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BIOCHEMICAL ANALYSIS AND EVALUATION OF ANTIMICROBIAL PROPERTIES OF THEAFLAVINS AND FLAVONOIDS RICH EXTRACT (TFE) AND ITS SILVER NANOCONJUGATES: A COMPARATIVE STUDY

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Antimicrobial Property, Biochemical Characterization, Black Tea, HPLC, Nanoconjugates, Silver, Theaflavins (TF2A, TF2B, TF3), Thearubigins, Theaflavins and Flavonoids rich Extract (TFE)

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ABSTRACT: Black tea, the most widely consumed aromatic non-alcoholic beverage second to water, is prepared by boiling cured leaves of *Camellia sinensis.* It is reported that black tea contains plenty of bioactive constituents, including complex polyphenols like theaflavins (TF_2A, TF_2B, TF_3) and thearubigins that exhibit a wide range of pharmacological properties and also contribute to its quality and bioactivity. The present study aims at biochemical characterization and evaluation of antimicrobial activity of Theaflavins and Flavonoids rich Extract (TFE) and their silver nanoconjugates. TFE was obtained by steeping black tea in boiling water at 90 °C for 5 min, subsequently extracted with ethyl acetate and dried for further use. The biochemical characterization of TFE was done using HPLC and other spectrophotometric methods. Next, silver nanoconjugates of the TFE were prepared using a green synthesis route, and antimicrobial property was evaluated with respect to its non-conjugated form. It was observed that the TFE is an antioxidant rich fraction and helps in metal-nanoparticle synthesis efficiently. The study highlighted that, in maximum cases, nanoconjugates gave better results than their non-conjugated form.

INTRODUCTION: Black tea is one of the most beloved drinks or beverages in the world, and almost 80% of humans consume it. Black tea differs from green tea physically by color, taste, and aroma, and chemically by main polyphenols and flavonoids constituent.

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Biochemical analysis reveals that catechins and its derivatives, present in tea leaves, are enzymatically oxidized to yield a complex mixture of oxidation products, including theaflavins (dimers with benzotropolone structures linked through the B-ring) [1] and more complex products such as the thearubigins during the production of black tea.

Black tea represents around 78% of world tea production and is greatly consumed in North America, Europe, Asia, and North Africa [3]. Numerous protective, as well as therapeutic impacts related to tea polyphenols, have been

investigated and reported as the counteractive action of different maladies including malignancy, coronary illness, atherosclerosis, stroke, and intestinal inflammation [4, 5]. The polyphenols exert their defensive impact against different oxidative stress-related disorders by neutralizing the free radicals [3].

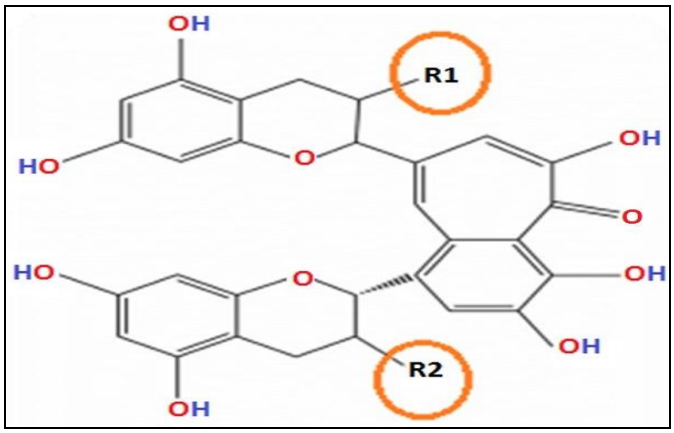

FIG. 1: STRUCTURE OF THEAFLAVINS AND ITS DERIVATIVES. BASED ON THE R1 AND R2 GROUP THEY CAN BE CLASSIFIED AS TF1 (R1= R2= OH), TF2a (R1= Gallate AND R2= OH), TF2b (R1= OH AND R2=Gallate), AND TF3 (R1= R2= Gallate) [2]

In the era of high-throughput technologies, nanoparticles derived from noble metals play a pivotal role because of their vast application in diagnostics and therapeutics. Silver (Ag) was chosen for this study due to its proven antibacterial activity. During the past few years, silver nanoparticles (AgNPs) are the most substantially investigated area in the field of biomedical sciences. Green synthesis being an eco-friendly, non-toxic, and inexpensive method was chosen where the plant extract acted as a reducing agent to form AgNPs. The present course of study aims at comparative evaluation of antimicrobial efficacy of Theaflavins and Flavonoids rich Extract (TFE) and it's Silver Nanoconjugates.

MATERIALS AND METHODS:

Reagents Used: Reagents used for quantitative estimation of phytochemical analysis such as total polyphenols and flavonoids content along with antioxidant studies were Ascorbic Acid (Sigma), Gallic Acid (Merck), Sodium carbonate (Merck), Sodium Hydroxide (Merck), Anhydrous Sodium Nitrite (Merck), DPPH (Merck), Aluminium Chloride (Merck), Methanol (Merck), Catechin (Sigma), Folin-Ciocalteu reagent (Merck). Silver Nitrate (Merck) was also used for the green synthesis of nanoparticles. Another set of chemicals used for the analysis of antimicrobial properties was Luria Broth (Merck) and Luria Agar (Merck). All chemicals were analytically graded, and HPLC grade water (Merck) was used throughout the experiment.

Sample Collection and Processing: Three brands of marketed black tea, such as GoodrickThurbo Darjeeling (B1); Castleton Vintage Darjeeling (B8), and Makaibari Darjeeling (B12) were purchased from a local market in Kolkata, West Bengal during the month of January 2019. After collecting the samples, they were ground using mortar and pestle, sieved, and processed to obtain fine sample powder, which was stored in amber-colored, glass airtight containers for further analysis.

Determination of Theaflavins, Thearubigins, and their Ratio in Tea Decoction: Quantitative estimation of Theaflavins was done by the standard method as described by Yao L.H. *et al*., 2006 and Yongwen J. *et al*., 2018 with slight modification [6, 7].

Preparation of Ethyl Acetate Extract: 2 g each of the processed samples was brewed in 100 ml hot water (90 ºC) for 5 min. Tea decoctions were filtered, collected, and partitioned using ethyl acetate (EA). The EA fractions were collected separately, dried, and stored in a cool, dry place in capped glass vials for further use.

Determination of Extractive Value: Dried EA fractions were measured, and extractive values were expressed as,

% Extractive value = (Weight of EA fraction × 100) / amount of tea samples [in g]

Phytochemical Analysis (Quantitative Assay):

Estimation of Total Polyphenols: The total polyphenols content was measured using the Folin-Ciocalteu method [8, 9] with slight modification. Gallic acid was used as standard, and the absorbance for the test solutions was determined against the reagent blank at 765 nm with a UV-VIS spectrophotometer. The content of polyphenols was expressed as mg of Gallic acid equivalents (GAE)/g of extract. Results were reported as mean ± SD (n=3).

Estimation of Total Flavonoids: Total flavonoid content was studied by the aluminum chloride colorimetric assay [10] with slight modification. Catechin was used as standard, and the absorbance was read at 510 nm against the reagent blank with a UV-vis spectrophotometer. The total flavonoid content was expressed as mg of Catechin equivalents (CE)/gm of extract. Data were reported as mean ± SD (n=3).

DPPH Radical Scavenging Assay: The free radical scavenging activity was studied by using DPPH (2,2-diphenyl-1-picrylhydrazyl)with slight modification [10]. Absorbance was read at 517 nm against methanol as blank with a UV-VIS spectrophotometer. Ascorbic acid was used as standard. DPPH scavenging activity expressed in terms of Ascorbic acid equivalents, as the percentage of inhibition calculated by the following formula.

% Inhibition of DPPH= [(OD of control – OD of the sample)/ OD of control] × 100

Final results were reported as mean ± SD in triplicates.

Determination of Catechin and its derivatives in TFE by HPLC: High Performance Liquid Chromatography (HPLC) was performed using instrument Agilent Technologies 1260 Infinity series with binary pump (G1312B) for solvent delivery, 20µl loop for injection and detector (DAD G1315D) and Open-Lab ChemStation software for data processing. The separation was achieved using reverse phase column, Phenomenex C18 column (250mm × 4.6mm, 5µm). Individual TFEs were prepared at a concentration of 1mg/ml and filtered through 0.2µm PDVF filter. Standards like, Gallic Acid (GA), Catechin, Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin (EGC) and Epigallocatechin gallate (EGCG) were prepared in methanol at a concentration of 1000ppm as stock solution. Further dilutions were made for calibration of each standard. The mobile phase contains methanol (solvent A) and 3% Acetic acid solution (Solvent B). The gradient elusion was 0% to 63% A, with flow rate of 1ml/min in 27 mins. The mobile phase composition back to initial condition and allowed to run for another 13 mins, before another injection of sample. The detection of compounds was performed using detector at 280 nm. Each compound was identified by its retention time under same conditions. The quantification of samples were done by the measurement of the integrated peak area and the content was calculated using the calibration curve of the respective standard sample [11].

Green Synthesis of Nanoparticle: Silver nitrate solution was prepared by dissolving 17 mg silver nitrate salt ($AgNO_3$) in 100 ml distilled water and subsequently filtered through syringe-filter. 5 ml of that metal solution was taken in a conical flask and placed on a hot plate magnetic stirrer. Equal volume of filtered solution of TFE was added drop wise to the boiling $AgNO_3$ solution and formation of silver nanoparticles were noticed by monitoring the colour change of the mixture. Characterisation of the nanoparticles were studied by UV-VIS, DLS, and FTIR [12].

Characterization of Silver Nanoparticles (AgNPs):
UV-VIS Spectroscopy: The AgNPs were characterized in a UV-VIS spectrophotometer (SHIMADZU Model: 2401PC) to know the size of the AgNPs. The scanning range for the samples was 200-800nm and the base line correction of the spectrophotometer was done by using a double distilled water as reference [12].

Dynamic Light Scattering Particle Size Analyzer: In order to determine particle size and size distribution of the nanoparticles, the silver nano-conjugates of TFE were diluted and analysed using the DLS instrument (Zetasizer Nano-S, By: MALVERN Instruments, Model: ZEN1600) [12].

Fourier Transform-Infra Red (FT-IR) Spectroscopy: Presence of different functional groups and their mode of binding with silver nanoconjugates were determined using FT-IR RX1-Perkin Elmer with transmittance in the range of (4000 to 450) cm^{-1} at a resolution of 4 cm^{-1} (PerkinElmer Model: Spectrum 100) [12].

Determination of Antimicrobial Property by Kirby Bauer Disk Diffusion Method: Sterile paper discs were soaked with the prepared sample TFEs and their nanoconjugates and placed at different spots on agar plates spread with the test bacteria - *Staphylococcus aureus* (Gram-Positive) and *Escherichia coli* (Gram Negative) [Kind gift from the Department of Microbiology, Calcutta University, West Bengal] following the Kirby-

Bauer Disk Diffusion Method [13]. 100 µl of each bacterium was subcultured in 5 ml Luria Broth and incubated at 37 ºC for 24 h. 20 µl of test bacteria from the log phase of freshly sub cultured tubes were spread on plates. 20 µl of each test sample (*i.e.* extracts and nanoconjugates) was given. The plates were then incubated at 37 ºC for 24 h followed by the measurement of the zone of inhibitions (in mm) obtained.

Statistical Analysis: Assays were performed in triplicate* and the mean ± standard deviation were calculated using MS Excel Software. Experiments were carried out a minimum of three times to ensure reproducibility, and data from a representative experiment are presented. A paired two-tailed T-test was performed to check whether the data was statistically significant and ANOVA was done to analyze the differences among group means.

*- In case of HPLC, single set of data was used.

RESULTS AND DISCUSSION:

Determination of Theaflavins, Thearubigins, and their Ratio in Tea Decoction: Quantitative estimation of %Theaflavins (%TF), %Thearubigins (%TR), and TF/TR ratio were done in black tea extracts using the standard procedure as described prior to extraction of theaflavins rich portion from tea. **Table 1** shows TF%, TR%, and TF/TR ratio of all the three marketed black samples.

TABLE 1: THEAFLAVINS, THEARUBIGINS CONTENT AND THEIR RATIO IN BLACK TEA SAMPLES (N=3). THE RESULTS PRESENTED HERE ARE REPRESENTATION OF THREE INDEPENDENT EXPERIMENTS. VALUES ARE MEAN ± SD

Sample name	Theaflavins (%)	Thearubigins (%)	TF/TR ratio
Goodrick Thurbo Darjeeling	0.60±0.03	7.01±0.55	0.0856
Castleton Vintage Darjeeling	0.96±0.06	9.52±0.43	0.1008
Makaibari Darjeeling	1.68±0.05	6.69±0.15	0.2511

Determination of Extractive Value: In **Table 2**, the Extractive Value (%) of the 5 min hot decoction of the three marketed black tea samples are shown.

TABLE 2: EXTRACTIVE VALUE OF TFE OF BLACK TEA SAMPLES (N=3)

Sample name	Tea taken for Extraction (gm)	Amount of dry TFE obtained (gm)	Extractive value (%)
GoodrickThurbo Darjeeling	2	0.11	5.5
Castleton Vintage Darjeeling	2	0.12	6
Makaibari Darjeeling	2	0.1	5

Phytochemical Characterization of Ethyl Acetate extract of Tea Decoction:

Total Polyphenolic Content: Polyphenolics are the important antioxidant species towards oxidation and it regulates human physiological activity. The oxidation process and free radical generation leads to diabetes, inflammations, cancer and other related diseases. The activity of polyphenols against oxidative stress-related processes can have therapeutic application in pharmaceutical and nutritional industry. The total polyphenol content of TFEs were quantified from the standard curve of Gallic Acid ($y = 10.478x+0.0495$; $R^2 = 0.9997$).

Among the three different black tea samples, the highest amount of polyphenolic compounds was observed in the TFE of Makaibari Darjeeling tea (B12) and the lowest amount was observed in the TFE of Castleton Vintage Darjeeling tea (B8) and it was 371.81±3.58 mg GAE/cup of tea and 281.14 ±3.58 mg GAE/cup of tea respectively **Table 3**.

Total Flavonoid Content: Flavonoids are the class of plant secondary metabolites with prominent antioxidant and chelating activities. Antioxidant properties of flavonoids depend on the structure and substitution way of hydroxyl groups. The antioxidative activities of flavonoid compounds are due to various mechanisms like scavenging of free radicals, chelation of metal ions (*e.g.* iron and copper), and inhibition of enzymes which are basically responsible for a free-radical generation. Depending on their specific structure, flavonoid compounds are able to inhibit all the possible ROS. The total flavonoid content of TFEs was quantified from the standard curve of Catechin ($y = 4.2865x – 0.004$; $R^2 = 0.9999$). The highest amount of flavonoids' content was observed in TFE of Castleton Vintage Darjeeling tea (B8) and the lowest amount was observed in the TFE of GoodrickThurbo Darjeeling tea (B1) and it was 52.57±0.71 mg CE/cup of tea and 50.24±4.48 mg CE/cup of tea respectively **Table 3**.

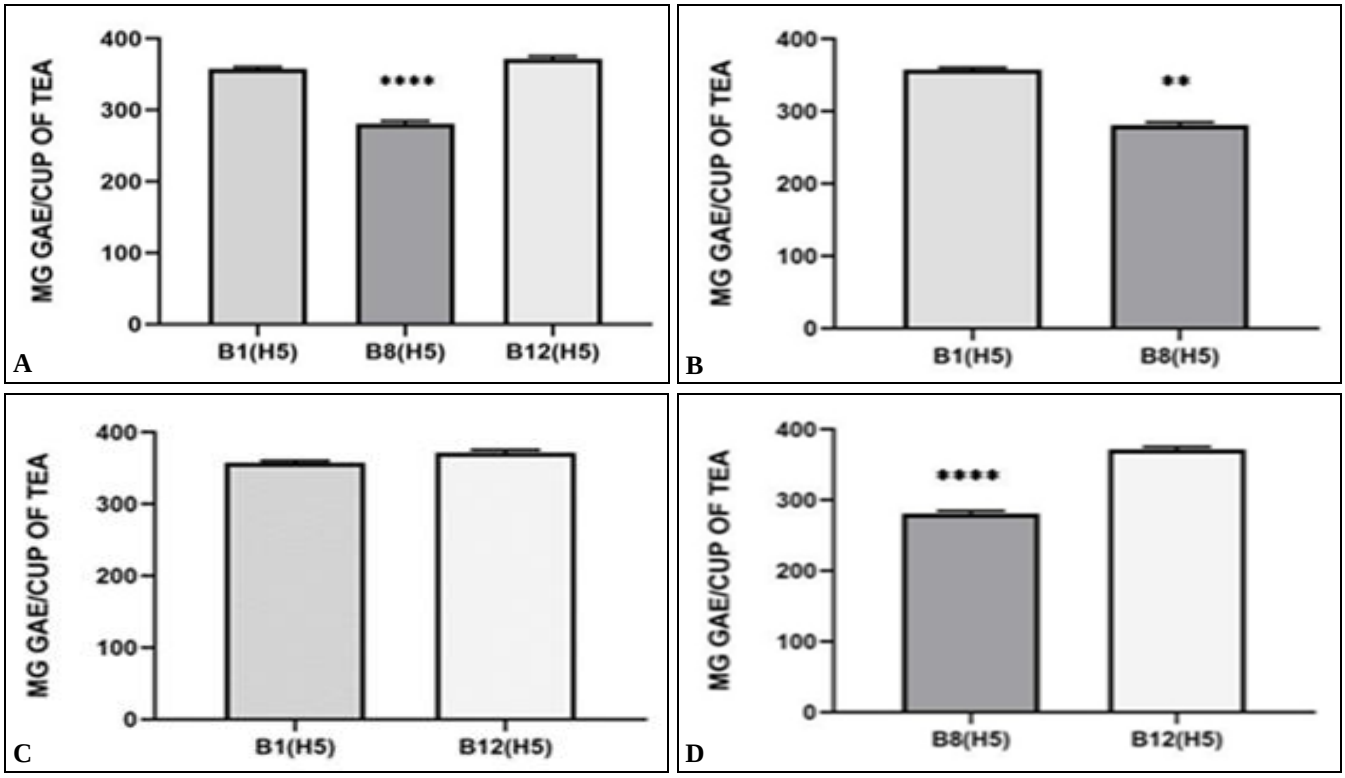

FIG. 2: (A) COMPARISON OF TOTAL POLYPHENOL CONTENT (mg GAE/cup OF TEA) OF THEAFLAVIN LAYER PER CUP OF BLACK TEA (n=3) BY ONE-WAY ANOVA TEST. THE P VALUE IS <0.0001. (B) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B8 WHERE P<0.01. (C) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B12 WHERE THE P>0.05. (D) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B8 & B12 WHERE THE P<0.0001

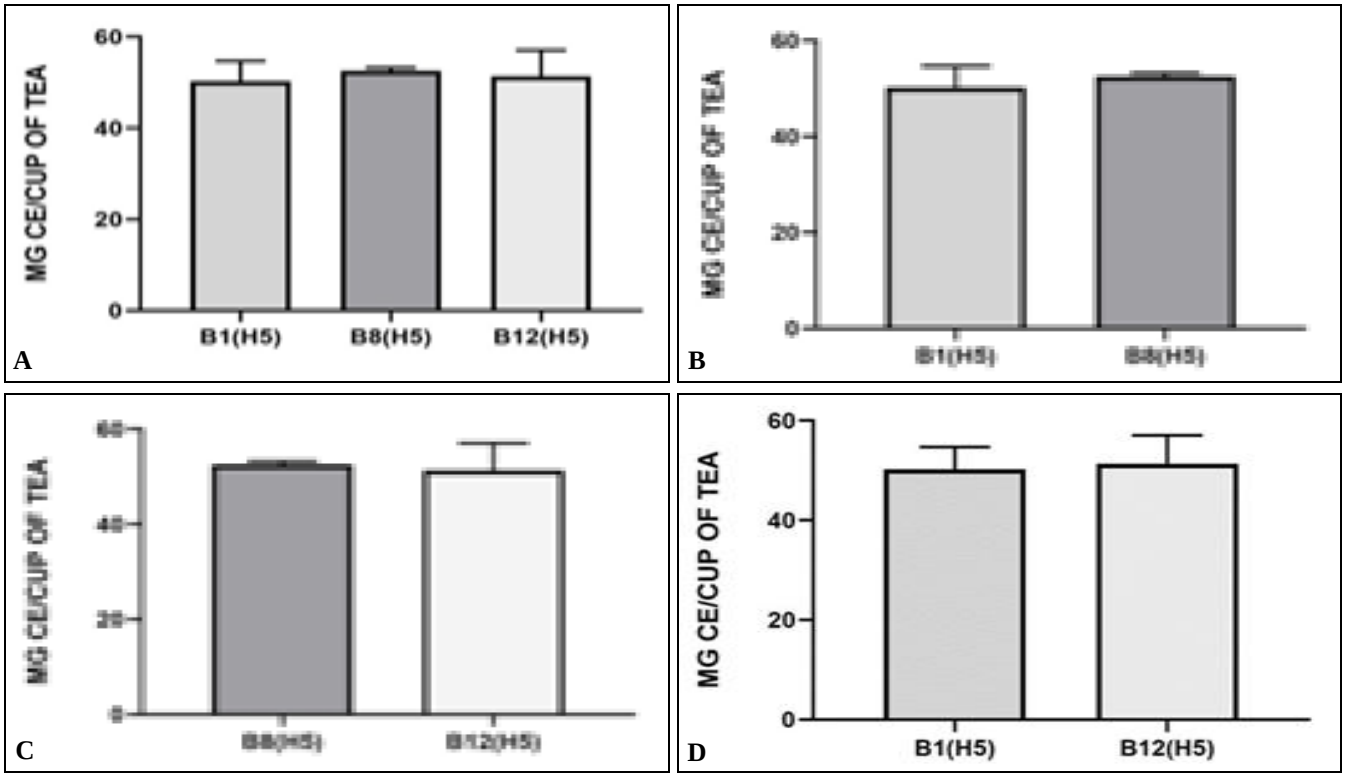

FIG. 3: (A) COMPARISON OF TOTAL FLAVONOID CONTENT (mgCE/cup OF TEA) OF THEAFLAVIN LAYER PER CUP OF BLACK TEA (n=3) BY ONE-WAY ANOVA TEST. THE P VALUE IS 0.8017 (ns). (B) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B8 WHERE P=0.4326 (ns). (C) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B8 & B12 WHERE P=0.7342 (ns). (D) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B12 WHERE P=0.3688 (ns)

DPPH Radical Scavenging Assay: Inhibition percentages and concentration values for different radical scavenging assays were studied. Inhibitory concentration is the amount of free radicals scavenged in the evaluation of antioxidant property. In the current study, the maximum inhibition percentage of DPPH radical scavenging assay was found to be 48.18±0.33% / cup of tea for the TFE of Goodrick Thurbo Darjeeling tea (B1) and the minimum was 38.64±0.17% / cup of tea for the TFE of Castleton Vintage Darjeeling tea (B8). The highest amount of antioxidant content was observed in the TFE of Goodrick Thurbo Darjeeling tea (B1) and the lowest amount was observed in the TFE of Castleton Vintage Darjeeling tea (B8), and it was 709.55±4.82 mg AAE/cup of tea and 570.75±2.41 mg AAE/cup of tea respectively **Table 3** which was determined from the standard curve of Ascorbic Acid ($y=343.44x - 0.5613$; $R^2= 0.9997$) and % radical scavenging activity. DPPH is a stable free radical and is highly utilized to evaluate the radical scavenging activity of anti-oxidant activities. This assay is based on the reduction of DPPH radicals in methanol in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical shape of DPPH-H. DPPH has the advantage over some factors like it is unaffected by side reactions, such as metal ion chelating and enzyme inhibitions.

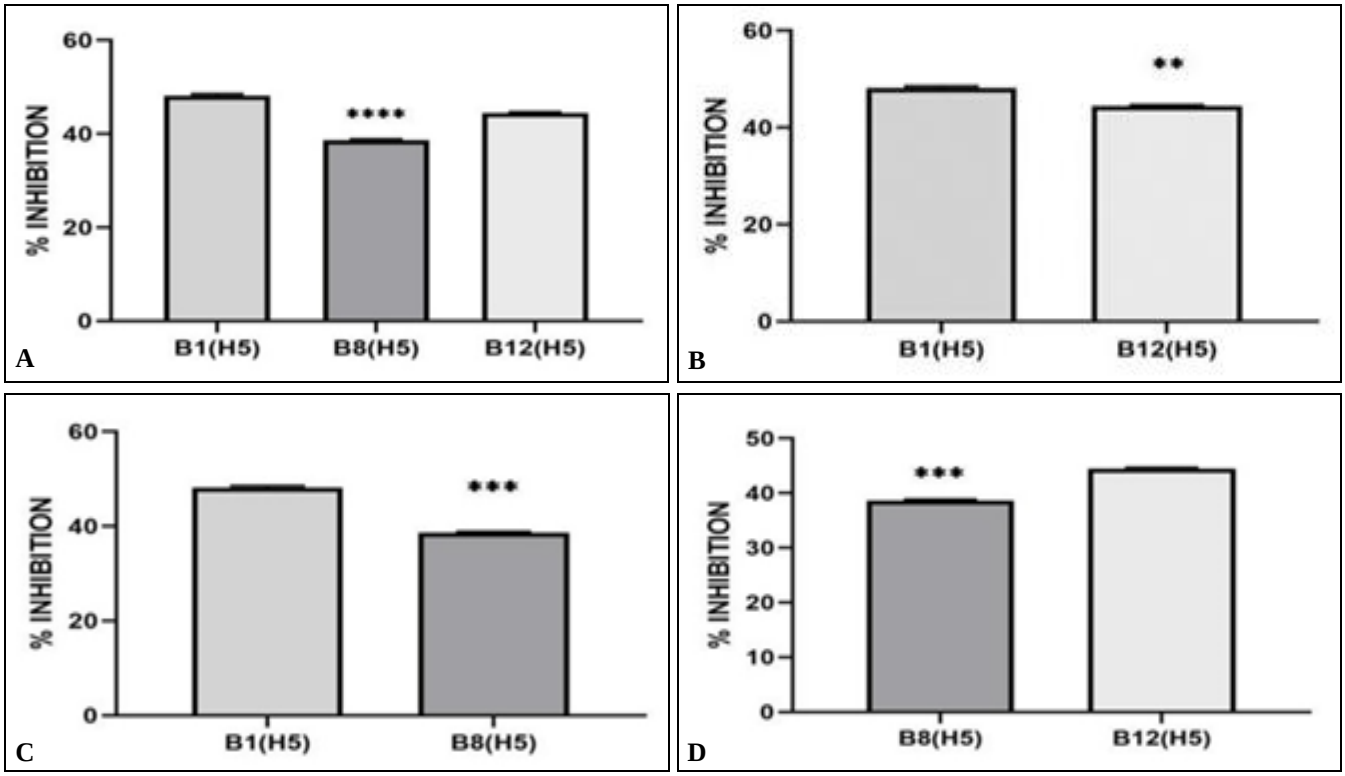

FIG. 4: (A) COMPARISON OF TOTAL ANTIOXIDANT CONTENT BY RADICAL SCAVENGING DPPH (% INHIBITION) OF THEAFLAVINS LAYER PER CUP OF BLACK TEA (n=3) BY ONE-WAY ANOVA TEST. THE P VALUE IS <0.0001. (B) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B12 WHERE P < 0.01. (C) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B1 & B8 WHERE P < 0.001. (D) PAIRED T TEST PERFORMED BETWEEN TWO BLACK TEA SAMPLES B8 & B12 WHERE P< 0.001

TABLE 3: THE TOTAL PHENOLIC, TOTAL FLAVONOIDAND DPPH % INHIBITION OF ALL THE THREE BLACK TEA VARIANTS AS MEAN ± SD (n=3). PHENOLICS AND FLAVONOIDS ARE EXPRESSED IN MG GALLIC ACID EQUIVALENT AND MG CATECHIN EQUIVALENT PER CUP OF TEA RESPECTIVELY

Sample name	TPC (mg GAE/ cup of tea)	TFC (mg CE/ cup of tea)	DPPH Scavenging Activity (% inhibition / cup of tea)
GoodrickThurbo Darjeeling	357.50±3.10	50.24±4.48	48.18±0.33
Castleton Vintage Darjeeling	281.14±3.58	52.57±0.71	38.64±0.17
Makaibari Darjeeling	371.81±3.58	51.32±5.73	44.42±0.22

HPLC Analysis of Phenolic Content of TFE: HPLC analysis was performed for B8 & B12 TFEs to quantify the main five catechins such as Gallic Acid (GA), Epigallocatechin (EGC), Catechin (C),

Epicatechin (EC) and Epicatechin gallate (ECG). Tea is rich in catechins, which is an essential bioactive compound. The chromatograms of the samples of Castleton Vintage Darjeeling tea and Makaibari Darjeeling tea under 280nm are shown in **Fig. 5**.

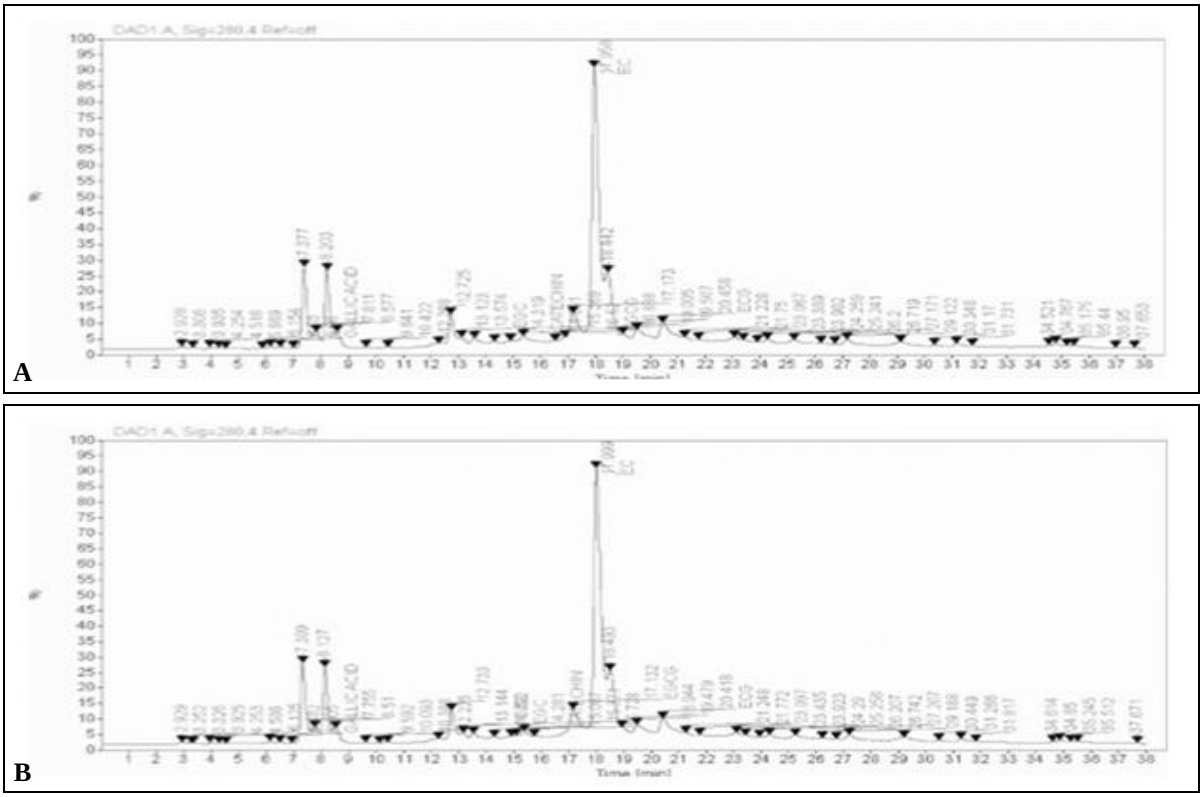

FIG. 5: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) CHROMATOGRAM UNDER 280 nm OF THE STANDARD COMPOUNDS AGAINST THE TFE OF (A) CASTLETON VINTAGE DARJEELING TEA AND (B) MAKAIBARI DARJEELING TEA

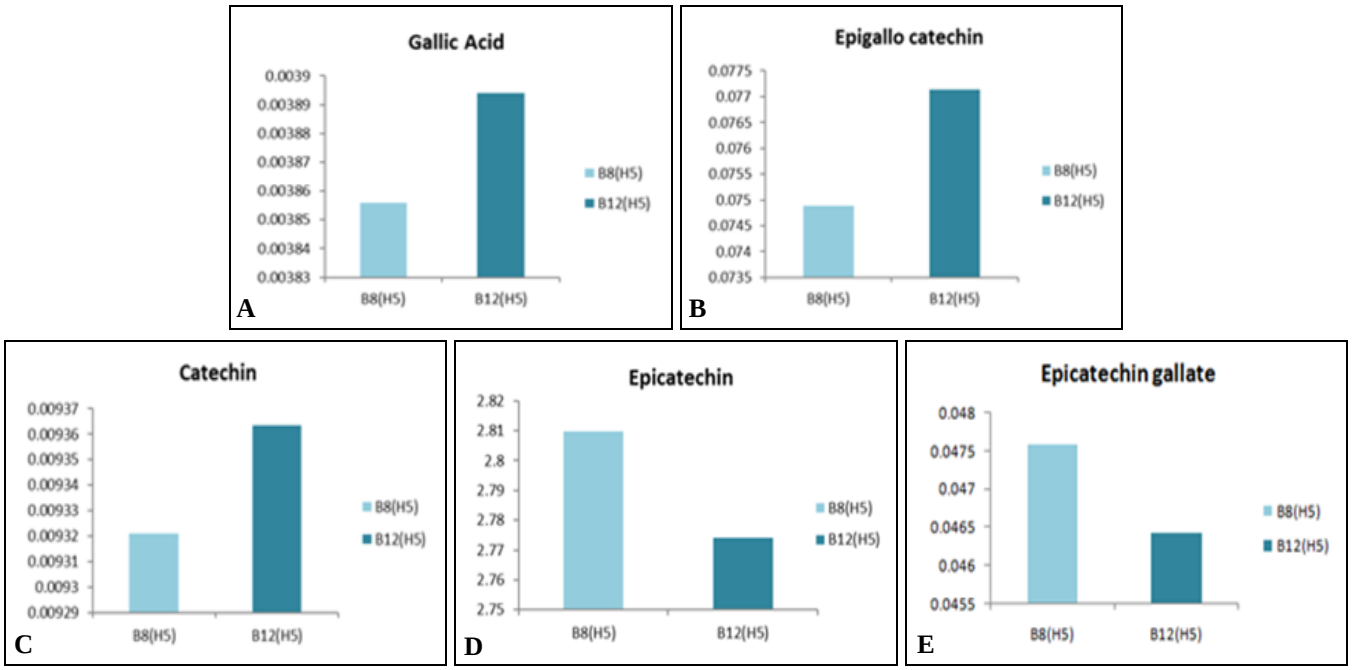

FIG. 6: GRAPHICAL REPRESENTATION OF HPLC ANALYSIS OF CATECHIN COMPOUNDS BETWEEN TFE OF BLACK TEA SAMPLES B8 & B12 (n=2). FOR RESPECTIVE B8 & B12 TFEs (A) GALLIC ACID CONTENT ARE 0.00386 mg/ml AND 0.00389 mg/ml, (B) (-) EPIGALLOCATECHIN CONTENT ARE 0.07489 mg/ml AND 0.07714 mg/ml, (C)(+) CATECHIN CONTENT ARE 0.00932 mg/ml AND 0.00936 mg/ml AND (D) (-) EPICATECHIN CONTENT ARE 2.80970 mg/ml AND 2.77419 mg/ml, (E) (-) EPICATECHIN GALLATE CONTENT ARE 0.04759 mg/ml AND 0.04644 mg/ml

Green Synthesis and Biophysical Characterization of Silver Nanoparticles:

UV-Vis Spectral Analysis: The reduction of silver ions resulted due to the addition of the TFE having antioxidants. The nanoparticles thus formed were subjected to UV–Vis spectroscopy. The reduction of pure Ag^+ ions was analyzed by using a UV-Vis spectrophotometer; the wavelength is set in the range of 200-1000 nm. The UV-Vis absorption spectrum of silver nanoparticles in the presence of TFE showed single peaks in the range of (400-500) nm in all the three tea samples. GoodrickThurbo Darjeeling tea (B1) gave a peak at 465.5 nm**;** Castleton Vintage Darjeeling tea (B8) gave a peak at 468 nm, and Makaibari Darjeeling tea (B12) gave a peak at 451.5nm. From **Fig. 7**, we can see that $AgNO_3$ (Standard) itself gives a peak at around 217 nm, whereas the Ag nanoconjugates give a peak at around (400-500) nm due to localized Surface Plasmon Resonance (LSPR) thus indicating the formation of nanoparticles.

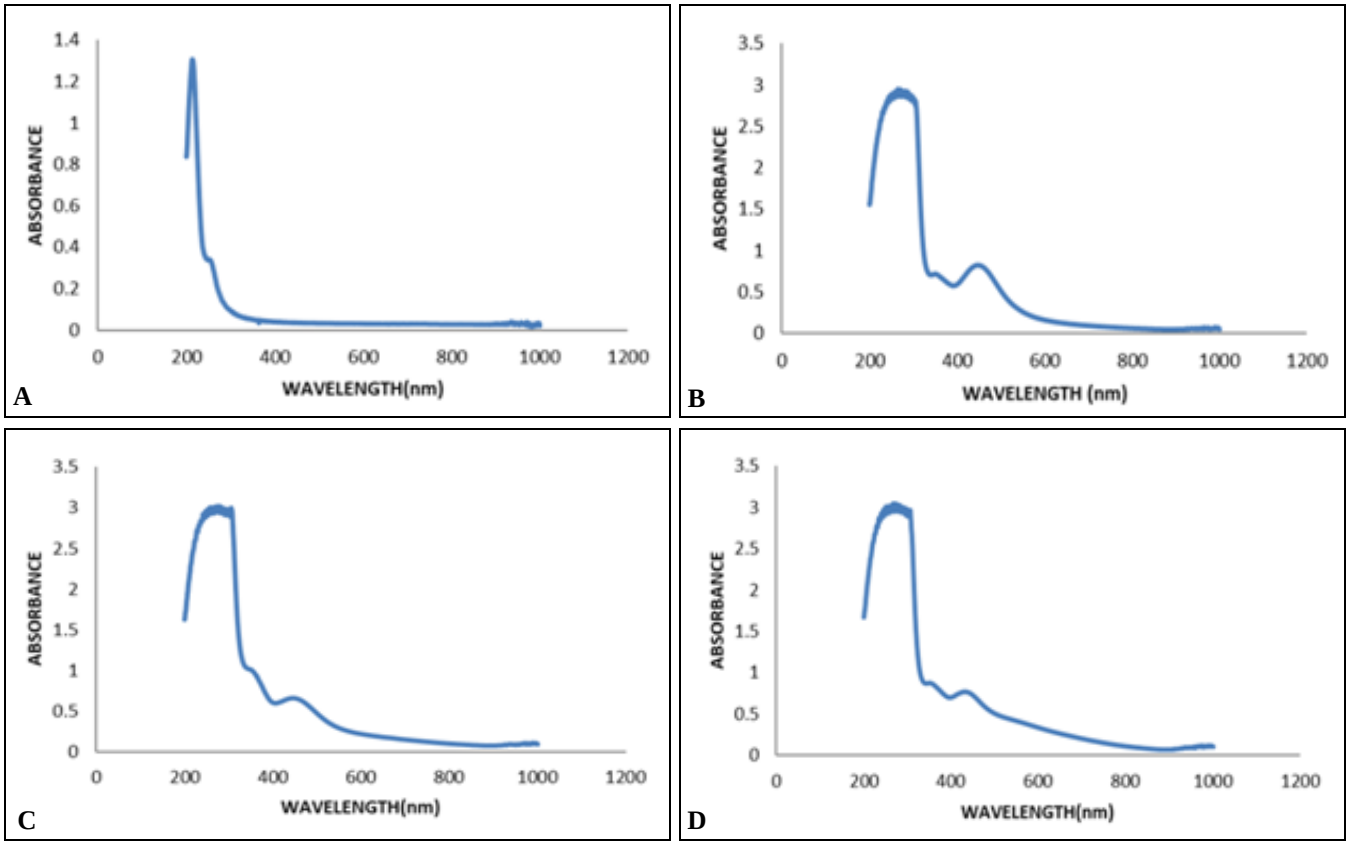

FIG. 7: UV-VISIBLE ABSORPTION SPECTRUM OF (A) $AgNO_3$ STANDARD, (B) B1 TFE STABILIZED SILVER NANOPARTICLE, (C) B8 TFE STABILIZED SILVER NANOPARTICLE AND (D) B12 TFE STABILIZED SILVER NANOPARTICLE

Dynamic Light Scattering (DLS) Particle Size Analysis: To assess whether material falls within the definition of 'nanomaterial', instead of the size range, 'size distribution based on the number' shall be considered. There is no universally accepted definition, but generally, we consider those as nanoparticles that have 50% of the forms within the size distribution that falls in the range (1-100) nm, depending on the field of application. In the pharmaceutical field, nanoparticles in the range of (10-1000) nm is usually considered. However, if particles are of near about 1000 nm, one may term it as microparticles.

The size of the silver nanoparticles synthesized by TFE of black tea was in the range of (13.54 - 295.3) nm **Fig. 8**.

TFE-Ag-nanoconjugates stability was checked by storing them in a cool, dry place and performing DLS after a week **Fig. 9**. Stability was more or less maintained. All the TFE-Ag-nanoconjugates showed an increase in size by two-fold, except for B12 **Fig. 9c** whose size got reduced, which can be attributed to the different composition of bioactive flavonoid compounds present in TFE.

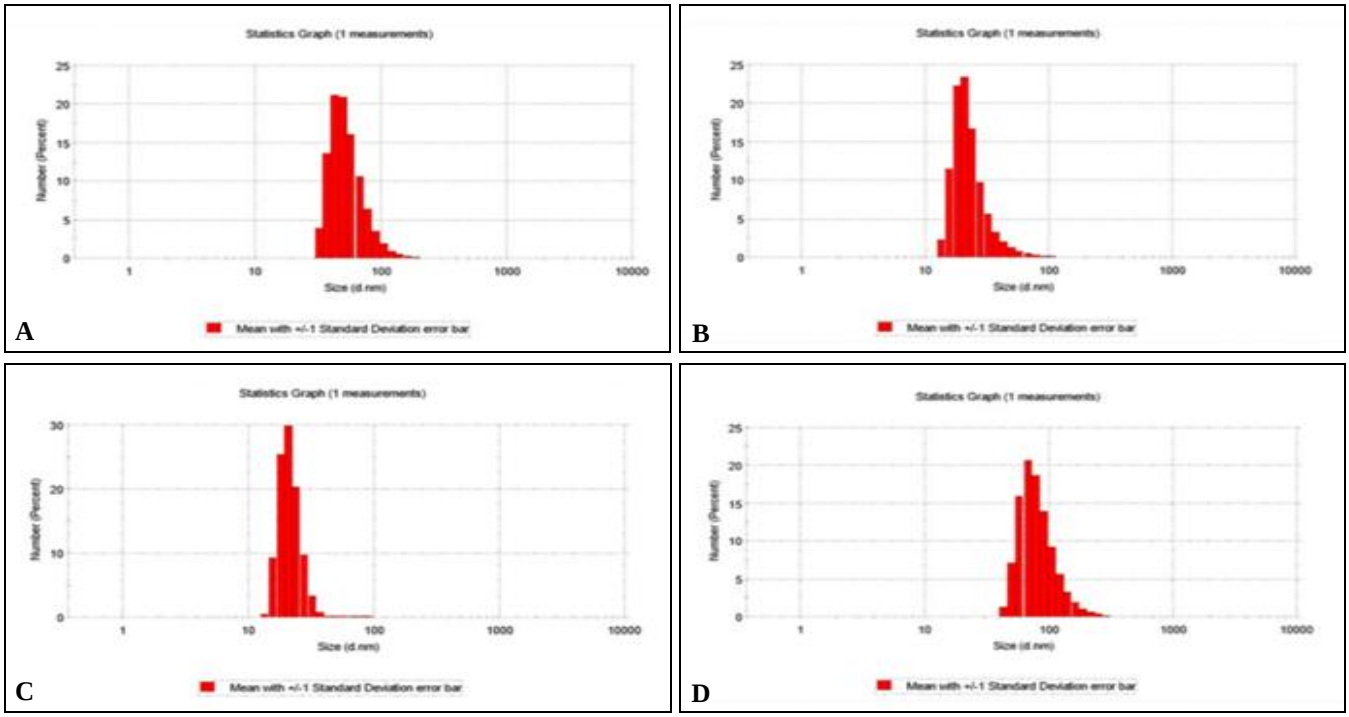

FIG. 8: SIZE DISTRIBUTION STATISTICS GRAPH OBTAINED FROM DYNAMIC LIGHT SCATTERING (DLS) ANALYSIS OF (A) $AgNO_3$ PARTICLES WHOSE SIZE RANGE WAS MEASURED AS (32.67 – 190.1) nm WITH MAJORITY (MEAN NUMBER PERCENT – 21.1) GIVING THE SIZE OF ABOUT 43.82 nm, (B) B1 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED AS (13.54 – 105.7) nm WITH MAJORITY (MEAN NUMBER PERCENT – 23.4) GIVING THE SIZE OF ABOUT 21.04 nm, (C) B8 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED AS (13.54 – 105.7) nm WITH MAJORITY (MEAN NUMBER PERCENT – 29.9) GIVING THE SIZE OF ABOUT 21.04 nm AND (D) B12 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED TO BE (43.82 – 295.3) nm WITH MAJORITY (MEAN NUMBER PERCENT – 20.6) GIVING THE SIZE OF ABOUT 68.06 nm

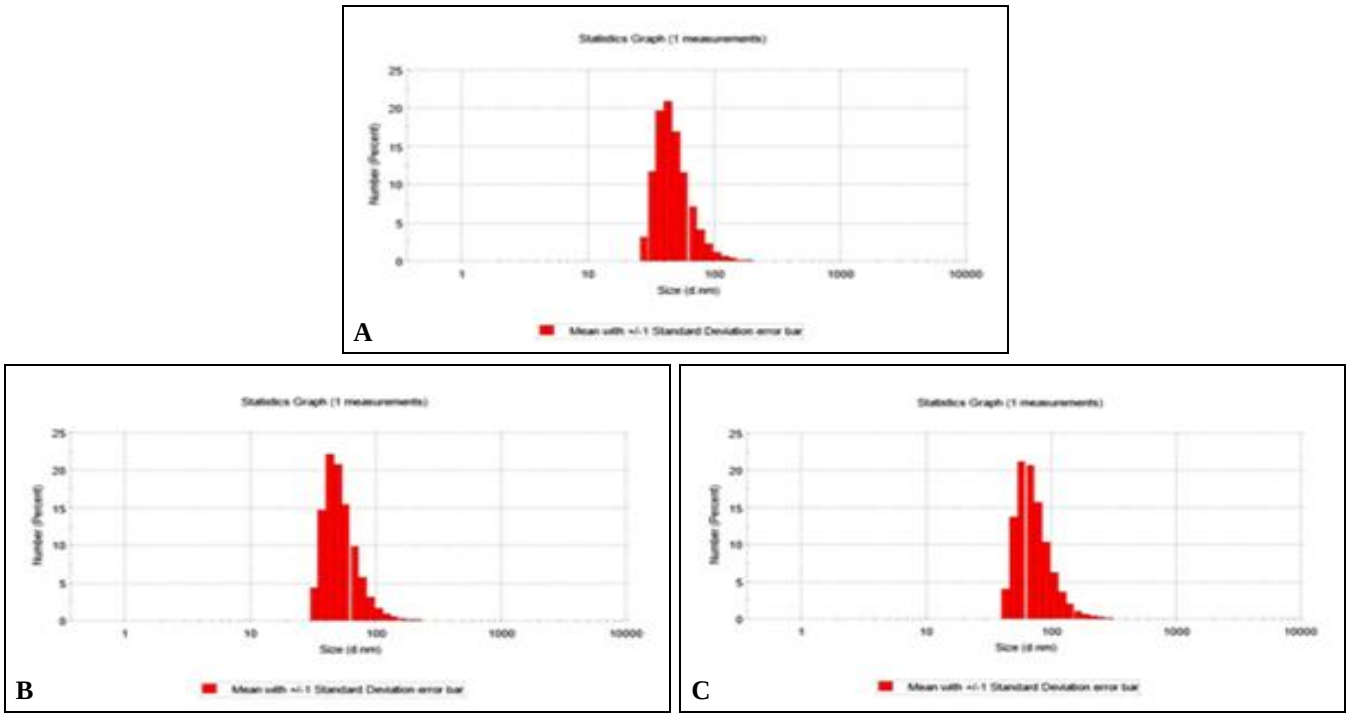

FIG. 9: SIZE DISTRIBUTION STATISTICS GRAPH OBTAINED AFTER ONE WEEK FROM DYNAMIC LIGHT SCATTERING (DLS) ANALYSIS OF (A) B1 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED AS (28.21 – 220.2) nm WITH MAJORITY (MEAN NUMBER PERCENT – 20.9) GIVING THE SIZE OF ABOUT 43.82 nm, (B) B8 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED AS (32.67 - 255) nm WITH MAJORITY (MEAN NUMBER PERCENT – 22) GIVING THE SIZE OF ABOUT 43.82 nm AND (C) B12 TFE NANOCONJUGATES WHOSE SIZE RANGE WAS MEASURED TO BE (43.82 – 295.3) nm WITH MAJORITY (MEAN NUMBER PERCENT – 21.2) GIVING THE SIZE OF ABOUT 58.77 nm

Fourier Transform-Infra Red (FT-IR) Spectroscopy Analysis: FT-IR analysis was executed to identify the possible functional groups involved in the synthesis of AgNPs using TFEs, and to determine the bioactive compounds present in the sample. FTIR spectrum of $AgNO_3$, TFE and silver nanoparticles stabilized by TFEs are shown in the following figure **Fig. 10**. The FT-IR spectrum of the TFE stabilized nanoparticles (TFE-AgNPs) were found to be not significantly different from their respective TFEs thus retaining the compound characteristics. The broad and strong bands are noticed at around (3358 - 3380) cm^{-1}, corresponding to the O-H stretching frequency. For TFE samples, the C-H bending can be noted from the peaks in the range (750 - 1000) cm^{-1} and the peaks of the TFE-AgNPs in the range (750 - 100) cm^{-1} are slightly shifted to higher wavenumbers. From all these interpretations, it can be inferred that a TFE layer is created on the nanoparticle surface, and this helps in the formation and stability maintenance of the AgNPs [14].

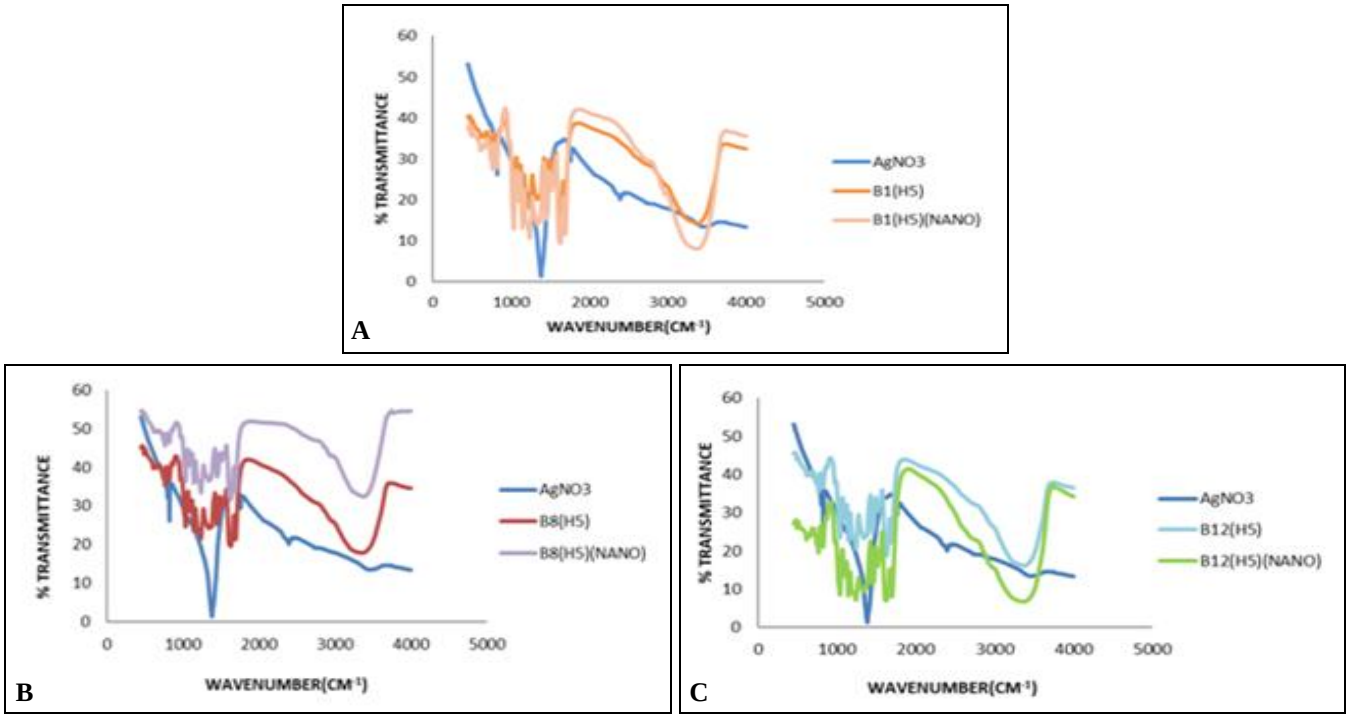

FIG. 10: FT-IR SPECTRUM OF RESPECTIVE TFE STABILIZED SILVER NANOPARTICLES OF (A) GOODRICK THURBO DARJEELING TEA, (B) CASTLETON VINTAGE DARJEELING TEA AND (C) MAKAIBARI DARJEELING TEA AGAINST THEIR RESPECTIVE TEA SAMPLES AND $AgNO_3$ (STANDARD)

Antimicrobial Analysis: Antibacterial potential of TFEs **Table 4** and its Ag-nanoconjugates **Table 5** were assessed in terms of zone of inhibition of bacterial growth against bacterial species – *S. aureus* (Gram-positive) and *E. coli* (Gram-negative).

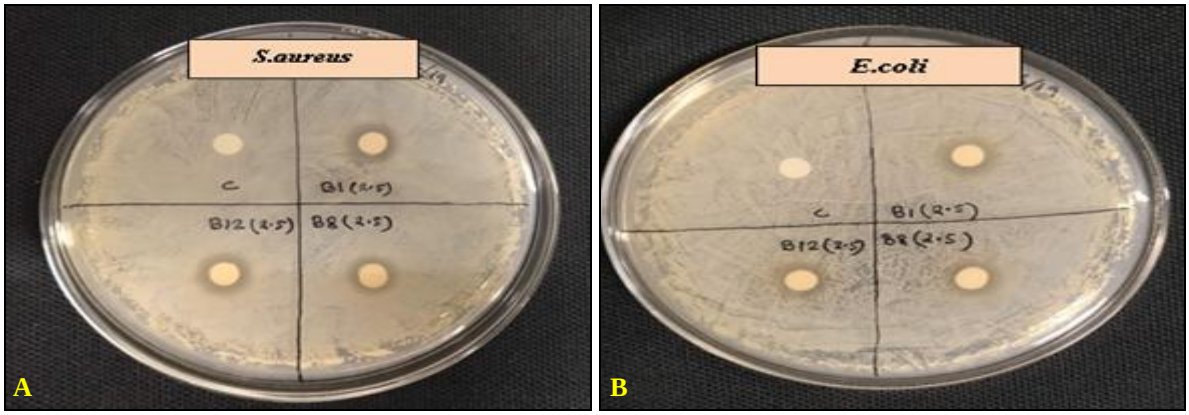

FIG. 11: ANTIMICROBIAL ACTIVITY OF TFE EXTRACTS USING KIRBY-BAUER DISK DIFFUSION ASSAY AGAINST (A) *STAPHYLOCOCCUS AUREUS* AND (B) *ESCHERICHIA COLI*

TABLE 4: VALUES ARE MEAN ± SD OF THREE PARALLEL MEASUREMENTS (n=3); - = NO ZONE OF INHIBITION

Sample (2.5mg/ml)	Concentration (µl)	Organism - *S. aureus*	Organism - *E. coli*	Control (C) (ddH_2O)
		Zone of Inhibition (mm)		
B1(H5)	20	8.78±0.51	8.00±0.33	-
B8(H5)	20	8.67±0.58	8.44±0.69	-
B12(H5)	20	8.89±0.51	8.33±0.33	-

TABLE 5: VALUES ARE MEAN ± SD OF THREE PARALLEL MEASUREMENTS (n=3)

Sample (nano - conjugates) (2.5mg/ml)	Concentration (µl)	Organism - *S. aureus*	Organism - *E. coli*	Control (C) ($AgNO_3$) (0.17mg/ml)
		Zone of Inhibition (mm)		
B1(H5)NC	20	10±0.33	10.33±0.33	8.67±0.47
B8(H5)NC	20	10.78±0.19	10.44±0.19	8.67±0.47
B12(H5)NC	20	10.22±0.51	9.44±0.19	8.67±0.47

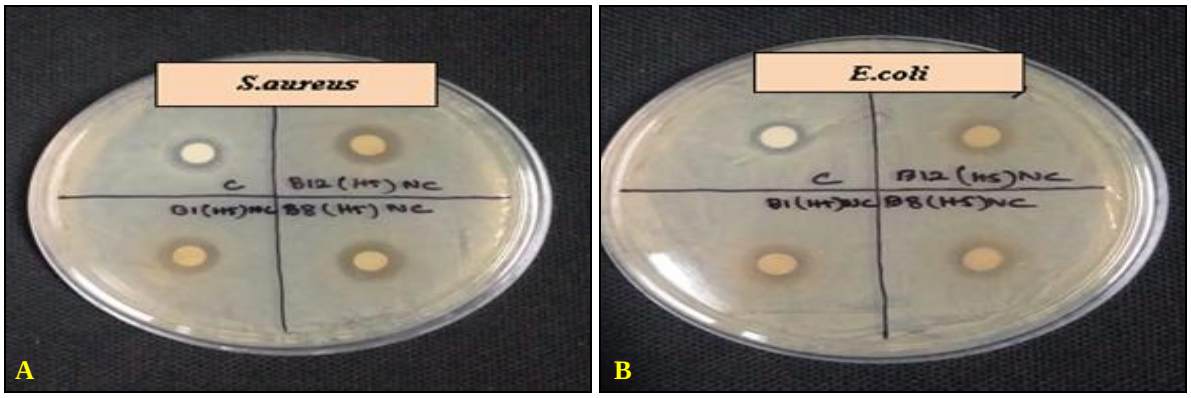

FIG. 12: ANTIMICROBIAL ACTIVITY OF TFE Ag-NANOCONJUGATES USING KIRBY-BAUER DISK DIFFUSION ASSAY AGAINST (A) *STAPHYLOCOCCUS AUREUS* AND (B) *ESCHERICHIA COLI*

The wide antimicrobial impact of silver ($AgNO_3$) is outstanding, and it has been utilized in various fields in medication for a considerable length of time. Because of their little size, nanoparticles have an enormous contact region, thus providing better contact with the microorganisms by binding to the cell membrane and furthermore infiltrating inside [15].

It can be clearly noted by comparing **Table 4** and **Table 5**, that the Ag- nanoconjugates gave better results than their respective TFE counterparts for the same concentrations (2.5mg/ml) against both organisms.

From **Table 5**, it can also be seen, that the silver nanoconjugates gave better results than $AgNO_3$ itself, thus proving enhanced antibacterial activity.

CONCLUSION: Black tea contains theaflavins which have antioxidant properties (theaflavins were shown to be able to scavenge superoxide radicals more efficiently and 10-times faster than EGCG) and are therefore of great interest to the food and biomedical industries [16].

This work mainly deals with the extraction of Theaflavins and Flavonoids rich Extract (TFE) from black tea, checking its antioxidant and other phytochemical status and finally synthesizing silver nanoconjugates and checking its antimicrobial activity against their respective TFEs. Significant levels of polyphenols, flavonoids and antioxidants were noted in the TFEs which may be involved in the reduction of the nanoparticles.

Formation of nanoconjugates is initially clearly evident from the colour change of the TFE during green synthesis, thus denoting spectral shift. On further analysis *via* UV-Vis spectrophotometer, the TFE stabilized silver nanoparticles gave peak ranging from (400-500) nm, confirming binding of silver with TFE to form nanoconjugates.

DLS analysis for size distribution proved that the size of silver nanoparticles synthesized by TFE of black tea were in the range of (13.54 – 295.3) nm, thus indicating its size in nano-regime and uniformity of shape and size in a stabilized condition. The nanoparticles were checked for stability and not much change in its size was noted after a week, specifying good stability. The FT-IR data further proved that the nanoconjugates retained their sample characteristics and had maintained stability.

Finally, these TFE-Ag-nanoconjugates were compared with their respective TFEs in terms of antimicrobial activity of the same concentration. The results clearly showed that the nanoconjugates gave a better zone of inhibition than their respective TFE counterparts and $AgNO_3$ itself, thus indicating enhanced activity.

Thus, we can conclude that the green synthesis of TFE stabilized silver nanoparticles yield better results than the TFE. Theaflavins and Flavonoids are the important antioxidant bioactive components, as well as non-cytotoxic to normal human cells, and hence TFE stabilized silver nanoparticles will serve as better antibacterial agents than TFE in our everyday lives. Thus, theaflavins would be another restorative material later on [17]. TFE conjugated nanoparticles may find application in pharmaceutical as well as garment industries due to their enhanced antimicrobial activity and non-toxic nature.

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REFERENCES:

1. Gosslau AL: Therapeutic connection between black tea theaflavins and their benzotropolone core structure. Curr Pharmacol Rep 2018; 4: 447-52.
2. GAO: Inhibitory effects of the four main theaflavin derivatives found in black tea on ovarian cancer cells. Anticancer Research 2016; 36: 643-51.
3. Armidin RP: Effectiveness of Rinsing Black Tea Compared to Green Tea in Decreasing Streptococcus mutans. Open access Macedonian Journal of Medical Sciences 2019; 3799-3802.
4. Bedran TBL, Morin MP, Spolidorio DP and Grenier D: Black tea extract and its theaflavin derivatives inhibit the growth of periodontopathogens and modulate interleukin-8 and β-Defensin secretion in oral epithelial cells. PLOS ONE. 2015; 10(11): e0143158.
5. Samad N and Javed A: Therapeutic effects of gallic acid: current scenario. Journal of Phytochemistry & Biochemistry 2018; 2(2): 1-6.
6. Yao LH: Phenolic compounds in tea from Australian supermarkets. Food Chemistry 2006; 96: 614-20.
7. Jiang Y, Hua J, Wang B, Yuan H and Ma H: Effects of Variety, Season, and Region on Theaflavins Content of Fermented Chinese Congou Black Tea [Research Article]. Journal of Food Quality; Hindawi 2018.
8. Singleton VL and RJ: Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American J of Enology and Viticulture 1965; 16: 144-53.
9. Yao MM: Optimization and validation of Folin–Ciocalteu method for the determination of total polyphenol content of Pu-erh tea. International Journal of Food Sciences and Nutrition 2017; 68: 913-18.
10. Bizuayehu DA: Determination of some selected secondary metabolites and their *in-vitro* antioxidant activity in commercially available Ethiopian tea (*Camellia sinensis*). Springer Plus 2016; 5.
11. Diky-Ramdani AS: Alkaloid and polyphenol analysis by HPLC in green and black tea powders and their potential use as additives in ruminant diets. AIP Conference Proceedings 2018; 192.
12. Vasantharaj NMS: Surface active gold nanoparticles biosynthesis by new approach for bionanocatalytic activity. J of Photo and Photob B: Biology 2018; 119-25.
13. Bauer AW: Single disc antibiotic sensitivity testing of Staphylococci. A.M.A. Arch Intern Med 1959; 208-16.
14. León A, Reuquen P, Garin C, Segura R, Vargas P, Zapata P and Orihuela PA: FTIR and Raman Characterization of TiO2 Nanoparticles Coated with Polyethylene Glycol as Carrier for 2-Methoxyestradiol. Applied Sciences 2017; 7(1): 49.
15. Mittal J, Singh A, Batra A and Sharma MM: Synthesis and characterization of silver nanoparticles and their antimicrobial efficacy. Particulate Science and Technology 2017; 35(3): 338-45.
16. Ibrahim H: Green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative micro-organisms. Journal of Radiation Research and Applied Sciences 2015; 265-75.
17. He HF: Research progress on theaflavins: efficacy, formation, and preparation. Food & Nutrition Research. 2017; 61(1).

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