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Studies on cytotoxic and clot lysis activity of probiotically fermented cocktail juice prepared using *Camellia sinensis* and *Punica grantum*

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Abstract. In the current research the effect of probiotic microorganisms viz; *Lactococcus lactis* and *Lactobacillus plantarum* on fermentation of *Camellia sinensis* and *Punica grantum* was studied. In vitro test were done to analyze the anticancer, antioxidant and atherosclerosis (clot lysis) properties of fermented juice. The juice was fermented for 48 and 96h, during which concentration of phenolic content, total acid content and free radical scavenging activity of the sample was analyzed by DPPH assay (α , α -diphenyl- β -picrylhydrazyl). Dropping of pH was observed after 48 h of fermentation. The clot lysis activity was found to be 80 % in 100 μ l concentration of fermented cocktail juice. The 96 h fermented sample has shown around 70% inhibition against colon cancer cell lines. Analytical study of HPLC proves the organic acid production such as ascorbic acid in superior amount for 96h of fermented sample, Based on the retention time, the corresponding peaks were detected at 4.919 and 4.831 min.

1. Introduction

During the past few years, we have observed that there is a significant change in people's idea about the usefulness of food. In this modernized era, there has been a great urge among peoples to develop economically affordable food products which can fight against lethal diseases like cardiovascular diseases, osteoporosis and cancer. Accordingly, a new term functional food was proposed. Functional food is basic and fundamental part of diet and has a broad range of health benefits and which can also reduce the risk of chronic disease along with their nutritional effects. The functional food containing a natural bioactive substances that include probiotics and antioxidant such as dietary fiber, foods and synthesized food material to traditional food. Among the functional components prebiotics and probiotics, plant antioxidants, omega-3poly unsaturated fatty acids, soluble fiber, conjugated linoleic acid, minerals, vitamins, proteins, phospholipids, peptides and amino acids are regularly declared. *Camellia sinensis* (green tea) and *Punica grantum* (pomegranate) are two such raw food products which has got a wide range of beneficial properties [1]. Various studies have already revealed that *Camellia sinensis* (green tea) possess anti-inflammatory, anti-mutagenic, anti-bacterial, anti-diabetic, hypocholesterimic and anti-tumor properties [9]. Moreover, pomegranate contain a compound called punicalagins which helps the heart and blood vessels. Punicalagins are the min and foremost component present in pomegranate which has a properties of antioxidant, reduce cholesterol level, reduce platelet aggregation, lower blood pressure and also function against atherosclerosis. *Punica grantum* (pomegranate) studies also proved that it has an inhibitory effect against prostate cancer, breast cancer, leukemia, colon cancer and to avoid vascular changes that endorse tumor growth in laboratory animals. The main objective of this study was to enhance the existing beneficial properties in *Camellia sinensis* and *Punica granatum* by probiotically fermenting the combined mixture of these two with a probiotics microorganisms viz; *Lactococcus lactis*



and *Lactobacillus plantarum* are a species of lactic acid bacteria. The objective of this research is mainly on the production of refreshing drink by using probiotics which can be act as a very good functional food with various beneficial properties and can be used against deadly diseases like cancer and atherosclerosis.

2. Materials and methods

2.1. Substrate preparation

One gram of *Camellia sinensis* (green tea) was boiled in 100ml of water, cooled and filtered by using sterile strainer. Twenty grams of *Punica granatum* (pomegranate) seeds were weighed and grinded in a sterile grinder. The juice obtained after grinding a pomegranate was added into 400ml of sterile water under aseptic condition. Cocktail juice was prepared by mixing 400ml of pomegranate juice and 100ml of green tea solution [6]

2.2. Fermentation

Prepared cocktail juice was divided into two equal parts and one used as a control and other was inoculated with 2.5ml of each *Lactococcus lactis* and *Lactobacillus plantarum* culture (freshly prepared overnight culture) into 250ml of juice under aseptic condition and fermented at room temperature for 48h and 96 h. After 96h of fermentation, the cocktail juice sample was stored in refrigerated condition. Sampling was performed periodically at 0thh (0th day), 48h (2nd day), 96h (4th day), 384h (8th day) and 1536h(12th day) respectively by centrifuging at 10,000rpm for 15 mintues at 4° C [6] and supernatant was used for further assays.

2.3. Microbial enumeration

The cells viability after 48h and 96h of cocktail juice fermentation were determined by pour plate method. Serial plate technique was used to dilute a 48 h and 96 h fermented cocktail samples. Plating was done on MRS agar media by using 10⁻⁴ dilution of fermented samples and incubated at 37°C for 24 h. Later, the total number of colonies were counted by using colony counter and recorded [4].

2.4. Determination of pH

The pH of the fermented broth was measured in periodic days of fermentation by using pH paper. The color change was observed and according to that pH was determined. pH of the control i.e; nonfermented cocktail was also measured to compare with fermented sample [4].

2.5. Acidity determination

The total amount of acid in a sample was analyzed by acid base titrimetic method. Standard solution of sodium hydroxide (NaOH) as a base and phenophthaline as an indicator used [5]. Total acid was calculated by determining the presence of total percentage of tartarate (non-volatile) and percentage of acetate (volatile) by using given formula as following:

$$\% \text{tartare} = \frac{\text{sample volume(ml)} \times \text{Nalkali(0.1)} \times 7.5 *}{2! \text{Vol. of alkali run down in burette(ml)}}$$

$$\% \text{tartare} = \frac{\text{sample volume(ml)} \times \text{Nalkali(0.1)} \times 6 *}{\text{Vol. of alkali run down in burette(ml)}}$$

2.6. Determination of total polyphenol compound

The total phenolic compounds in non-fermented and fermented cocktail juice was established by Folin-Ciocalteu method [7], with slight modification. Test was done by diluting 0.1 ml of sample in 3 ml of deionised water followed by addition of 1200µl of 1N Folin-Cioceltau solution and equilibrated for 5 mins. Later, 7% (w/v) of 1000µl of Na₂CO₃ were mixed to reaction mixture, absorbance was measured spectrophotometrically at 750nm after 90 mintues of incubation in dark. A standad was used as a Gallic

acid and total phenolics compound were expressed as gallic acid equivalents from the standardization curve.

$$Y = A + B X C$$

Where; Y= Absorbance of the test sample

A= Y intercept (0.08867)

B= Slope (constant value 0.000833)

C= Unknown concentration

2.7. Free radical scavenging assay

Anti-oxidant activity were done by diluting 0.025 ml of fermented cocktail juice in 4ml of methanol followed by the addition of 0.6 ml of freshly prepared DPPH. The reaction mixtures were incubated in dark for 30 minutes and OD value was noted at 520 nm [6]. DPPH was prepared by dissolving 0.002% in methanol (w/v) and 0.2mg/ml of ascorbic acid were used as a standard compound [8].

Free radical ability of a fermented cocktail were measured by standard formula:

$$\text{DPPH inhibition \%} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

2.8. Detection of compounds by HPLC

The HPLC (high performance liquid chromatography) technique was used to determined the presence of organic acid viz; ascorbic acid, lactic acid and citric acid after 96 h fermented sample. The fermented sample was centrifuged and clear supernatant was collected for analysis. For carrying out the HPLC, 2.50 mM of sulphuric acid was used as mobile phase and prepared standard of citric acid, lactic acid and ascorbic acid. The injection volume was set at 20µl and mobile phase flow rate was 0.2 ml/min [3].

2.9. Blood clot lysis assay

Clot lysis test was done by withdrawing 10ml of blood from a healthy person and from that 1ml of blood was transferred into 10 different pre weighed and sterilized eppendroff tubes . After addition of blood, tubes were weighed again and incubated for at 37°C for 30 minutes. Clot weight was determined by removing the serum carefully from clotted blood without any disturbance. Tubes containing clot were labeled (1 to 10) and different concentration of sample (25µl to 100µl) was added in the labeled tubes respectively. Tubes were incubated at 37°C and observed for lysis after an interval of 18 hours and the extra fluid was removed. The lysis was observed by keeping the tubes in inverted condition. Weight of each tube was taken again to determine the difference in weight after disruption of clot at different concentrations of the sample. The resulted difference in weight before and after lysis of blood clot were expressed in percentage of clot lysis. Blood clot lysis activity were calculated by the following formula [2]

$$\% \text{ of clot lysis} = \frac{\text{weight of released clot}}{\text{Clot weight}} \times 100$$

2.10. Anticancer activity - colon tumor cell lines

As 96h fermented sample has shown the maximum activities in all the previously performed tests this sample were centrifuged. The supernatant were taken in a new tube and examined the cytotoxic activities against colon tumor cell lines HT- 29. The reagents used for this assay are MIT (3-4,5 [dimethyl thiazole 2-yl] 2,5 diphenyl tetrazolium bromide: 0.5mg/ml of serum free DMEM medium, Dimethyl sulfoxide and PBS. The cells were overlaid in a 96 Microtiter plate with concentration of 105 cells/well. After 24h, the cells were washed with 25µl of serum free medium and starved at 37° C. After starvation cells were treated with various concentrations of test sample for 24h. At the end of the period the medium was aspirated and serum free medium containing MIT (0.5 mg/ ml) was added and incubated for 4h at 37°C. The MIT medium was discarded and cells were washed with PBS (200µl). The crystals were dissolved in 100µl of DMSO and this was mixed properly by pipetting up and down. The absorbance value of the

purple blue formazan dye was measured in a microplate reader at 570nm in Spectrophotometer. Cytotoxicity was measured by graph pad prism software 5.

3. Result and discussion

3.1 Microbial enumeration

The number of colonies counted with respective periods of fermented sample was given in Table 1 and Figure 1.

Table 1. Number of colonies counted with respective periods of fermented sample

Hours	Number of colony counted cfu/ml
0 th h	3.9×10^4
48 th h	12.5×10^4
96 th h	24.0×10^4
384 th h	10.7×10^4
1536 th h	No growth

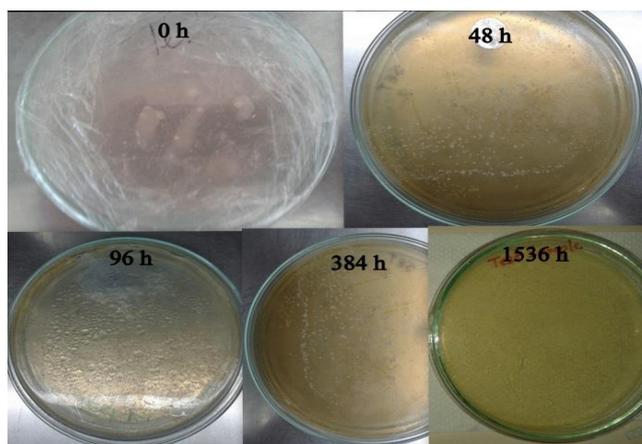


Figure 1. Number of colonies counted with respective periods of fermented sample

3.2 Determination of pH

pH of non-fermented cocktail juice was found to be 5.0 and analysis of fermented cocktail juice for 48h (2nd day), 96h (4th day), 388h (8th day) and 1536h (12th day) was found 3.0. This may be due to activities of probiotic microorganisms on breakdown of sugar molecule and production of organic acids. Figure 2 indicates the change of color which was because of decreasing of pH than that of non-fermented cocktail juice.

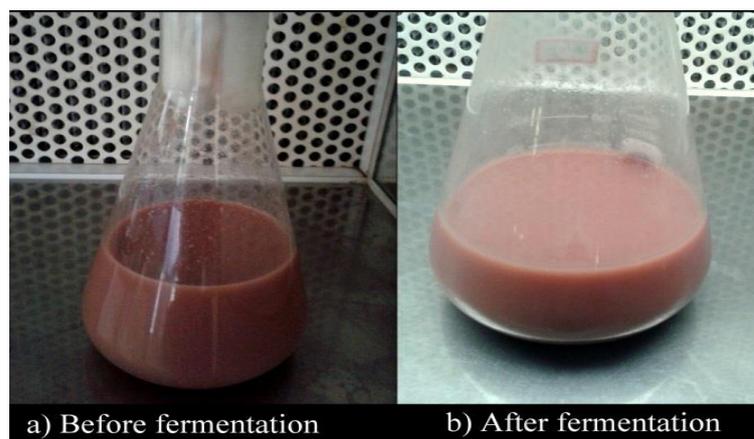


Figure 2. Change of Colour during fermentation indicates decrease of pH

3.3 Acidity determination

The percentage of total acid content in fermented and non-fermented cocktail juice was observed as given in table 2.

Table 2. Determination of Acidity

Hours	pH	% tartrate	% acetate
0h	5	0.37	0.3
48h	3	0.75	0.6
96h	3	0.75	0.6
388h	3	0.75	0.6
1536h	3	0.75	0.6
Control	5	0.37	0.3

3.4 Determination of total polyphenol content

Total concentration of polyphenol in fermented cocktail juice has increased during the ongoing days of fermentation. But once it has reached the 1536h (12th day) of fermentation the concentration of polyphenol has become constant. The concentration was found less in the un-inoculated sample i.e., control as the probiotic bacteria were not present there to ferment. The comparatively less concentration of polyphenol which was found in control was all because of its own property of having polyphenol present in it. The data has been tabulated in the following table 3. This result proves that the growth of probiotic bacteria which have been inoculated i.e., *Lactococcus lactis* and *Lactobacillus plantarum* has reached the static or stationary phase and the fermentation process has stopped.

Table 3. Determination of total phenol content

Hours	O.D at 750 nm	Concentration $\mu\text{g/ml}$
0h	0.725	906
48h	0.844	1885
96h	0.680	1910
388h	1.659	2017
1536h	1.769	2017

3.5 DPPH - Free radical scavenging assay

The free radical scavenging ability of a fermented cocktail in 0h (0th day), 48h (2nd day), 96h (4th day), 388h (8th day) and 1536h (12th day) are 12.5%, 87.5%, 89.2%, 65% and 50% respectively. Figure 3 shows that the free radical scavenging ability has increased initially along with the fermentation period and decreased once the microorganism's activity has stopped because as the growth of probiotic microorganism is reducing, their capability of increasing the radical scavenging compounds of the sample is also getting decreasing and even the existing radical scavenging property of the sample before fermentation is also getting reduced due to the oxidation of water. Figure 3 is showing the color of the sample after forming a complex with DPPH. Test tube 1 was the standard ascorbic acid and test tubes 2, 3, 4, 5, 6 were the sample of 0 h, 48 h, 96 h, 388 h and 1536 h respectively. Test tube 7 was the non-fermented cocktail.



Figure 3. Free radical scavenging assay

3.6 Clot lysis activity

Clot lysis activity of the 96h fermented sample was observed. Plate 4 and 5 shows that clot lysis activity has increased with the increase of the concentration of the sample. The maximum lysis of about 75% and 80% was observed after adding 75 μ l and 100 μ l respectively. The percentage of respective concentration of the sample has been tabulated in the table 4. From the result it can be said that 100 μ l of the fermented cocktail juice will act as a potent probiotic drink or medicine against various heart diseases which occur due to clotting of blood in heart.

Table 4. Clot lysis activity

Concentration of sample (μ l)	% of clot lysis
25	50
50	60
75	75
100	80



Figure 4 & 5. Clot lysis before and after addition of fermented cocktail juice

3.7 Analytical study – HPLC for the presence of organic acid

HPLC study established the organic acid production like ascorbic acid in a superior quantity at 96h fermented sample, Retention time of peaks were detected at 4.919 and 4.831 showed in figure 6: (C) and (D) respectively, as compared to raw sample, the respective peaks were detected to be 4.715 & 6.601 showed in figure (A) and 4.712 showed in figure (B). After fermentation the peak having the retention time 6.601 was not observed which may be due to the degradation of the compound resulted from fermentation. The increase in the value of the peak having 4.715 and 4.721 in raw sample to 4.919 and 4.831 respectively in fermented sample implies the increase in amount of organic acid after fermentation. The retention time of ascorbic acid is 5.1 which is very near to the observed peak value 4.919 and 4.831 that indicates the organic acid form after fermentation is may be ascorbic acid.

Figure A and B

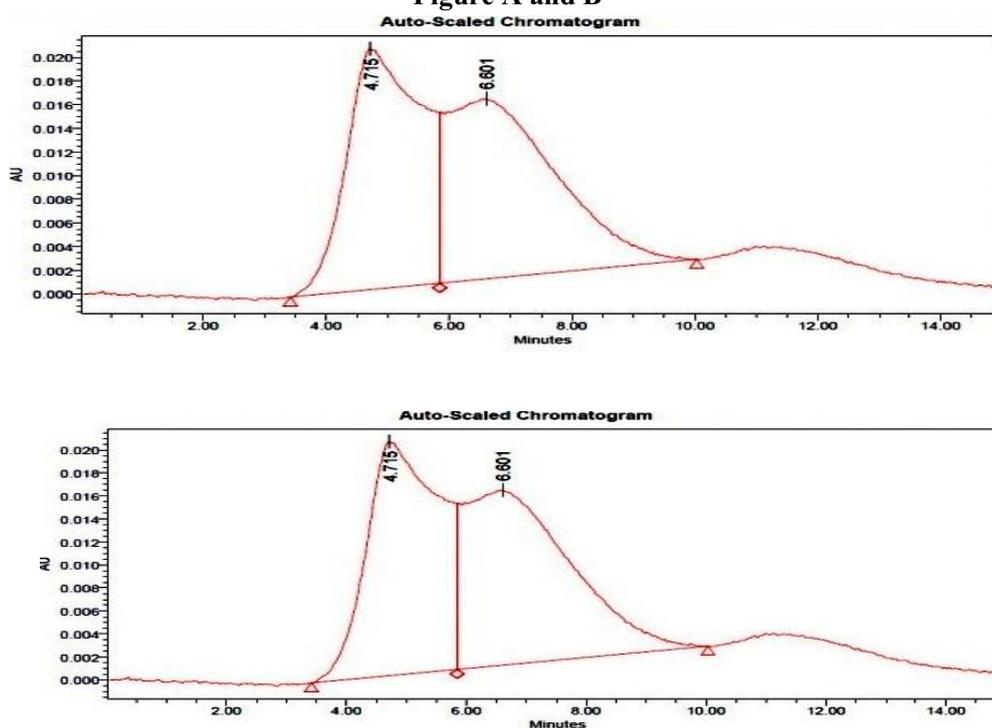
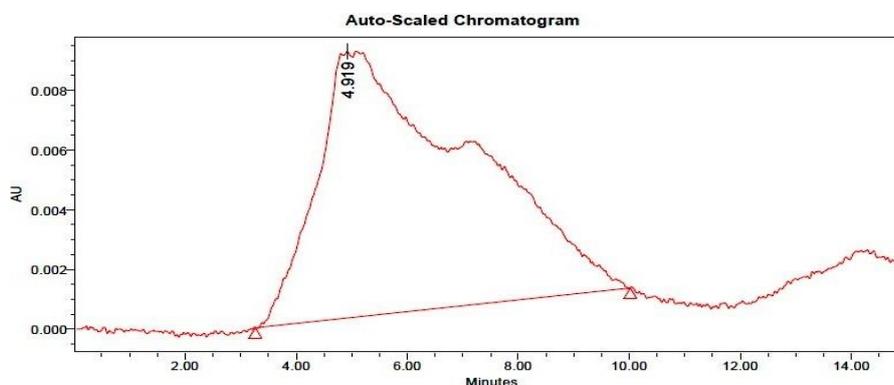


Figure 6. A and B represents the chromatogram for raw cocktail juice

Figure C and D



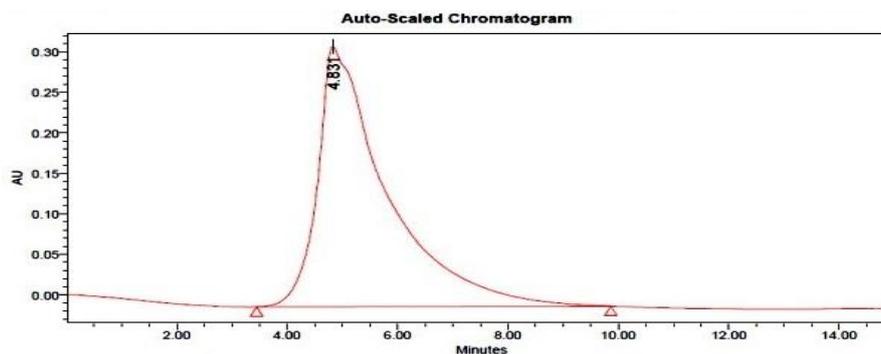


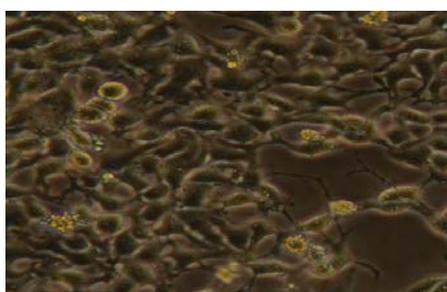
Figure 7. C and D Represents the chromatogram for 96 h fermented juice

3.8 Anticancer activity against human colon cancer cell line

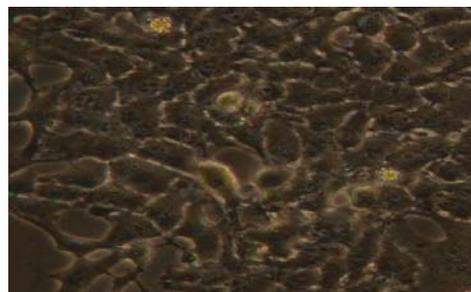
The sample of fermented cocktail juice of 96h was analysed for cytotoxicity activity against human colon cancer cell line HT – 29cell, whereas maximum cell lysis was observed at concentration of 250 $\mu\text{g/ml}$ when compared to 12, 25, 50 and 100 $\mu\text{g/ml}$ shows result in table 5 and Figure 8. The highest concentration showing maximum percentage to destroy cancer cell is given in the Figure F. When compared to the positive control (cyclophosphamide) in Figure 3 (E) the test sample has shown less cell lysis Figure 3 (F) but the positive control used is a chemical drug which has many side effects to human health and also it is an expensive product. So, by increasing the concentration of the concerned cocktail sample a better amount of cell lysis can be obtained which will have some effective benefits like cheaper than chemical drug thus availability will be easy for common men. Another benefit which is involved in using the fermented cocktail is that it is a natural product so it will not have any side- effects to human body. Both *Punica grantum* and *Camellia sinensis* have an antioxidant and artherosclerotic properties and from these test it has been also proved that fermented cocktail juice of these two viz; *Punica grantum* and *Camellia sinensis* are active against colon cancer.

Table 5. Anticancer activity against human colon cancer

Concentration of sample($\mu\text{g/ml}$)	% of cell viability
12	52.05
25	77.3
50	71.06
100	53.625
250	31.595



A



B

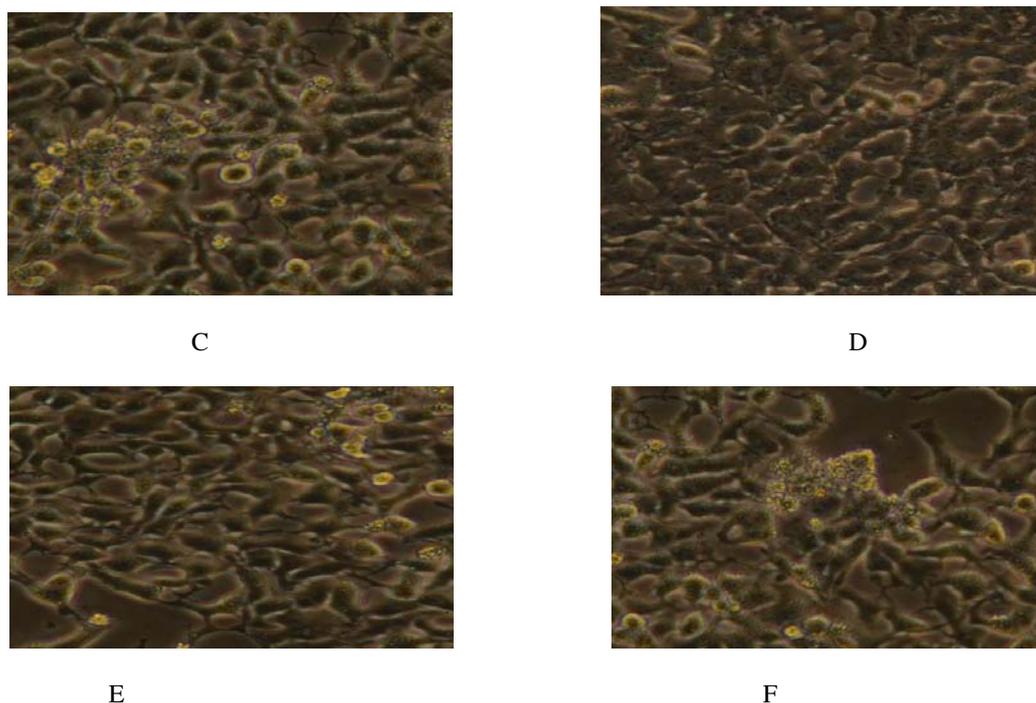


Figure 8. (A) Control cell, (B) Cancer cells treated with 12 $\mu\text{g/ml}$, (c) Treated with 25 $\mu\text{g/ml}$, (D) Treated with 50 $\mu\text{g/ml}$, (E) Treated with 100 $\mu\text{g/ml}$, (F) Treated with 250 $\mu\text{g/ml}$ of 96 h fermented cocktail juice

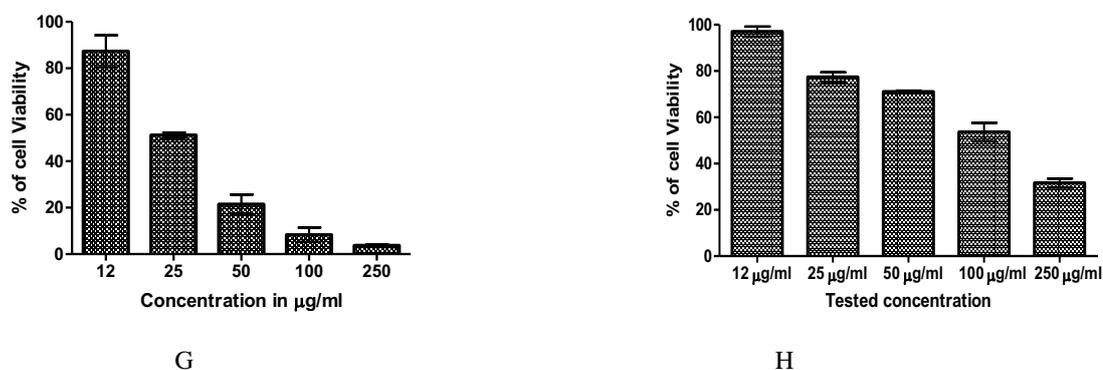


Figure 9. (G) Positive control readings (Cell viability for Cyclophosphamide for HT -29 cells) and (H) Tested sample (Cell viability for 96 h fermented cocktail juice) at different concentration.

4. Conclusion

It is concluded that the probiotically fermented cocktail of pomegranate juice and green tea have exhibited increased radical scavenging activity, Increased polyphenol content and anti-microbial activities. The quantity of activity depends upon culture period and starter origins, which in turn determined the forms of their metabolites. Although antioxidant properties have exposed the time-dependent profiles, prolonged fermentation was not recommended because of accumulation of organic acids, which may achieve harmful levels for direct consumption. Metabolic manipulations are one of the effective methods to elevate the

antioxidant activities and fermentation efficiency of the concerned fermented cocktail. Thus this type of probiotics fermentation of this cocktail has enhanced the existing beneficial properties of both the ingredients of the cocktail in an enormous way which in turn has made it a very potent and useful functional food.

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