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Comparative cytotoxic and phytochemical analysis of aqueous and ethanolic extract of *Manihot esculenta* Crantz raw root tuber

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Abstract
Medicinal plants exist as the backbone of traditional medicine since Vedic times. Cassava (*Manihot esculenta* Crantz,) is drought resistant staple food in subtropical and tropical regions and which contain starch in higher amount. The high starch content of cassava root is responsible for its suitability in wide range of food industries, textile, pharmaceutical, detergent, and feed industries. Recent investigations showed that many plants used as food or traditional medicine have cytotoxic and genotoxic effects and mutagenic effects *in vitro* and *in vivo* assays. This raises concern about the potential genotoxic mutagenic or hazards resulting from the long-term use of these plants.
Even though *Manihot esculenta* Crantz, root tuber is used in medicine and as food, because of its neurotoxic effects in animals, it is essential to study the cytotoxicity also. However, cyanogenic glycosides secreted in cassava produce hydrogen cyanide and are accounting for its safety concerns and toxicity. From this experiment it has been concluded that *Manihot esculenta* Crantz root tuber raw has high cytotoxic activity. From this it is clear that *Manihot esculenta* Crantz, root tuber extract has mito-depressive activity on cell division. As the concentration of extract increases mito- depressive activity also increases. As the concentration decreases, the cytotoxicity also decreases. So as a food or medicine it can be used in very low concentration. From the phytochemical analysis it is clear that the extract of *Manihot esculenta* Crantz, root tuber raw contain Protein, carbohydrates, Alkaloid, Flavonoids, Glycosides etc.

Keywords: Cassava, root tuber, cytotoxic, genotoxic, cyanogenic glycosides

Introduction

Plants are a silent but vital part of life on Earth. Medicinal plants as well as medicinal herbs have been identified and are used since ancient times. Herbs have played a fundamental and essential role in the human's life due to both their nutritive and curative properties. It improves the sensory characteristics of food also. (Nidal Jaradat et.al.2021) [8]. Medicinal plants are the backbone of traditional medicine and more than 3.5 billion people in the world use medicinal plants on a regular basis. But there is an end for the blind dependence on chemicals and people are returning to herbals with the hope of security and safety (Davidson, 2000).

The results from the WHO global survey on traditional, alternative, and herbal medicines showed that the market for these medicines is growing worldwide. Recently, many people have been using these formulations in different national healthcare systems. Moreover, many patients often use herbal medicines to complement treatment with conventional medicines. (Bustanji, et.al.2010) [2]. Medicinal herbs have been used in folk medicine for millennia. It is known that green plants in general are a primary source of natural toxic agents as well as antimutagens, and many plants contain cytotoxic and genotoxic substances. (El-Sharkawy, 1993) [4]. Recent investigations have revealed that many plants used as traditional medicine or as food in have mutagenic effects, genotoxic effects and cytotoxic and *in vitro* and *in vivo* assays. This raises concern about the potential genotoxic hazards or mutagenic resulting from the long-term use of such plants. Many plants contain carcinogenic substances and their use has been correlated with high rate of tumor formation. (Tülay Aşkin Çelik* and Ozlem Sultan Aslanturk, 2010) [14].

Currently there are thousands of species of plants with with therapeutic properties a high diversity of chemical compounds. During the last decades, a great interest was given to medicinal plants that are used in folk medicine for cancer treatment, leading to discoveries of many antitumor agents (Prasad R, Koch B, 2014) [10]. An important example is, Cassava (Manihot esculenta Crantz,) is drought resistant staple food in subtropical and tropical regions and containing starch in higher amount.

Cassava starch, being abundantly available and cheap, is capable of providing food security of the world. High starch content of cassava root accounts for its suitability in wide range of pharmaceutical, detergent, textile, plastic industries, feed industries and food industries (Uchechukwu-Agua et.al., 2015) [15]. Cassava secreted Cyanogenic glycosides for self-defense produce hydrogen cyanide on hydrolysis and are accounting for its safety concerns and toxicity. (Anil Panghal et.al. 2019) [1]. However, a limiting characteristic for the consumption of cassava is its content of cyanogenic glycosides which release cyanide. Cyanide inhibits several cellular enzymes, including cytochrome oxidase, a key enzyme in the cellular respiratory chain (Sharma et.al. 2010) [13].

Allium cepa assay is used to initiate the search for drugs from plants. For the screening of environmental mutagens and carcinogens, the antimitotic activity of the extracts was tested by the *Allium cepa* root meristem assay. This is considered widely as a practical and reliable system. The patterns of divisions in onions cells and animal somatic cells are similar, an extract which is able to inhibit the cell

division in *Allium cepa* root cells, will be effective in human or animal cells. Thus, it is possible that chemicals that affect plant chromosomes will also affect the chromosomes of animals also. Hence, these meristematic cells of plants can be used for preliminary screening of antimitotic or anticancer activity of extracts. (Fiskc - jo et. al., 1985).

The extracts are caused to identify different kinds of chromosome aberrations in dividing and non-dividing cells of *Allium sepa* such as micronucleus, non congression metaphase multinucleated cells in the interphase stage, bridges, laggards, stickiness, polyploidy and disturbed anaphase etc. (Jaradat N et.al.,2017) [7]. The main compounds found in plants correspond to four major biochemical classes: Polyphenols, glycosides, terpenes, and alkaloids. Plants synthesize these compounds for a variety of purposes, including protection of the plant against fungi and bacteria, defense against insects and attraction of pollinators and dispersal agents to favor the dispersion of seeds and pollens. (Nieto G., 2020) [9].

The main aim of our study was to evaluate the extracts or their toxic effects and primary and secondary metabolites present, before it can be used for applications that are of importance to the human being.

Objectives of the study

- To compare the chromosomal abnormalities of onion root tip (*Allium cepa* L.) in different concentrations of ethanol and aqueous extract of *Manihot esculenta* Crantz root tuber, Raw.
- To know about the phytochemical composition of *Manihot esculenta* Crantz root tuber, raw in ethanolic and aqueous extract.

Materials and Methods

Materials used

Manihot esculenta Crantz

Systematic position:

Kingdom: Plantae Order: Malpighiales Family: Euphorbiaceae Genus: Manihot Species: *M. esculenta*

Manihot esculenta Crantz, commonly known as bitter cassava, tapioca, yuca or manioc is a milky- sapped tropical shrub. Shrubs; bark smooth, yellowish grey. Leaf- lobes 7-17 x 2.5-5.5 cm, oblanceolate, apex shortly acuminate; petiole 12-23 cm long, light greenish to red. Male flowers: perianth greenish- white with reddish-white bands inside; yellow anthers; disc yellow to light orange. Female flowers: green perianth with red margin; disc pink; ovary with 6 longitudinal ridges. Capsule sub globose, 3-winged when young, smooth later. Seeds up to 12 mm long. (indiabiodiversity.org).

***Manihot esculenta* Crantz - as food:** Manihot esculenta Crantz have been a source of food and medicine for many people who live in proximity to these plants. These plants are well-known to be highly poisonous and must be carefully processed to remove toxins, before they are edible. If they are not properly processed, they can cause liver damage, vomiting, and even death, there is also evidence they have neurotoxic effects.

***Manihot esculenta* Crantz – as medicine:** Yet, it is known that cassava contains flavonoid glycosides and alkaloids with medicinal values as well as fiber which can be translated for medical nutrition therapy management of diabetes and its cardiovascular complications including heart disease (Padmaja et.al. 1995) [11].

Materials used for cytotoxic experiments

Hydrochloric acid solution (HCl), Ethanol, Acetocarmine stain solution, Forceps, Microscope slides, Onions bulbs (*Allium cepa* L.), Water, Paper towels, Compound microscope, Pipettes, Scalpel, Cover slips, etc.

Methods used for cytotoxic experiments

Extraction: Collected tubers of *Manihot esculenta* Crantz, raw were shade dried and ground into fine powder. 50 gm dried powder of seed was mixed in 300 ml of ethanol and in water in Soxhlet extractor. Kept the extract in Soxhlet extractor for 5 days. After these days, the extract was poured into Petri dishes equally and dried. Prepared different concentrations like 20%, 40%, 60%, 80% and 100% of the dried extract. Took a set as control.

Preparation

Actively growing onion root tips are required for this activity. Allow at least 2–4 days for new roots to grow. To grow root tips, obtain 5–6 onion bulbs. Remove any dried, old root growth from the bottom of the bulbs. Place each onion bulb into a wet sand which is sterilised. After two days take out the onions, wash with distilled water and then put into solution of different concentrations like 20%, 40%, 60%, 80%, 100%, control respectively. After two hour the onions taken out and did cytotoxicity experiment.

Procedure

1. Cut roots from onion plant using a scalpel.
2. Trim the tip of each root to 1 cm; use only the tapered end of the root tip.
3. Use forceps to place 2–3 root tips (use only the 1-cm tips) on a glass microscope slide.
4. Place them in a solution containing Hydrochloric acid and ethanol in 1:1 concentration
5. Allow the root tips to soak in the mixture for 1 minutes.
6. After one minute, Put the material in water and wash off the solution.
7. Place the root tip on a slide. Use a paper towel to blot away excess water.
8. Using a clean, graduated pipette, add 2–3 drops of Aceto carmine stain to the root tip.
9. Allow the root tips to soak in the stain for 3 minutes.
10. Use a paper towel to blot away excess stain.
11. Place a cover slip on the root tissue. Gently squash the root tissue.
12. Using low magnification on the microscope, focus on the root cells. Switch to medium power or high power as necessary to easily visualize the inside of the onion root cells.
13. Study all of the squashed tissue to locate cells in each stage of the cell cycle.

Repeat same procedure for all concentrations. (Udo, 2015) [16]

a. Preparation of crude extract in ethanol and water

Solvent extract *Manihot esculenta* Crantz was prepared in ethanol and water (solvents) at room temperature by Soxhlet extraction method.

b. Mitosis in onion root tip

An onion (Allium cepa L.) cell possess eight chromosomes. It belongs to Liliaceae family. Cell division occurs rapidly in growing root tips of sprouting seeds or bulbs.

The most commonly used root tips in labs to study mitosis are onion, wheat, lentil, barley and alfalfa. An onion root tip is a rapidly growing part of the onion and thus many cells will be in different stages of mitosis. The onion root tips can be prepared and squashed in a way that allows them to be flattened on a microscopic slide, so that the chromosomes of individual cells can be observed easily. The super coiled chromosomes during different stages of mitosis present in the onion root tip cells can be visualized by treating with DNA specific stains, Acetocarmine stain.

Mitotic index

The percentage of cells undergoing mitosis or it is defined as the ratio of no. of cells in the dividing phase to the total number of cells observed. This will help to identify the region of most mitotic activities. Mitotic index helps us to quantify the cell division. The meristematic region in the root tip is the actively growing region and thus the mitotic index is high.

Mitotic index = n/N × 100

N: Total number of dividing cells observed

N: Total number of cells in the field of microscope

Mitotic index is used to quantify the differences in cell division when environmental parameters are changed. (Sadaqa et.al. 2016) [12]

Different types of Chromosomal Aberrations

Nuclear lesions, Chromosome Bridge, Ring chromosome, Micronuclear interphase, Chromosome clumping, Strap shaped nuclei, Polyploid cells, disoriented metaphase, Leggad, Disturbed metaphase, Disturbed anaphase, Disturbed telophase etc.

Preliminary Phytochemical analysis

Detailed preliminary phytochemical analysis was done for the ethanol and water extract of root Tuber of Manihot esculenta Crantz, Raw. The details are as follows:

Plant materials used

The tuber of Manihot esculenta Crantz are the selected as material for the study. They were collected from various localities of Kannur District. They were shade dried at room temperature for 30-40 days. Later they were made into fine powder. This powder was stored in dry and airtight container and used for further studies.

Chemicals used

Dragendorff's reagent, distilled water, Sodium Hydroxide, Ferric chloride, concentrated Sulphuric acid, concentrated Hydrochloric acid and glacial Acetic acid were used for the chemical analysis.

Methods

a. Preparation of crude extract in ethanol

Solvent extract was prepared in ethanol and water (solvents) at room temperature by Soxhlet extraction method. Collected *Manihot esculenta* Crantz Raw was shade dried and ground to fine powder. 50 gm dried powders of seed Manihot esculenta Crantz Raw was mixed in 300 ml of ethanol and water in Soxhlet extractor. Took extract by keeping for 5 days.

b. Preliminary Phytochemical analysis

The primary and secondary metabolites present in Manihot esculenta Crantz Raw in aqueous and ethanol were identified by following preliminary phytochemical analysis.

1. Test for Carbohydrates

Molisch's Test: In a test tube, add 2 ml of the test solution and 2 drops of α-naphthol solution. Carefully incline the tube and pour dropwise conc. H2SO4, using a dropper, along the sides of the tube. Observe the violet color at the junction of the two liquids would indicate presence of Carbohydrate.

2. Test for Proteins

Add 1 ml of sodium hydroxide solution and 1% copper (II) sulphate solution dropwise (one drop at a time) to the sample. They range from no color change (blue) to pink to deep violet would indicate presence of Protein.

3. Test for Alkaloids

To 1ml. of each extract in separate test tubes 2-3 drops of Dragendorff's reagent was added.

An orange red precipitate or turbidity would indicate presence of alkaloid.

4. Test for Flavonoids

Alkaline reagent test: Two to three drops of sodium hydroxide were added to 2 mL of extract. Initially, a deep yellow color appeared but it gradually became colorless by adding few drops of dilute HCL, indicating that flavonoids were present.

5. Test for Glycosides

Keller Kiliani test: To the 2ml. of extracts, 1ml. of glacial acetic acid with ferric chloride and concentrated sulphuric acid was added. The appearance of blue colour indicates the presence of glycosides.

6. Test for Tannins

2ml. of each extract was diluted with dilute water in separate test tubes and 2-3 drops of 5% ferric chloride solution was added. A green black or blue-black coloration indicates presence of tannins.

7. Test for Saponins

One ml of each extract was taken in separate test tubes. And 5ml. of distilled water was added and vigorously shaken. A persistent froth indicates presence of saponin.

8. Test for Phenols

5ml. of concentrated extracts were taken and 2ml. of neutral ferric chloride solution was added. Appearance of violet color indicates presence of Phenol. (Harborne, 2005) [6]

Results and discussion

Result of cytotoxic analysis

***Manihot esculenta* Crantz root tuber, Raw in Ethanol solution**

Mitotic index of control set of onion root tip (in pure water) is 48.5. 20% concentration shows a decrease in mitotic

index i.e., 40.5. 40% concentration shows a decrease in mitotic index i.e., 35.4. 60% concentration shows a decrease in mitotic index i.e., 28.6. 80% concentration shows a decrease in mitotic index i.e., 14.5 and 100% concentration shows further decrease in mitotic index 6.8 As the concentration of solution increases the mitotic index also decreases. From this it is clear that *Manihot esculenta* Crantz, root tuber extract has mito-depressive activity on cell division. As the concentration of extract increases mito-depressive activity also increases.

In control only a very little chromosomal aberration is noticed. Only nuclear lesions are visible. The percentage of abnormality is very low. But in 20% concentration, there is high inter phase abnormalities like Lesions, Strap shaped nucleus, etc. Prophase abnormality and metaphase abnormality are also prominent like Clumping, Polyploid nuclei, Disorientation etc. In anaphase chromosomal bridges are also observed. The most prominent abnormality in anaphase stage wasthe formation of chromosome or Chromatid Bridge. Such bridges sometimes persisted during telophase. The separation of the daughter chromosomes to the polar became incomplete and remained connected together by these chromatic bridges.

In 40% concentrated solution, inter phase and prophase abnormalities are at higher rate. Lesions, Strap shaped nucleus, micronuclei can be observed. Chromosomes fragmentation was induced and was quite clear at interphase as micronuclei. The formation of unbalanced daughter nuclei leading to somatic instability. Extract involves disruption of the spindle apparatus or interference with normal chromatid separation. In interphase stage the occurrence of micronuclei and multinuclei were frequently noticed. The production of micronuclei may be as a result of laggard chromosome fragments which may be surrounded by nuclear membrane, such micronuclei may be persisted in the following prophase. Metaphase clumbing and polypoid cells can be observed. But anaphase abnormalities could not be observed.

In 60% concentrated solution, almost every types of abnormalities are observed. In 80% concentration, anaphase and telophase abnormalities are very low. In 100% concentration. Only inter phase and prophase abnormalities can be seen. They are at higher rate. This is because the cells are get arrested at interphase or prophase itself. So the metaphase, anaphase and telophase abnormalities are very low.

As the concentration of the solution increases, the inter phase abnormalities increases. Prophase abnormality decreases from 20% to 60% but in 80% and 100% it is higher. Metaphase abnormality decreases as concentration of solution increases. Anaphase and telophase abnormalities are very low.

Manihot esculenta Crantz root tuber, Raw in Aqueous solution

Mitotic index of control set of onion root tip (in pure water) is 48.5. 20% concentration shows a decrease in mitotic index i.e., 42.3. 40% concentration shows a decrease in mitotic index i.e., 38.7. 60% concentration shows a decrease in mitotic index i.e., 27.6. 80% concentration shows a decrease in mitotic index i.e., 16.8 and 100% concentration shows further decrease in mitotic index 8.5. As the concentration of solution increases the mitotic index also decreases. From this it is clear that *Manihot esculenta* Crantz, root tuber aqueous extract has mito-depressive activity on cell division. As the concentration of extract increases mito-depressive activity also increases.

Aqueous extract also shows similar abnormalities, but it is lower than in ethanolic solution. In 20% concentration, there is high inter phase abnormalities like Lesions, Strap shaped nucleus, etc. Prophase abnormality and metaphase abnormality are also prominent like Clumping, Polyploid, and Disorientation etc. In anaphase chromosomal bridges are also observed.

Similar to ethanolic extract, anaphase and telophase abnormalities are very low in 80% concentration. In 100% concentration, only inter phase and prophase abnormalities can be seen as in the case of ethanolic extract. They are at higher rate. This is because the cells are get arrested at interphase or prophase itself. So, the metaphase, anaphase and telophase abnormalities are very low. As the concentration of the solution increases, the inter phase abnormalities increases. But in 80% and 100% it is higher. Metaphase abnormality decreases as concentration of solution increases. Anaphase and telophase abnormalities are very low.

Result of phytochemical analysis

The extract of *Manihot esculenta* Crantz, root tuber raw in ethanolic extract shows the presence of Carbohydrate and Glycosides in higher concentration. Flavonoids, Saponins, Alkaloids and Protein in medium concentration. Phenol and Tannins are completely absent.

The extract of *Manihot esculenta* Crantz, root tuber raw in aqueous extract shows the presence of Carbohydrate, protein and Flavanoids in higher concentration. Alkaloids in medium concentration. Glycosides, Phenol and Tannins are completely absent.

The extract of *Manihot esculenta* Crantz, root tuber raw in aqueous extract shows the presence of Carbohydrate and Protein in higher concentration. Saponins in medium concentration. Flavonoids in low concentration. Alkaloids, Tannins, Phenol and Glycosides are completely absent.

Conclusion

When we compared *Manihot esculenta* Crantz root tuber, raw in ethanolic extract with *Manihot esculenta* Crantz root tuber, raw in aqueous extract, *Manihot esculenta* Crantz root tuber raw in ethanolic extract had showed high cytotoxic activity.

From this experiment it has been concluded that *Manihot esculenta* Crantz root tuber raw has high cytotoxic activity.Even though *Manihot esculenta* Crantz, root tuber is used as food and in medicine, because of its neurotoxic effects in animals, it is necessary to study the cytotoxicity also. From the cytotoxicity assay it is clear that, higher concentration of *Manihot esculenta* Crantz, root tuber raw aqueous and ethanolic extract causes higher level of cytotoxicity. As the concentration decreases, the cytotoxicity also decreases. So as a food or medicine it can be used in very low concentration. So, it is better to boil the root tuber and remove the toxic solvent while consumption.

From the phytochemical analysis it is clear that the extract of *Manihot esculenta* Crantz, root tuber raw contain Protein, carbohydrates, Alkaloid, Flavonoids, Glycosides etc. As a

future work it is necessary to do quantitative analysis of *Manihot esculenta* Crantz, root tuber raw as well.

Chart 1: Cytotoxic effect of different concentration of *Manihot esculenta* Crantz root tuber, Raw in Ethanol solution

Concentration	Time (Hrs)	Mitotic index	Interphase	Lesions	Strap shaped nucleus	Micronuclei	Total interphase abnormalities	Prophase	Total prophase abnormalities	Metaphase	Clumping	Polyploid cells	Disorientation	Total metaphase abnormalities	Anaphase	Bridge	Total anaphase abnormalities	Telophase
Control	2	48.5	88	2	0	0	2	80	0	20	0	0	0	0	3	0	0	5
20%	2	40.5	97	71	22	0	53	45	32	34	12	1	5	18	4	1	5	3
40%	2	35.4	132	45	10	33	98	18	12	21	5	5	1	11	0	0	0	2
60%	2	28.6	156	112	8	0	120	18	14	21	7	4	0	11	3	0	3	0
80%	2	14.5	168	102	34	18	154	51	36	13	2	2	0	4	0	0	0	0
100%	2	6.8	164	64	0	98	162	30	30	0	0	0	0	0	0	0	0	0

Chart 2: Cytotoxic effect of different concentration of *Manihot esculenta* Crantz root tuber, Raw in aqueous solution

Concentration	Time (Hrs)	Mitotic index	Interphase	Lesions	Strap shaped nucleus	Micronuclei	Total prophase abnormalities	Prophase	Total prophase abnormalities	Metaphase	Clumping	Polyploid cells	Disorientation	Total metaphase abnormalities	Anaphase	Bridge	Total anaphase abnormalities	Telophase
Control	2	48.5	88	2	0	0	2	80	0	20	0	0	0	0	3	0	0	5
20%	2	42.3	131	36	4	5	45	54	22	18	5	2	2	9	8	4	4	2
40%	2	38.7	120	51	10	10	71	44	14	18	7	4	1	12	4	3	3	1
60%	2	27.6	152	65	19	11	95	24	18	25	9	4	0	13	2	2	2	0
80%	2	16.8	114	58	32	22	112	30	25	15	3	1	0	4	0	0	0	0
100%	2	8.5	165	64	36	60	160	47	41	0	0	0	0	0	0	0	0	0

Chart 01: Phytochemical analysis of *Manihot esculenta* Crantz root tuber, Raw in aqueous and Ethanol solution (RAW)

Solvents	Carbohydrates	Protein	Alkaloid	Saponin	Tannin	Phenol	Flavanoids	Glycosides
WATER	+++	+++	---	+++	---	---	+++	++-
ETHANOL	+++	++-	++-	++-	---	---	++-	+++

- - - indicates low concentration + + + indicates high concentration + + - indicates medium concentration + - - indicates low concentration

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