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Research Article

EVALUATION OF PHYTOCHEMICAL AND ANTICANCER POTENTIAL OF CHLOROFORM EXTRACT OF *TRICHOSANTHES TRICUSPIDATA* LOUR ROOTS (*CUCURBITACEAE*) USING IN-VITRO MODELS

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ABSTRACT

Objective**:** The present study was carried out to evaluate the anticancer activity for chloroform extract of *Trichosanthes tricuspidata* roots. The present research work had carried out on laboratory level assay to avoid the use of different animal models. Preliminary phytochemical tests of successive extraction of *Trichosanthes tricuspidata* roots powder had performed to find out the different chemical moieties as per standard procedures.

Methods: The dried roots *of Trichosanthes tricuspidata* were successively extracted with petroleum ether, chloroform and water. Chloroform extracts are subjected to in vitro Antimitotic and Antiproliferative assay by Allium cepa root inhibition and yeast model. The successive chloroform extract was subjected to in vitro anticancer activity by SRB assay PC3, MCF7 and L1210 cell lines. The antimitotic assay by onion root method was selected because this is easy to done and give fastest promising results. Onion was selected for the antimitotic assay which shows the root growth inhibition that compared with standard antimitotic drug (Methotrexate).

Result: folin – ciocalteu method for total phenolic contain and total tannin contain clears that ($CHCl_3$) extract of plant contain amount of phenolic compound i.e. contain 32 mg/gm of gallic acid and 28.5mg/gm of tannic acid respectively. The chloroform extract exhibited potent cytotoxicity with an IC50 of 42.88, From the data obtained, it was observed that $CHCl_3$ extract effective against L1210 and MCF-7 where as extract have moderate effect on PC3 comparable to the standards adriamycin.

Conclusion: Chloroform extract of *Trichosanthes tricuspidata* roots powder shows most promising anticancer activity.

Keywords: *Trichosanthes tricuspidata,* Antimitotic (Allium cepa root inhibition), Antiproliferative , Anticancer by SRB assay.

INTRODUCTION

Cancer is one of the most fatal diseases in human population and one of the most frequent causes of death worldwide. To prevent the cancer, synthetic and natural sources are used in alone or combination.

Today due to resistance of different allopathic medicines natural source is preferred mainly to block the development of cancer in human. Plant shows different chemical moiety including flavonoids [1,2,3] terpenoids [1,2,3]and steroids [1,2,3]which have the Pharmacological properties like antiulcer [1], antihyperlipidemic [2], antioxidant, cytotoxic as well.

Ayurveda is conventional medicinal systems of Indian's. Now the whole world is interested in India's *Ayurveda* and other traditional medicine systems. The demand of medicinal plants is increasing day by day in both developing as well as developed countries as a result of recognition of the non-narcotic nature, lack of side effects and easily availability of many herbal drugs. Most often the medicinal plants are collected from the wild. There are numerous data on the uses of medicinal plants. The therapeutic potential of various herbal plant have need to be explore for its medicinal use. In this present paper we have attempted to briefly summarize the information available on the potency of *Trichosanthes tricuspidata* because of its immense medicinal potential it is a very important medicinal plant.[3]

Trichosanthes tricuspidata (cucurbitaceae) generally found at an altitude of 1200 to 2300 m. It ranges from eastern Himalayas in India and southern China through southern Japan, Malaysia, and tropical Australia. In India it is a large climber. Evan though the plant is known for a large number of biological activities such as The fruits are carminative, purgative, abortifacient and violent hydragogue; useful in asthma, earache, ozoena, inflammations, epilepsy and rheumatism; cures hemicrania, weakness of limbs, ophthalmic and leprosy. Literature survey reveals lack of any scientific cytotoxic investigation of this plant. Hence in present study the plant was subjected to evaluation of antimitotic, antiproliferative and cytotoxicity on cancer cell lines.

MATERIALS AND METHODS

Plant material

Fresh roots of *Trichosanthes tricuspidata* collected from Kolhapur District, Maharashtra. The fresh roots were shade dried and ground into powder with the aid of blender and stored in air tight bottles at room temperature till use.

Physicochemical evaluation of *Trichosanthes tricuspidata* roots

Ash values: Table no. 1

Ash values are indicative to some extent of care taken in collection and preparation of drug for market and of foreign matter content of natural drug. The object of ashing is to remove all traces of organic material interfering in an analysis of inorganic elements.

The residue remaining after incineration is the ash content of the drug, adhering to it, or deliberately added to it as a form of adulteration. Many a time, the crude drugs are admixed with various mineral substances like sand, soil, calcium oxalate, chalk powder or other drugs with different inorganic contents.

Ash value is a criterion to judge the identity or purity of drug part of *Trichosanthes tricuspidata roots* was obtained by reported methods.[7,8,24]

Total Ash

This method is designed to measure total amount of material remaining after ignition. It includes both physiological ash and non physiological ash. The physiological ash is derived from plant tissue itself and non-physiological ash is residue of extraneous matter (e.g. Sand and soil) adhering to plant surface. Total ash usually consists of carbonate, phosphate, silicates, and silica.

Procedure:

2gm of accurately weighed air-dried powder drug was taken in tarred platinum crucible. Spread the drug material in fine even layer

at bottom of the platinum crucible. This platinum crucible with drug material was kept in muffle furnace for ignition at high temperature. Temperature of furnace increased gradually up to 450°C. the material was kept at this temperature for 6 hours till complete ignition of drug occurred ,that is till complete white colored ash was obtained, intermittent weighing was also done and heating continued till constant weight of crucible . Crucible was then taken out from furnace, cooled and weighed.

The total ash was calculated by subtracting the weight of crucible with ash of drug after ignition from weight of crucible with drug powder before ignition. Percentage of total ash was calculated with reference to air-dried drug.[7,8]

Acid insoluble ash

Acid-insoluble ash, which is a part of total ash insoluble in dilute hydrochloric acid, is also recommended for certain drugs . Adhering dirt, sand as well as variation caused by calcium oxalate may be determined by acid-insoluble ash content.

It is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present especially as sand and siliceous earth.

Procedure:

To the crucible containing the total ash, 25 ml of dilute hydrochloric acid was added, covered with a watch glass and boiled gently for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water until the filtrate is neutral. It was dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 30 minutes and then weighed without delay.[7,8]

Water-soluble ash

It is that part of the total ash which is soluble in water. It is good indicator of either previous extraction of the water-soluble salts in the drug or incorrect preparation. It is expressed as a minimum value.

Procedure:

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water, and ignited in a crucible for 5 minutes. The weight of this residue was subtracted from the weight of total ash.[7,8]

Extractive Values

The extractives obtained by exhausting crude drugs are indicative of approximate measures of their chemical constituents. Taking into consideration the diversity in chemical nature and properties of contents of drugs, various solvents are used for determination of extractives. The solvent used for extraction is in a position to dissolve appreciable quantities of substances desirable.

It is employed for material to which yet no suitable chemical or biological assays exist. Extracts were prepared with various solvents by standard methods. Percentage of dry extract was calculated in terms of air dried powder drug part.

Water-soluble extractive value

This method is applied to drug that contains water-soluble active constituents of crude drugs such as tannins, sugars, plant acids, mucilage, glycosides and etc.

Procedure**:**

Accurately weighed 5gm of powdered drug in the glass stopper conical flask. Macerated with 25 ml of distilled water 6 hours with frequent shaking, and then allowed to stand for 18 hrs.

After completion of 18 hours filtered the contents of flask and transferred the filtrate in tarred flat bottom porcelain dish. Then filtrate was evaporated to dryness on water bath and dried at 105°C for 6 hours cooled in desiccators for 30 min and weighed. Calculated content of extractable matter in milligrams per gram of air dried material.[7,8]

Alcohol-soluble extractive values

Alcohol is an ideal solvent for extraction of various chemicals like tannins, resins etc. therefore

Procedure:-

Accurately weighed 5 gm of powdered drug placed in the glass Stoppard conical flask and macerated with the 25 ml of ethanol (95%) for 6 hour with frequent shaking, mixture allowed to stand for 18 hours.

After completion of 18 hours, filtered rapidly taking care not to lose any solvent. Transferred the filter in the tarred flat bottom porcelain dish. Filtrate was evaporated to dryness on water bath, dried at 105°C for 6 hours cooled in desiccator for 30 min and weighted. Calculate content of extractable matter in milligram per gram of air dried material.[7,8]

Qualitative Tests for Determination of Inorganic Elements

Total ash was prepared, as per method mentioned above and added with 50 **%** ^{V}/vHCl and kept for 1hour, filtered. The filtrate was taken to perform qualitative tests listed below. Table no.2,

Extraction

Hot continuous extraction, Soxhlet process was used for the extraction of the plant material with solvent Solvents were choose according to increasing order of its polarity like petroleum ether and chloroform. In Cold maceration procedure water is use as a solvent. For experimental study chloroform extract ($CHCl_3$) of roots of plant were used. Shown in table no.3

Preliminary Phytochemical Analysis of chloroform extract ($CHCl_3$) of roots

Phytochemical analysis was performed using the methods described by Harbone (1973) and Trease and Evans (1983) were used to identify glycoside (cardiac glycoside), alkaloids, tannins and phenolic compound, triterpenoid, steroids and proteins. [9,10,23] shown in table no. 4

Determination of Total Phenolic content of chloroform extract

The amount of total phenolics in ($CHCl_3$) extracts of roots was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). Concentration of 20,40,60,80 and 100 mcg/ml of gallic acid were prepared in ethanol. Concentration of 0.1 mg/ml of plant ($CHCl_3$) extract were also prepared in ethanol and 0.5ml of each sample were introduced into test tubes and mixed with 2.5ml of a 10 fold dilute Folin- Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was read at 760nm spectrometrically. All determinations were performed in triplicate. The Folin-Ciocalteu reagent being sensitive to reducing compounds including polyphenols is producing a blue color upon reaction which is measured spectrophotometrically. [3,4] Shown in fig. no.1

Determination of Total Tannin content of chloroform extract

The tannins were determined by Folin and Ciocalteu method. Concentration of 20,40,60,80 and 100 mcg/ml of tannic acid were prepared in ethanol. 0.1mg/ ml of the sample extract was added with 7.5 ml of distilled water and adds 0.5 ml of Folin Phenol reagent, 1 ml of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid is treated in the same manner as described earlier and read against a blank. The results of tannins are expressed in terms of tannic acid mg/g of extract.[5,6] Shown in fig. no.2

In-vitro Anticancer Activity *Trichosanthes tricuspidata*

Antimitotic activity

***Allium cepa* Root Tip Meristem Model**

Locally available onion bulbs (*Allium cepa* 50 ± 10 g) were grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm length. The base of each of the bulbs were suspended on the extract inside 100 ml beakers, root length (newly appearing roots not included) and root number at 0, 48, 96 hrs for each concentration of extract and control was measured. The percentage root growth inhibition after treating with Chloroform extract at 48 and 96 hrs was determined. Methotrexate (standard) as well as extract of roots was used at 10 mg/ml concentration . The extract of Trichosanthes tricuspidata roots produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of extract and standard produced a growth retarding effect that was associated with a decrease in the root number [7,20]. Table no.5, fig. no. 3

Antiproliferative activity

Antiproliferative study was evaluated by yeast Saccharocymes cerevisiae model according to the previously reported method of Julian et al .

1. Preparation of Yeast inoculums

The yeast was inoculated with sterilized potato dextrose broth and incubated at 37 ^{o}c for 24hrs, referred as seeded broth. The seeded broth diluted with sterilized distilled water, in order to get 25.4x10^{4}cells (average).

2. Cell viability count

For cell viability count solution containing 2.5 mL of potato dextrose broth and 0.5 mL of yeast inoculums were prepared in four separate test tubes. In the first and second tubes, 1 mL of Chloroform extract and Water extract mixed while third tubes standard Methotrexate was added. The fourth tubes kept control without extract. All tubes were incubated at 37 ^{o}c . In the above cell suspension, 0.1% methylene blue dye was added in all tubes and they were observed under low power microscope. The no. of viable cells, those does not stain and look transparent with oval shape while dead cells get stained and appeared blue in colour were counted in 16 chambers of hemocytometer and the average no. of cell was calculated. [16]Fig no.6

Cytotoxicity study by SRB assay

Principle

Sulphorodamine B (SRB) is a bright pink Aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37oC for 72 hours in 5% CO2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4oC for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10mM Tris base was then added tothe wells to solubilise the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540nm.[17,18,19]·[21,22]Table no.7 The percentage growth inhibition was calculated using following formula, The percentage growth inhibition was calculated using following formula,

$$\%\text{cell inhibition} = 100-\{(Ti-Tz)/ (C-Tz)\}x100$$

Where, Ti = Absorbance value of test compound

Tz = Absorbance value of blank

C =Absorbance value of control

RESULT AND DISCUSSION

Qualitative Tests for of Inorganic Elements were conclude that roots contain magnesium chloride, nitrate and aluminium.

Phytochemical investigations clears that Trichosanthes tricuspidata roots extract contain Carbohydrate, proteins, Glycosides, alkaloids, steroid, triterpenoid, tannin and phenolic compound.

folin – ciocalteu method for total phenolic contain clears that plant contain large amount of phenolic compound i.e. ($CHCl_3$) extract contain 32 mg/gm of gallic acid.

folin – ciocalteu method for total tannin contain clears that plant contain large amount of tannin compound i.e. ($CHCl_3$) extract contain 28.5mg/gm of tannic acid.

To the best of our knowledge we are the first which exploring the anticancer properties of this plant. Cancer is a disease recognised by seven hallmarks: unlimited growth of abnormal cells, self sufficiency in growth signals, insensitivity to growth inhibitors, evasion of apoptosis, sustained angiogenesis, inflammatory microenviroment, and eventually tissue invasion and metastasis . A survey of the literature revealed that no studies on the anticancer activity of extracts of roots of plants was carried out hence above mentioned, had been undertaken on the three human cancer cell lines. It is known that different cell lines might exhibit different sensitivities towards an anticancer compound, so the use of more than one cell line is therefore considered necessary in the detection of anticancer compounds. From the data obtained, it was observed that both $CHCl_3$ extract and water extract effective against L1210 and MCF-7 where as extract have moderate effect on PC 3 comparable to the standards adriamycin.

CONCLUSION

Chloroform extract of *Trichosanthes tricuspidata* roots powder shows most promising anticancer activity.

Table 1: Selection of appropriate extraction method for plant material

S. No.	Solvent	Percentage of Extracts
1.	Pet ether extract	2.32 % w/w
2.	Chloroform extract	5.59 % w/w
3.	Water extract	6.22 % w/w

Table 2: Ash value and extractive value

Ash value (%)	Total ash	5.32
	Acid insoluble ash	1.67
	Water-soluble ash	4.25
Extractive value (%)	Water soluble	12.53
	Alcohol soluble	10.07

Table 3: Qualitative tests for inorganic elements

S. No.	Element	
1	Calcium	-
2	Magnesium	+
3	Sodium	-
4	Potassium	-
5	Iron	-
6	Sulphate	-
7	Phosphate	-
8	Chloride	+
9	Carbonate	-
10	Nitrates	+
11	Zinc	-
12	Aluminium	+

(+ Presence, - absence)

Table 4: chemical test of extracts - phytochemical screening of different extract of roots

S. No.	Name of Chemical Test	$CHCl_3$ Extract
A.	**Test for Carbohydrate**	
1)	Molish test	-ve
B.	**Test for Proteins**	
1)	Biuret test	+ve
2)	Xanthoproteic test	+ve
C.	**Test For Steroids**	
1)	Liebermann burchard reaction	+ve
2)	Rosenheim test	+ve
D.	**Test for triterpenoid**	
1)	Salkowski test	-ve
E.	**Test for Glycosides**	
1)	Cardiac Glycosides	-ve
2)	Anthraquinone Glycosides	-ve
3)	Saponin Glycosides	-ve
F.	**Test for flavonoids**	
1)	Shinoda test	
G.	**Test for alkaloids**	
1)	Dragendroffs test	+ve
2)	Hager's test	+ve
3)	Wager's test	+ve
H.	**Test for tannins & phenolic compounds**	
1)	Lead acetate solution	+ve
2)	Ferric chloride	+ve

+ve: indicates presence of constituents; -ve: indicates absence of constituents

Table 5: Observations for *Allium cepa* root length and root number attained following incubation with Chloroform extracts of *Trichosanthes tricuspidata* roots and standard drugs.

S. No.	Type of plant extract	Findings of phytochemical screening.
1	Chloroform extract	proteins, steroid, alkaloids, tannin, phenolic compound

Allium cepa bulbs showing the effect of different extracts of roots of *Trichosanthes tricuspidata* on root length following 96 h of incubation.

Groups	Concentration	Roots length in (cm)		
		0 hr	48 hr	96 hr
Control (Water)	--	3.33 (n=16)	4.12 (n=22)	4.63 (n=28)
Chloroform Extract	10 mg/ml	2.56 (n=18)	2.33 (n=14)	2.23 (n=11)
Std (Methotrexate)	10 mg/ml	2.32 (n=13)	2.29 (n=17)	2.15 (n=21)

Table 6: Result of Antiproliferative activity

Sample	IC_{50} (mg/ml)
Methotrexate	17.89
Chloroform extract	42.88

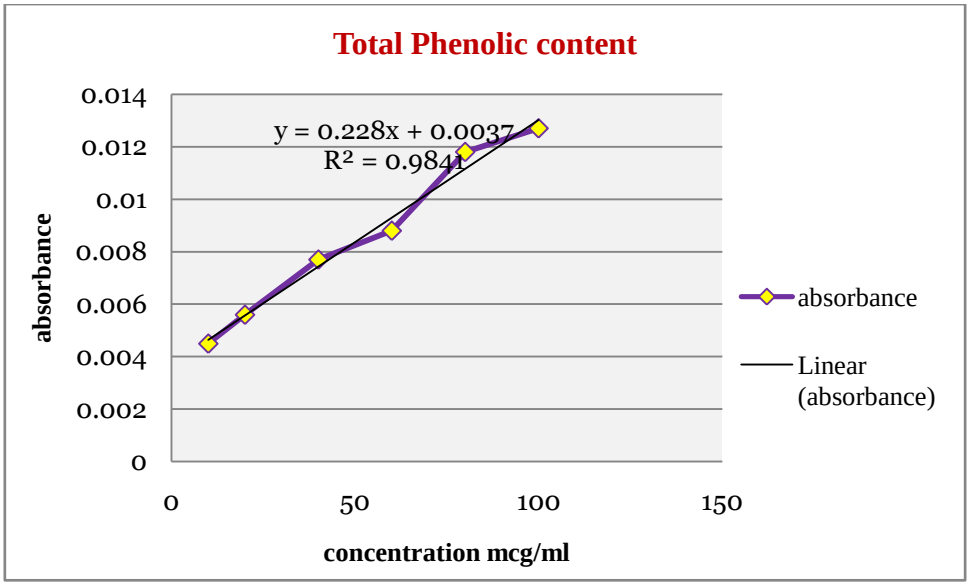
IC_{50} value of $CHCl_3$ extract on Antiproliferative activity

Table7: Cytotoxicity study by SRB assay GI50 value of Sulforhodamine B (SRB) assay on different cell lines

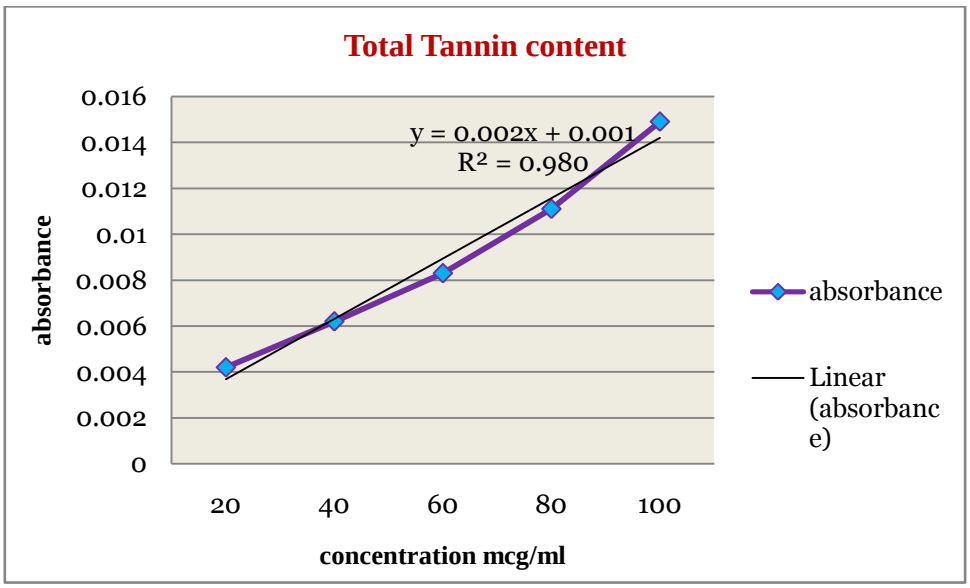
Tissue of origin	Cell lines	Concentration (mcg/ml)		
		$CHCl_3$ extract	Compound separated by pH gradient of $CHCl_3$ extract	Adriamycin
Human Breast	MCF-7	11.6	27.0	<10
Human Prostate	PC 3	30.8	40.9	<10
Murine Leukemia	L1210	30.5	25.4	48.8

Note: The in vitro testing for Anticancer Activity in cell lines were tested in TATA MEMORIAL ADVANVCED CENTRE FOR TREATMENT, RESEARCH AND EDUCATION IN CANCER (ACTREC)

- All GI50 values are averages of 3 experiments.
- Compound with GI50 values of ≤ 10^-6 (i.e. 1 µmole) or ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 20µg/ml is considered to demonstrate activity.
- Growth inhibition of 50 % (GI50) calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, drug concentration resulting in a 50% reduction in the net protein increase
- Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50% cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = -50.

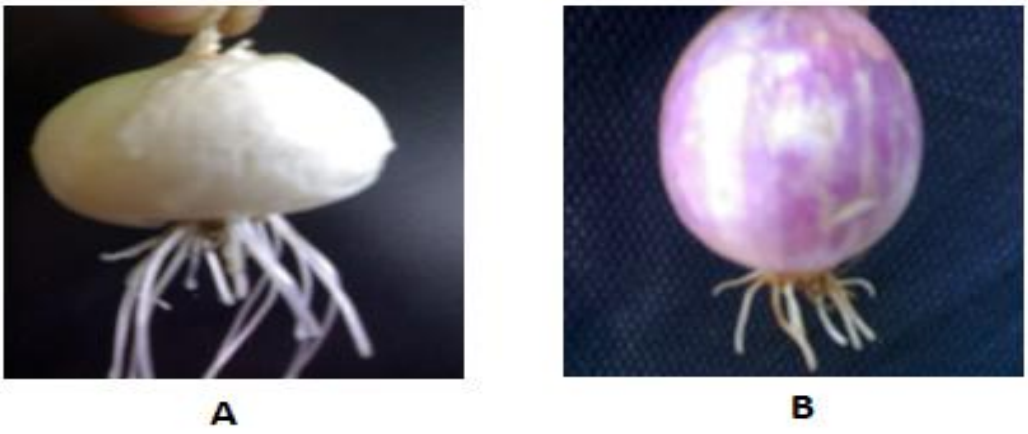


(a)



(b)

Fig. 1: (a) Results of Total Phenolic content (b) Results of Total Tannin content



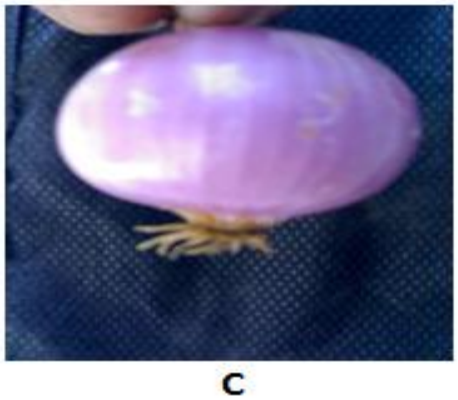


Fig. 2: Result of Antimitotic activity

Allium cepa bulbs showing the effect of chloroform extracts of roots on root length following 96 h of incubation.

(A) Control (water) (B) Chloroform extract (C) Std (Methotrexate)

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