Purification of Quercetin by HPLC from green tea leaves and its application in cancer therapy

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ABSTRACT

Quercetin is the subject of intense research on the basis of its antioxidant, anti-inflammatory and anti-cancer activities. Antioxidant potential of Green tea (*Camellia sinensis*) leaves was checked by various antioxidant methods. The estimation of Flavonoid content with the help of Quercetin Standard curve and the total amount of polyphenolic contents in all extracts of Tea leaves was determined with Aluminum chloride colorimetric method for flavonoids determination. Total Phenolic Content assay was performed on standard Gallic acid and various extracts for calculating the amount of Phenolic content present. Nitric Oxide Scavenging Activity Assay and DPPH Scavenging Assay were performed for L-Ascorbic Acid as Standard along with phytochemical screening. HPLC method was employed for quantitative examination of flavonoid quercetin from various plants and the time of Retention was calculated. Antitumor properties of the extract of Green tea was determined by using Cytotoxicity assay.

KEYWORDS: Quercitin, flavonoids, phenolic, scavenging assay

1. INTRODUCTION

Flavonoids are the compounds of polyphenolic group that are being distributed in large group. Near about 6000 flavonoids are being recognized, most of which are found in fruits, vegetables and beverages and are dietary antioxidants. The flavonoids have produced great awareness just because of their possible valuable effects on human health[1,2]. Quercetin pentahydroxy flavone belongs to a widespread class of polyphenolic compounds, particularly flavonoids which are almost abundant in plants and plant food sources[3-5]. Quercetin is a compound which is largely present in food products and plants, including those of fruits, seeds, vegetables, tea, coffee, bracken fern, and natural dyes. Quercetin is generally obtained from hydrolysis of rutin (quercetin-3-rutinoside), a naturally occurring flavonoid glycoside although it can also be synthesized[6,7]. Quercetin has been found to be a stronger antioxidant because of its capacity to scavenge free radicals and bind them with the transition metal ions[8]. All these properties allow it to obstruct lipid peroxidation[9,10]. Green Tea, which is an older and widespread therapeutic beverage consumed around the globe, is obtained from the leaf of the plant called "*Camellia sinensis*". It can be prepared as a drink, which can have many systemic health effects or an "extract" that is used as medicine. Green tea has been reported to contain number of bioactive ingredients that are almost contributed by polyphenols playing a major role in inhibition and treatment of many diseases[11]. So, the present study has been planned to explore the Quercetin therapeutic index.

2. MATERIAL AND METHODS

Cancer cell lines HL-60 (Acute Lymphoblastic Leukaemia) were bought from NCCS, Pune. Plant specimens (green tea leaves) were collected from the fields of Palampur (Himachal Pradesh). All other chemicals used in the present study were of analytical grade and purchased from Himedia Laboratory Pvt. Ltd, SD Fine Chemicals and Ranbaxy.

Preparation of Leaf Extract: Leaves were taken from healthy plant and were washed in water containing Tween -20 solution for 15 minutes and then with distilled water. 70% ethanol treatment was given to leaves for 90 seconds, rinsed again with distilled water 2-3 times. Surface sterilized leaves were air dried at room temperature and were grinded into fine powder.

Aqueous extract: 5 grams of the powder leaves sample was soaked in 150 ml of distilled water, stirred for 16 h at magnetic stirrer and filtered by Whatman filter paper. The filtrate was evaporated at 40 ºC.

Ethanol extract: 5 grams of finely ground powder of leaves were soaked in 150ml of 100% ethanol, stirred for 16 h at magnetic stirrer and filtered by Whatman filter paper. The filtrate was evaporated at 50º C. The extract was collected in powdered form.

Ethyl acetate extract: Ethyl acetate extract was prepared with the help of soxhlet assembly. 15 g of powder (sample) was wrapped in Whatman filter paper no.1. 130 ml of ethyl acetate was poured into the assembly. Half of round bottom flash was filled with acetate. The assembly was run at 75º C for 24 h. After 24 h,crude extract was collected in round bottom flask. Extract was evaporated in the water bath at 70º C and collected in the powder form.

Antioxident study: Flavonoid estimation: Aluminum chloride colorimetric method was being used for flavonoids determination. 0.5 mL of extract of the plant was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate

and 2.8 mL of distilled water. The above solution was then allowed to stand at room temperature and the absorbance of the above mixture was calculated at 415 nm. The calibration curve was then obtained using quercetin at concentrations of 12.5 to 100 µg/mL in methanol[12].

Total polyphenolic contents: Mixed 1.0 ml sample of various concentrations with 1.0 ml of ethanol. Added 15 ml distilled water and 0.5 ml of 50% Folin - Ciocalteu Reagent. Reaction mixture was allowed to react for 5 minutes and added 5% Sodium Carbonate solution. Reaction Mixture was mixed thoroughly and placed in dark place for 2 hours and the absorbance was measured at 765 nm on UV – Spectrophotometer. Dilution of Gallic Acid was made with solvent (Ethanol) and standard calibration curve of Gallic Acid was prepared[13].

Nitric oxide scavenging activity assay: 2 ml of 10mM Sodium Nitroprusside and 0.5 ml of Phosphate Buffer saline (pH 7.4) were mixed with 0.5 ml of extract of various concentrations. Reaction mixture was incubated at 25°C for 150 minutes. 0.5 ml was taken from incubated reaction mixture and added into 1.0 ml of Sulfanilic Acid Reagent (0.33% in 20% Glacial Acetic Acid) and incubated for 5 minutes at room temperature. 1 ml Naphthylenediamine Di-hydrochloride was mixed and incubated at room temperature for 30 minutes. Absorbance was taken at 540 nm with UV – Spectrophotometer[14]. Nitric Oxide Scavenging Activity was measured by following equation:

%Inhibition=[(Ao-Ai)/Ao x 100]

Where, Ao = Absorbance of the control (Blank) and Ai = Absorbance in presence of extract

Diphenyl picryl hydrazyl (DPPH) scavenging activity assay: Vaious concentration of extract was mixed with 0.8 ml Tris HCL Buffer (pH 7.4) and 1.0 ml of DPPH was added in this reaction mixture. Reaction mixture was shaken vigorously and left for 30 minute and absorbance was measured at 517 nm with UV–Spectrophotometer[15]. Scavenging Activity was calculated by following equation:

$$\% \, \boldsymbol{Inhibition} = \left[\frac{(\boldsymbol{Ao} - \boldsymbol{Ai})}{\boldsymbol{Ao}} \times \mathbf{100}\right]$$

Where, Ao = Absorbance of the control (Blank) and Ai = Absorbance in presence of extract

Phytochemical Screening: Test for alkaloids: Dragendorff reagent was used to test the presence of alkaloids. The presence of alkaloids was indicated by the appearance of yellow precipitate when few drops of chemical was added to the solution[16].

Test for saponins: Development of foamy lather was observed when the test solution alongwith the 2 drops of distilled water was taken in a test tube, indicating the presence of saponins.

Test for Tannins: About 0.5 g of the dried powdered samples was heated in 10 ml of water and then it was being filtered. 0.1% ferric chloride was being added to the above solution and was being noticed for brownish green or a blue-black colouration[16].

Test for Phlobatannins: The aqueous extract of the plant samples was heated with 1% aqueous HCl. Development of red precipitates showed the presence of phlobatinins.

Test for Anthraquinones: About 0.5 g of extract was taken in a test tube and then added 10 ml of benzene. Filter the solution and add 5ml of 10 % ammonia solution, the mixture was shaken vigorously. In the ammonical layer, presence of pink, red, or violet color indicates the presence of free anthraquinones[16].

Test for Terpenoids: 2 ml of chloroform was taken in a test tube and added 3 ml of concentrated sulphuric acid. Added 5 ml of each extract into the solution. Formation of reddish brown interface showed the presence of terpenoids.

Test for Flavonoids: Aqueous filtrate of the plant extract was taken in attest tube and added 5 ml diluted ammonia solution along with the concentrated sulphuric acid. Yellow florescence indicates the presence of flavonoids (16). **Test for Steroids:** 2 ml of acetic anhydride was added to 0.5 g extract of the sample along with 2 ml H2SO4. The change in color from violet to blue or green indicates the presence of steroids[16].

HPLC Conditions: Mobile Phase (A): filtered and degassed mixture of 1.2 ml Orthophosphoric acid in 1000 ml of purified water. Mobile Phase (B): Acetonitrile (HPLC grade). Flow: 1.0ml/min. Wavelength: 280 nm. Injection volume: 10µl. Colum temp: 30ºC. Gradient Program: 3:1. Table 1 shows the percent mobile phase at different time intervals.

Table.1. Percent Mobile phase at different time interval

Time (minute)	Mobile phase(A)%	Mobile Phase(B)%
0.01	95	5
35	70	30
36	70	30
40	95	5

Standard preparation: Weigh 25mg of Green tea extract working standard in 50 ml volumetric flask. Add 35 ml of 0.1% H_3PO_4 and sonicate. Add 5ml of Acetonitrile, sonicate for 2 min and cool. Dilute up to the mark with 0.1% H_3PO_4.

Procedure: Reference standard solutions and samples were prepared. Single injections for blank and standard were injected and prepared a linearity curve for the standard, using peak area vs. concentration (mg/mL). Linear regression analysis was performed on the data. The R2 value must be ≥0.999 for each analyte's calibration curve.

Determination of Anti-Tumour properties using cytotoxicity assay: The tissue containing cancerous cell (previously washed in buffer) was disaggregated in media containing RPMI 1640 and sodium bicarbonate. The disaggregation leads to formation of suspension containing large amount of cells, which were then added to culture flasks containing media, hormones and antibiotics. They were incubated for 24 hours and were sub cultured. The sub culturing involves culture flasks containing fresh media and addition of inoculum to them in a sufficient amount, which leads to formation of cell lines from sub culturing of primary culture[14]. Micro titer plates of Elisa reader were used by calculations of table 2.

Table.2.Calculations for micro titer experiment for Rhodamine assay

Sample	1	2	3	4	5	6	7	8	9	10	11	12
A	E	C	10	10	10	90	90	90	E	E	E	E
B	E	C	20	20	20	100	100	100	E	E	E	E
C	E	C	30	30	30	110	110	110	E	E	E	E
D	E	C	40	40	40	120	120	120	E	E	E	E
E	B	IC_{50}	50	50	50	140	140	140	E	E	E	E
F	B	IC_{50}	60	60	60	160	160	160	E	E	E	E
G	B	IC_{90}	70	70	70	180	180	180	E	E	E	E
H	B	IC_{90}	80	80	80	200	200	200	E	E	E	E

*C=control, cancer cells; B=blank, media; E= Empty

Test samples at different concentration (90 µl), about 10µl of rhodamine dye and 100µl of cancerous cells were placed from well no. 3 to 10. After the preparation of micro titer plate, readings were noted on Elisa reader. OD was noted twice for each well at 450nm and 630nm and calculated for:

Test mean = addition of all triplets OD / 3

Blank OD = OD of each (four) well / 4

Control OD = OD of each (four) well / 8

% inhibition = Test OD – Control OD / Control OD

3. RESULTS

Flavonoid Estimation: Absorbance was noted at different concentrations of quercitin and a standard plot was drawn between concentration and absorbance from 12.5 to 100 µg/ml in methanol (Fig1). It can be clearly seen from the figure that absorbance is directly proportional to concentration. The amount of Flavonoid content in green tea leaves is shown in fig 2. Keeping methanol quantity (1.5ml), $Alcl_3$ (0.1ml), Potassium Acetate (0.1ml), and distilled water (0.8ml) to be constant, absorbance was calculated for HPLC fractions. Ethyl acetate extract showed highest flavonoid amount than ethanol and water extract. HPLC of Std Quercitin was done from range 200-1000ppm and the retention time has been calculated.

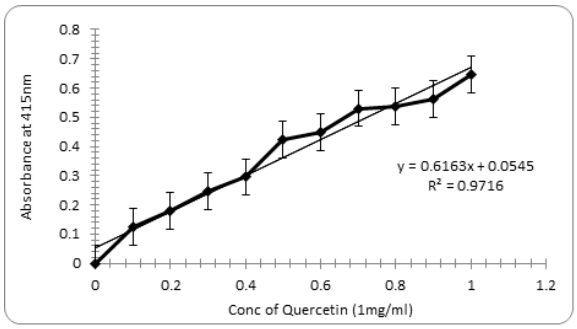

Figure.1. Standard calibration curve of Quercetin

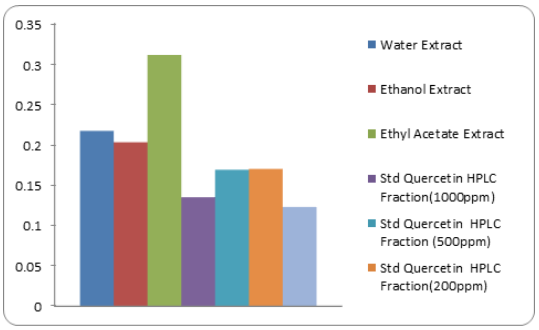

Figure.2. Flavonoid content in green tea leaves

Estimation of Total Phenolic content: Absorbance was noted at different concentrations of gallic acid (100-1000ppm) and standard plot was made between absorbance and concentration. It was found that the absorbance is directly proportional to concentration (fig 3).

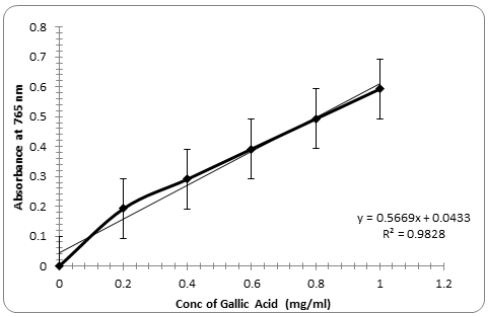

Figure.3. Standard curve of Gallic Acid

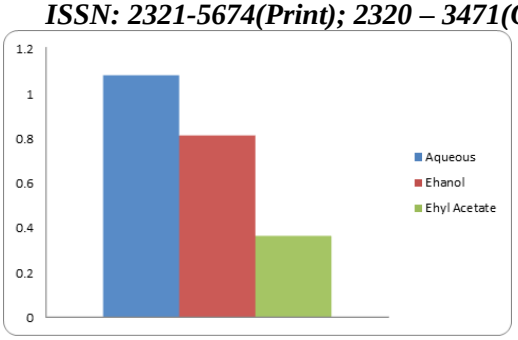

Figure.4. Total phenolic content in Green Tea Extracts

Total phenolic content was estimated in green tea leaves. Absorbance was noted for all the extracts and was plotted as in figure 4. Total Phenolic Content in decreasing order was:

Aqueous extract > Ethanolic extract > Ethyl acetate extract

Thus, the aqueous extract contained maximum amount of total phenolic content.

Nitric Oxide (NO) Scavenging Activity Assay: NO Scavenging activity was maximum at 1mg as shown in table 3. More than 50% inhibition of cancer cell lines was obtained at 1 ml of aqueous extract (fig 5). Similar results were obtained from ethanol extract (fig 6) but the percent inhibition was decreased to 44% in case of ethyl acetate extract (fig 7).

Table.3.NO Scavenging % for L-Ascorbic acid as standard (100ppm)

Test Tube	Concentration of L-Ascorbic Acid (mg)	Absorbance at 540 nm	NO Scavenging
Ao	0.0	-	-
1	0.2	0.562	12.324
2	0.4	0.551	14.040
3	0.6	0.517	19.345
4	0.8	0.484	24.493
5	1.0	0.437	31.825

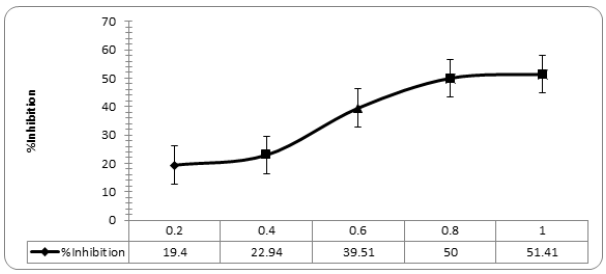

Figure.5.Percent inhibition of Aqueous Extract

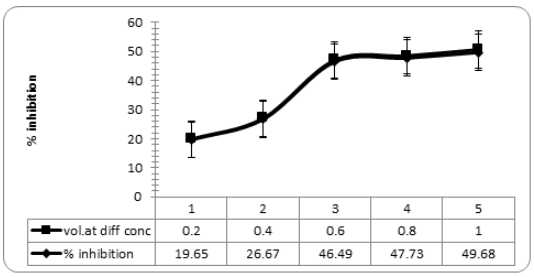

Figure.6.Percent inhibition of Ethanol Extract

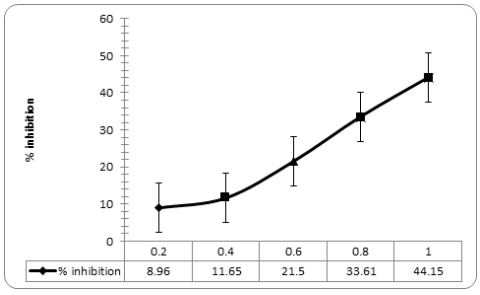

Figure.7.Percent inhibition of Ethyl Acetate Extract

DPPH Scavenging Activity Assay: Increase in the concentration of L-Ascorbic acid decreases the absorbance while there is correspondence increase in the DPPH scavenging as shown in table 4. Standard curve was plotted between DPPH Percent inhibition and concentration of l-ascorbic acid obtaining regression

coefficient value equal to 0.9474 as seen in figure 8. 1 ml of aqueous extract gave 68% inhibition (fig 9) in the viability of HL-60 cancer cell lines. In case of ethanol extract percent inhibition increased to around 60% (fig 10) and remained constant thereafter while for ethyl acetate extract percent inhibition increased to 80% (fig 11) at the same concentration.

Table.4.DPPH scavenging % for L-Ascorbic Acid as standard

Test Tube	Concentration of L-Ascorbic Acid (mg)	Absorbance At 517 nm	DPPH Scavenging
Ao	0.0	--	--
1	0.2	0.246	54.86
2	0.4	0.231	57.61
3	0.6	0.207	62.01
4	0.8	0.188	65.50
5	1.0	0.198	65.75

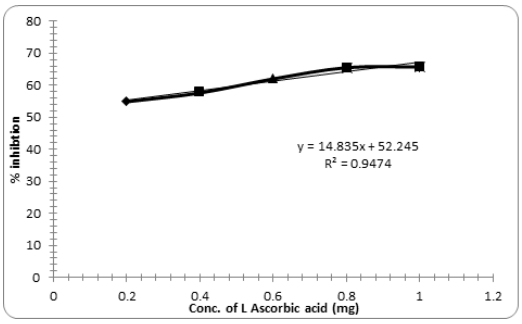

Figure.8.Calibration curve of DPPH L-Ascorbic acid as standard

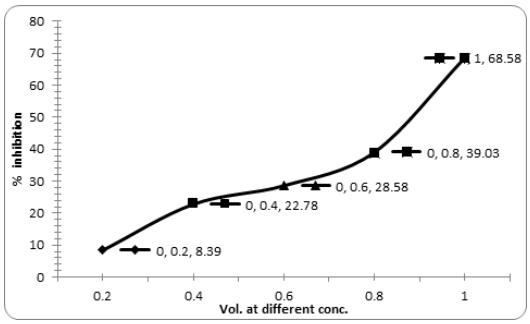

Figure.9.Percent inhibition for aqueous extract

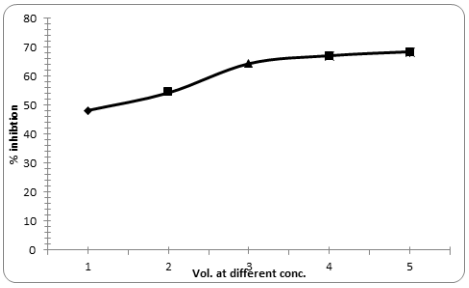

Figure.10.Percent inhibition of for Ethanol Extract

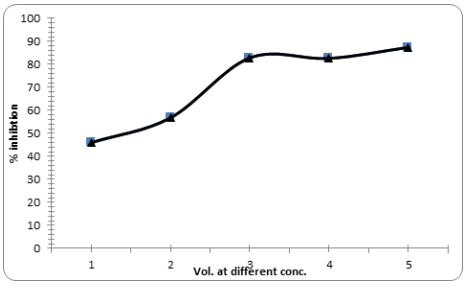

Figure.11.Percent inhibition of for Ethyl Acetate Extract

Phytochemical screening: By phytochemical screening it was observed that the green tea contained alkaloids, saponins, phlobataninns, steroids, terpenoids and flavonoids but not tannins and anthraquinones (table 5).

Table.5.Results for Phytochemical Screening

Test	Result
Alkaloids	Positive
Saponins	Positive
Tannins	Negative
Phlobataninns	Positive
Anthraquinones	Negative
Steroids	Positive
Terpenoids	Positive
Flavonoids	Positive

HPLC: By repeated experiment of HPLC having concentrations 200, 500, 1000 ppm the retention time obtained was same ~15 min as shown in figure 12-16. From the above results aqueous extract was used for further experiment.

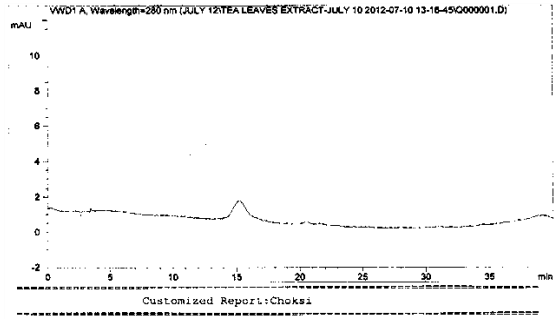

Figure.12.HPLC Curve for blank

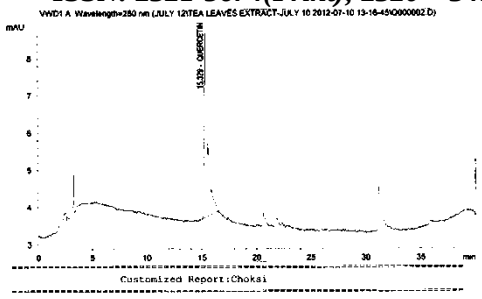

Figure.13.HPLC Curve for Standard quercetin (200ppm)

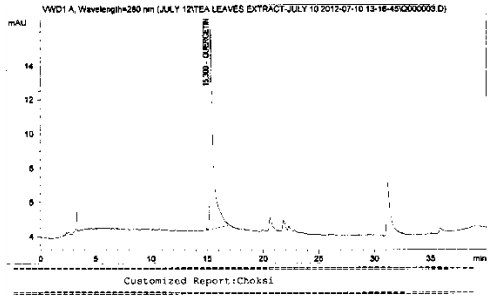

Figure.14.HPLC Curve for Standard quercetin (500ppm)

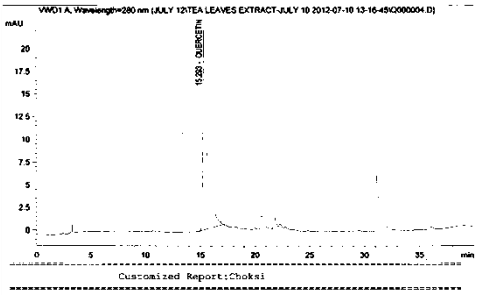

Figure.15.HPLC Curve for Standard quercetin (1000ppm)

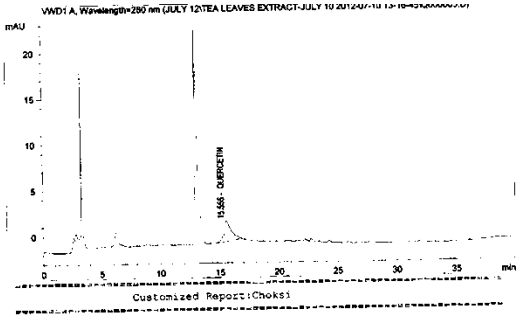

Figure.16.HPLC Curve for Green Tea Leaves extract (aqueous)

Determination of antitumor using cytotoxicity assay: Viable cancer cells were seen under flourescent microscope at 100x and the results are seen in figure 17. Inhibition activity of Standard Quercetin and Aqeous extract increase exponentially with the increase in concentration of extract. Thus, it showed antitumor properties. Standard Quercetin showed maximum inhibition (figure 18) at 80µl and the amount of Quercetin present is 16µg/ml. While Aqueous extract showed maximum inhibition at 160µl and the amount of Quercetin present is 5.9µg/ml. Thus, it can be concluded that even at this concentration it is very effective against cancer.

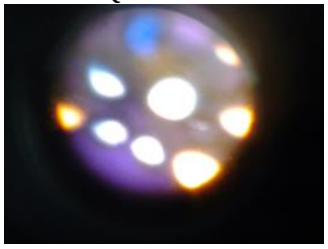

Figure.17.Viable Cancer cells seen under Flourescent Microscope at 100x

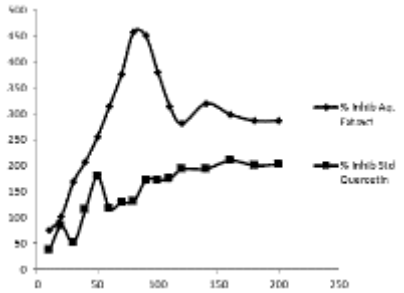

Figure.18.Comparison for Percent inhibition for aqueous extract and standard quercetin

DISCUSSION

Quercetin, the phenolic compound is the matter of strong research on the basis of its antioxidant, anti-inflammatory and anti-cancer activities. Quercetin

along with other flavonoids, have the ability to act as strong antioxidants and have often proved the same *in vitro*. Antioxidant potential of Green tea (*Camellia sinensis*) leaves was checked by various antioxidant methods.

Antioxidant activity is directly associated to the presence of Total phenolic content and Flavonoid content. The Flavonoid content was estimated with the help of Standard curve of Quercetin and the amount of total polyphenolic contents in all extracts of Tea leaves was determined with Aluminum chloride colorimetric method for flavonoids determination. The amount of Flavonoid content in Ethyl acetate extract and Aqueous Extract showed highest flavonoid amount then Ethanol. Total Phenolic Content assay was performed on standard Gallic acid and various extracts to determine the amount of Phenolic content present. Total Phenolic Content assay depicted that the leaves of *Camellia sinensis* are rich in the phenolic contents. Nitric Oxide Scavenging Activity Assay and DPPH scavenging assay were performed for L-Ascorbic Acid as Standard. Aqueous extract showed the higher amount of inhibition then Ethanol extract and Ethyl Acetate extract for both the assays. Phytochemical screening also showed positive results for the presence of Alkaloids, Saponins, Phlobataninns, Steroids, Terpenoids and Flavonoids. High Performance Liquid Chromatography was used for quantitative analysis of flavonoid quercetin from various plants and the Retention time of Green Tea Leaves Aqeouse extract was found to be 15.55. Antitimor properties of Green tea extract was determined by using Cytotoxicity assay i.e. Rhodamine assay and the results showed that the inhibition activity of Standard Quercetin and Aqeous extract increased exponentially with the increase in concentration of the extract. Thus, it shows antitumor properties.

4. CONCLUSION

From the present study, it was observed that leaves of Green tea (Camellia sinensis) have antioxidant and antitumor properties. *In vitro* research have shown quercetin for the ability to have a number of separate and independent mechanisms of anti-tumor action.

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