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Comparative Study on Phytochemical, Antioxidant and Anticancer Properties of *Centellaasiatica* and Their Impact on P53 Expression in MCF-7 Cells

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ABSTRACT

Centellaasiatica (Linn.) is traditionally used as medicine from ancient days and reported for its wide therapeutic properties. It is widely used for leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhoea, fever, amenorrhea, anxiety etc. Thus present study had done on screening of phytochemicals, determination of antioxidant potential and their anticancer properties of two different traits in *Centellaasiatica* ($C_1$and C_2). The preliminary screening of phytochemicals by ethanol, chloroform and aqueous extract revealed a broad spectrum of secondary metabolites. Among the extracts ethanol extract contains rich in flavonoid content and it exhibited potential antioxidant activity at a concentration of 100 mg/mL 63.41% in C1 and 65.21% in C2. The anticancer study showed IC50of 187.78µg mL for C1 and 86.11 µg mL for C2 against MCF-7. The extract against L929 showed no cytotoxic effect towards normal cells with 698.55 µg/mL. Further analysis on non-toxic behaviour showed ethanol extracts of C2 is less toxic than C_1. The test compound namely C_2 showing p53 expression at 63.40 in terms of mean fluorescence intensity (MFI). The present study affirms that the leaf extracts of two different traits of *Centellaasiatica* has enormous potential and effective as natural antioxidant and anticancer. The phytochemicals present may responsible for antioxidant and other pharmacological effects of the plants. Based on the results it can be concluded that the C2 ethanol extracts holds significant antioxidant and anticancer properties.

Keywords: *Centellaasiatica*, phytochemicals, antioxidant, anticancer.

Introduction

Centellaasiatica (Linn.) Urban is a prostrate, perennial, creeping runner belongs to the family Apiaceae (Umbelliferae). The generic name *Centella* is derived from the Greek word centum and asiatica because of its native continent Asia. This plant was originally named as *Hydrocotyleasiatica* by Carl Linnaeus and later it is reclassified in the valid botanical systematics as *Centellaasiatica* (Linn.) Urban. It is also named as Gotukola in English, Pegaga (in Malaysia), Daunkakikuda (in Indonesia) and Brahmamanduki or Mandukaparni in Sanskrit. The species is native to India, China, Pakistan, Sri Lanka, Indonesia, Malaysia and South Africa and found throughout wet sandy areas near streams, rivers or other watercourses. It contains several bioactive constituents like triterpenoid, saponins such as, made cassoside, centelloside and asiaticoside [1].

Plants have ability to synthesize a wide variety of biologically functional compounds, e.g. primary and secondary metabolites and can be used to effectively treat human diseases[2].*C. asiatica* possesses wide range of pharmacological effects mainly used for wound healing, mental disorders, antibacterial, antioxidant, anticancer, ulcer preventive [3], anti-depressive sedative [4],wound healing, skin problem and digestive disorders [5].In India it is used

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as ethnomedicine, ayurveda, unani and in the traditional Indian medicinal systems for thousands of years for different ailments like asthma, skin disorders, ulcers and body aches [6], improving memory, nervine tonic and in treatment of dropsy, elephantiasis, gastric catarrh, kidney troubles, leprosy, leucorrhoea, urethritis [7], dog bite, asthma, carminative, itching, leucorrhoea, malaria, tumour and wounds [8].

The genetic variation of *C. asiatica* was reported in different samples from different localities in China[9] and India [10], but genetic characterization of closely related species is lacking. Although, previous studies suggested the influence of plant origin or growth conditions on the chemo type variation in the production of the active secondary metabolites [11]. There is no much more studies had been undergone on assessing this variation at the species level or its association to genetic variation. Thus our aims of our study is to differentiate the *C.asiatica* with different leaf size and analyze their properties based on phytochemical compositions, antioxidant and anticancer properties.

Materials and methods

Collection of plant material

The leaves of *C. asiatica* were collected from Kurunthenkodu of Kanyakumari District, Tamilnadu, India. It was identified and authenticated by Dr. P. Nagerndra Prasad, Head, Department of Biotechnology, Sri Paramakalyani College, Alwarkurichi, Tirunelveli. The plant was grouped into two groups based on the leaf size. The small leaf size is grouped as C_1 and big leaf size is grouped as C_2 based on morphology.

Preparation of plant extract

The plant leaves were cleaned, shade dried, chopped to fine particles using blender. The powdered leaves were subjected to soxhlet extraction with three different solvents ethanol, chloroform and aqueous for 24 hours. The extract was filtered and concentrated at 45°C using rotary vacuum evaporator and the extract were used for further investigation.

Qualitative analysis of phytochemicals

Phytochemical screening of leaf extracts of *C. asiatica* were subjected to qualitative phytochemical test for the analysis of various classes of active chemical constitutes such as carbohydrate [12], protein [13], steroids , saponins , tannin [14], terpenoid , glycosides , alkaloid , flavonoid [15] and phenol using standard procedures.

Quantification of phytoconstituents

Phytoconstituents present in the extracts were used to estimate quantitatively by various methods. For estimation of carbohydrate [16], protein [17], steroids [18], saponins [19], tannin [20], terpenoid [21], glycosides [22], alkaloid [23], flavonoid [24] and phenol [25] were used.

Determination of antioxidant activity

Ferrous ions chelating ability

The ferrous ion chelating potential of the extracts was evaluated by [26]. The reaction mixture contained 1.0 ml of various concentrations of the extracts (2-10 mg/ml) and 0.05 ml of 2 mM $FeCl_3$. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. The reaction mixture was shaken vigorously and left standing at room temperature for 10 minutes and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicated a higher ferrous ion chelating ability. The control contained all the reagents except sample. Ascorbic acid was used as standard for comparison.

$$\text{Percentage of inhibition} = [(\text{Control- Test})/\text{control}]\times 100$$

Nitric oxide radical scavenging assay

Nitric oxide generated from sodium nitroprusside interacts with oxygen to produce nitrite ions which was measured by the Griess reaction. This assay was done by the procedure described by [27]. The reaction mixture contained 3.0 ml of 10 mM sodium nitroprusside in phosphate buffered saline (pH 7.4) and various concentration of

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(2-10 mg/ml) extracts. The resulting solution was then incubated at 25°C for 60 minutes. To the incubated sample 5.0 ml of Griess reagent (1% sulphanilamide, 0.1% NEDD in 2% H_3PO_4) was added and the absorbance of the chromophore formed was measured at 546 nm against a reagent blank. Percentage inhibition of the nitrite ions generated is observed. The standard ascorbic acid and BHT was used for comparison. The free radical scavenging activity was determined by evaluating % inhibition as above.

Cytotoxicity assay

The mouse fibroblast cell lines procured from NCCS, Pune were cultured and maintained until they reach 70% confluence in T25 flask at 37°C in a 5% CO_2incubator. Afterwards, 200 µl cell suspension was seeded in a 96-well plate at the required cell density (20,000 cells per well), without the test agent and allowed the cells to grow for about 24 hours. Appropriate concentrations of *C. asiatica* leaf extract (25, 50, 100, 200 and 400 µg/ml) dissolved in DMEM media high glucose (Cat No. AL111, Himedia) were added and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Camptothecin with the concentration of 25µm is used as a positive control for the study. After the incubation period, remove the spent media and add 100 µl of MTT reagent (Cat No: 4060, Himedia) was added and incubated for 3 hours at 37°C. After incubation period, the formed formazan crystals were dissolved with 100 µl of DMSO (Cat No.1309, Sigma) and the absorbance readings were taken by ELISA Reader (ELX 800, Biotek) at 570 nm and the IC_{50} value is calculated using linear regression equation i.e. Y =Mx+C derived from the cell viability graph. The viability of the cells was determined by the following formula.

Percentage of viability = (OD of test compound treated cells/OD of untreated cells) x 100

Expression study of p53

Cell culture of MCF7 cells in 6 well plate at density of 3 x 10^5 cells/2 ml were incubated in CO2 incubator for overnight at 37°C for 24 hours. Remove the utilized media and treat the cells with C_1 (Test sample or experimental compounds) 86.11µg/ml, standard camptothecin 10µM and controls, in 2 ml of culture medium and incubate the cells for 24 hours. After treatment the medium from the well will be removed and washed with PBS and add 200 µl of trypsin-EDTA solution and incubate at 37°C for 3-4 minutes. Add about 2 ml of culture media and harvest the cells directly in 12 x 75 mm polystyrene tubes. Centrifuge the tubes at 300 x g at 25°C for five minutes. Decant the supernatant and wash the cells twice with PBS and remove PBS completely. Fix in 1 ml of prechilled cold 70% ethanol and add cell pellet by vortexing. Fix for at least 30 minutes on ice and pellet cells at high speed for 5 minutes, remove the supernatant. Wash twice with PBS and add 5 µL of FITC p53 antibody and incubate in dark room at 20-25^{0}C for 30 minutes. Wash with 1X PBS with 0.1% sodium azide and add 0.5 mL of PBS, and analyze by Flow Cytometry.

Results

The phytochemical screening of *C. asiatica* showed the presence of many phyto constituents in ethanol extract. In C_1 variety ethanol extract showed presence of carbohydrate, protein, flavonoid, glycoside, phenol and terpenoid. In C_2varietycarbohydrate, protein, flavonoid, glycoside, steroid and tannin was present. Alkaloid, steroid, saponin and tannin was absent in C_1 ethanol extract. In C_2 alkaloid, saponin, phenol and terpenoid was absent. The result obtained were tabulated in table 1.

The quantitative estimation of phytochemicals in different extracts was carried out and was tabulated in Table 2. Ethanol extract of $C_1$and C_2leaves showed the presence of 60.7 mg/gand 85.5 mg/g of flavonoids respectively. Whereas, minimum amount of saponin12 mg/gpresent in chloroform extract of C_1 and steroid 7.1 mg/g present in ethanolic extract of C_2.

Nitric oxide scavenging assay of all extracts was determined at three different concentrations 25, 50 and 100 µg/ml and results obtained for C_1 is tabulated in table 3 and C_2 in table 4. The results showed that scavenging is higher of 63.1% and 55% by the higher concentration of ethanol extract of C_1 and C_2 respectively.

In the same trend ferrous iron chelating assay was measured with three concentrations 25, 50 and 100 µg/ml of all extracts. The result showed increased activity with increased concentration. The chelating power was 52.1% and 65.1 by the ethanol extract of C_1 and C_2.

The crude extract of *C. asiatica* do not exhibited cytotoxic potential properties against the L929 cell lines by

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MTT assay. The results obtained in C_1 and C_2 extract are tabulated in table 5 and 6.

The cytotoxicity effect against MCF7 showed varied in their activity. Cells treated with concentrations of 25, 50, 75 and 100 µg/ml showed9.26% cytotoxicity in 25 µg/mland62.26 % toxicity at 400 µg/ml concentrations of the test compound C_1. Result obtained was tabulated in table 7.

The cytotoxicity against MCF7 by the test compound C_2 showed increased in their toxic effect when the treated concentration increases. The toxic effect at 25 µg/ml showed 20.26 %, at higher concentration 400 µg/ml toxicity was 87.5 %. Results were tabulated in table 8. The microscopic images of cell death observed is shown in figure 1 and 2.

Cells treated with different concentrations of 25, 50, 100, 200 and 400 µg/ml showed varied in their cytotoxicity. The inhibitory concentration IC_{50}value of L929 cells with C_1 treated sample showed 698.55 µg/ml and 620 µg/ml for C_2 treated cells. MCF-7 cells treated with C_1 showed IC 50 value at 187.78 µg/ml, In C_2 treated cells the inhibitory concentration is 86.11 µg/ml. Results obtained were tabulated in Table 9.

Expression study of p53against MCF7

The study investigated by changes in p53 expression in MCF-7 cells. The relative mRNA expression levels of the genes were determined using a flow cytometry. The fold increase or decrease in the expression of the genes was evaluated, relative to the calibrator. It was observed that C_2 extracts showed a significant fold increase in the p53 expression (Table 10 and Figure 3)

The statistical observation of p53 expression study by flow cytometry suggesting that the expression of p53is very low in untreated MCF7 cells (9.28 MFI) compared to stranded drug Camptothecin (10uM) showing 75.14MFI. The test compound namely C_2 showing p53 expression at 63.40 in terms of mean fluorescence intensity (MFI).

Discussion

In phytochemical analysisof*C. asiatica* in ethanol, chloroform and aqueous extracts of two different variety C_1 and C_2 showed varied in the presence. In summarized result of qualitative analysis revealed the phyto constituent presence was higher in ethanol extract of C_2 when compared with all other extracts. The results by [28] in *C.asiatica*also showed the constituents such as alkaloids, glycosides, flavanoids, phenols, saponins, tannins andterpenoids.

The phyto constituent's variationsin their availability insamespeciessuggest that variation in the phytochemical composition depends mainly on the genetic background. This can be a reason from the morphological characteristics of the species such as leaf size of *C. asiatica*. In the same way differences in secondary metabolite contents was observed in *C. asiatica* from different geographical regions was reported previously [29].

Quantitative estimation study reveals the ethanol extract of C_2 showed better phyto constituents when compared with all other extracts. The variation in their availability between C_1 and C_2 may depends on the environmental factors, nutrients in the soil etc. Flavanoid content in the ethanol extract showed higher in their availability in C_2. The flavonoid presence can help to have anioxident, anticancer property. Related to our statement a review states flavonoid content are rich in anticancer property [30].

Antioxidant activity of *C. asiatica*showed statistically significant results by the presence of free hydroxyls and several phenolic constituents such as flavonoids and tannins. This can help to possess an ideal structure for the scavenging of free radicals. The same result was observed in ethanolic extract of *C. asiatica* [31]. Flavanoid content in the extract helps to have highest antioxidant property. Our results coincides results obtained by [32], in aqueous extract of *C. asiatica.* Phenolic compounds found in leaf extract may help to have good antioxidant activity.

Extract of C_2 variety showed good and promising anticancer property.Previous research report reveals *C. asiatica*has good anticancer property either alone or combined form [33].*Terminaliabellirica*extract induces anticancer activity through modulation of apoptosis [34].

Extract of *C. asiatica* C_1 and C_2against L929 cell lines showed no harmful effects. Thus the compounds present in the extract has no lethal effectagainstnormal human cells.Similarly*Piper cubeba* extract against L929 cell lines showed same results [35].

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Cytotoxic effect against MCF7 showed good results in C_2ethanol extract when compared to C_1. Methanol extract of *Elaeisguineensis*exhibited significant cytotoxic effects on MCF-7 cell line [36].This may be due genetically changed in their species variety or some environmental changes. The viability of cells was 12.5 % at 400 µg/ml concentration. Thus it has good competency with cancer cells and able to kill at minimal dosage.

Expression of p53 gene by cytometry study reveals lowest fluorescence intensity in untreated cells and increased in stranded drug camptothecin followed by C_2 extract. When comparing fluorescence intensity of camptothecin and C_2 extract, mean fluorescence intensity showed slight higher in camptothecin.Anticancer effects of polyisoprenoid from *Nypafruticans*leaves showed controlled expression of p53 gene [37].

Thus C_2 extract has a therapeutic potential against human breast cancer and derived diseases. Further research studies on pharmacokinetics, clinical studies can help to develop a successful drug.

Conclusion

The result of the study shows that the ethanolic extraction showed presence of different phyto constituents in two different traits of *C. asiatica.* Antioxidant activity among DPPH, nitrous oxide and ferrous ion chelating assay sowed very good reduction in ethanoic extract of *C. asiatica*. Cytotoxic effect against and L929 showed low harmful effect by C_1trait and in MCF-7 result implies C_2showed good activity by competing cancer cells at lower concentration. Thus utilization of herbal medicinal plants for human treatment can help to live without any side effects.

Acknowledgement

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Conflict of Interest

The authors declare that no conflict of interest exists in the course of conducting this research. All authors had final decision regarding the manuscript and the decision to submit the findings for publication.

1. Phytochemical analysis of *Centellaasiatica* leaf extracts

Tests	C_1			C_2		
	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform	Aqueous
Carbohydrate	+	-	+	+	-	+
Protein	+	+	-	+	+	-
Alkaloid	-	-	+	-	-	+
Flavanoid	+	-	+	+	-	+
Glycoside	+	-	+	+	+	+
Steroid	-	-	-	+	-	-
Saponin	-	+	-	-	+	-
Phenol	+	+	+	-	-	-
Tannin	-	-	-	+	-	+
Terpenoid	+	-	-	-	-	-

Table 2. Quantitative estimation of phytoconstituents of *C. asiatica* leaf extracts

Test (mg / G)	C_1			C_2		
	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform	Aqueous
Carbohydrate	43.6	-	36.9	57.6	-	57.8
Protein	12.8	44.1	-	14.2	56.9	-
Alkaloid	-	-	53.8	22.2	-	46.8
Flavanoid	60.7	-	40.2	85.5	-	74.5
Glycoside	38.3	-	29.3	44.5	60.6	22.3

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Terpenoid	33.1	-	-	-	-	-
Tannin	-	-	26.1	35.2	17.8	-
Phenol	36.9	62.1	-	-	-	-
Saponin	-	12	-	-	25.3	-
Steroid	-	-	-	7.1	-	-

Table 3. Nitric oxide scavenging activity of *C. asiatica* leaf extracts

Concentration	C_1			C_2			Standard (Ascorbic Acid)
	Percentage of Nitric oxide scavenged (%)			Percentage of Nitric oxide scavenged (%)			
	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform	Aqueous	
25 µg/ ml	33.2	30.0	26.1	25.1	18.5	23.0	56.3
50 µg/ ml	48.0	45.1	37.8	33.5	25.5	31.6	69.5
100 µg/ ml	63.1	57.1	55.0	55.0	48.4	52.5	92.5

Table 4. Ferrous ion chelating activity of *C. asiatica* leaf extracts

Concentration	C_1			C_2			Standard (Gallic acid)
	Percentage of Ferrous Ion chelated (%)			Percentage of Ferrous Ion chelated (%)			
	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform	Aqueous	
25 µg/ ml	35.2	18.1	21.2	38.8	25.2	33.1	55.4
50 µg/ ml	47.5	25.3	12.0	48.1	37.9	35.2	74.9
100 µg/ ml	52.1	44.1	25.4	65.1	55.0	58.8	93.4

Table 5. Cytotoxic effect of *C. asiatica*extract (C_1) against L929

	Blank	Untreated	CPT 25 µM	25 µg	50 µg	100 µg	200 µg	400 µg
Reading 1	0.04	0.838	0.487	0.826	0.811	0.756	0.702	0.617
Reading 2	0.046	0.834	0.485	0.825	0.81	0.753	0.705	0.612
Mean	0.043	0.836	0.486	0.825	0.810	0.754	0.703	0.614
Mean OD-Mean B		0.793	0.443	0.782	0.767	0.711	0.660	0.571
Standard Deviation		0.0028	0.0014	0.0007	0.0007	0.0021	0.0021	0.0035
Standard error		0.0020	0.0010	0.0005	0.0005	0.0015	0.0015	0.0025
Viability %		100	55.86	98.67	96.78	89.72	83.291	72.06

Table 6. Cytotoxic effect of *C. asiatica*extract (C_2) against L929

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	Blank	Untreated	CPT 25 µM	25 µg	50 µg	100 µg	200 µg	400 µg
Reading 1	0.04	0.838	0.487	0.819	0.799	0.734	0.685	0.578
Reading 2	0.046	0.834	0.485	0.817	0.802	0.732	0.681	0.574
Mean	0.043	0.836	0.486	0.818	0.800	0.733	0.683	0.576
Mean OD-Mean B		0.793	0.443	0.775	0.757	0.69	0.64	0.533
Standard Deviation		0.0028	0.0014	0.0014	0.0021	0.0014	0.0028	0.0028
Standard Error		0.0020	0.0010	0.0010	0.0015	0.0010	0.0020	0.0020
Viability %		100	55.86	97.73	95.52	87.01	80.70	67.21

Table 7. Cytotoxic effect of *C. asiatica*extract (C_1) against MCF-7

	Blank	Untreated	CPT 25 µM	25 µg	50 µg	100 µg	200 µg	400 µg
Reading 1	0.04	0.872	0.465	0.795	0.634	0.571	0.429	0.356
Reading 2	0.046	0.878	0.46	0.793	0.638	0.565	0.432	0.358
Mean	0.043	0.875	0.462	0.794	0.636	0.568	0.430	0.357
Mean OD-Mean B		0.832	0.419	0.751	0.593	0.525	0.387	0.314
Standard Deviation		0.0042	0.0035	0.0014	0.0028	0.0042	0.0021	0.0014
Standard Error		0.0030	0.0025	0.0021	0.0020	0.0030	0.0015	0.0010
Viability %		100	50.42	90.74	71.27	63.10	46.57	37.74

Table 8. Cytotoxic effect of *C. asiatica*extract (C_2) against MCF-7

	Blank	Untreated	CPT 25 µM	25 µg	50 µg	100 µg	200 µg	400 µg
Reading 1	0.04	0.872	0.465	0.708	0.583	0.424	0.271	0.145
Reading 2	0.046	0.878	0.46	0.705	0.589	0.427	0.276	0.149
Mean	0.043	0.875	0.462	0.706	0.586	0.425	0.273	0.147
Mean OD-Mean B		0.832	0.419	0.663	0.543	0.382	0.230	0.104
Standard Deviation		0.0042	0.0035	0.0021	0.0042	0.0021	0.0035	0.0028

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Standard Error		0.0030	0.0025	0.0015	0.0030	0.0015	0.0025	0.0020
Viability %		100	50.42	79.74	65.26	45.97	27.70	12.5

Table 9. Percentage of cell viability and IC_{50} values of cell lines treated with *C. asiatica*extract (C_1 and C_2)

Cell lines	Sample	Percentage (%) of cell viability					IC_{50}valueμg/ml
		25μg	50μg	100μg	200μg	400μg	
L929	C1	98.67	96.78	89.72	83.291	72.06	698.55
	C2	97.73	95.52	87.01	80.70	67.21	620.00
MCF-7	C1	90.74	71.27	63.10	46.57	37.74	187.78
	C2	79.74	65.26	45.97	27.70	12.5	86.11

Table 10. Mean fluorescence intensity of p53-FITC against the untreated, standard and drug treated MCF7 cells and overlay of the results plotted in bar graph as below.

p53	Relative mean fluorescence intensity
Cell control	9.28
Std control	75.14
Test trait	63.40

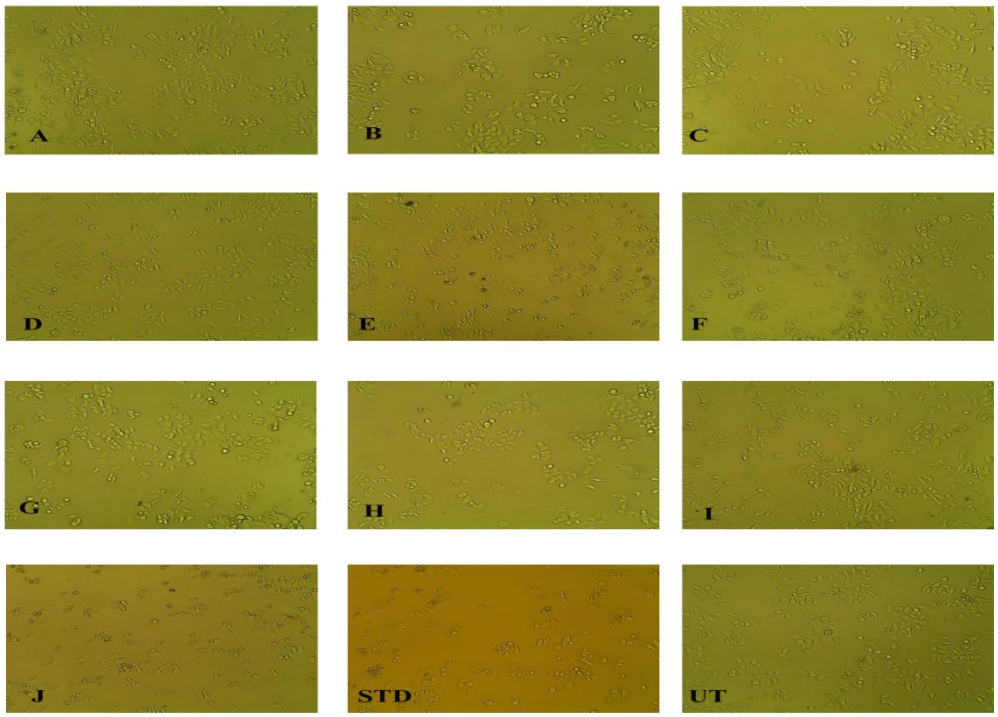

Figure 1. MTT assay of MCF 7 cell line at different concentrations with both C1(A) 25 (B) 50 (C) 100 (D) 200 (E) 400 and C2 extract. (A) 25 (B) 50 (C) 100 (D) 200 (E) 400 (UT) cell control (STD) Standard Drug.

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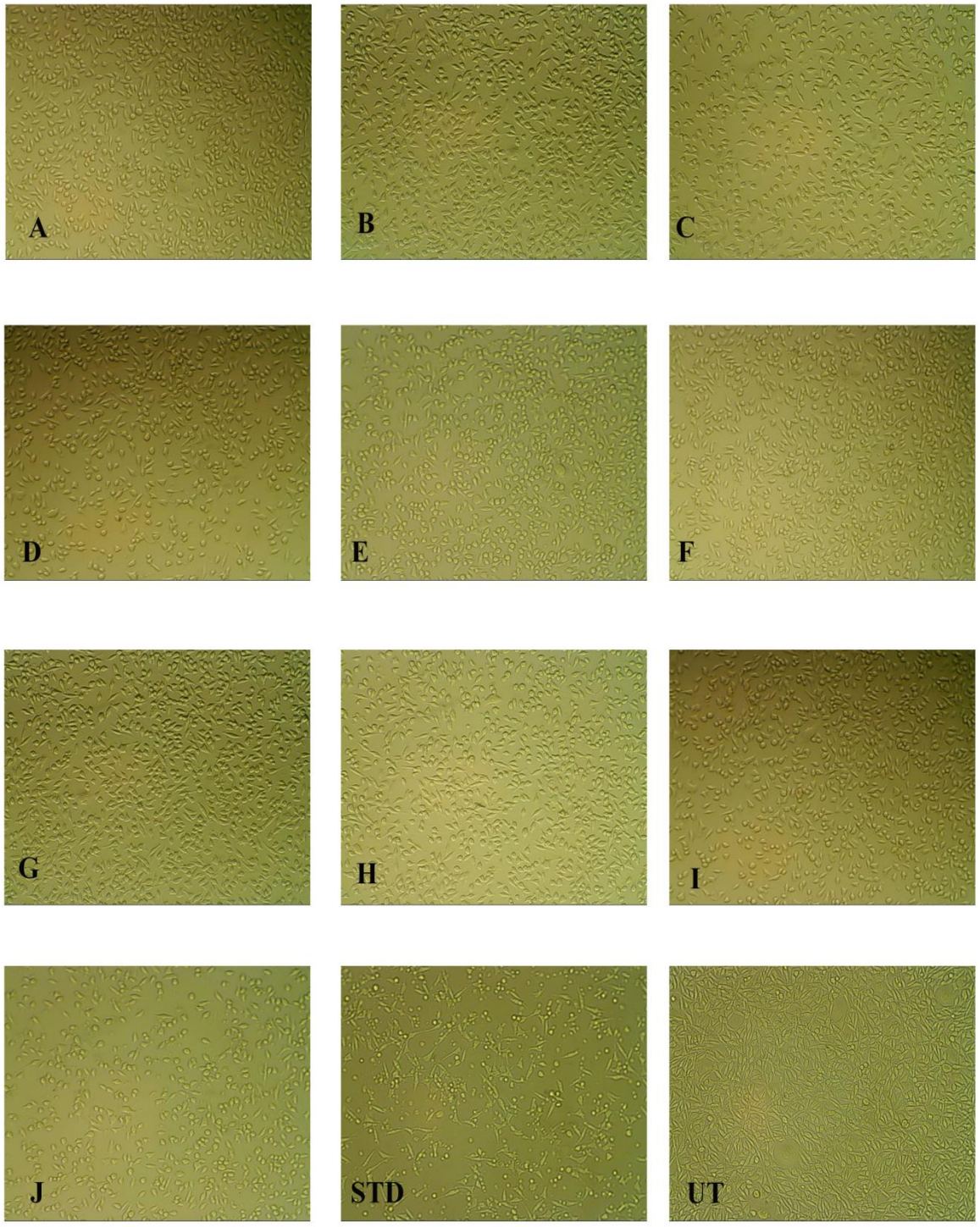

Figure 2. MTT assay of L929 cell line at different concentrations with both C1(A) 25 (B) 50 (C) 100 (D) 200 (E) 400 and C2 extract. (A) 25 (B) 50 (C) 100 (D) 200 (E) 400 (UT) cell control (STD) Standard Drug.

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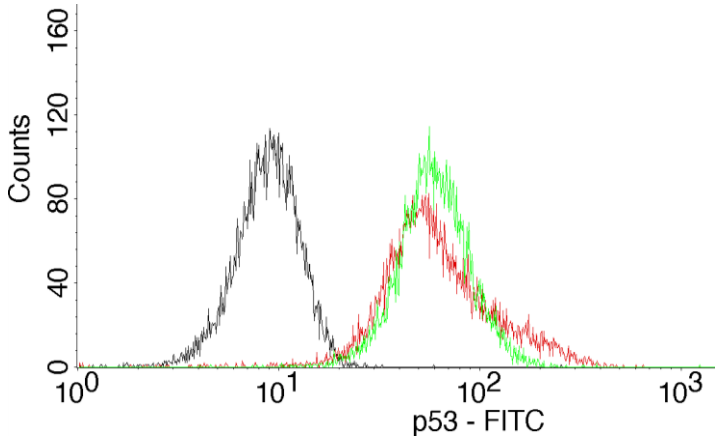

Figure 3. Overlay histogram vs MCF7
Black : Untreated MCF7 cells
Red : Standard drug camptothecin with 10uM treated MCF7 cells
Green : Test compound C_2 treated MCF7cells

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