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Cytotoxic and genotoxic effects of *Centella asiatica* extract in the cultured human peripheral blood lymphocytes

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Centella asiatica (CA) is a medicinal herb which has been valued in ayurvedic medicine for its different activities. In the present studies, CA methanolic extract was prepared by Soxhlet extraction and then evaluated for the cytotoxicity and genotoxicity in cultured human peripheral blood lymphocytes. Mitotic Index (MI) Cell Proliferation Kinetics (CPK) and Sister-chromatid exchanges (SCE) were scored to measure the cytotoxic and genotoxic effects of the CA extract in cultures set up from the three different healthy donors. The treatment of the cell culture was done employing two different CA methanolic extract concentrations (500 & 1000 µg/ml) and the control (did not receive any additive). Our present studies revealed that the MI, CPK and SCES for control are 4.28, 0.49 and 6.08 respectively where as the CA extracts has the MI, CPK and SCES for 500µg/ml are 4.07, 0.47 and 5.84 respectively and for 1000 µg/ml are 3.96, 0.46 and 5.52 respectively. From the results, we can conclude that the MI and frequency of SCES of CA methanolic extracts are almost similar to that of control which indicates that the CA plant extracts has no significant cytotoxicity and genotoxicity effects in cultured human peripheral blood lymphocytes.

Key words : *Centella asiatica*, Soxhlet Extraction, Cytotoxicity, Mitotic Index, Genotoxicity, Sister-chromatid exchanges.

Medicinal herbs have been used in folk medicine for millennia. Simply, in recent times, scientific study of their effects has flourished. Nevertheless, some of them can cause adverse effects or have the potential to interact with other medications (Zink and Chaffin, 1998). Moreover, there is little information on the potential risk to health of such herbs. Based on their long-term use by humans, one might expect herbs used in traditional medicine to have low toxicity. It is known that green plants in general are a primary source of antimutagens as well as natural toxic agents (Plewa and Wagner, 1993) and many plants contain cytotoxic and genotoxic substances. Recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic, cytotoxic and genotoxic effects in vitro and in vivo assays (Schimmer *et al.*, 1994).

Centella asiatica is a perennial creeper, faintly aromatic and a valuable medicinal herb of which is distributed throughout tropical and subtropical regions of World such as India, China, Nepal, Madagascar, Srilanka, and Indonesia etc. Traditionally, *Centella asiatica* has been valued for centuries in ayurvedic medicine for the treatment of leprosy, ulcer,

asthma, bronchitis, elephantiasis, eczemas, anxiety, urethritis, cataract, eye troubles, diarrhoea among children, skin diseases, wound healing and for revitalizing the nerves and brain cells, hence primarily known as a "Brain food" or "Memory enhancer" in India (Seema chaitanya *et al.*, 2011). Phytochemical analysis of *Centella asiatica* plant extracts revealed the presence of various biochemical compounds such as alkaloids, flavonoids, glycosides, triterpenoids and Saponins etc. Several pharmacological studies on *Centella asiatica* reported that it has anti-inflammatory (Seema chaitanya *et al.*, 2011), antibacterial, antimicrobial (Minija *et al.*, 2003), antispasmodic, antioxidant (Seema chaitanya Ch *et al.*, 2011), antitubercular, immunomodulatory (Jayathirtha *et al.*, 2004), antiarthritic (Seema chaitanya *et al.*, 2011), anticancer and antiulcer activities. In this study, cytotoxic and genotoxic effects of *Centella asiatica* in cultured human peripheral blood lymphocytes have been evaluated.

Mitotic index (MI) as a measure of Cytotoxicity

Cell population growth occurs as cells pass through interphase and mitosis to complete the cell cycle. Many cells lose the capacity to divide as they mature or divide only rarely. Other cells are capable of rapid cell division. Cytotoxicity is a quality of being toxic to cells. Treating cells with a cytotoxic compound can result in a variety of cell fates. Examples of toxic agents are a chemical substance, plant extract, an immune cell or some types of venom. By quantifying aspects of a dividing cell population; we can examine how cells differ in their capability to divide. Experimentally, we can change properties of the cell's environment and quantify the effects on cell division "as discussed by Kannan *et al.*, (Kannan *et al.*, 2006). The way to quantify cell division is by using the mitotic index. If you administer colchicine or other colchicine-derivative medications (i.e. colcemid) you can arrest the cell cycle at this point leaving the chromosomes in their visible form. Colchicine disrupts the microtubule formation which is necessary for the spindle fibers to separate the chromosomes during anaphase. Mitotic index is a measure for the proliferation status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. The mitotic index can be worked out from a slide, even with light microscopy. It is the number of cells containing visible chromosomes divided by the total number of cells in the field of view.

Sister Chromatid Exchange Test as a measure of Genotoxicity

Genotoxicity is a quality of being toxic to cell's genetic material affecting its integrity. Genotoxic substances are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumors. This includes certain chemical compounds, plant extracts and certain types of radiation. The Sister Chromatid Exchange (SCE) assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. The SCE test is usually performed on human peripheral blood lymphocytes. As peripheral lymphocytes are in the resting G₀ stage of the cell cycle, they have to be stimulated to divide by an aspecific antigen, like phytohaemagglutinin. To collect a sufficient number of mitotic cells, a spindle inhibitor like colcemid may be added shortly before fixation (at 72 hours), to block cells in (pro) metaphase of the second mitosis. To allow for a differential staining that enables the researcher to distinguish both chromatids, BrdU (bromo-deoxy-uridine) is added to the culture medium for the duration of two complete cell cycles. The Giemsa staining was able to stain due to the presence of bromodeoxyuridine analogous base which was introduced to the desired chromatid.

Chromatids in which only one strand of DNA incorporated BrdU show a normal dark Giemsa staining, whereas those with two substituted strands stain less darkly. If an exchange occurred, this can be seen as the dark part changes to the other arm: "harlequin chromosomes". The technique can also be used to estimate the division rate, as first metaphases stain uniformly dark. (Kannan *et al.*, 2006; Maria *et al.*, 1997).

Materials and methods

Extraction and Processing of the Plant Material

The fresh whole plant of *Centella asiatica* was collected from Araku Valley, Near Visakhapatnam, Andhra Pradesh, India. The plant material was garbled and air dried under the shade upto 48 days. By using the kitchen blender the plant material was grounded to a fine powder form. Powdered plant material (5g) was mixed with 500 ml of methanol. Using the soxhlet apparatus continuous extraction was done for 24 hrs. The extract was filtered and methanol was evaporated on a rotary evaporator under vacuum at a lower temperature of 50°C to dryness. These plant extracts were weighed and preserved at 4°C in desiccator.

Blood sample collection

Three fresh blood samples from the volunteers were collected, generally from the arm by venipuncture, and placed into a heparinised tube.

Peripheral Blood Culture Initiation

Few drops of whole blood (0.5mL) are cultured in medium RPMI 1640 and a mitogen phytohemagglutinin (PHA) 100µL was added. *Centella asiatica* plant extracts of 3 different doses (500 µg/ml and 1000 µg/ml) were added. And for mutagen cultures we were added Mitomycin-c 0.1 µg. After 24th hour of the cultures BrdU was added. Peripheral blood cultures were placed in a 37°C incubator for 72 hrs.

Harvesting of the blood cultures

By administering colchicine or other colchicine-derivative medications (i.e. colcemid) you can arrest the cell cycle at this point leaving the chromosomes in their visible form. Colchicine disrupts the microtubule formation which is necessary for the spindle fibers to separate the chromosomes during anaphase. To arrest the cell division at metaphase, 0.1 ml (1 µg) of colcemid was added to the cultures just 90 minutes before harvesting. After addition of colcemid the cultures were incubated for 40 minutes and centrifuged at 1200 RPM for 10 minutes. To the pellet pre-warmed hypotonic solution (0.75M KCl) of 6ml was added. Incubated for 10 minutes and centrifugation was done at 1200RPM for 10 minutes.

Fixation of blood cultures

To the pellet 5 ml of fixative solution (3:1 ratio of methanol and acetic acid) was added and centrifuged. 4-5 times of fixative washes were given. The fine pellet was dropped onto microscopic sterile and chilled slides.

Preparation of metaphase chromosome spreads

Chromosome spreads were prepared by gently dropping the cell suspension from a height of 1 foot on to a clean grease free chilled slide and air-dried.

Mitotic Index (MI) Assay

The slides were stained with 10 per cent Leishman's stain in phosphate buffer saline (pH 6.8) for 5 minutes. The slides were dried, mounted and the chromosomes were observed under

the microscope (Kannan *et al.*, 2006; Maria *et al.*, 1997). Slides were prepared from each blood culture and a total of 1000 lymphocytes were counted per culture to determine the mitotic index. The mitotic index was used to determine if the *Centella asiatica* plant extracts produced any cytotoxicity. It is a measure for the proliferation status of a cell population. It can be defined as the ratio between the number of cells in mitosis and the total number of cells or the number of cells containing visible chromosomes divided by the total number of cells in the field of view. It can be calculated as follows:

$$\text{Mitotic Index (MI)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

Cell Proliferation Kinetics (CPK)

Cell-cycle specific patterns were determined by M₁, M₂, and M₃ metaphases. These were defined by the number of cell cycles completed. M₁ metaphases substituted BrdU unifilarly in both chromatids without differential staining. Chromosomes of M₂ metaphases contained a bifilar substitution of BrdU in one chromatid, which, consequently, was lightly stained, thus displaying the typical sister chromatid differentiation pattern. Bifilar incorporation of BrdU in the third cell cycle yielded light staining of both chromatids. Such metaphases were classified as M₃ metaphases. The cell proliferation kinetic index is calculated using

$$\text{Cell Proliferation Kinetics (CPK)} = \frac{M_1 + 2M_2 + 3M_3}{100}$$

Whereas M₁ are Dark stained metaphases, M₂ are Differential stained metaphases and M₃ are Light stained metaphases.

Sister Chromatid Exchange assay

After the third day of the preparation of the slides, then they were used for Sister Chromatid Exchange assay. For this assay we kept a drop of Hoechst 3325 solution onto the slide and mounted with cover slip for 25 minutes. Washed the slides with water and kept for drying in the dark chamber and then placed the slides in petridish which contains 2XSSC, for 25 minutes and exposed to UV for 40 minutes. Washed the slides with water and dried for few minutes. Incubate the slides in 2XSSC at 56°C for 2hrs. Stain the slide with 2% Giemsa's stain with Sorenson's buffer (Kannan TP *et al.*, 2006). The sister chromatid exchanges were used to determine if the *Centella asiatica* plant extracts produced any genotoxicity. Slides were prepared from each blood culture and No. of sister chromatid exchanges in the centromere was counted in 1000 M₂ Metaphases in the chromosome spreads per culture was assessed to score for any chromosomal aberrations that may be induced due to the *Centella asiatica* plant extracts. SCEs can be calculated using the formula as follows:

$$\text{SCE} = \frac{\text{Total no. of sister chromatid exchanges observed}}{\text{Total no. of metaphases observed with sister chromatid exchanges}} \times 100$$

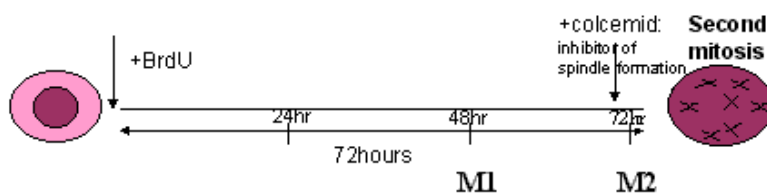


Fig.1 Principle of sister chromatid exchange test

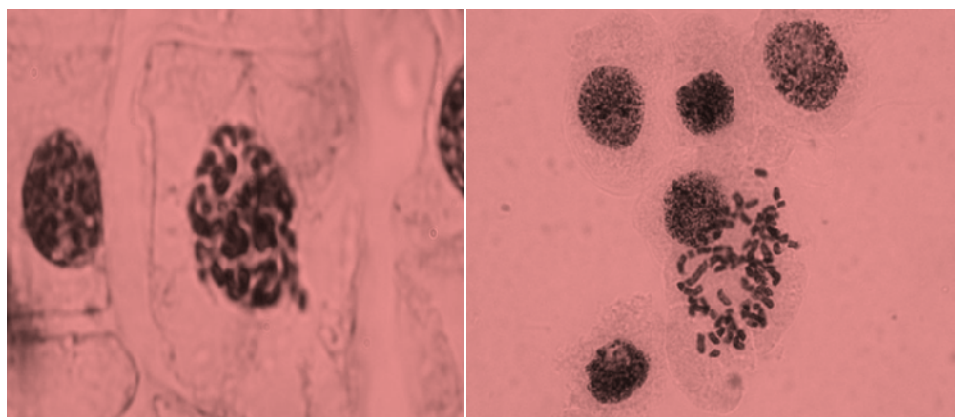
Results and discussion

Determination of Mitotic Index (MI)

For the calculation of mitotic index, number of dividing cells has to be counted for every 1000 cells counted.

From the slides, we have observed the 2 types of dividing cells

1. Prophase - stage of mitosis in which the chromatin condenses (it becomes shorter and fatter) into a highly ordered structure called a chromosome in which the chromatin becomes visible. This process, called chromatin condensation
2. Metaphase - stage of mitosis in the eukaryotic cell cycle in which condensed & highly coiled chromosomes, carrying genetic information, align in the middle of the cell before being separated into each of the two daughter cells.



a) Prophase observed

b) Metaphase observed

Fig. 2 Images of the dividing cells observed under microscope

From the observed slides, dividing and non dividing cells were counted to determine the mitotic index for the assessment of the cytotoxicity in the control and *Centella asiatica* plant extracts of concentration 500 & 1000 $\mu\text{g}/\text{ml}$ using three blood samples for the accuracy. The Mean Mitotic Index (MI) values of the cultures which are exposed to two doses of plant extracts and control are obtained as shown in the Table 1. The mean value of MI of the control is 4.28 where as the *Centella asiatica* extracts have the mean value of MI of the 500 $\mu\text{g}/\text{ml}$ is 4.07 and the mean value of MI of the 1000 $\mu\text{g}/\text{ml}$ is 3.96. MI of *Centella asiatica* plant extracts were not much different from the MI of control.

From the Fig 4 of MI Vs Type of culture, obtained equation was $y = -0.145x + 4.503$ and $R^2 = 0.9809$, which indicates that MI of *Centella asiatica* plant extracts were not significantly different from the MI of control.

Table 1. Mitotic indexes of *Centella asiatica* plant extracts (500 & 1000 µg/ml) and control using three blood samples.

Type of Cultures	Control			CA (500 µg/ml)			CA (1000 µg/ml)		
	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3
No. of the Prophases	01	02	03	02	03	04	04	07	05
No. of the Metaphases	47	49	46	45	44	38	39	38	34
No. of the Dividing cells	48	51	49	47	47	42	43	45	39
No. of the Cells observed	1037	1182	1068	1063	1123	1064	1004	1106	1003
Mitotic Index (MI)	4.64	4.32	4.17	4.43	4.19	3.95	4.28	4.07	3.89
Mean MI	4.28			4.07			3.96		

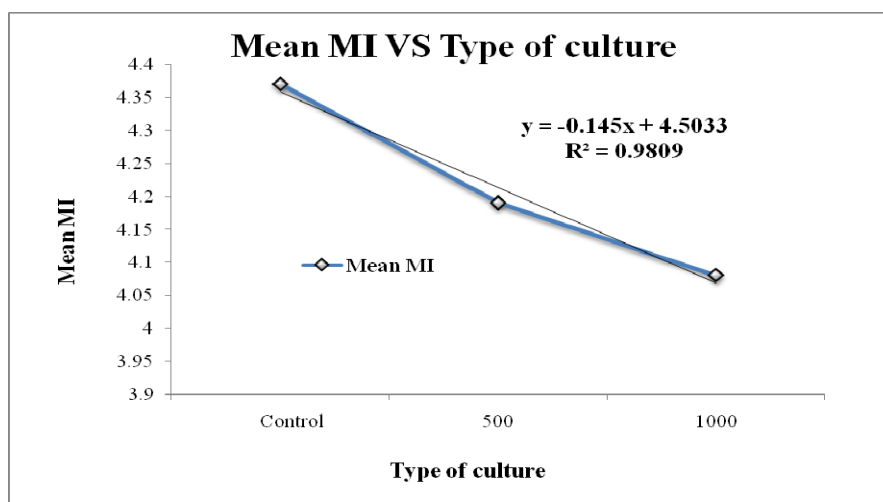


Fig. 4 Graphical representation of the Overall Mean Mitotic Index (MI) of *Centella asiatica* extracts (500 & 1000 µg/ml) and control

Determination of the frequency of Sister Chromatid Exchanges (SCES):

Slides were prepared from each blood culture and 1000 metaphase chromosome spreads were counted per culture was assessed to score for any chromosomal aberrations (sister chromatid exchanges) that may be induced due to the *Centella asiatica* plant extract of concentration 500 & 1000µg/ml using three blood samples for accuracy.

The Mean Sister chromatid exchanges (SCES) values of the cultures which are exposed to two doses of plant extracts and control are obtained as shown in Table 2. The mean value of SCES of the control is 6.08 where as the *Centella asiatica* extracts have the mean value of SCES of the 500µg/ml is 5.84 and the mean value of SCES of the 1000 µg/ml

is 5.52. SCES of *Centella asiatica* plant extracts were not much different from the SCES of control.

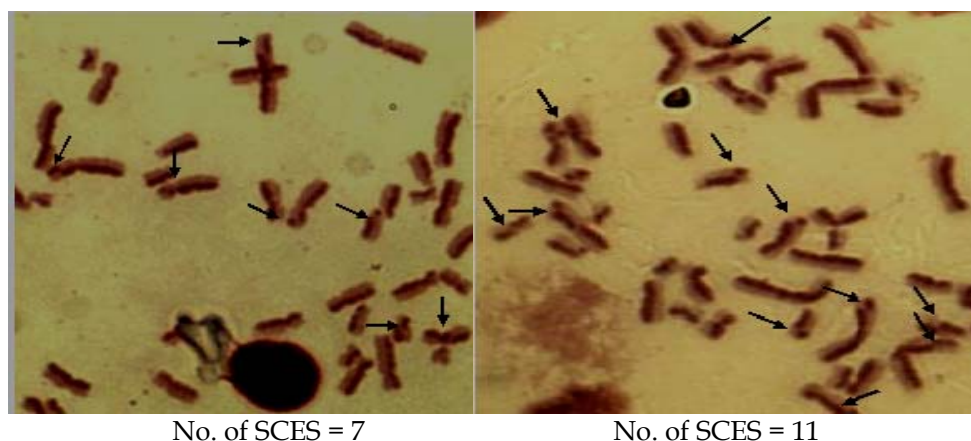


Fig. 3 Images of the sister chromatid exchanges observed in the metaphases of the *Centella asiatica* plant extracts 500 & 1000 µg/ml.

Table 2 Sister Chromatid Exchanges (SCES) of *Centella asiatica* plant extracts (500 & 1000 µg/ml) and control using three blood samples.

Type of Cultures	Control			CA (500 µg/ml)			CA (1000 µg/ml)		
	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3
Total no of sister chromatid exchanges observed	6136	5763	6377	5924	5361	6246	5783	5048	5962
No. of metaphases observed	1000	1000	1000	1000	1000	1000	1000	1000	1000
SCES	6.13	5.76	6.37	5.92	5.36	6.24	5.78	5.04	5.96
Mean SCES	6.08			5.84			5.52		

From the Fig 5 of SCES Vs Type of culture, obtained equation was $y = -0.28x + 6.3733$ and $R^2 = 0.9932$, which indicates that the frequency of SCES in *Centella asiatica* plant extracts were not significantly different from the SCES of control.

Determination of Cell Proliferation Kinetics (CPK)

Slides which were prepared for genotoxicity studies are also used to assess cell proliferation kinetics (CPK) and 25 metaphase chromosome spreads per culture were counted to determine the cell proliferation kinetics (CPK) in the control and *Centella asiatica* plant extracts of concentration 500 & 1000 µg/ml using three blood samples for the accuracy. The Mean Cell proliferation kinetics (CPK) values of the cultures which are exposed to two doses of plant extracts and control are obtained as shown in Table 3. The mean value of CPK of the control is 0.49 where as the *Centella asiatica* extracts have the mean value of CPK of the 500µg/ml is 0.47 and the mean value of CPK of the 1000 µg/ml is 0.46. CPK of *Centella asiatica* plant extracts were not much different from the CPK of control.

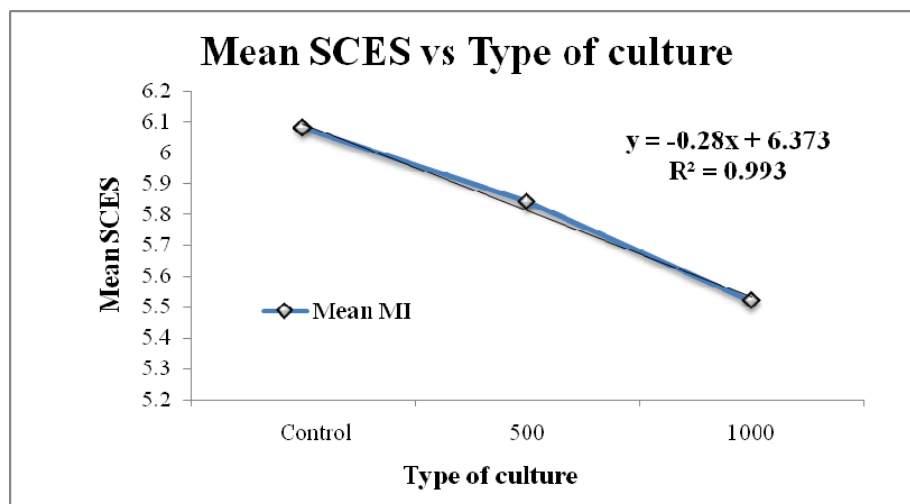


Fig. 5 Graphical representation of the overall mean Sister chromatid exchanges (SCES) of *Centella asiatica* extracts (500 & 1000 µg/ml) and control.

Table 3 Cell Proliferation Kinetics (CPK) of *Centella asiatica* plant extracts (500 & 1000 µg/ml) and control using three blood samples

Type of Cultures	Control			CA (500 µg/ml)			CA (1000 µg/ml)		
	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3	Blood sample 1	Blood sample 2	Blood sample 3
Dark stained metaphases M1	4	6	6	4	8	8	6	6	7
Differential stained metaphases M2	19	15	14	18	13	11	16	18	15
Light stained metaphases M3	3	4	5	3	4	6	3	1	3
Cell Proliferation Kinetics (CPK)	0.51	0.48	0.49	0.49	0.46	0.48	0.47	0.45	0.46
Mean CPK	0.49			0.47			0.46		

From the Fig 6 of CPK Vs Type of culture, obtained equation was $y = -0.015x + 0.5033$ and $R^2 = 0.9643$, which indicates that CPK of *Centella asiatica* plant extracts were not significantly different from the CPK of control.

Conclusion

In the present studies, *Centella asiatica* methanolic crude extract was prepared by soxlet extraction and then evaluated for the cytotoxicity and genotoxicity in human peripheral blood lymphocyte cultures. We used in this study crude extracts of *Centella asiatica*. Studying with crude extracts is appropriate because traditional medicinal herbs are generally used as crude extracts. However, working with crude extracts also means working

with complex mixtures of biologically active compounds. Some of these compounds can be cytotoxic and/or genotoxic; others can be cytoprotective and/or antigenotoxic.

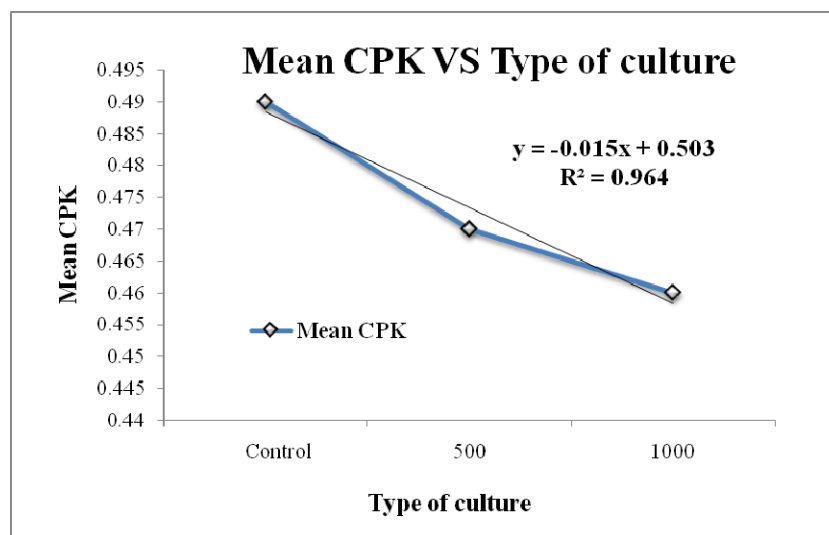


Fig. 6 Graphical representation of the overall means Cell Proliferation Kinetics (CPK) of *Centella asiatica* extracts (500 & 1000 µg/ml) and control.

Mitotic Index (MI), Cell Proliferation Kinetics (CPK) and Sister-chromatid exchanges (SCE) were scored to measure the cytotoxic and genotoxic effects of the *Centella asiatica* extract in cultures set up from the three different healthy donors. From the results, we can conclude that the MI and frequency of SCEs of *Centella asiatica* methanolic extracts are almost similar to that of control which indicates that the *Centella asiatica* plant extracts has no significant cytotoxicity and genotoxicity effects in cultured human peripheral blood lymphocytes.

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