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In Vitro Evaluation of *Berberis aristata* for Cytotoxic Activity

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Abstract:
To evaluate the in vitro anti cancer activity of Berberis aristata root of chloroform, methanol and aqueous extracts against Brine Shrimp, Allum cepa root model, and MTT assay. amongst these the 'Methanol extracts' of Roots had shown significant cytotoxicity, in Three reported models, data shows consistant results and confirms the cytotoxic activity of Methanolic and other extract. preliminary phytochemical screening for detection of chemical constituents Present in them shows that it contains Alkaloids, Flavonoids, Proteins,Tannins etc. .

Keywords: Anticancer activity, Berberis aristata

INTRODUCTION.

Cancer is group of disease characterized by uncontrolled growth (division beyond the normal limits) and spread of abnormal cells (metastasis). If metastasis is not controlled, it can result in death. In the world cancer remains the major cause of morbidity and mortality and the management of cancer is also a very difficult and not up to the mark. Disease can be managed by surgical removal, chemotherapy and radiotherapy. Although numerous drugs are currently in use for cancer chemotherapy, they exhibit cell toxicity, induces genotoxic, carcinogenic, and teratogenic effects in non-tumor cells. Therefore, the search for novel drugs that are both effective and non-toxic bioactive plant products has been increased.[1]

The use of plants, plant extracts and pure compounds isolated from natural sources provided the foundation to modern pharmaceutical compounds. The well known Indian systems of medicines, namely, the Ayurveda ,

Siddha and Unani use predominantly plant based raw materials. Most of this traditional preparations and formulations have been found to be reservoir of pharmaceuticals.[2]

The majority of evidence has indicated that flavonoids by their antioxidant nature found to inhibit membrane lipid peroxidation (LPO) and intake of flavonoid containing drugs reduces the tumour burden which suggests a protective antioxidant action imparted by flavonoids. Interaction between flavonoids and ascorbic acid has also been documented. Ascorbate is reported to have flavonoid protective and flavonoid enhancing activities .In turn, vitamin C present in sub optimal concentration is stabilized by flavonoids. As flavonoids and phenolic compounds prominently available in Berberis aristata, they could be a useful strategy to improve anticancer activity.[1]

MATERIALS AND METHODS

Collection of plant material

The Roots of *Berberis aristata* were collected from a supplier and authenticated by National Institute of Science Communication Resources (NISCAIR) Delhi. The collected plant roots were dried and pulverized into coarse material. The coarse plant material was subjected to different physiochemical parameter testing.

Procurement of chemicals and Standard drugs:

All the chemicals used for experimental purpose were of laboratory grade. Distilled water was used in studies. Brine shrimp (*Artemia Salina*) eggs were purchased from Matsyakanya Aquarium, 480/58, Cannada Corner, Nasik-5, Maharashtra. and the standards drugs i.e. Cyclophoshpamide purchased from Medical Stores, Nagpur, M.S.

Extraction process:

Soxhlet apparatus was used for continuous extraction of the powdered crude drug. The material was packed in the apparatus and allowed to get extracted with hot solvent that continuously percolates from top to bottom. Condensed Fresh solvent percolates every time through the powder and is the major advantage with this technique. The powdered drug was extracted using organic solvent petroleum ether (60-80 C), methanol, and chloroform respectively for 24 hrs. The ratio of powdered to solvent was 10:100.

Petroleum ether (60-80º) extraction:

Dried powder was charged in soxhlet apparatus and first extracted with petroleum ether to remove fatty material. After the extraction process, solvent was distilled off and the extract was dried at 50 ºC. Dried extract was stored in desiccators till further use.

Chloroform extraction:

Mark obtained from petroleum ether extraction was air dried and extracted with chloroform. After the extraction process, solvent was distilled off and the extract was dried at 50 ºC. Dried extract was stored in desiccators till further use.

Methanol extraction:

Mark obtained from ethyl acetate extraction was air dried and extracted with methanol. After the extraction process, solvent was distilled off and the extract was dried at 50 ºC. Dried extract was stored in desiccators till further use.

Water extraction:

Mark obtained from ethanol extraction was air dried and macerated at room temperature. The extract was dried at 50 ºC after the maceration process. Dried extract was stored in desiccators till further use

Evaluation of Physical parameter

In Physical parameter foreign organic matter, loss on drying, ash value, Total ash, sulphated ash, acid - insoluble ash were determined.[3,4]

Determination of Extractive value

Different extractive values like alcohol soluble extractive, water soluble extractive values were performed by standard method. [3,4]

Preliminary Phytochemical analysis

The extracts were then subjected to preliminary phytochemical screening to detect the presence of various phytoconstituent. [3,4]

Determination of Antioxidant Activity:[5,6,7,8,9]

Antioxidant activity of different extracts of *Berberis aristata* roots was determined by two different in vitro methods- DPPH (1,1-Diphenyl, 2-picryl-hydrazyl) antiradical activity and Nitric oxide (No) scavenging activity.

DPPH radical scavenging

The free radical scavenging activity of *berberis aristata* roots extracts and ascorbic acid was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. 0.1mM solution of DPPH in methanol was prepared and 1.0ml of this solution was added to 3.0 ml of extract solution in water at different concentration (10-100 µ1/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using following equation:

Scavenging Effect (%) = [Abs. of control – Abs. of sample/Abs. of control] x 100

The antioxidant activity of the extract was expressed as IC_{50} value was defined as the concentration (in µg/ml) of extract that indicates the formation of DPPH radical by 50 %.

An accurately weighed quantity of extract was dissolved in respective solvents and volume was made up to 100ml with respective solvent (100 µg/ml).

The aliquot portion of stock solution of test extract solution were diluted appropriately with representative solvent to obtain a concentration range of 10 -100 µg/ml.

Nitric oxide (No) scavenging activity.

Nitric oxide was generated from sodium nitroprusside and measured by the Griess Reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generated nitric oxide, which interact with oxygen to produce to nitrite ions that can be estimated by use of Griess Reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide.

Sodium nitroprusside 5mM in phosphate buffer saline was mixed with different conc. Of each extract (10, 20, 40, 60, 80,100µg/ml) dissolved in respective solvent incubated at 25 C for 150 min. The same reaction mixture without extract but equivalent amount of ethanol served as control. At interval, sample (1.5 ml) of the incubation solution were removed and diluted with (1.5 ml) Griess Reagent (1 5 sulphanilamide, 2 % H3PO4 and 0.1 % Napthyl ethylene diamine dihydochloride). The absorbance of the chromo pore formed during diazotization of nitrite with sulphanilide subsequent coupling with NEDA was read at 546 nm. Ascorbic acid was use as a standard.

***IN VITRO CYTOTOXIC* ACTIVITY**

Model I : Brine Shrimp Lethality Bioassay (BSLB) : [10,11,12,13,14,15,16,17]

Brine Lethality bioassay was carried out to investigate the cytotoxicity of extracts of medicinal plant *Berberis aristata.* Artemia Salina L. (Artemiidae), the brine shrimp, is an invertebrate of the fauna of saline aquatic and marine ecosystems. It plays an important role in energy flow of the food chain and it can be used in laboratory bioassay in order to determine toxicity through the estimation of medium lethal concentration (LC_{50} values) which has been reported for series of toxins and plant extracts. This method, which determines the LC_{50} values of the active compound and extracts in saline medium in µg/ml has been used in research on medicinal plants carried out in different countries in order to evaluate toxicity, anticancer, and other biological actions, which in some cases have been related to pharmacological studies carried out for different chemical compounds as a screening method mainly for product of plant origin.

Procedure for BSLB:

The 150 mg of the eggs were kept for hatching in a conical shaped vessel (1L), filled with sterile artificial sea water (prepared using sea salt 38 g 1-1) and adjusted to pH 8.5 using 1N NaOH to avoid risk of death to the *Artemia larvae* by decrease of pH during incubation, under constant aeration for 72 h. After 48 hours of hatching 15ml of yeast solution 0.06% was added to vessel for every liter of salt water in order to feed larvae after 72 hours hatching takes place, active nauplii free from egg shells were collected and used for the assay.

For the plant extract, ten concentrations were tested in order to determine dose response relationship, and a control group was set with vehicle used for dilutions. Tested concentration of samples were 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml.

Ten nauplii were drawn through a glass capillary and placed in test tube containing sample, filled with 5 ml total volume of artificial sea water. Experiment were conducted along with control (vehicle treated) at above mentioned concentrations of test substances in a set of three test tubes per dose. After 24 hours, live nauplii were counted and Lc_{50} value was estimated.

Model II : Allium Cepa Root Model [18,19,20,21]:

Allium cepa root tip meristems have been widely used for the evaluation of cytotoxic activity of various compounds. Onion bulbs or garlic root tips are often used. Here, Allium Cepa root meristem model was used to evaluate the cytotoxic activity.

Growing Allium cepa meristems

Locally available Onion bulb (*Allium cepa* 50 ± 10 g) were obtained and grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm. The water was changed daily. For the root growth inhibition,. The base of each of the bulbs was suspended on the extracts inside 100 ml beakers in the dark and the length of the roots of all onion bulbs with the best growth i.e. Length and Root number at 0, 48, 96 hr. for each concentration of extract and control was measured (in cm) with a ruler. The percentage root growth inhibition in relation to the negative control and the root growth after treating with different at 48 and 96 hr.extracts was determined.

Conditions for drug Incubation

Working dilutions of all the drugs were made in tap water. Cyclophosphamide as well as different extract of drug and was used at 1 mg/ml and 10 mg/ml concentration. The bulbs with root tips grown unto 2-3 cms. were placed over different extract and incubation was carried out at ambient temperature. The length of roots

grown in drug solution (newly appearing roots not included), root number and root length were recorded at 0, 48 and 96 h and compared with that of control bulbs placed over tap water.

Observation of root :

Number of roots = n

$$\textbf{Average number of roots} = \frac{\textbf{Sum of all roots Length}}{\textbf{Number of roots (n)}}$$

Model III: 3-**(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT)[22-24]:**

Cell Proliferation activity of various extracts of *Berberis aristata* Roots carried out by MTT Assay, which estimated the effect of various extracts on the growth of cell in vitro. Measurement of cell viability and proliferation forms is used as basis for this in vitro assay.

Procedure:

Monolayer cultures was Incubated in microtitration plates in a range of drug diffrent concentrations. The drug was removed and feeded the plates daily for 2-3 PDTs, then again plates was feeded and MTT added to each well. Incubate the plate in dark and then the medium and MTT was removed. The water-insoluble MTT-formazan crystals were dissolved in DMSO, buffer was added to adjust the final pH, and recorded the absorbance.

The Methanolic extract and Water extract of *Berberis aristata* had been send for 'MTT' evaluation at "Deshpande Laboratories, Bhopal."

Further experimental analysis had been carried out, and Result had been provided by "Deshpande Laboratories".

RESULTS AND DISCUSSION

1. Physicochemical parameters:

The shed dried, crushed roots of Berberis aristata were subjected to physicochemical parameters for confirming identity and purity. The parameter evaluated were foreign organic matter (0.13 ± 0.01), total ash value (8.33 ± 1.04), Acid insoluble ash (3.06 ± 0.41), alcohol soluble extractives (11.2 ± 1.22), water soluble extractive values (10.8 ± 1.74) and loss on drying (4.67 ± 0.22) found to be with in specified limit.
Table No: 3

Table No.1: Result of physiochemical parameters

No	Physiochemical parameters(% w/w)	Mean ±S.D
1.	**Foreign organic matter**	0.13 ± 0.01
2.	**Total ash value**	8.33 ± 1.04
3.	**Acid insoluble ash value**	3.06 ± 0.41
4.	**Loss on Drying**	4.67 ± 0.22
5.	**Alcohol soluble extractive**	11.2 ± 1.22
6.	**Water soluble extractives:**	10.8 ± 1.74

2. Extraction and preliminary phytochemical screening:

Powdered roots were subjected to successive solvents extraction by using solvents such as Petroleum ether (60-80 c) .Methanol, Chloroform and Water. The percentage Value was obtained to be 0.76, 4.70, 1.64 and 5.66 percentages. Table No.4

Table No.2: Extractive values of *Berberis aristata* roots extracts

Sr.No.	Solvent	Extraction process	% value
1.	**Petroleum ether**	Soxhlation	**0.76**
2.	**Chloroform**	Soxhlation	**1.64**
3.	**Methanol**	Soxhlation	**5.66**
4.	**Water**	Maceration	**4.71**

The Extracts obtained were subjected to preliminary phytochemical screening for detection of chemical constituents Present in them. The extract contains Alkaloids, Flavonoids, Proteins,Tannins. The result of Phytochemical screening are shown in Table No.5

Table No.3: Preliminary Phytochemical analysis

Plant nstituents	Test /Reagent	Petroleum ether	Chlorofor	Methan	Water
Sterols	salkowski test	_	_	_	_
Fats and oils	solubility test	+	+	_	_
Tannins	Ferric chloride test	_	_	+	+
Flavonoids	shinoda test	_	_	+	+
	lead acetate test	_	_	+	+
Alkaloids	dragendorffs test	_	+	+	+
	mayer reagent	_	+	+	+
Proteins	biuret test	_	_	+	+
	precipitation test	_	_	+	+

3. Antioxidant Assay:

a. DPPH radical scavenging activity:

DPPH is nitrogen centered free radical that shows strong absorbance at 517nm. Deep solution changes to yellow colour in presence of DPPH radical scavengers .DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule . Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colours in the form of IC50 values. Lower IC50 values represent higher antioxidant activity. The antioxidant activity was compared with Ascorbic acid as standard.

The IC50 values of Ascorbic acid, Methanol, water, chloroform were found to be 40.19, 43.89, 47.85, and 80.65 respectively. Table no: 6 The Methanol extract has lower IC50 values among the extracts and hence maximum antioxidant activity of other extract was in the order Ascorbic acid > Methanol> Water>chloroform. Comparatively, the Methanolic extract of root has maximum antioxidant activity, as compare to other extract of Berberis aristata roots.

Table No.4: Results of DPPH radical scavenging activity of extracts

Concentration (µg/ml)	% Inhibition			
	ASC	MTH	WAT	CHE
10	35.38 ± 0.50	29.25 ± 0.96	26.99 ± 0.46	9.98 ± 1.91
20	37.99 ± 4.19	33.35 ± 4.87	32.27 ± 1.02	17.09 ± 1.40
40	49.63 ± 0.20	45.56 ± 0.97	41.79 ± 0.53	26.02 ± 0.50
60	70.81 ± 0.84	67.10 ± 0.76	58.40 ± 1.50	36.68 ± 0.42
80	84.20 ± 5.47	74.89 ± 1.67	70.32 ± 1.90	49.58 ± 0.95
100	93.61 ± 0.42	90.48 ± 2.02	83.97 ± 1.07	53.51 ± 0.82
IC_{50} Values	40.19	43.89	47.85	80.65

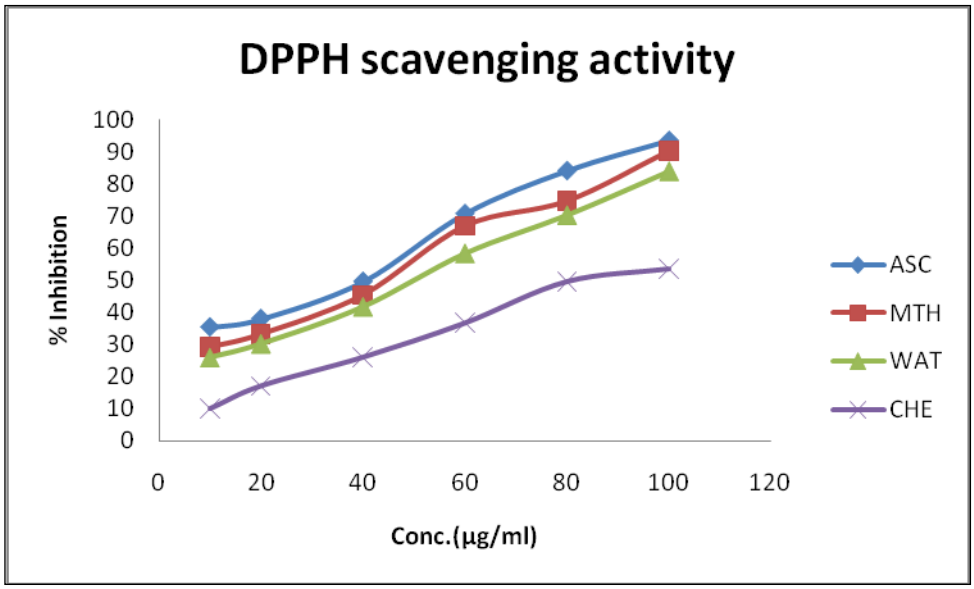


Figure:1 DPPH radical scavenging activity of extracts

b.Nitric oxide scavenging activity:

Sodium nitropruside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of Griess Illosvoy reaction. Extent of nitric oxide radical scavenged was determined by the decrease in intensity of pink coloured chromophore in the form of IC50 values. The absorbance of pink coloured chromophore was determined at 540 nm. Lower IC50 values represent higher antioxidant activity. The antioxidant activity was compared with Ascorbic acid as standard. The IC50 values of Ascorbic acid, Methanol, water, chloroform were found to be 52.57, 57.90, 84.38, 91.64.Respectively. Table no.7 the Methanol extract has lower IC50 values among the extracts and hence maximum antioxidant activity of other extract was in the order Ascorbic acid > Methanol>

Water>chloroform. Comparatively, the Methanolic extract of root has maximum antioxidant activity, as compare to other extract.

Table No.5: Result of Nitric oxide scavenging activity of extracts

Concentration (µg/ml)	% Inhibition			
	ASC	**MTH**	**WAT**	**CHE**
10	21.86 ± 1.35	8.76 ± 2.34	9.1 ± 4.18	6.43 ± 0.50
20	26.50 ± 2.54	3.40 ± 0.95	6.92 ± 2.13	0.56 ± 1.37
40	41.69 ± 3.43	8.97 ± 1.55	2.11 ± 2.27	5.02 ± 0.43
60	57.12 ± 2.42	1.81 ± 1.90	8.43 ± 1.09	1.61 ± 1.35
80	67.12 ± 2.45	3.07 ± 0.85	7.15 ± 1.89	5.97 ± 3.07
100	84.5 ± 0.5	6.91 ± 1.82	1.48 ± 0.73	4.56 ± 0.24
IC_{50} Values	52.57	57.90	84.38	91.64

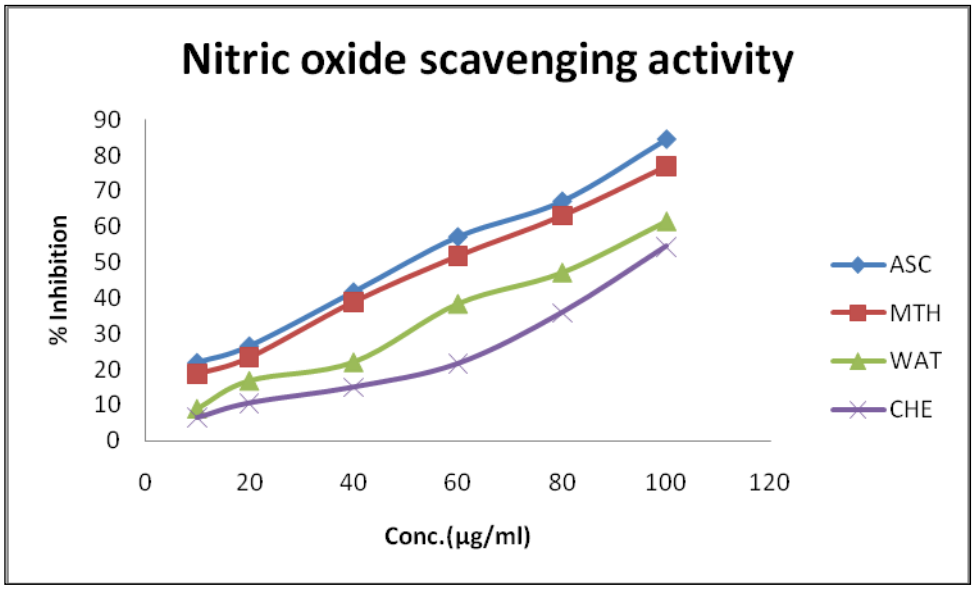


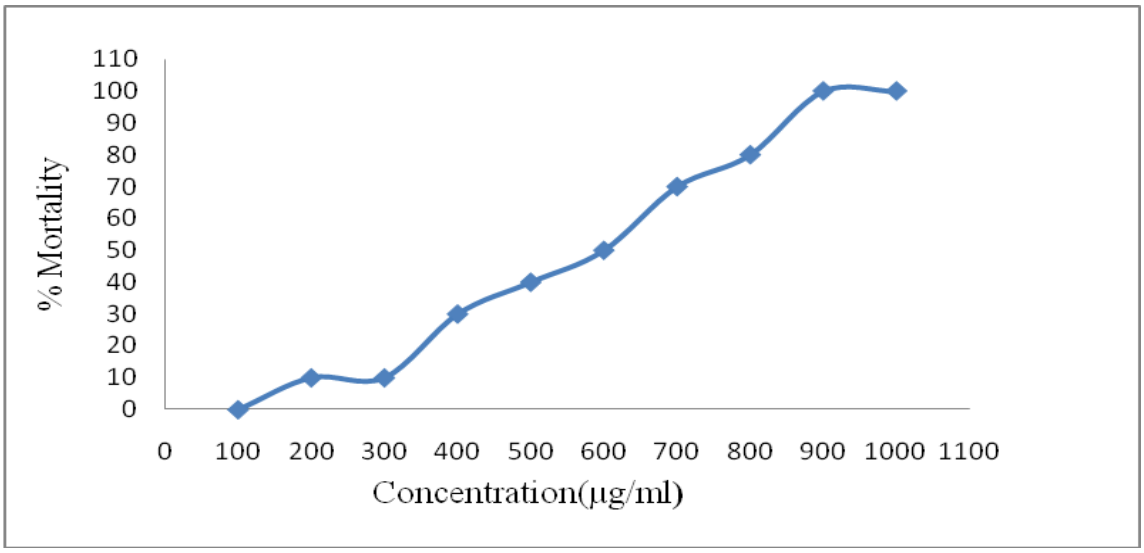
Figure: 2 Nitric oxide radical scavenging activity of extracts

3. Cytotoxic Activity:

a.Brime Shirmp Lithality bioassay (BSLP)

In the present bioactivity study, all of the extracts of berberis aristata (methanol, water, chloroform,) showed positive results indicating that the test samples are biologically active. Plotting concentration versus percent mortality (% Mortality) for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC50, the concentration at which 50% mortality of brine shrimp nauplii

occurred) were determined. LC50 value was found to be methanol 500(µg/ml), Water 500(µg/ml) and Chloroform 735(µg/ml), All the values were compared with standard cytotoxic agent cyclophosphamide, who's LC50 was found to be 250(µg/ml). Table no. 8,9,10,11,12



Firgure:3 Average mortality and LC_{50} value: Methanol extract

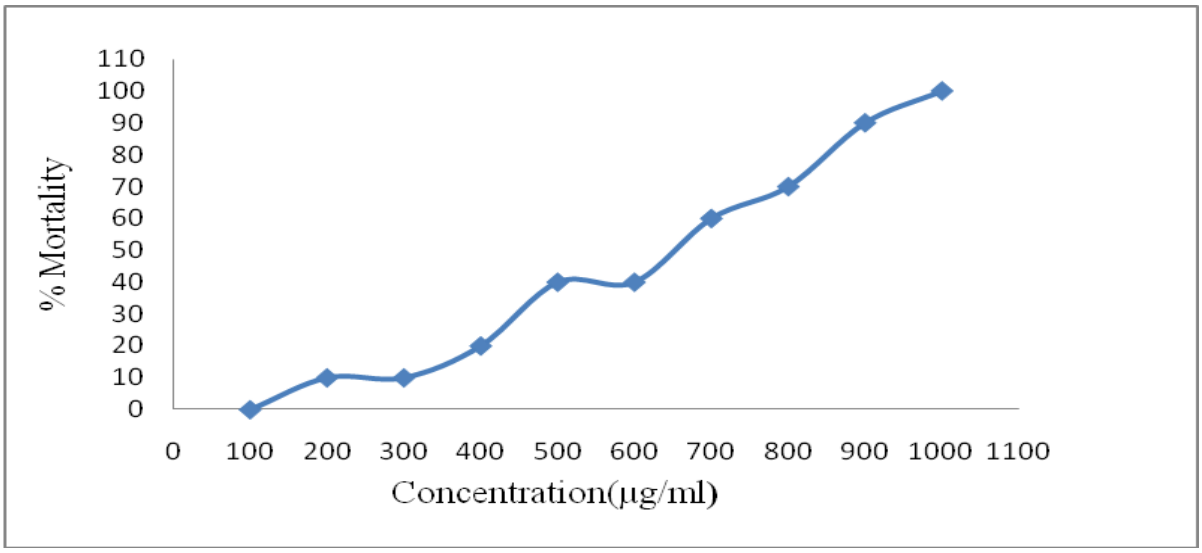


Figure: 4 Average mortality and LC_{50} value: Water extract

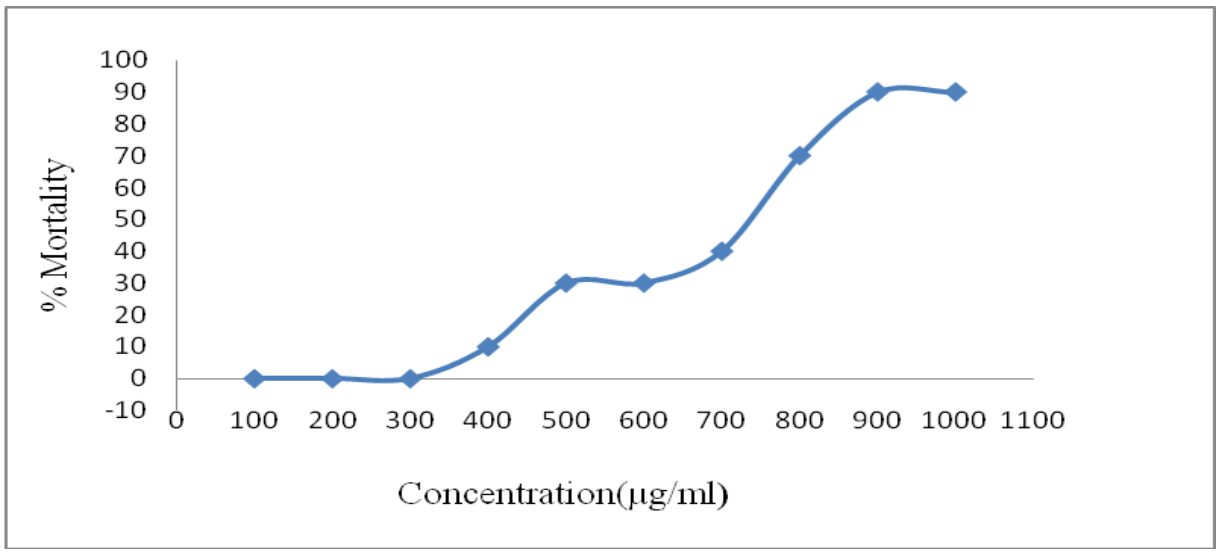


Figure: 5 Average mortality and LC_{50} value: Chloroform extract

Table No.6: Result of LC 50 Values of Respective Extract(s)

LC 50 Value of Standard (Cyclophosphamide)	250(µg/ml)
Name of Extract(s)	**LC 50 (µg/ml)**
Chloroform extract	**500**
Aqueous extract	**650**
Methanol extract	**735**

b.Allum cepa root model

Allum cepa root model have been widely used for the evaluation of cytotoxic potential. In the present study we have tested the cytotoxic effect of extracts of berberis aristata roots, with the help of standard anticancer drug cyclophosphamide.

Cytotoxic effect of berberis aristata is evident in the form of shortening and decaying of roots which has been quantified as decrease in extracts treated group compared with the control.

A progressive increase in root number and root length was observed in control group. The root length in control group shown in table no: 13 The inhibitory effect of extracts of berberis aristata was evaluated on the growth of Allum cepa root meristems and the effect was compared with standard anticancer drug cyclophosphamide.

Incubation of Bulbs in different concentration of cytotoxic agents produced a growth retardation effect that was associated with a decrease in root number. Both extracts specially the Methanolic extract and cyclophosphamide arrested the root growth.

However among the different extract of berberis aristata roots which were tested , Methanolic extract has shown the maximum growth retarding effect at 10 mg / ml when compared to standard drugs. Which is analogous to the result obtained in BSLB method. The root length after 0, 48, 96 hr. with significance at 10 mg /ml was found to be 3.75 ± 0.71 (n = 17), 2.88 ± 0.91 (n = 12), 1.97 ± 0.12 (n = 8).

Table No.7: Allum cepa root length and root number attained following incubation with different extract of *Berberis aristata* roots with concentration 1mg / ml

Sr. No.	Group	0 hrs		48 hrs		96 hrs	
	Con. 1 mg each	**ot no.**	**ot length**	**ot no.**	**ot length**	**ot no.**	**ot length**
1.	**Control**	14	34 ± 0.39	26	4.19 ± 1.08	35	4.48 ± 0.93
2.	**Methanol**	12	2.51 ± 0.77	23	2.38 ± 0.64	16	'.10 ± 0.51*
3.	**Water**	19	2.43 ± 0.71	34	2.80 ± 0.66	42	3.14±0.85
4.	**Cyclophosphamide**	15	3.52 ± 0.52	12	2.86 ± 0.28	08	1.95 ± 1.13

* indicates significant difference at P<0.05 when compared to control

Data Analyzed by one way ANOVA

Table No 8: Allum cepa root length and root number attained following incubation with different extract of *Berberis aristata* roots with concentration 10 mg / ml

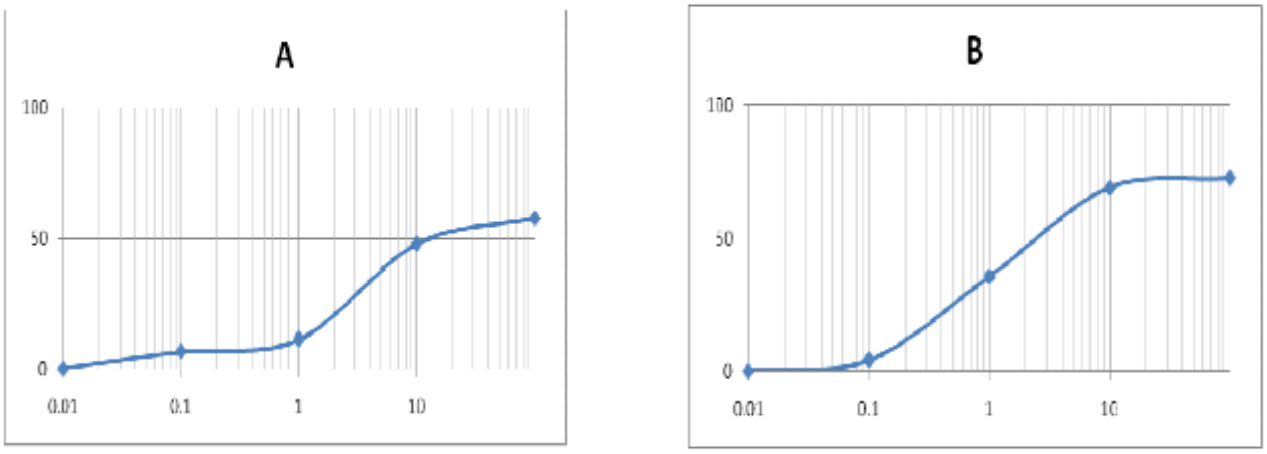
Sr. No.	Group	0 hrs		48 hrs		96 hrs	
	Con. 1 mg each	**ot no.**	**ot length**	**ot no.**	**ot length**	**ot no.**	**ot length**
1.	**Control**	15	5 ± 0.61	22	7 ± 0.64	25	1 ± 0.89
2.	**Methanol**	16	6 ± 0.64	12	7 ± 0.41	08	1 ± 0.46**
3.	**Water**	13	8 ± 0.67	13	1 ± 0.89	12	5 ± 0.58*
4.	**Cyclophosphamide**	12	2 ± 0.75	08	8 ± 0.55	04	7 ± 0.09**

c. MTT Assay:

Cell Proliferation activity of various extracts of Berberis aristata roots carried out by MTT Assay, which estimated the effect of various extracts on the growth of cell in vitro. Measurement of cell viability and proliferation forms is used as basis for this in vitro assay. The Methanol extract of roots and Water extract of roots of Berberis aristata had been send for 'MTT' evaluation at "Deshpande Laboratories, Bhopal."

Analogus to the results obtained in previous models, Methanol extract was found to be active with IC 50 value of 2.5 µg/ml. and also Water extract was found to be active with IC 50 value of 10.0 µg/ml.

Figure:3 Dose response curves for compounds against A549.



X axis – concentration in micro molar

Y axis- % growth inhibition

A – Water Extract

B – Methanol Extract

Table No. 9: IC 50 values of respective extract(s) of *Berberis aristata.*

Name of Extract	IC50	Status
Water Extract	10	Active
Methanol Extract	2.5	Active

CONCLUSION

From the preliminary screening, we have identified extract(s) of Berberis aristata stems shows cytotoxic activity by using Brine Shrimp, Allum cepa root model, and MTT assay. We have screened three different extracts (Chloroform, Methanol and Aqueous) of Berberis aristata amongst these the 'Methanol extracts' of Roots had shown significant cytotoxicity, in Three reported models, data shows consistant results and confirms the cytotoxic activity of Methanolic and other extract .

Based on the possible relationship between all these models used and plant bioactivity, this work could serve for further pharmacological research viz, isolation of constituents from the extracts and finding out the constituent(s) responsible for activity.

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