1	400	115	285	71.25
	10	400	105	295	73.75
	100	400	97	303	75.75
ARL-EtAc	0.1	400	167	233	58.25
	1	400	117	283	70.75
	10	400	227	173	43.25
	100	400	131	254	67.25
ARL-Hx	0.1	400	159	241	60.25
	1	400	87	313	78.25
	10	400	141	259	64.75
	100	400	119	281	70.25

A significant ($p < 0.05$) inhibition effect of all three concentrations of *Annona reticulata* ethanol extract (ARL-EOH) on MI (Table S7) was observed. All phase of cell cycles contributed to the overall decrease in MI. In control group, the MI was above 63.5% which fell significantly to as low as 26.5% with 10 mg/mL ARL-EOH extract exposure. The cells gained the potential to divide when allowed to recovery in water; however, the potential decreased as the concentration increased. 100 mg/mL extract exposure the MI was 34.75 mg/mL which fell significantly mitodepressive effect as compared to control and standard.

Similarly, the study showed a significant ($p < 0.05$) decrease in the mitotic index of all three concentrations except *Annona reticulata* Hexane extract (AR-Hx) than *Annona reticulata* Ethyl acetate extract (AR-EtAc), *Annona reticulata* Acetone extract (AR-Ac), (Table 7). The reduction in the MI was significantly increased with increasing concentration of the extract. The overall decrease in MI was contributed to by all phases of cell cycles. The MI was above 60% in control group which dropped significantly ($p < 0.05$) to as low as 1% with 10 g/L *Annona reticulata* Hexane extract exposure. The recovery rate had relatively decreased as the concentration of the extract increased.

The leaf extracts were evaluated for their different phytochemical constituents. Among the four Hexane < Ethyl acetate < Acetone < Ethanol < Aqueous) leaf extracts, the ethyl acetate and methanolic leaf extracts were found to contain major phytochemicals. Phenolic compounds, flavanoids, tannins and emodols were abundantly present in methanol, acetone and ethyl acetate leaf extracts. Aqueous extracts recorded less separation of compounds from the leaf extracts. On the other hand, other components like carbohydrates, starch, quinones, cardiac glycosides, alkaloids, saponins, volatile oils and terpenoids were also found to be present in varying amounts in the different extracts of leaves (Table 2). Their studies on leaf extracts also revealed the presence of carbohydrate, fats and oils, terpenoids, flavonoids, amino acids, tannins and phenolic compounds, alkaloids, glycosides and steroids.

In the present investigation mitosis (Figure 4) was found to be normal when the Allium cepa roots were treated with distilled water (control) which clearly showed chromosomes at the metaphase stage shown in stages of mitosis (Table 4). A wide spectrum of abnormalities were recorded in the Allium cepa roots after treating with extracts of seven species of *Annona reticulata* having polar and non-polar compounds and plant extracts having polar fractions alone Plant extracts induced severe effects on cell division. The cell division was arrested and the percentage of dividing cells reduced with increase in concentration (Tables 3–6). The percentage of cytotoxicity and mitotic inhibition was found to be different in plant extracts comprising both polar and non-polar components and the plant extracts having polar compounds alone.

The present study revealed that treatment of Allium cepa root meristems with extracts containing both polar and non-polar fractions of *Annona reticulata leaves* had a detrimental effect on the test material Allium cepa. Treatment not only brought down the frequency of dividing cells but also produced a good number of anomalies in the mitotic cells. There was a marked decrease in the mitotic index (Table 7).

5. Conclusions

One of the very potent acetogenins proves to be effective in vivo models (Kedari and Khan, 2014). This could be due to chromosomal aberrations being observed mostly after prophase stage since *A.* reticulata restricted most of the cells to proceed beyond prophase. Hence, only interphase and prophase stages were seen. Hence, it can be said that *A.* reticulata has compounds which affect the proteins in the cells and hinders cell division. The most hindrance was caused by hexane extract at the highest concentration which could be having the most compounds with antioxidant properties. The leaf extracts did contain significant amount of phenols and flavonoids. Further studies could be carried out using mammalian cancer cells to encourage its consumption as a medicine.

Conflicts of Interest: The authors have not declared any conflict of interests

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