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Comparative pharmacological and phytochemical studies of the dried and fresh green tea leaves grown in Bangladesh

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Summary

The crude methanolic extracts (dried & fresh) were investigated to compare for possible phytochemical, antibacterial and biomedical activities. Phytochemical analysis, antioxidant test, hemolytic activity, haemagglutination inhibition test and *in vitro* anti-inflammatory tests were performed. Moderate antioxidant activities were found in case of fresh tea leaves methanolic extract whereas the dried tea leaves methanolic extract showed poor antioxidant activities.

The crude methanolic extract of fresh tea leaves showed poor anti-microbial activity whereas dried tea leaves extracts showed no antibacterial activities. Hemagglutination inhibition activities were observed against human red blood cells (RBCs) in both fresh tea leaves and dried tea leaves crude extracts.

The crude methanolic extract of dried and fresh tea leaves also showed to have prevented haemolysis of RBCs. However, higher anti-inflammatory effects were found in dried methanolic tea leaves extracts. The presence of caffeine and Epigallacto catechine Gallate (EGCG) were also compared in dried and fresh tea leaves where we found that fresh tea leaves contained a higher amount of EGCG and lower amount of caffeine than those of dried tea leaves. Therefore, this study will help to understand phytochemical, biological and microbiological observations of tea leaves locally grown in Bangladesh.

Keywords: Camellia Sinensis, Antioxidant activity, Hemolysis activity, Flavanoids, Antimicrobial activity, Bangladesh

Introduction

Camellia sinensis is native to India, distributed widely in sub-Himalayan regions and southward to Godavari. It is also widely distributed in Bangladesh, Indo-Pak subcontinent and South-Korea Asia, tropical and sub-tropical South East Asia. It grows in Japan and China also. It is also found in little amount in Madagaskar (Africa) [1, 2]. Tea leaves contain many compounds, such as polysaccharides, volatile oils, vitamins, minerals, purines, alkaloids (eg.caffeine) and polyphenols (catechins and flavonoids). Although all three tea types have antibacterial and free radical capturing (antioxidising) activities, the efficacy decreases substantially the darker the variety of tea is. This is due to lower contents of anti-oxidising polyphenols remaining in the leaves. Flavonoids (polyphenols) [3]. Proven medicinal properties include antioxidant, anti-inflammatory, anti-allergic, antibacterial and antiviral effects. They also have the ability to strengthen veins and decrease their permeability. It is widely believed that the antioxidising effects of both black and green varieties are reduced when taken with milk. This is thought to be due to the effective binding of flavonoids by proteins. However, a recent *ex vivo* study concluded that flavonols are absorbed from tea and their bioavailability is not affected by milk [4]. Tea tannins called catechins (polyphenols) appear to be the most potent therapeutic plant-derived chemicals, in that, aside from their antiseptic and antioxidant properties, they are able to form complexes with other molecules, thereby detoxifying the system. Catechins include gallocatechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (EGC) and epigallocatechin gallate (EGCG). Catechins make up approximately one-quarter of fresh dried green tea leaves, of which EGCG comprises 60 %. Vitamin C. A recent study showed that Black, Green and Oolong tea are all extremely good sources of vitamin C. They found that 1 or 2 cups a day provide the equivalent of three glasses of orange juice or two capsules (200mg) of vitamin C [5, 6]. Water and methanol extracts of persimmon leaf tea were studied for antioxidant and radical-scavenging activities and results showed that persimmon leaf tea could be considered as a natural antioxidant source [1]. The possible cancer preventive activity of tea has received much attention in recent years. Human intervention trials are warranted to determine the possible prevention of cancer of specific sites by preparation of tea constituents. [2] The antibacterial activity of the methanol and aqueous extract of *Camellia sinensis* was investigated and found that the methanol extracts of the test plant produces larger zones of inhibition against the bacteria than the water extract.

In this study we have shown the content of polyphenols and flavanoids present in the fresh leaves and dried leaves found in Bangladesh. We have also shown the comparisons of antioxidants, antibacterial and anti-inflammatory effects of fresh tea leaves and dried tea leaves grown in Bangladesh.

Materials and methods

2.1. Plant collection and identification

The fresh tea leaves of the plant were collected from The plant sample was collected from Srimangal, under Sylhet division on 20th June, 2012 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka as *Musa sapientum var. sylvesteris*. A voucher specimen of the plant has been deposited (Accession No.: DACB 37902) in the herbarium for further reference.

2.2. Extraction of the plant material

Fresh tea leaves and sun-dried and powdered plant material (750 gm x 2) was extracted with fresh distilled water and methanol by cold extraction process. In case of extracting the aqueous extract the fresh tea leaves were boiled at 100°C for 30 minutes. The extracts were stored at 4°C until used.

2.3. TLC analysis

The extracts were analyzed by performing TLC to

determine the composition of each extract. TLC was done under polar basic solvent consisted of ethyl acetate, ethanol, and water (8:1.2:0.8). After completion of TLC, the plates were exposed to UV light for compound detection and identification. For charring the plates were exposed to 10% sulphuric acid solution, dried and then heated to 80-90°C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible. For detection of flavanoids the plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place. For detection of polyphenols the plates were washed with Folin-ciocalteu reagent and dried. Epigallactocatechin gallate (EGCG) and caffeine were used as reference standard for this purpose. EGCG was given from the gift of ITOEN, Japan and caffeine was purchased from MERCK, Germany.

2.4. Chemical Analysis of the extract

Ultraviolet (UV) spectroscopy scanning of the diluted extract was performed within 200nm to 400nm using a Lambda UV spectrometer (Shimadzu, Japan) [7]. EGCG and caffeine were used as reference standard for this purpose.

2.5. Determination of caffeine in fresh and dried tea leaves by UV-spectroscopy

Different concentrations (0-50 µg/ml) standard caffeine was dissolved in distilled water and boiled at 100 °C to make a clear caffeine solution. A standard curve was prepared using various concentrations of caffeine solutions by measuring the absorbance at 273 nm in ultraviolet (UV) spectroscopy (Shimadzu, Japan). By plotting the values of concentration and absorbance a standard curve is prepared. From the standard curve the concentration of caffeine in fresh and dried tea leaves were measured.

2.6. Determination of total phenolic content

The total phenolic content of extracts was determined using Folin-Ciocalteu method using gallic acid as standard [8]. The extracts were oxidized with 10% Folin-Ciocalteu reagent (Merck, Germany), and were neutralized with 700 mM sodium carbonate solution. The absorbance of the resulting blue color was measured at 765 nm after 60 minutes. The total phenolic contents were determined using a standard curve prepared with gallic acid. The estimation of the phenolic compounds was carried out in triplicate. The results were mean ± standard deviations and expressed as milligram of gallic acid equivalent/g of extract.

2.7. Total Flavonoid Assay

The total flavonoid compounds in each extract were determined as previously described by Jothy et al. 2011 [8]. An aliquot (1.5 ml) of methanolic extract was added to 6ml of deionized water and then 0.45 ml 5% (w/v) $NaNO_2$ and incubated for 6 min. 0.45 ml 10% (w/v) $AlCl_3$ and 6 ml 4%(w/v) NaOH was added and the total volume was made up to 15 ml with distilled water. The absorbance was measured at 510 nm by using visible spectrophotometer. The results were expressed as mg rutin equivalents/g. The experiments were performed in three times.

2.8. DPPH radical scavenging activity

The free-radical scavenging activity of the extract were measured by decrease in the absorbance of methanolic solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) [9]. The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the plant extract (10, 50, 100, 200 & 500 µl/ml, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg /ml). Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant. After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the following equation:

DPPH antiradical scavenging capacity (%) = (Absorbance of sample – Absorbance of blank) × 100/Absorbance of blank

Methanol plus different concentration of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control. IC_{50}values denote the concentration of the sample required to scavenge 50% of DPPH radicals.

2.9. Total reducing assay

The reducing power of the extracts of MSS extract was measured using the potassium ferricyanide reduction method. Various amount of extracts (10, 50, 100, 200 & 500 µl/ml, in water) and L-ascorbic acid (0-1000 mg) were taken in different test tubes as previously described by Oyaizu et *al.*, 1986 [10]. Then 2.5 ml of distilled water and 2.5 ml of potassium ferricyanide $[K_3Fe_3(CN)_6]$ solution were added in all test tubes and mixed well. After incubation at 50°C for 20 min, 2.5 ml of trichloro acetic acid(TCA)[10% w/v] was added in all test tubes and centrifuged at 3000 rpm for 10 min. Afterwards, upper layer of solution(5ml) was mixed with 5 ml distilled water. Then 1ml of $FeCl_3$ was added each test tube. Then from each test tube we collect 1ml of solution and mixed it with 9 ml of distilled water. Then the solution was incubated at 35°C for 10 min. the formation of perls prussian color was measured at 700 nm in a spectrometer. Increased absorbance of the reaction mixture indicates increasing reducing power. L-ascorbic acid was used as a standard. The analysis was performed in twice.

2.10. Antibacterial assay

In order to screening the antimicrobial assay, five different bacterial strains of gram negative, three different strains of gram positive bacteria and three different strains of fungi were used to carry out this assay. The gram negative bacteria are *P. aureus*, *S. boydii*, *V. parahaemolyticus*, , *S. typhi, E. coli;* the gram positive bacteria are *S. lutea, S. aureus, B. subtilis*; the fungi are *C. albicans, A. niger, S. cerevisiae.* Nutrient agar was used as the culture media. Stocks of these bacterial solutions were revived in nutrient agar by incubating at 37°C for 24 hrs. A single disk diffusion method was used to assess the presence of antimicrobial activities of the methanolic extract of dried and fresh tea leaves. Whatman's filter paper was punched, and 6 mm disks were collected in a beaker. The beaker was covered with foil paper and autoclaved. 20 µl of different concentration extracts (0-10 mg/ml) were loaded per disk. The revived test organisms were plated onto nutrient agar plates. The disks were then placed equidistant on all plates for all extracts. Standard disc (Himedia, India) of Azithromycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. After incubation at 37°C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

2.11. Hemagglutination inhibition assay

Hemagglutination activity of seed extracts, fresh juice and pulp extract were tested against human erythrocyte blood groups ABO as previously described by Saha et *al.* 2009 [11]. Stock solution of the test samples was prepared at concentration of 5 mg/ml and each solution was serially diluted. Fresh blood was collected from healthy persons, centrifuged and the erythrocytes were separated. 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) of all blood groups. 1 ml of the test sample dilution was taken with 1 ml of 1% erythrocyte and incubated at 4°C. After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

2.12. In-vitro anti-inflammatory test (Erythrocytes membrane stabilization method)

*In vitro*anti-inflammatory test has been perfor-

med as previously described with slight modifications [12]. Alsever solution was prepared by 2% (w/v) dextrose solution, 0.8% (w/v) sodium citrate0.05% (w/v) citric acid, and 0.42% (w/v) sodium chloride dissolved in distilled water then the solution was sterilized. Blood was collected from cubital vein of healthy volunteers (HRBC). The collected blood was mixed with equal volume of sterilized alsever solution. The blood was centrifuged at 3000 rpm for 5 minutes and packed cells were washed with isosaline and a suspension of 10 %(V/V) isosaline was made. Various concentrations of seed extracts, fresh juice and pulp extract were prepared in 1 ml phosphate buffer (pH 6.8), 2ml hyposaline and 0.5ml HRBC suspension. Diclofenac sodium was used as standard drug. The assay mixtures were incubated at 37°C for 30 minutes and centrifuged at 3000 rpm for 5 minutes. The hemoglobin content in the supernatant solution was estimated using UV analysis at 560 nm.the percentage haemolysis was calculated by assuming heamolysis produced in the presence of distilled water as 100%.the percentage of HRBC membrane stabilization produced was calculated by using the following equation.

2.13. Anti hemolytic assay

Inhibition of H_2O_2 induced red blood cell (RBC) hemolysis of methanolic extract was examined by the *in vitro* method described previously by Tavazzi et *al*. 2001 [13]. The erythrocytes from human blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of fresh and dried tea leaves extract sample (0-25 ìg /ml) with saline or buffer were added to 2 ml of the suspension of erythrocytes and the volume was made up to 3.5 ml with saline or buffer. This mixture was pre incubated for 120 min and then 0.5 ml H_2O_2 solutions of appropriate concentration in saline or buffer were added. The concentration of H_2O_2 in the reaction mixture was adjusted so as to bring 90% hemolysis of blood cells after 120 min incubation. Incubation was concluded after these time intervals by centrifugation during 5 min at 3×1000g and the extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation. Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{Antihemolytic activity (\%)} = (\text{Control}_{540\ nm} - \text{Sample}_{540nm}) \times 100/\text{Control}_{\ 540\ nm}$$

where, Sample 540 nm was the absorbance of the sample and Control 540 nm was the absorbance of the control.

Results and Discussions

3.1. Phytochemical screening

In order to perform phytochemical screening of tea leaves extract TLC analysis were done as described in materials and methods and shown in Fig. 1. The plate was observed under UV light (indicated as 1 in each plate). It showed some spots which indicate the presence of different compounds in that sample. After charring of the TLC plate with sulfuric acid (indicated as 2) has revealed many spots in the TLC plate. Spraying of the DPPH solution in the TLC plate shows moderate violet yellow color which indicates the presence of flavanoids in the extract (indicated as plate 3).

From the UV-spectroscopy scanning of Caffeine and the aqueous extract of *Camellia sinensis* the absorbance maximum was measured from 200-800 nm and found the presence of caffeine in both fresh tea leaves and dried tea leaves (Fig. 2A) and it was about 273 nm. Again, UV spectroscopy scanning of EGCG and the methanolic extract of *Camellia sinensis* the absorbance maximum was measured from 200-800 nm and found the presence of EGCG in both fresh tea leaves and dried tea leaves (Fig. 2B) and it was about 270 nm.

3.2. Total caffeine, phenolic and flavanoids content

Quantitative analysis of caffeine, polyphenols and flavanoids were performed as described in the

method section. The amount of caffeine was measured from a standard curve, Y=105.5 X, R2=0.858. 1 gram of dried tea leaves and fresh tea leaves extract contain caffeine about 0.42 mg and 0.20 mg respectively. In case of polyphenols quantifiacation as standard curve was used where the equation is y = 1.932X, R2= 0.973. From the standard curve, the total phenolic compounds as tannic acid equivalent (TAE) of the dried tea leaves and fresh tea leaves extract was 0.26 mg and 0.11 /100 mg sample respectively. In case of flavanoid quantification a standard curve was used where the equation is y=0.002X+0.318, R2=0.9989. From the standard curve the amount of quercitin present in the dried tea leaves and fresh tea leaves extract is 6.6 and 25.16 mg of quercitin respectively equivalent per gram of sample.

3.3. DPPH radical scavenging activity

From the analyses of Figure 3, we can conclude that the scavenging effect of fresh tea leaves extracts is higher than that of dried tea extract. L Ascorbic acid was used as a positive control and the 50% inhibitory effect was 1.65 mg/ml. Whereas, the 50% inhibitory effect of the fresh tea leaves and dried tea leaves extract was calculated from the curve and it was 0.0021 mg/ml and 0.0068 mg/ml respectively. This maybe because the drying has caused some of its scavenging power to lose.

3.4. Total reducing assay

The reducing properties are generally associated with the presence of reductants which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The reducing power ability of crude methanolic extract of *Camellia Sinensis* (leaves) was determined using L-ascorbic acid as positive control. Total reducing assay of the dried tea leaves and fresh tea leaves extract was investigated in compared with L-ascorbic acid as shown in Figure 4. The comparison graph above clearly shows that the reducing power of fresh tea leaves is slightly higher than that of the dried tea leaves.

3.5. Anti inflammatory test

The crude methanolic extracts of *Camellia sinensis* (leaves) were subjected to determine Anti- inflammatory Activity as shown in Figure 5. In this experiment, the dried tea leaves showed better anti-inflammatory activity than the fresh tea leaves.

3.6. Hydrogen peroxide induced hemolytic inhibition activity

Hemolysis caused by hydrogen peroxide was inhibited by the extract at various concentration has shown in Figure 6. 300 µl of H_2O_2 was used for complete lyses of RBC. Here, again, the fresh tea leaves showed better anti-haemolytic activity than the fresh tea leaves. The fresh tea leaves were the raw extracts, which obviously contained more active compounds than those of the dried leaves.

3.7. Hemagglutination inhibition assay

Various concentrations of fresh tea leaves and dried tea leaves extracts (0-5 mg/ml) were taken to investigate hemagglutination inhibition activity on different types of human blood groups. The results in the titer plate above show that the haemagglutination activities of the fresh and dried tea leaves are almost the same. It showed best results in case of blood groups A+ and moderate activity in B+ and O+ blood groups. AB+ blood group showed less positive result. So, the values for minimum concentrations at which the extracts will give positive result for different blood groups are shown in Table 1.

3.8. Antimicrobial assay

The methanolic crude extract of dried and fresh leaves of *Camellia sinensis* was subjected to the various bacterial cultures and from that zones of inhibition were measured as shown in Tab.2. From the result we can conclude that

methanolic extracts of dried tea leaves may have little anti microbial properties against some micro-organisms but methanolic extracts of fresh tea leaves have very poor or no anti microbial activities.

Conclusion

The phytochemical and biological investigations of methanolic extracts of dried and fresh tea leaves was of great importance as because we have found some immense differences between the dried and green tea. In past few years, a number of studies including phytochemical and pharmacologically have been conducted in different countries to prove such efficiency. Our results were an attempt to compare the major photochemical compounds and pharmacological effects of fresh green tea leaves and dried tea leaves.

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Blood Group	Minimum concentration of positive activity (mg/mL)
A+	0.156
AB+	1.25
B+	0.313
O+	0.313

Tab.1: Hemagglutination inhibition activities of tea leave extract

Microorganisms	Dried tea leaves	Fresh tea leaves	Azithromycin	Negative control
	Zone of inhibition (mm)			
Sarcina lutea	8	6	16	-
Aspergillus. niger	6	-	24	-
Candida albican	-	-	30	-
Staphylococcus aureus	-	-	32	-
Pseudomonas aureus	-	-	29	-
Saccharomyces cerevisiae	8	-	20	-
Bacillus subtilis	7	6	20	-

Tab. 2: Antibacterial activities of dried and fresh tea leave extract

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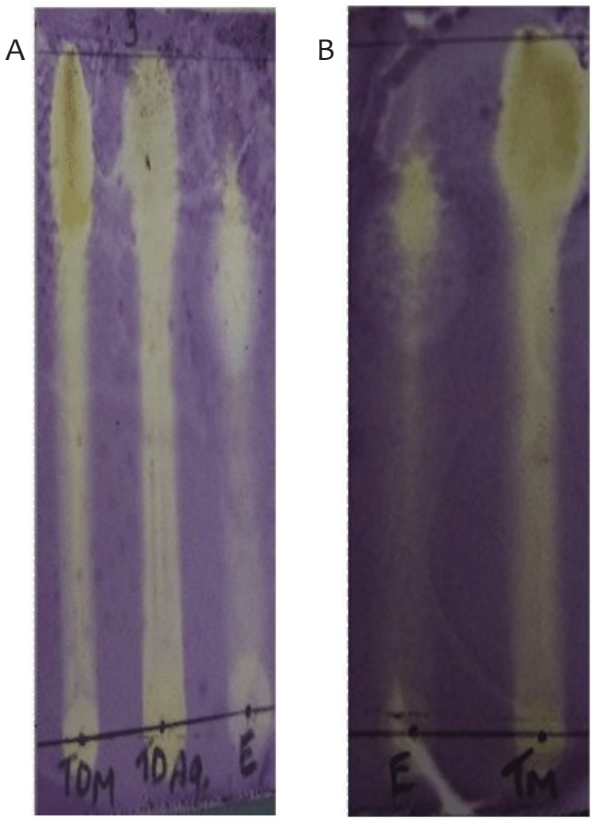

Fig. 1: TLC analysis of dried and fresh tea leaves extract after DPPH staining. Fig 1A. Yellow spots of dried tea leaves methanolic extract, aqueous extract in comparison with EGCG (from left to right). Fig 1B. Yellow spots of EGCG and fresh tea methanolic extract (from left to right).

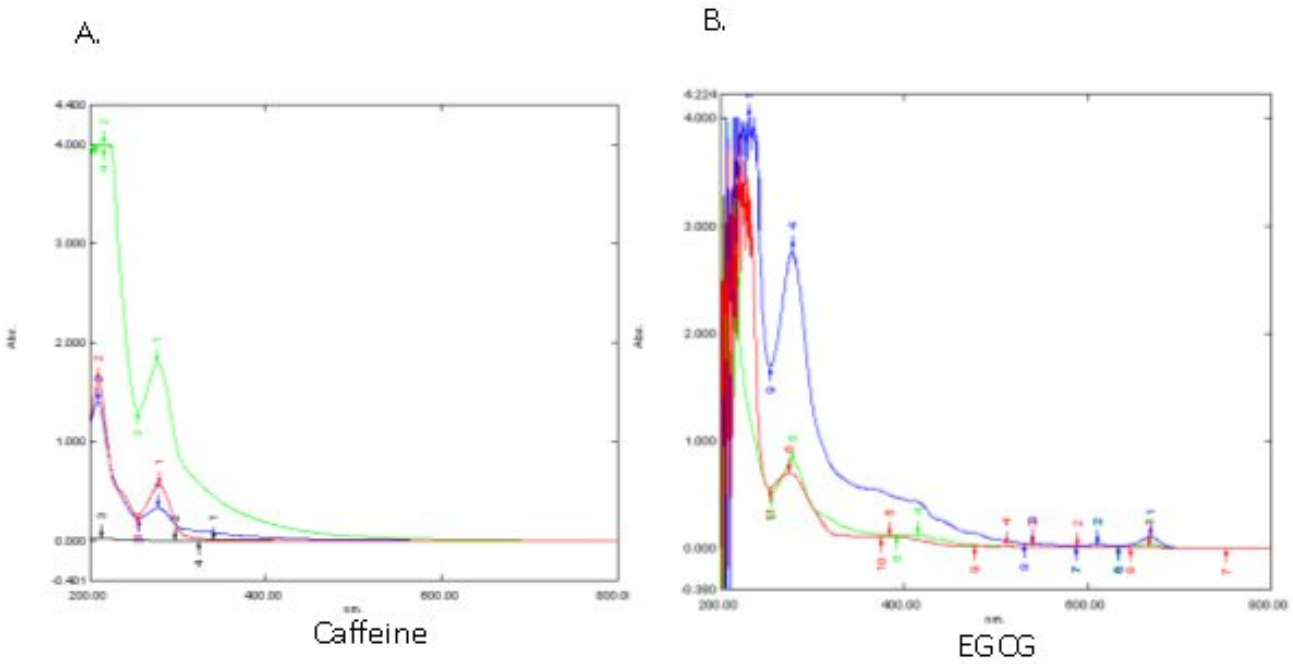

Fig 2: UV-scanning of aqueous and methanolic extract of dried and fresh green tea leaves. Fig 2A. Presence of caffeine in aqueous tea extract. Green color (top most) indicates dried tea leaves extract, Red color (middle) indicates standard caffeine solution, Blue color (bottom most) indicates fresh tea leaves extract. Fig 2B. Presence of EGCG in methanolic tea extract. Green color (medium) indicates dried tea leaves extract, Red color (bottom most) indicates standard EGCG solution, Blue color (top most) indicates fresh tea leaves extract.

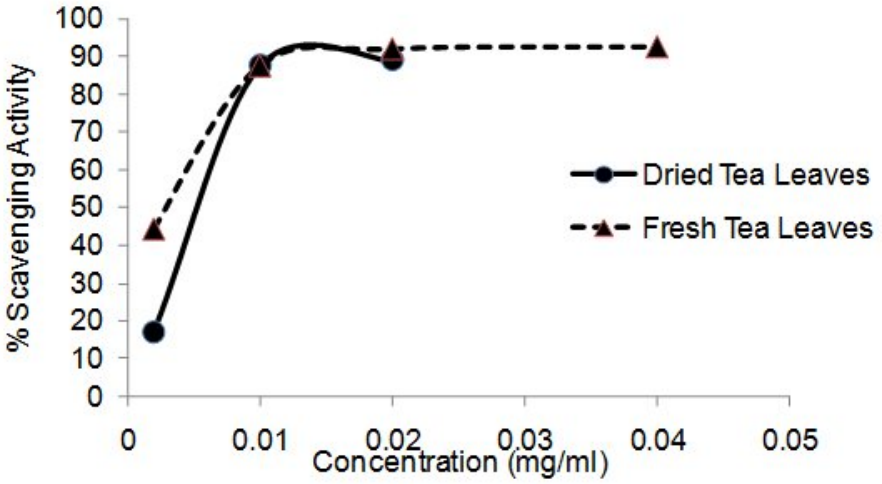

Fig 3: DPPH scavenging activities of fresh and dried tea leaves. Closed circle with solid line indicates the effect of dried tea leaves extract and triangular dotted line indicates the effect caused by fresh green tea leaves extract.

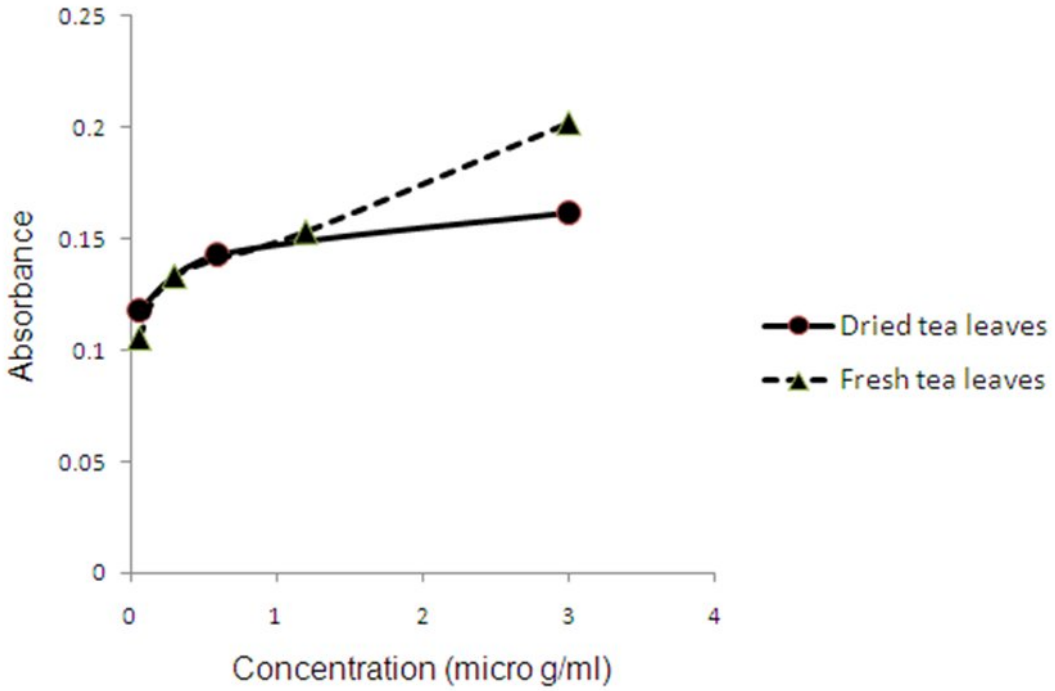

Fig 4: Total reducing activities of fresh and dried tea leaves. Closed circle with solid line indicates the effect of dried tea leaves extract and triangular dotted line indicates the effect caused by fresh green tea leaves extract.

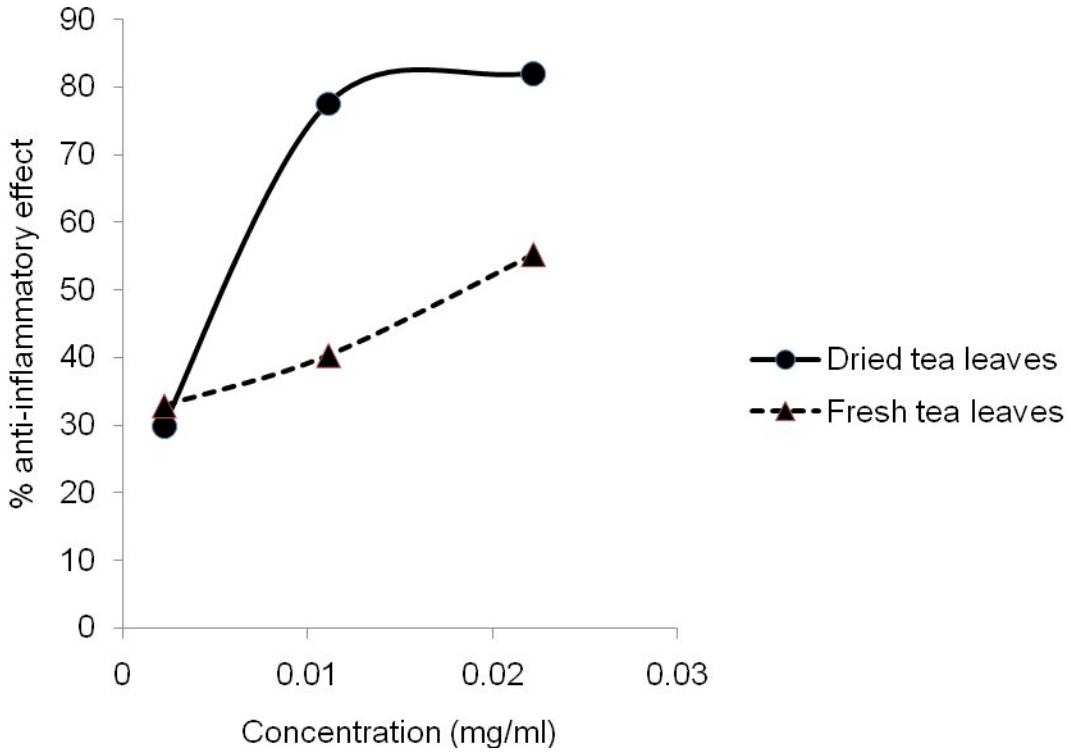

Fig 5: Anti-inflammatory activities of fresh and dried tea leaves. Closed circle with solid line indicates the effect of dried tea leaves extract and triangular dotted line indicates the effect caused by fresh green tea leaves extract.

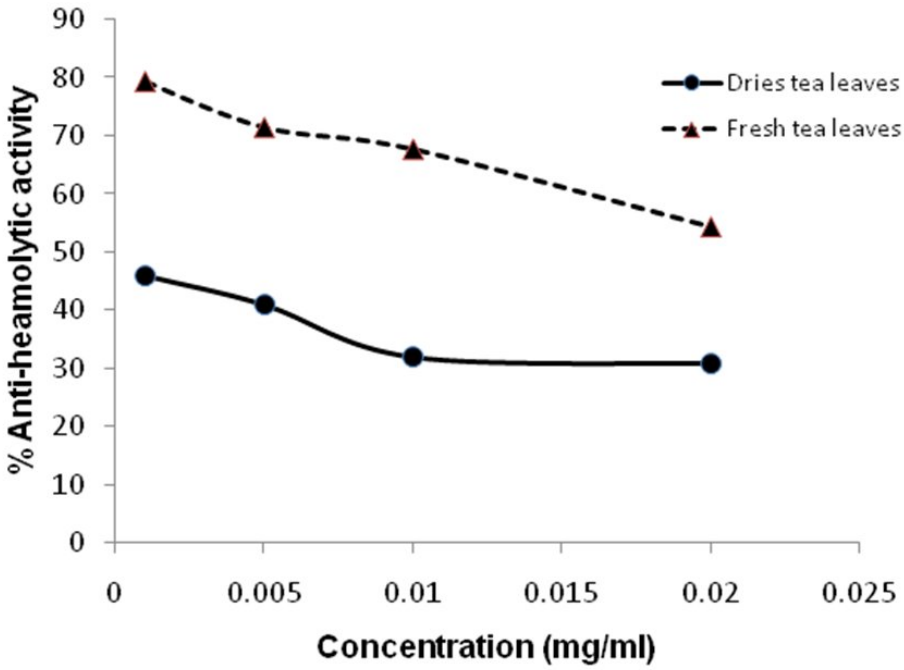

Fig 6: Hydrogen peroxide induced antihemolytic activities of fresh and dried tea leaves. Closed circle with solid line indicates the effect of dried tea leaves extract and triangular dotted line indicates the effect caused by fresh green tea leaves extract.