“Radio-protective activity of *Mentha piperita* extract: In vitro analysis of amelioration of Ionizing Radiation induced DNA damage”

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BY

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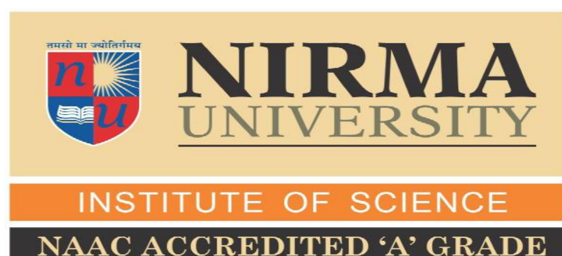

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1. ABSTRACT

Human exposure to radiation occurs in many forms, including cancer treatment, diagnosis, food sterilization, etc. Accidental exposures to radiation have also been reported, for example incidences like Fukushima or Chernobyl. Generally, any radiation above a certain level can be harmful to human due to its effect on biological systems like Central Nervous System, respiratory, and other normal healthy cells. In order to prevent these effects, radioprotective agents are required. Till now only two radioprotective drugs have been approved by FDA (Amiphostine and Palifermin), but these have side effects too. Hence, there is a need to explore naturally available sources as radio protectors. It is well known that free radicals can cause DNA damage. As flavonoids from plants can act as potent free radical scavengers, they can yield in production of ideal radio protectors.

Mentha piperita (peppermint) which is rich in flavonoids such as luteolin, eriocitrin, hesperidin, rosmaniric acid, etc. was chosen for this study. Two different concentration of the ethanolic extract of *M. piperita* were used 7µl and 10µl. An in vitro assessment of Radioprotective effect of this extract was carried out, the laboratory end points were: Cytokinesis Blocked Micro-Nucleus assay & COMET assay (Single cell gel electrophoresis) to assess the effect on DNA damage.

The study conducted indicates that M. piperita extract may be used as a potential radioprotective agent. It suggests that higher concentration of the extract shows significant reduction of DNA damage.

2. INTRODUCTION

2.1 Radiations

Ionizing radiation is a type of energy which is released by atoms in the form of electromagnetic waves (gamma or X-rays) or particles (alpha, beta, and gamma).

Human beings are exposed to radiation in several ways such radiotherapy as well as accidental exposure. Radiation plays an important role in several fields. Some of the uses of radiation are listed as follows:

Ionizing radiation can modify physical, chemical, and biological properties of biological materials. The main R&D activities of the Applications of Ionizing Radiations [14]

1) Irradiation of Food and Agricultural Products

2) Radiation and Radioisotopes Applications in Industry and Environment;

3) Radioactive Sources and Radiation Applications in medical sciences; and

4) Radioactive Facilities and Equipment for Nuclear Techniques Applications

2.2 Need for Radioprotectors

Exposure to radiation above a certain level is harmful for humans including radiotherapy which affect normal cells along with the cancer cells for which it is used. Long term exposure to UV rays leads to skin cancer, and 80% of cancer patient require radiotherapy at certain stage of treatment. Radiotherapy targets the tumor cells but it also affects the other normal healthy neighboring cells which surround the tumor cells. This leads to damage to those normal healthy cells.

Two drugs are approved by FDA- Palifermin and Amifostine which are synthetic radio protective agents. But these drugs have certain drawbacks thus there is a need to explore more radioprotective agents.[1]

Amifostine is effective in certain types of cancer whereas it does not protect central nervous system from the side effect of radiotherapy. Also extent to which it provides protection to other tissues varies and is not constant thus, dose selection becomes difficult.[15]

Such drawbacks lead to other side effects. Thus, there is a need to explore the world of natural radioprotective agents.

2.3 Natural Radioprotective Agents

- Plants are naturally gifted fight against excess UV rays by producing certain compounds in large amount thus the act as natural radioprotective agents.
- Some of the phytochemical or herbal extracts are reported to prevent the toxic effects of ionizing radiations on biological systems, these are known as Natural Radio-protectors
- Flavonoids like vicenin, luteolin, orientin, etc. are reported to be playing a major role in reducing against radiation induced damages.
- There are several plants whose radioprotective activity has been reported in biological systems. Some of them are listed below:-

PLANT	COMPOUND	REFERENCE
Malphigia glabra	antioxidants	Dusman E. et al; 2014
Tinospora cordifolia	Cordifolioside A	Patel A, Bigoniya P; 2013
Rosa roxburghii	flavonoids	Xu P, et al;2015
Citrus sinensis	L-ascorbic acid	Cimino et al., 2007
Solanum Lycopersicum	Carotenoids – lycopenes	Griffiths et al., 2005; Saraf and Kaur, 2010; Ravichandran et al., 2005
Phyllanthus amarus	Flavonoids	Londhe JS et al;2009
Mangifera indica	L-ascorbic acid	Song et al., 2013
Trifolium pretense	Isoflavone – Genistein	Widyarini et al., 2001

2.4 Properties of Ideal Radioprotector

A substance with immunomodulatory, free radical scavenging or anti-stress, anti-inflammatory, antimicrobial, antioxidant, properties may act as a potential candidate as a radioprotector

- Preservation of the anti-tumor efficacy of radiation
- High therapeutic ratio
- Wide window of protection against all types of toxicity
- High efficacy/toxicity profile (Low intrinsic toxicity profile)
- Easy and comfortable administration
- Cost-effective

2.5 Mechanism of action of radioprotectors

([Yamini K., Et al (2010)]

The Radioprotectors exhibit their protective actions by several mechanisms such as:-

1. Inhibit activation of protein kinase, nitrogen activated protein kinase, cytochrome P-450, nitric oxide.
2. Delay of cellular division and inducing hypoxia in the tissues.
3. Activate mRNAs of antioxidant enzymes such as catalase, glutathione transferase, glutathione peroxidase, superoxide dismutase.
4. Promoting the recovery of hematopoietic and immune functions.
5. Scavenging of free radicals (antioxidant mechanisms).
6. Triggering the DNA repair enzymes.
7. Detoxifying the radiation induced reactive species
8. Reduction in lipid peroxidation and elevation in non-protein sulfhydryl groups.

2.6 Introduction To *Mentha piperita*

2.6.1 Taxonomical Classification of *M. piperita*

Kingdom: Plantae

Order: Lamiales

Family: Lamiaceae

Genus: Mentha

Species: piperita

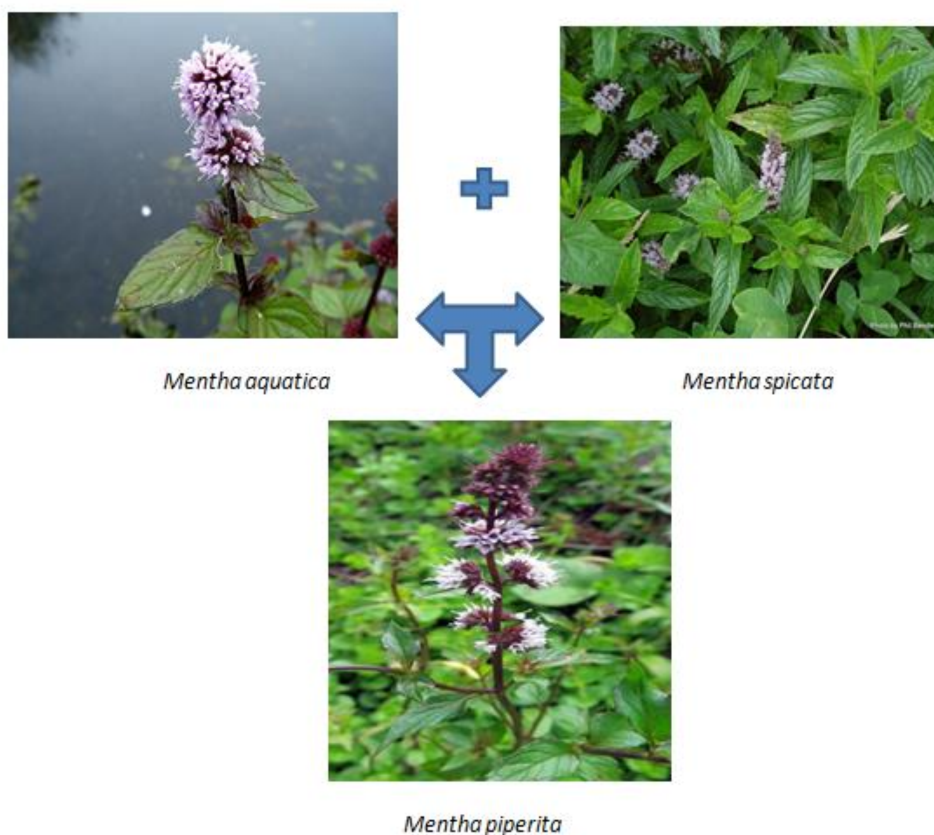
(**http://www.flowersofindia.net/catalog/slides/Peppermint.html)**

2.6.2 Vernacular Names

LANGUAGE	NAME
English	Peppermint
Hindi	Vilayati pudina
Sanskrit	Paparaminta
Gujarati	Phudino
Malyalam	Karppoora tulasi
Assamese	Poduna

2.6.3 Botanical Description

- *Mentha piperita* (peppermint) is a hybrid plant species of *Mentha aquatica* (water mint) and *Mentha spicata* (spearmint).



Mentha piperita

(http://nhb.gov.in/Horticulture%20Crops%5CMint%int1.
http://botanical.com/botanical/mgmh/m/mints-39.html)

- The leaves of peppermint are shortly but distinctly stalked, 2 inches or more in length, and 3/4 to 1.5 inches broad, finely serrated, their surfaces smooth, both above and beneath, or only very slightly hispid on the principal veins and mid-rib on the underside. The stems, 2 to 4 feet high, are quadrangular, often purplish.
- Flowers are borne in axillary and terminal verticillaster, abundant in number, purplish in colour and rarely bear seeds. The entire plant has a very characteristic odour, due to the volatile oil present in all its parts. [4]

2.6.4 Phyto-Constituents of *M. piperita*

Plants of *Mentha* genus are rich in flavonoids, particularly in flavones and flavanones. Luteolin and its derivatives are the main flavones described in *Mentha piperita.* The components eriocitrin, luteolin-7-*O*-glucoside, naringenin-7-*O*-glucoside, isorhoifolin, eriodictyol, luteolin, and apigenin are also reported to be identified.[3]

2.6.5 Geographical Distribution

M. piperita is an aromatic perennial herb and grown mostly in the Northern hemisphere. In India, it is largely confined to North India in the States of Uttar Pradesh, Punjab and Haryana. Temperate to tropical climate is suited for plant growth. Sunny weather with moderate rain is conducive to its luxuriant growth. A deep soil, rich in humus which can retain moisture, is suitable for mint cultivation. [11]

2.6.6 Uses of M. piperita

M. piperita serves a variety of purposes. Such as:

1. It is used for digestive problem including heartburn, nausea, vomiting, morning sickness, irritable bowel syndrome (IBS), cramps of the upper gastrointestinal (GI) tract and bile ducts, upset stomach, diarrhea, bacterial overgrowth of the small intestine, and gas.
2. It is antiseptic and analgesic in nature.
3. Useful for treatment of bronchitis.
4. Flowers of peppermint provide nectar to produce the largest amount of honey.[5]
5. M. piperita is a reported antioxidant and free radical scavenger in mice[6]

As *M. piperita* is reported antioxidant and free radical scavenger there is a need to test the efficacy of these plant extract using different assays.
In this study the assay performed are-

1) CBMN assay
2) Comet assay

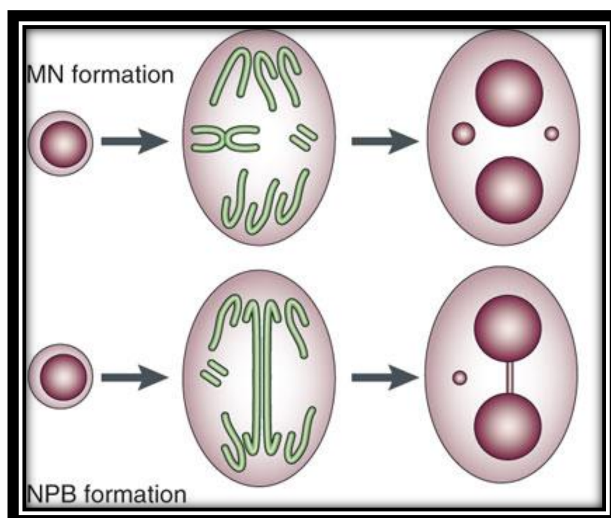
   CBMN Assay performed here helps in analyzing the genetic damage during the cytokinesis phase which indicates the damage in new generation of cells.

2.7 Cytokinesis Block Micronuclei Assay

The peripheral blood lymphocyte MN assay was first described by Countryman and Heddle. However, in this original method no attempt was made to determine whether the cells scored had actually completed nuclear division in vitro.

Yet another reliable approach was developed on the basis of the use of the cytokinesis inhibitor, cytochalasin-B. With the use of cyto-b Fenech and Morely demonstrated that cells finished one complete nuclear division and could be considered as a binucleated cell and that MNi could be scored efficiently in these BN cells while excluding non-dividing mononuclear cells.[7]

Acentric chromosome fragments are formed as result of activity of ionizing radiation. It also leads to mal-segregation of whole chromosomes. Such acentric chromosomal fragments and whole fragments can’t interact with the spindle fibers and remain left over at anaphase. Hence, these fragments are not included in the main daughter nuclei but, form a separate smaller nuclei known as micronuclei. The presence MNi can be considered as a marker of damage caused by ionizing radiation. [8]



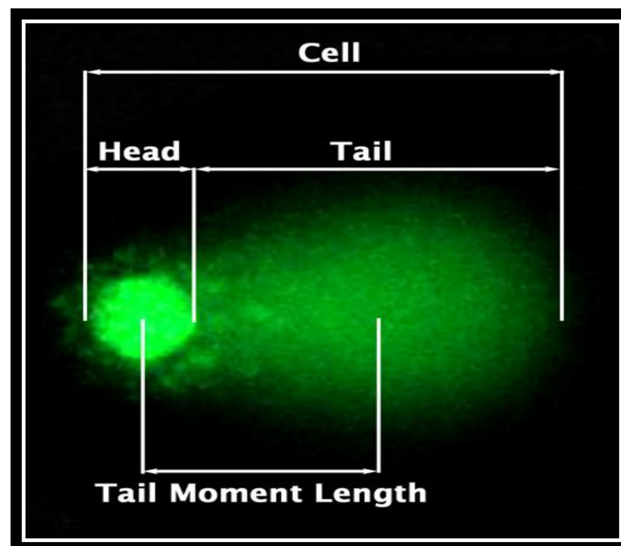
(Cytokinesis-block micronucleus cytome assay Michael Fenech)

- Another method to test the efficacy of the plant extract needs to be done. DNA damage can be quantified using Comet assay.

2.8 Comet Assay

Comet assay also known as single cell gel electrophoresis is a method for quantifying DNA damage, specifically strand breaks, in eukaryotes. The basic principle of this assay is cells are embedded in between two layers of agar, thus the name sandwich assay. These gel layers are laid on slides and lysed with detergents and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Alkaline treatment or electrophoresis at higher pH leads to unwinding of DNA strand. This unwinding of DNA leads to formation of tail of the comet. The comet like structure can be observed under fluorescence microscope. The head of the comet is the nucleus whereas, the tail indicates the DNA damage.

The assay has applications in testing novel chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, and fundamental research in DNA damage and repair. [9]



https://www.mybiosource.com/prods/Assay-Kit/OxiSelect-Comet

3. MATERIALS AND METHODS

3.1 Method for Preparation of M. piperita Leaf Extract

3.1.1 Identification and Authentication of *M.piperita*

Fresh Leaves of *Mentha piperita* were collected from Jamalpura, Ahmedabad, Gujarat. Authentication was done by the taxonomist Dr. Henna Patel Botany department of Gujarat University, Ahmedabad, Gujarat.

3.1.2 Preparation of Leaf Powder

Leaves were shade dried and ground to obtain fine powder. This powder was stored in airtight container and used for phytochemical screening.

3.1.3 Preparation of sterile leaf extract of M. piperita

Dried Leaf powder of *M. piperita* (10 gm) was mixed with 100 ml of 30% ethanol. It was refluxed for 1 hr on heating water bath and filtered through Whattman filter paper of the size 0.45 µm. These steps were repeated till flavonoid detection showed negative result. This extract was reduced by evaporation, it was then filtered using 0.45 µm syringe filter and diluted up to 10 ml. The diluted sample was further filtered through 0.22 µm syringe filter and tested for sterility.(Patel M., et al,2016)

Extraction Steps

3.1.4 Estimation of Flavonoid (By $AlCl_3$)

[Patel M; et al, 2016]

Dried Leaf powder 0.1gm was extracted with 100ml methanol by maceration for 24h and filtered. The final volume of the filtrate was made up to 100ml by methanol. 1ml of this extract was diluted up to 10ml with methanol and used for the estimation of flavonoids.

Method:

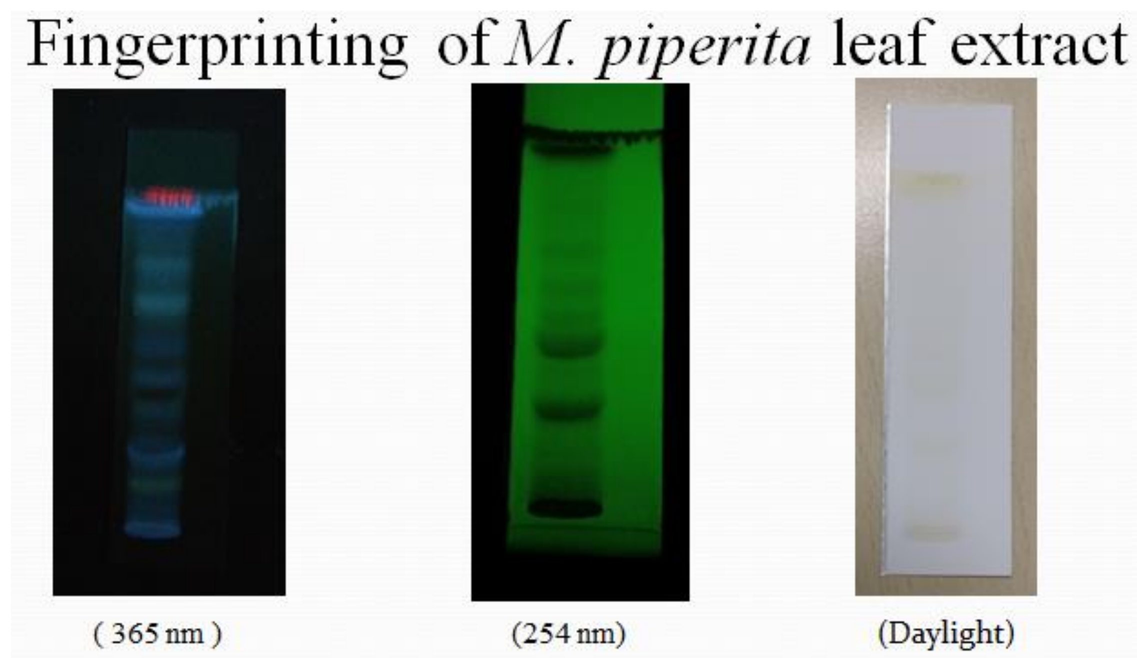
To 3ml of the ethanolic extract, add 3ml of methanolic $AlCl_3$. Take absorbance reading at 430 nm after 10 min. Results were expressed in g/100g of dry matter with respect to Rutin, serves as a standard.

Estimation of Total Flavonoid Content

The total flavonoid content estimated by AlCl3 method was found to be 92mg/g in *M. piperita.*

3.1.5 Detection of Flavonoids by TLC

Thin layer Chromatography was performed to detect the presence of flavonoids.



3.2 CBMN Assay

3.2.1 Reagent Preparation for CBMN Assay

[Patel M; et al, 2016]

1) **Cytochalasin B**

- 5 mg of solid powder Cyto-B dissolved in 8.33 ml of sterile DMSO to give solution of Cyto-B concentration of 600 µg/ml as follow :
- Cyto-B vial was removed from -20°C and it was allowed to attend room temperature. The top seal was sterilized with Ethanol
- 8.33 ml of DMSO was pipetted into 15ml sterile falcon tube. Using a 5ml syringe and sterile needle, 4ml of 8.33 ml DMSO was injected into rubber sealed vial through the 0.22 µm sterile filter.
- From the vial 4ml was removed and it was ejected into another 15ml tube labeled as I.
- The remaining amount of 4.3 ml of DMSO was aspirated as before into the vial and again ejected into sterile tube I
- The content was mixed properly and 700µl was dispensed into 1.5 ml eppendorfs. The aliquots were then stored at -20° c

2) **Hypotonic solution**

- 0.56 mg of KCl was added to 100 ml of DW and mixed well to prepare 0.56% KCl

3) **Fixative**

- Acetic acid & Methanol in ratio of 1:3 were mixed to prepare fixative and stored at 4° C.

4) **10% GIEMSA stain**

- 4.0 ml of GIEMSA stock was added to Sorensen's buffer (pH=7) and stored in the coupling jar.

3.2.2 Methods for CBMN Assay

1. **Blood Collection**

- Blood was collected from the cubital vein of a normal, healthy individual using a vacutainer into a heparin coated vial.
- A person who's not under any medication, doesn't smoke/drink and is not affected by any disease is considered normal and healthy for blood collection with their personal consent.

2. **Radiation Exposure**

- Blood collected was divided into different falcon tubes on the basis test groups under sterile conditions by using LAF.
- The concentration of the extract was decided on the basis of literature survey and OECD guidelines.
- The test groups are as follows:

i) Positive control – Blood was irradiated with gamma rays for 10 seconds and taken as positive control.

ii) Negative control – Normal blood was considered as negative control, which did not undergo any treatment.

iii) Blood+ Radiation+ Extract(7µl) – 7µl Extract was added to blood and this was irradiated with gamma rays for 10 seconds

iv) Blood+ Radiation+ Extract(10µl) - 10µl Extract was added to blood and this was irradiated with gamma rays for 10 seconds

v) Blood + Extract(7µl) - 7µl Extract was added to blood but, this sample was not irradiated

vi) Blood+ Extract(10µl) - 10µl Extract was added to blood but, this sample was not irradiated

- 1ml of each of these samples was added to 10ml of RPMI-1640 media for culturing it further and incubated in BOD incubator at 37^0C.
- These cultured blood falcons were mixed at regular interval by gentle shaking.

3. Cytochalasin-B Addition

Cyt-b addition was done 48th hour of culturing. A concentration of 6µg/ml was added to each tube, in order to block the cells at cytokinesis stage.

4. Harvesting

- Harvesting of cultured samples is done at 72th hour.
  Firstly, 5ml hypotonic solution (KCl) is added to each tube, so that the cells swell and scoring can be done easily, at 37^0C.
- After 2 minutes of addition of KCl, 1ml Carnoy's fixative is added to each sample tube.
- Addition of chilled and freshly prepared fixative is done slowly so that the cells do not undergo any shock.
- Further the sample was mixed well and centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and pellet was washed with fresh fixative.
- This washing step was repeated until a clear pellet was obtained.
- Pellet was suspended in 1ml of fixative and further used for slide preparation.

5 Slide Washing

- Slides were soaked in conc. Sulphuric acid overnight followed by wash with running tap water.
- Further these slides were given 3-4 washes with distilled water.

6 Slide Preparation [Patel M; et al, 2016]

- 2-3 drops of cell suspension were dropped on clean, pre-chilled clean slides and was allowed to dry.
- The slides were stained in 10 % Giemsa stain for 8 mins, which were then thoroughly rinsed in distilled water and allowed to dry then these slides were observed under microscope.

7 Scoring Criteria [8]

- The cytokinesis blocked cells that may be scored for MN frequency should have the following characteristics:

(a) The cells should be binucleate.

(b) The two nuclei in a BN cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.

(c) The two nuclei in a BN cell should be approximately equal in size, staining pattern and staining intensity.

(d) The two nuclei within a BN cell may be unconnected or may be attached by one or more fine nucleoplasmic bridges which are no wider than 1/4th of the nuclear diameter.

(e) The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable.

(f) The cytoplasmic boundary or membrane of a BN cell should be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

- MNi are morphologically identical to but smaller than the main nuclei. They also have the following characteristics:

(a) The diameter of MN in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.

(b) MN are non-refractile and can therefore be readily distinguished from artifacts such as staining particles.

(c) MN are not linked or connected to the main nuclei.

(d) MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

(e) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

3.3 COMET ASSAY

3.3.1 Reagent Preparation for Comet Assay

1. **PBS(Ca++,Mg++free)**

Dulbecco's PBS -1 L packet 990ml of dH2O was added, pH was adjusted to 7.4 q.s. to 1000ml and stored at room temperature.

2. **Lysis solution**:

2.5M NaCl weigh 146.1gm, 100mM EDTA weigh 37.2gm and 10mM trizma Base weigh 1.2gm were added 700ml dH2O ~8gm of NaOH was added and allowed to dissolved. The pH was adjusted to 10.0 using Conc. HCl or NaOH q.s. to 890ml with dH2O. it was stored at room temperature.

3. **Final Lysis solution:**

1% of triton X was added to 10% of DMSO and was refrigerated for at least 30 mins prior to slide addition.

4. **Electrophoresis buffer (300mM NaOH/1mM EDTA)**

The stock was prepared from 10N NaOH (200g/500 ml dH2O) and 200mM of EDTA (14.89gm/200ml dH2O pH 10). Both were stored at room temperature. For 1X buffer 30ml of NaOH and 5.0 ml of EDTA, q.s too 1000ml and was mixed properly. The pH of buffer should be >13 prior to use.

5. **1% NMA**

100mg NMA was added to 10ml distilled water and was further boiled and then used as base layer.

6. **1% LMPA**

100mg LMPA was added to 10ml distilled water and was boiled and cooled, under tap water, to room temperature.

3.3.2 Methods for Comet Assay

1. **Slide Washing**

- Slides were soaked in conc. Sulphuric acid overnight followed by wash with running tap water.
- Further these slides were given 3-4 washes with distilled water.

2. **Base Slide Preparation**

- 1% NMA was used as base layer.
- 350µl of NMA was poured on a clean slide using micropipette.
- Parafilm was used to cover the base layer so that a thin layer is obtained and the moisture remains intact in the agarose layer.
- Base slides was stored overnight at room temperature and used next day.

3. **Second Layer**

- 1% LMPA was used for second layer.
- 40µl of blood was taken for each sample and mixed with 360µl of 1% LMPA and poured on the base layer, after removing the parafilm.
- After this second layer was laid on the slide it was covered with clean coverslip and incubated at 4^0C for 10 mins.

4. **Third Layer**

- After removing the coverslip third layer laid of 300µl 1% LMPA.
- Again the slide was covered with coverslip and incubated at 4^0C for 10-15 mins.

5. **LYSIS**

- Slides were then incubated in lysis buffer for 2hrs in order to lyse the RBC present in the second layer.

6. **ELECTROLYSIS**

- Slides were then incubated in electrolysis buffer, for 45 mins, which acts as unwinding buffer. Here unwinding of DNA takes place.
- After this 45 minute incubation slide were electrophoresed in the same buffer for 30 mins at 24volts and 300 amp current.

- After electrophoresis the slides were washed with Dulbecco's PBS solution.

7. SLIDE OBSERVATION

- Slides were stained with DNA staining dye Propidium iodide, and observed in fluorescence microscope at 40x magnification.

4. RESULT AND DISCUSSION

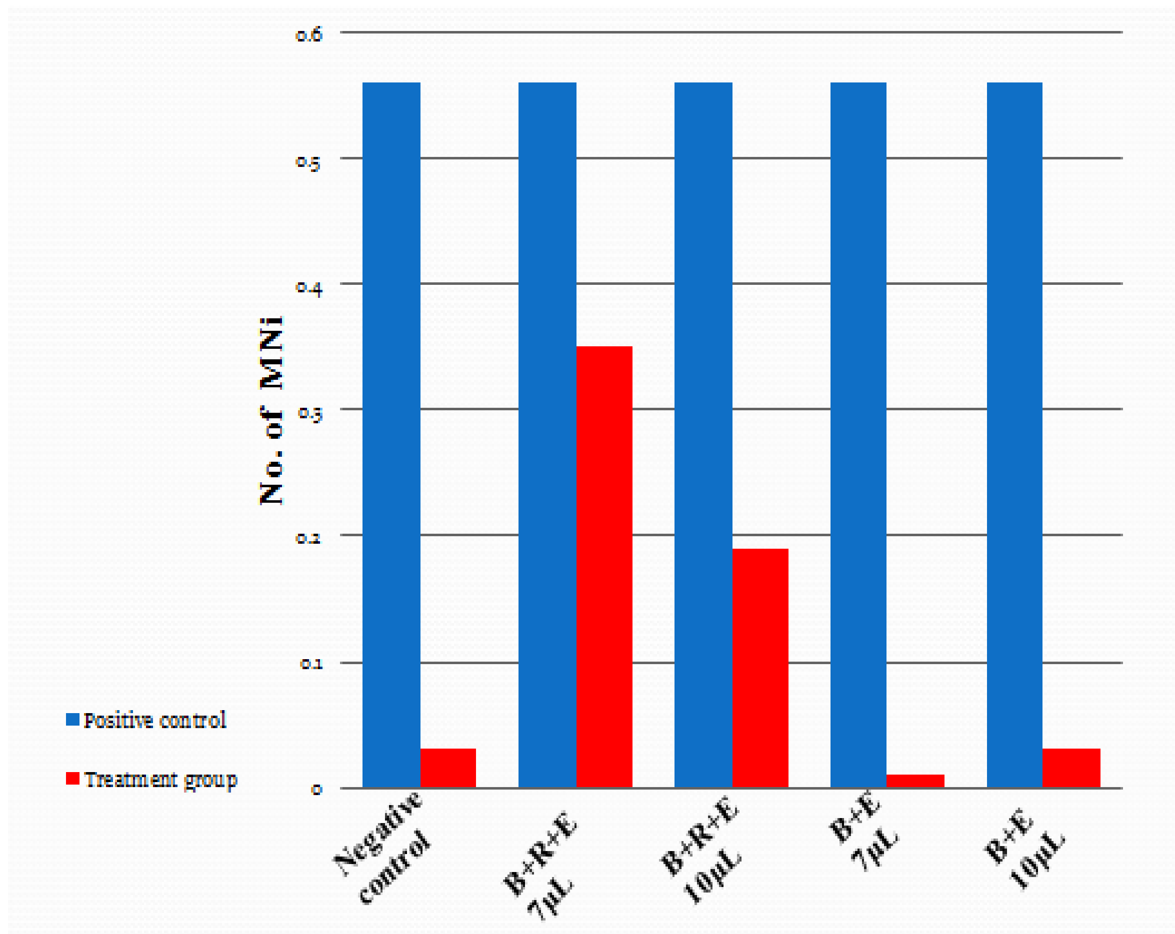
4.1 CBMN RESULTS

P $\geq$ 0.10	Not Significant
P $\leq$ 0.10	Marginal
P $\leq$ 0.05	Fair
P $\leq$ 0.01	Good
P $\leq$ 0.001	Excellent

STATISTICAL ANALYSIS

- The tables show the mean and S.E value of each of the two sets and also based on the Student t test the P- values obtained is ≤ 0.001.
- P-value is the number between 0 to 1. It is the probability of getting the observed value of test or more than that.
- Smaller P-value (typically ≤ 0.05) indicates strong evidence against the null hypothesis so, we reject the null hypothesis.
- Larger P-value (typically ≥ 0.05) indicates weak evidence against null hypothesis so fails to reject the null hypothesis.
- Here on the above set of all the treated groups the P- value obtained is ≤ 0.001 and so the values obtained are significant and hence the null hypothesis can be rejected as the value proves the strong evidence against null hypothesis

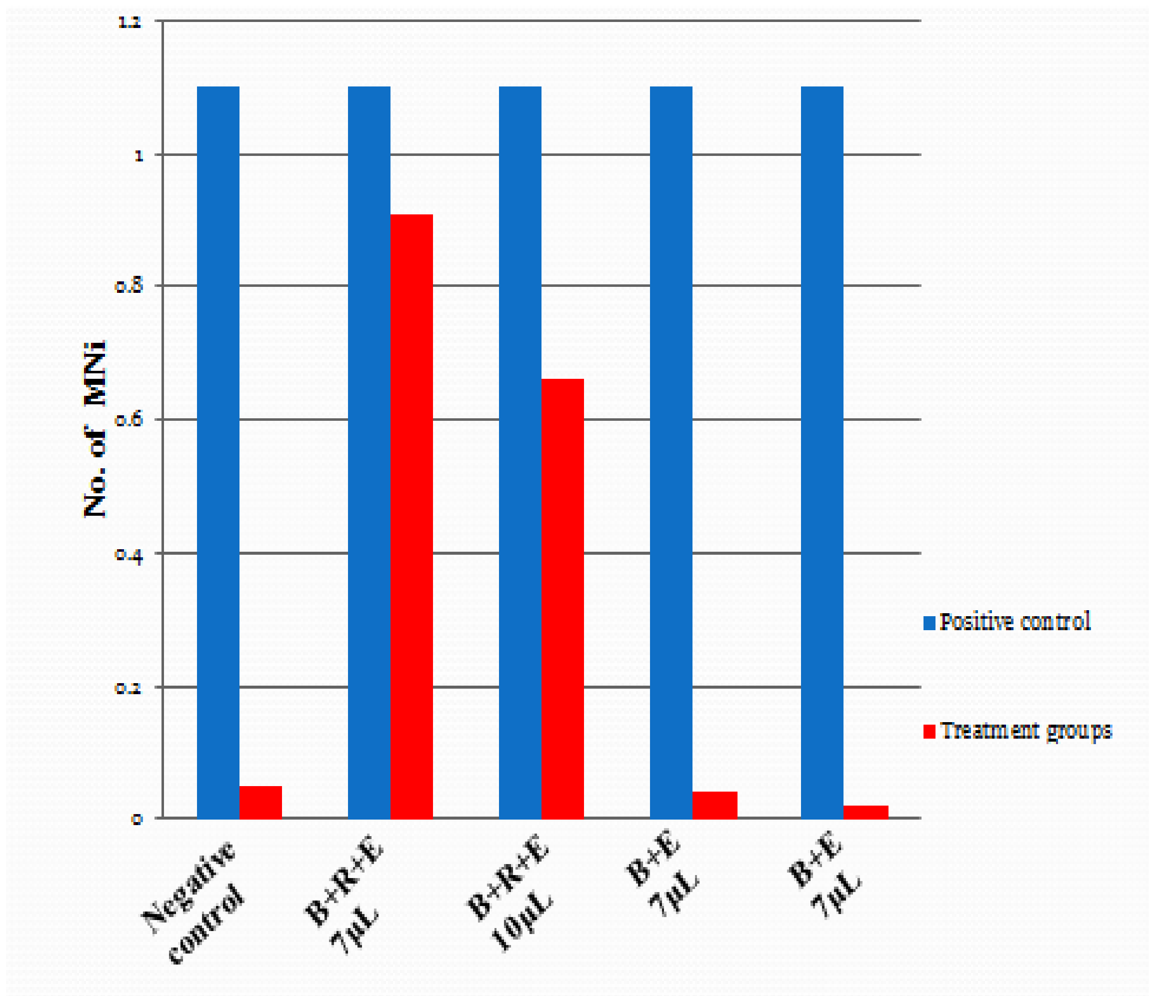
4.1.1 Set 1



Sample	Mean ± SE	P value
Positive control	0.56 ± 0.092	
Negative control	0.03± 0.022	>0.0005
B+R+E (7.14µl)	0.35± 0.070	>0.01
B+R+E (10µl)	0.19± 0.053	>0.0005
B+E (7.14µl)	0.01± 0.01	>0.0005
B+E (10µl)	0.03± 0.022	>0.0005

Table1

4.1.2 Set 2



Sample	Mean ± SE	P value
Positive control	1.1± 0.088	
Negative control	0.05± 0.026	>0.0005
B+R+E (7.14µl)	0.91± 0.093	>0.0005
B+R+E (10µl)	0.66± 0.098	>0.05
B+E (7.14µl)	0.04± 0.019	>0.0005
B+E (10µl)	0.02± 0.014	>0.0005

Table 2

- From the entire set of each of the samples total 100 cells were being counted and values were further plotted on graph and statistical analysis was carried out.
- The positive control shows highest number of micro- nuclei because of being exposed to only radiation and compared to that two test samples of 7µl and 10µl showed decrease in micro-nuclei number in culture sample treated with extract.
- The graphical representation shows that with increase in concentration of extract there is decrease in the number of micronuclei.
- Both the set showed similar trend of results that is frequency of BN cells with MNi decreases as the concentration of extract increases.

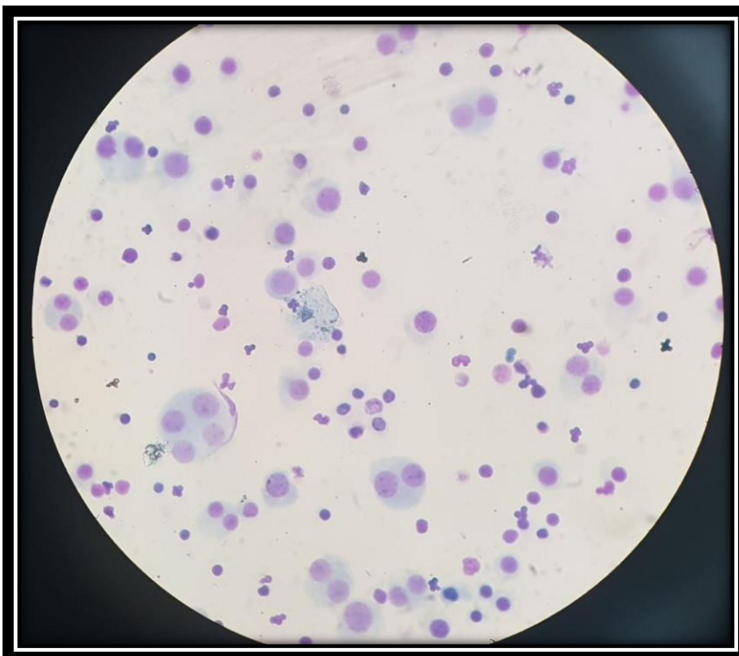


Fig: *Field of cells with mononucleated, binucleated as well as tetranucleated cell*

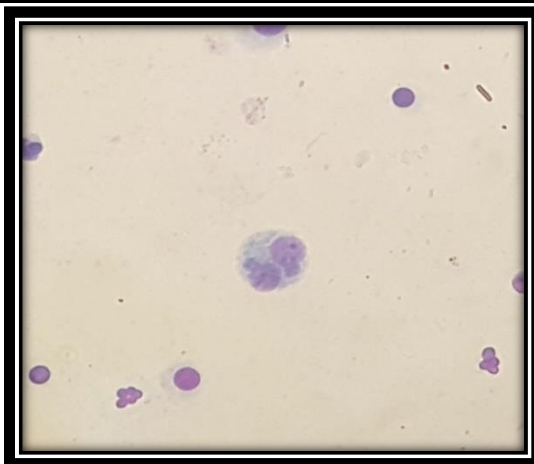


Fig: *Binucleated cell with nucleoplasmic bud*

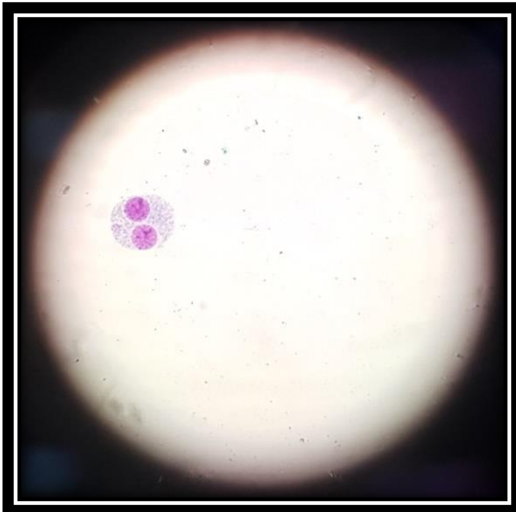


Fig: *BN cell with 1 MNi*

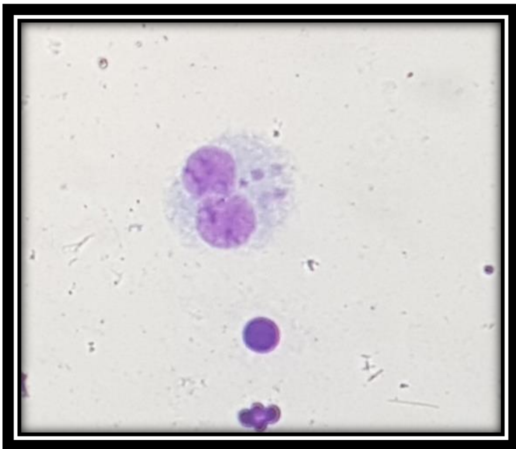


Fig: *BN cell with 5 MNi*

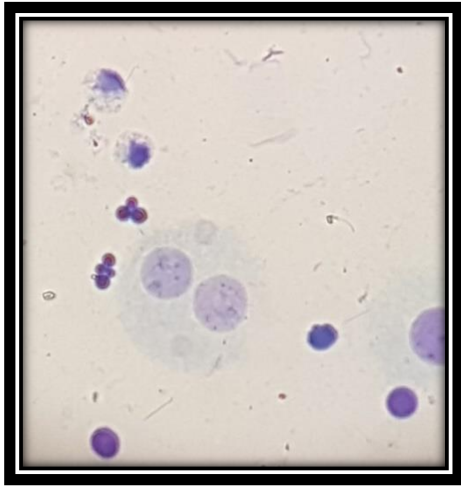


Fig: *BN with 2 MNi*

5. CONCLUSION

The result table as well as statistical analysis indicates that the flavonoid rich extract of *M. Piperita* may act as a potential radioprotective agent and can be further explored and researched.

According to the CBMN results obtained *M. piperita* may act as potent radioprotective agent

The results for comet assay are still awaited

6. SUMMARY

Preliminary phytochemical analysis of the leaves of the M. Piperita revealed the presence of metabolites like tannins, phenolics, alkaloids, saponins and mainly flavonoids

The total flavonoid content of *M. piperita* was found to be 92mg/gm.

The results of CBMN Assay using the leaf Extract of *M. piperita* showed significant reduction in the frequency of BN cells with MNi.

7. FUTURE SCOPE

As the results indicated *Mentha piperita* can be potent radioprotective agent, further studies can be conducted by changing and increasing the concentration of the extract.

Comet assay needs to be repeated performed so that better result can be obtained

Also, different assays can be performed such as CA, SCE, etc. or different radiation dose can be provided to the blood samples.

8. ABBREVIATION

M. piperita	*Mentha piperita*
DNA	Deoxyribonucleic Acid
CONC.	Concentration
CBMN	Cytokinesis Block Micronuclei Assay
BN	Bi Nucleated cell
MNi	Micronuclei
NMA	Normal Melting Agarose
LMPA	Low Melting Point Agarose
Cyt-b	Cytochalasin- b
amp	Ampere
PBS	Phosphate Buffer Saline
KCl	Potassium Chloride
DMSO	Di Methyl Sulphoxide
RPMI	Rosewel Park Memorial Institute
rpm	Rotation per minute
Min	Minutes
LAF	Laminar Air Flow
OECD	Organization for Economic Co-operation and Development
mg	Milli gram
µL	Micro litre
µg	Micro gram
^{0}C	Degree Celsius
S.E.	Standard Error

9. REFERENCES

1. Zbigniew Sroka*, Izabela Fecka, and Wojciech Cisowski; Antiradical and Anti-H2O2 Properties of Polyphenolic Compounds from an Aqueous Peppermint Extract Z. Naturforsch; March 15/May 4, 2005, **60c**, 826D832 (2005)
2. Yamini, K., & Gopal, V. (2010). Natural Radioprotective Agents against Ionizing Radiation – An Overview, *2*(2), 1421–1426
3. Chemical Composition and Biological Activities of *Mentha* Species
   Fatiha Brahmi[1]*, Madani Khodir[1], Chibane Mohamed[2] and Duez Pierre
4. http://www.flowersofindia.net/catalog/slides/Peppermint.html
5. http://www.homeremediess.com/medicinal-herb-peppermint-benefits
6. Ravindra m. Samarth, meenakshi panwar, madhu kumar & ashok kumar
7. FENECH, J., MORLEY, A.A., Cytokinesis-block micronucleus
   method in human lymphocytes: Effect of in vivo ageing and low-dose
   X-irradiation, Mutat. Res. 161 (1986)
8. Cytogenetic Analysis for Radiation Dose Assessment A Manual
9. Collins AR, The comet assay for DNA damage and repair, principles, applications, and limitations; Mol Biotechnol. 2004 Mar;26(3)
10. Mansi Patel,[a] Priti Mehta,[a,*] Sonal Bakshi,[b] and Shikha Tewari[b]
    Effect of *Phyllostachys parvifolia* leaf extract on ionizing radiation-induced genetic damage: A preliminary *in vitro* cytogenetic study, 2016
11. http://www.indianspices.com/spice-catalog/mint-1
12. "Phytochemical analysis and estimation of radioprotective activity of bamboo species growing in Gujarat" Mansi Patel; et al
13. Fenech, M. (2000). The in vitro micronucleus technique, *455*, 81–95.
14. https://www.ipen.br/portal_por/conteudo/documentos/201205161528420.6.ionizing_radiations_completo.pdf
15. http://www.cancernetwork.com/review-article/role-amifostine-radioprotector-1