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Original Research

# *In vitro* antioxidant and antibacterial potential of tea leaves harvested from small tea plantations of sub-Himalayan Terai region

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## Abstract

Tea has long been praised for its antioxidant and antimicrobial properties and other biological activities. In this research, we collected the leaves of ten different tea clones from small tea growers in the sub-Himalayan Terai region of West Bengal, India. Most of the collected samples were Toklai Vegetative (TV) tea clones established by the Toklai Tea Research Institute; some Garden-series clones were also included. Samples were extracted using three different solvents based on a wide range of polarity: petroleum benzene, acetone and methanol. Extracted samples were tested for *in vitro* antioxidant activity using DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay, Nitric Oxide (NO) assay and Ferric Reducing Power (FRP) assay.

Antibacterial activity against four pathogenic bacteria was tested using agar well-diffusion method. Results clearly showed that tea clones used by small tea growers in Terai are potentially bioactive. Moreover, TV9, which is originally a Cambod tea clone, performed better than all other samples in all tests. Of the non-polar extracts, AV2 recorded the best results, which confirms the biological active potential of its non-polar volatile aroma imparting molecules. Given that AV2 is a China-type Darjeeling tea clone, this result is valid and assuring. Total phenol and flavonoid quantification were also assessed. TV1 (an Assam-China hybrid) and TV9 again showed significant results. For a detailed antioxidant evaluation, fractionation by column chromatography was done with two of the best samples selected upon results of preliminary antioxidant analysis. Fractions of clones exhibited variable antiradical activity with changes in solvent's polarity. However, further detailed and more comprehensive studies on biochemical analysis, using tools of metabolomics to evaluate which components of tea clones are responsible for bioactive properties, are needed.

**Keywords:** Tea, antioxidant, antibacterial, total phenol, flavonoids, column chromatography

# Introduction

Tea, the most consumed beverage after water, is prepared by pouring hot or boiling water over the manufactured leaves of the tea plant (*Camellia sinensis* L. Kuntze) [1]. This refreshing, aromatic and bioactive drink is reported to contain nearly 4000 medicinally active compounds [2]. In addition to its medicinal importance, tea is one of the most important cash crops in India. Tea plantations in India are mainly large holdings first cultivated by the British in the first half of 19th Century. However, in the last quarter of 20th Century smaller holdings of tea plantations began to emerge as potential partners in the tea market, producing almost half of the total produce. These small-scale tea plantations began in the south Indian states of Tamil Nadu, Kerala and Karnataka before expanding to northeast and eastern states such as Assam and West Bengal [3]. Initially, these smaller tea growers faced several problems regarding farmland and tea cultivation management issues as most of the land available for cultivating tea was used for paddy cultivation. Against the odds, 50,000 small tea growers in North Bengal now have a share of approximately 62% of the total manufactured tea in West Bengal [4]. The Terai region of sub-Himalayan West Bengal is an ideal place for tea cultivation; it offers suitable soil status, humidity, temperature, rainfall status, topography and other conditions required for tea growing. The soil of this region is predominantly sandy loam with acidic pH [5]. It experiences heavy rainfall from April to September, with occasional rainfall during the rest of the year. Temperatures range from 5.5°C to 37°C [5]. This combination of favourable factors helped the expansion of small tea plantations in every corner of the region.

Despite supporting the socio-economic status of the Terai region, few reports or literature are available on the biochemistry of tea grown in the smaller plantations of this region. Labar *et al.*, 2019 [6] has reported the biochemical properties of some tea clones from the Terai region. AV2 contains a high amount of flavour-inducing aroma compound (volatile flavour oil) [7]. AV2 tea clone is popular for its flavour [8] and antioxidant activity [9]. A methanolic extract of the TV23 clone has shown high polyphenol content [10]. Our research was designed to explore the biochemistry of leaves from different tea clones harvested from Terai's small tea plantations with insights into antioxidant and antimicrobial potential.

# Material and methods

## Clone selection and sample collection

The tea clones were collected from small tea growers of the Terai region who conserved authentic tea clones in their gardens. Ten tea clones (TV1, TV2, TV9, TV20, TV22, TV23, TV29, AV2, Sanyasithan-8 [SNT8] and TEEN ALI-17/1/54) were selected for the study. Among the collected clones, TV1 is a hybrid clone of Assam and China-type tea; TV2 is of Assam variety; TV9, TV20, TV22, TV23 and TV29 are Cambod clones. AV2 clone is the only clone that is a Garden-series clone (China-type) from Darjeeling. SNT8 and TEEN ALI-17/1/54 are Garden-series clones mainly found in the sub-Himalayan plains or Terai region.

## Sample extraction

Three different solvents (petroleum benzene, acetone and methanol) were selected based on polarity, from non-polar (petroleum benzene) to polar (methanol), for use in sample extraction. Disease-free, young tea clone shoots were collected from small tea growers, washed thoroughly under running tap water and surface dried with tissue paper. Approximately

10 g of each sample was crushed with liquid nitrogen. Further samples were extracted with 10 ml of each solvent. The samples remained in the solvents undisturbed for 48 hours, after which time they were centrifuged at 10,000 rpm for three minutes and supernatants were collected for further experiments.

## DPPH free radical scavenging assay

DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenging activity was assessed using the protocol prescribed by Majumder *et al.*, 2021 [11] with slight modification as described here. 200 µl of each sample extract was added to 2800 µl of methanolic DPPH solution and the mixture was incubated for 30 minutes in darkness. Absorbance was measured at 517 nm in a UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis). Ascorbic acid was used as the reference to prepare a standard curve. Percentages of the DPPH scavenging activity were calculated using the formula:

$$Percentage\ of\ free\ radical\ scavenging = \frac{Absorption\ of\ Control - Absorption\ of\ Sample}{Absorption\ of\ Control} \times 100$$

## Quantification of total phenol compound

Total phenolic content (TPC) was analyzed by the protocol of Labar *et al.*, 2019 [6]. Sample extracts (100 µl) were taken in clean test tubes and incubated at room temperature for five minutes after adding 400 µl of 10% Folin-Ciacalteu reagent. Following incubation, 1 ml of 5% $Na_2CO_3$ was added and the mixture was incubated at room temperature for 120 minutes. Absorbance was measured at 730 nm in a UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis). Quantification was done from a standard curve prepared from gallic acid.

## Quantification of flavonoids

A sample extract (250 µl) of each tea clone was taken in clean test tubes, to which 1.25 ml of double-distilled water, then 75 µl of 5% $NaNO_2$ was added. Samples were left for five minutes, after which 150 µl of 10% $AlCl_3$ was added. The samples were kept at room temperature for six minutes. First, 500 µl of 1 mM NaOH was added, then 275 µl of double-distilled water was added. Finally, after 30 minutes of incubation, the absorbance was measured using UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis) at 510 nm. A standard curve was prepared using quercetin to quantify the flavonoids, which were expressed as mg QE or quercetin equivalent/ml of sample.

## Ferric reducing power assay and nitric oxide reducing activity

The ferric reducing power (FRP) of samples was analyzed by following the protocol of Ghosh *et al.*, 2020 [12]. Extracts of each sample with three different concentrations (50 µl, 100 µl, 150 µl) were added to 500 µl phosphate buffer (pH 7.4). Then, 1% of 500 µl K2Fe(CN)6 was added and incubated for 20 minutes at 50°C. After incubation, 500 µl of 10% trichloroacetic acid was added to the test tube followed by 1.5 ml double-distilled water. 300 µl of 0.1% ferric chloride was added and the mixture was diluted with 700 ml phosphate buffer. Absorbance was taken at 700 nm by using UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis). Recorded values of absorbance were plotted in a graph (MS Excel 2010) where samples showing increasing absorbance with increasing concentrations were considered as samples having FRP.

The nitric oxide (NO) reducing activity followed the protocol of Ghosh *et al.*, 2020 [12] and Bhattacharya *et al.*, 2009 [13] with slight modification as described. Three different concentrations (50 µl, 100 µl, 150 µl) of tea shoots extract were taken, and 10 mM of 0.5 ml sodium nitroprusside was added. The mixture was incubated at 25°C for 150 minutes. Next, 1.2 ml Griess reagent was added, and

absorbance was taken at 546 nm by using UV-Vis spectrophotometer (Agilent Technologies Cary 60 UV-Vis). Recorded values of absorption were plotted in a graph (MS Excel 2010) where samples showing increasing absorbance with increasing concentrations were considered as samples having NO reducing activity.

FRP and NO activity were experimented in three different concentrations (50 mg/ml, 100 mg/ml and 150 mg/ml). The tests results (absorbance) were further plotted in graphs depicting these activities following the protocol of Ghosh *et al.*, 2020 [12] and Chakraborty *et al.* 2021 [14].

## Antibacterial activity

Antibacterial activity analysis was carried out using petroleum benzene, acetone and methanolic extract and followed the protocol of Ghosh *et al.*, 2020 [12]. 0.5 ml of different samples were dried and dissolved in 1 ml dimethyl sulfoxide (DMSO). The antibacterial activity of the tea extracts was tested against four bacteria: *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP). These four microorganisms were pour plated in Mueller Hinton Agar (media). Using a sterile cork borer, wells were made in the plate and antimicrobial activity was studied by well-diffusion method [8]. 100 µl of extracts in different solvents were added and incubated overnight at 37°C. A zone of inhibition measurement was used to determine antibacterial activity.

## Fractionation of selected samples by column chromatography and antioxidant activity

Fractionation or separation of components through column chromatography was performed following the protocol of Majumder *et al.* [11] and Bhattacharya *et al.*. [13] Three samples were selected based on the best results found in *in vitro* assays. The dried samples were loaded on silica gel (Merck, 200–400 mesh size) and a series of non-polar to polar solvents (hexane, benzene, chloroform, ethyl acetate, acetone, ethanol, methanol and water) were passed through the column to obtain a total of 33 fractions (60 ml each): hexene, hexane:benzene (2:1), hexane:benzene (1:1), hexane:benzene (1:2), benzene, benzene:chloroform (2:1), benzene:chloroform (1:1), benzene:chloroform (1:2), chloroform, chloroform:diethyl ether (2:1), chloroform:diethyl ether (1:1), chloroform:diethyl ether (1:2), diethyl ether, ethyl acetate:acetone (2:1), ethyl acetate:acetone (1:1), ethyl acetate:acetone (1:2), ethyl acetate, ethyl acetate:acetone (2:1), ethyl acetate:acetone (1:1), ethyl acetate:acetone (1:2), acetone, acetone:ethanol (2:1), acetone:ethanol (1:1), acetone:ethanol (1:2), ethanol, ethanol: methanol (2:1), ethanol:methanol (1:1), ethanol:methanol (1:2), methanol, methanol:water (2:1), methanol:water (1:1), methanol:water (1:2), water (expressed as 1–33 in the results).

Each fraction was vacuum evaporated at low temperature, dried and dissolved in 2 ml methanol for further antioxidant assay to assess potential fraction behind those activities. The free radical scavenging activity through DPPH assay was performed following the method described by Majumder *et al.* 2021 [11] with minor modifications where antiradical activity was measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH (D9132 from Sigma-Aldrich) brought about by the sample. To 2800 µl of DPPH (100 µM) solution in methanol, 200 µl of each sample was added and incubated for 30 minutes in a dark room at room temperature. Decreases in the absorbance in presence of the sample were noted at 517 nm by UV-Vis spectrophotometer (Cary 60 UV-Vis Spectrometer by Agilent Technologies). Results were expressed as a percentage of DPPH inhibition (% inhibition) occurring due to exposure of samples.

# Results

## DPPH free radical scavenging assay

Through DPPH scavenging assay, it was found that methanol and acetone extracts of all leaf samples showed promising activities as shown in **Table 1**. Petroleum benzene extracts showed less antioxidant activity compared with acetone and methanol. Comparatively, the highest and lowest DPPH activity was 48.25% in AV2 (790.47±9.28 µg AAE/g of leaf sample) and 3.25% in TV1 (57.85±0.58 µg AAE/g of leaf sample) respectively for petroleum benzene extracts. Interestingly, only one petroleum benzene extract showed a good result (48.25% by AV2). Furthermore, no notable variations were found in the activities exhibited in the acetone and methanolic extracts. Results of the assay are given in **Table 1**.

## Total phenol compound

The highest value of TPC was found in acetone extract. The quantified values of phenol content for each sample dissolved in petroleum benzene, acetone and methanol are given in **Table 2**. According to the result of this assay, acetone and methanol were found to be the most effective in extraction of phenolic compounds. Acetone extract of TV1 (955.26±8.907 mg GAE/g of leaf sample) clone showed the highest TPC value. Petroleum benzene extract of TV2 (75.07±3.726 mg GAE/g of sample) clone recorded the lowest TPC value. Results of the TPC assay are given in **Table 2**.

**Table 1** DPPH scavenging percentage of plucked tea shoots in petroleum benzene, acetone and methanolic extracts

| Clone | Petroleum Benzene Extract | | Acetone Extract | | Methanol Extract | |
|---|---|---|---|---|---|---|
| | DPPH scavenging% | µg AAE/g of leaf sample | DPPH scavenging % | µg AAE/g of leaf sample | DPPH scavenging % | µg AAE/g of leaf sample |
| TV1 | 3.25 | 57.85±0.58 | 88.52 | 1676.68±51.06 | 86.10 | 1623.47±41.76 |
| TV2 | 4.43 | 83.85±0.52 | 87.71 | 1658.87±35.33 | 86.36 | 1629.27±17.52 |
| TV9 | 8.58 | 175.27±0.60 | 86.47 | 1631.67±76.79 | 87.83 | 1661.47±31.34 |
| TV20 | 4.34 | 81.85±0.41 | 85.78 | 1616.48±48.22 | 87.65 | 1657.47±29.80 |
| TV22 | 7.30 | 147.07±0.93 | 86.28 | 1627.47±30.35 | 87.32 | 1650.27±18.28 |
| TV23 | 22.25 | 218.34±1.26 | 87.10 | 1645.47±27.88 | 87.92 | 1663.47±20.53 |
| TV29 | 29.33 | 374.18±0.84 | 85.54 | 1611.06±12.99 | 86.54 | 1633.07±22.82 |
| SNT 8 | 7.80 | 158.07±0.72 | 87.47 | 1653.67±29.48 | 87.96 | 1664.27±31.53 |
| TEEN ALI-17/1/54 | 22.01 | 212.74±9.85 | 87.82 | 1661.27±26.54 | 88.08 | 1667.08±16.90 |
| AV2 | 48.25 | 790.47±9.28 | 87.28 | 1649.47±19.52 | 88.51 | 1676.48±13.67 |

Data communicated as a mean of five replicates ±SD. Percentage of DPPH scavenging was measured by the ascorbic acid equivalent (µg AAE/g leaf sample). It was calculated from the standard curve: y=0.876x+6.591; $R^2$=0.993

## Quantification of flavonoids

Flavonoid quantification was determined using of µg quercetin equivalent (mg QE/g) in fresh tea shoots extract in petroleum benzene, acetone and methanol. Acetone and methanolic extracts were the most effective (**see Table 2**). Acetone solvent extract of TV9 (7.16±0.006 mg QE/g of sample) and TV22 (7.16±0.007 mg QE/g of sample) clones recorded the highest number of flavonoids. Petroleum benzene solvent extract of TV2 (1.18±0.004 mg QE/g) clone recorded the lowest number of flavonoids. Results of flavonoid quantification are given in **Table 3**.

**Table 2** Quantification of total phenol was expressed by the mg gallic acid equivalent (GAE)/g of plucked tea shoots.

| Clone | Petroleum benzene (µg GAE/g of leaf sample) | Acetone (µg GAE/g of leaf sample) | Methanol (µg GAE/g of leaf sample) |
|---|---|---|---|
| TV1 | 107.54±1.615 | 955.26±8.907 | 860.56±8.041 |
| TV2 | 75.07±3.726 | 930.93±5.463 | 863.26±7.119 |
| TV9 | 111.35±2.428 | 824.65±4.203 | 842.56±6.147 |
| TV20 | 109.28±4.549 | 884.93±6.756 | 855.40±6.808 |
| TV22 | 128.58±5.311 | 843.56±3.350 | 880.74±3.702 |
| TV23 | 104.63±3.844 | 843.37±3.362 | 938.63±5.363 |
| TV29 | 160.51±6.975 | 856.07±4.825 | 920.93±3.454 |
| SNT 8 | 95.72±5.157 | 923.30±4.153 | 921.37±4.577 |
| TEEN ALI- 17/1/54 | 90.70±3.501 | 875.72±3.312 | 845.02±4.197 |
| AV2 | 340.72±6.856 | 851.23±3.128 | 920.63±3.607 |

Data communicated as a mean of five replicates ±SD. Total phenol was measured by the gallic acid equivalent (µg/g). It was calculated from the standard curve: y = 0.0043; x = 0.1672; $R^2$ = 0.998

**Table 3** Quantification of total flavonoid expressed as mg quercetin equivalent (QE)/g of sample used for extraction

| Clone | Petroleum benzene (mg QE/g of leaf sample) | Acetone (mg QE/g of leaf sample) | Methanol (mg QE/g of leaf sample) |
|---|---|---|---|
| TV1 | 1.22±0.014 | 6.29±0.004 | 4.50±0.006 |
| TV2 | 1.18±0.004 | 5.75±0.008 | 3.54±0.006 |
| TV9 | 1.20±0.003 | 7.16±0.006 | 5.99±0.006 |
| TV20 | 1.34±0.003 | 6.87±0.005 | 4.09±0.009 |
| TV22 | 1.55±0.003 | 7.16±0.007 | 4.36±0.009 |
| TV23 | 1.27±0.004 | 5.08±0.007 | 5.25±0.005 |
| TV29 | 1.46±0.004 | 5.41±0.005 | 5.21±0.008 |
| SNT 8 | 1.32±0.003 | 6.11±0.005 | 2.56±0.006 |
| TEEN ALI- 17/1/54 | 1.33±0.003 | 6.72±0.005 | 3.13±0.007 |
| AV2 | 1.35±0.003 | 4.03±0.006 | 2.59±0.008 |

Data communicated as a mean of five replicates ±SD. Flavonoid content was measured by the quercetin equivalent (mg QE/g). It was calculated from the standard curve: y = 0.207, x = 0.204; $R^2$ = 0.962.

## Ferric reducing power and nitric oxide reducing assay

FRP and NO reducing activity are expressed in **Fig. 1** and **Fig. 2** respectively. Samples of clones TV9, TV22, TV29, SNT8 and TEEN ALI- 17/1/54 for petroleum benzene extracts; TV2 for acetone extracts and TV1, TV2 and TV9 for methanolic extracts showed FRP activity (**Fig. 1**). These results are discussed below. NO reducing activity, as recorded positive results in all samples (**Fig. 2**); graphs (bar charts in **Fig. 2)** of increasing absorbance spectra were high rising and sharp for all sample extracts.

**Figure 1** Graphical representation of FRP assay shown by different samples extracted in petroleum benzene (A), acetone (B) and methanol (C)

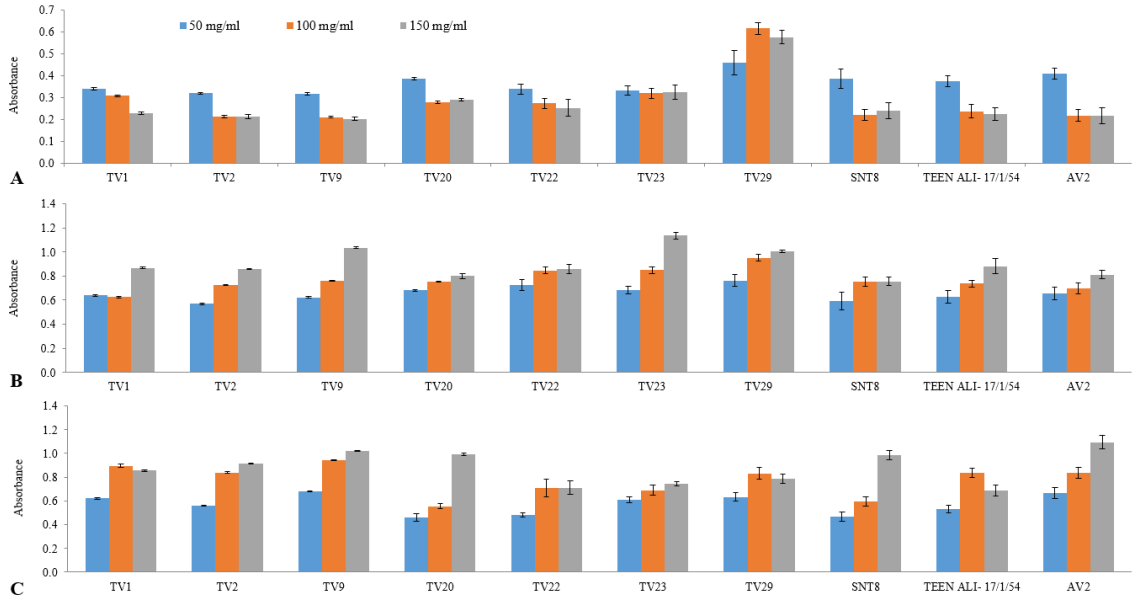
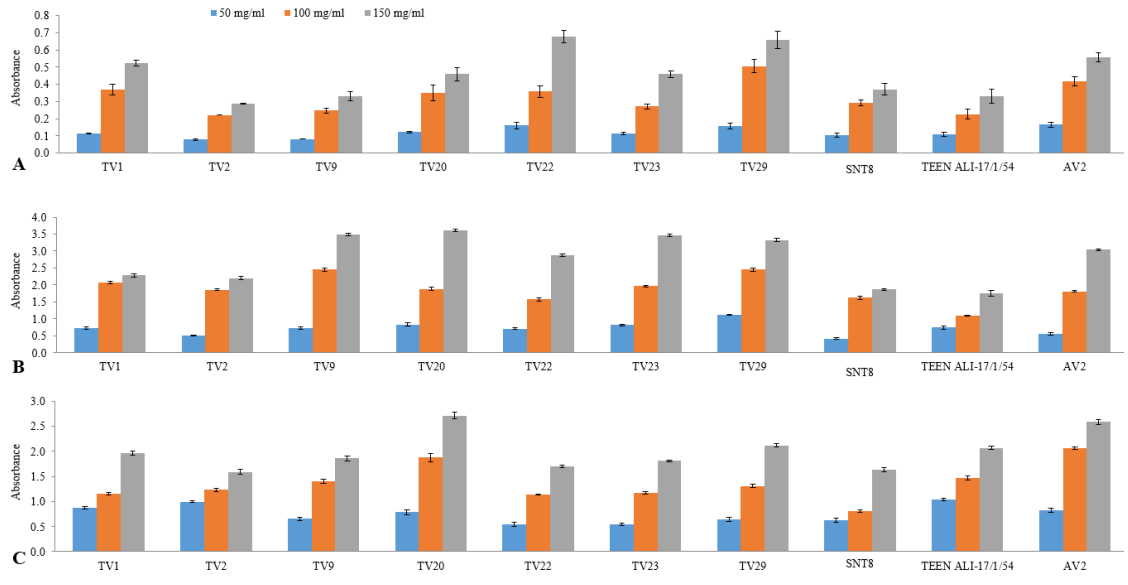



**Figure 2** Graphical representation of NO assay of different samples extracted in petroleum benzene (A), acetone (B) and methanol (C)



## Antibacterial activity

To examine the antibacterial activity of different tea clones in petroleum benzene, acetone and methanolic extractions, two gram-positive (BS and SA) and two gram-negative (EC and KP) bacteria were used. Evidence of antibacterial activity are shown in **Fig. 3** and **Table 4**. Petroleum benzene extract of TV1, TV2, TV9, TV20, TV22, TV23, TV29 and AV2 were found to inhibit growth of BS, while TV9, TV20, TV22, TV23, TV29 and AV2 clones inhibited growth of KP as shown in **Fig. 3**. Petroleum benzene extracts of Garden-series clones SNT8 and TEEN ALI- 17/1/54 did not show any antibacterial activity. Petroleum benzene extracts of TV9, TV20, TV22, TV23, TV29 and AV2 exhibited antibacterial activity against KP and BS. Petroleum benzene extracts of TV1 and TV2 also showed antibacterial activity against BS. All clones extracted in acetone exhibited activity against EC and SA except TV23, which showed no antibacterial activity against SA. Acetone extract of TV1 showed the highest inhibition zone against EC (16 mm) and SA (14 mm) (see **Table 4**). Petroleum benzene extract of TV9 (15 mm) and TV23 (15 mm) recorded the highest activity against KP while TV20 (14 mm), TV22 (14 mm) samples recorded the highest inhibition against BS for this extract (see **Table 4**). Tests on methanolic extracts showed that TV9 inhibited the growth of EC, BS and SA remarkably (see **Fig. 3**); TV1 recorded the same result against KP.

**Figure 3** Inhibition zones against bacteria produced by different solvent extracts. Petroleum benzene extracts (A), Acetone extracts (B), Methanol extracts (C)

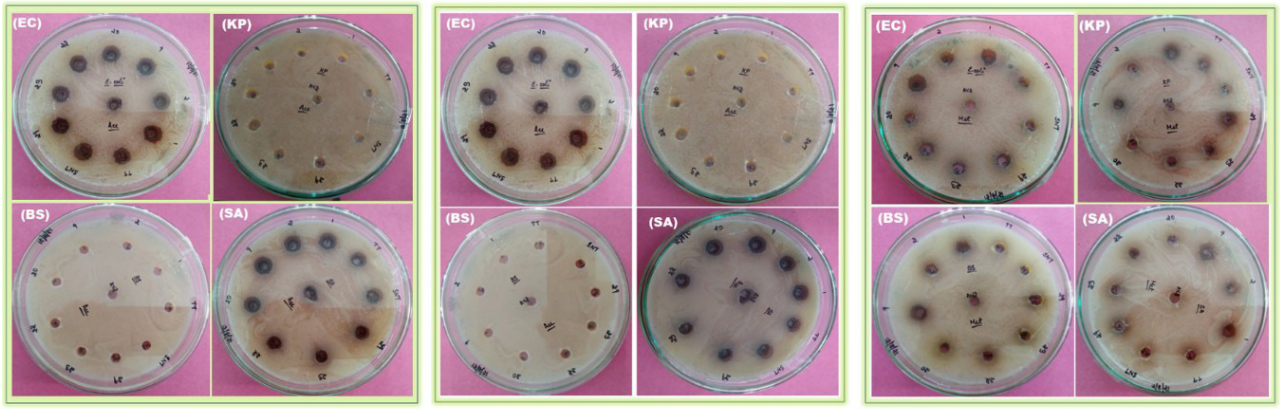



**Table 4** Inhibition zones (mm) against bacteria produced by different sample extracts

| Solvent extract | Bacteria | TV1 | TV2 | TV9 | TV20 | TV22 | TV23 | TV29 | SNT8 | TEEN ALI-17/1/54 | AV2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Petroleumbenzene** | *Escherichia coli* | - | - | - | - | - | - | - | - | - | - |
| | *Bacillus subtilis* | 10 | 13 | 13.5 | 14 | 14 | 12 | 10 | - | - | 10.5 |
| | *Staphylococcus aureus* | - | - | - | - | - | - | - | - | - | - |
| | *Klebsiella pneumoniae* | - | - | 15 | 12 | 12.5 | 15 | 12 | - | - | 10 |
| **Acetone** | *Escherichia coli* | 16 | 14 | 15.5 | 14.5 | 13.5 | 12.5 | 12.5 | 14.5 | 13.5 | 11.5 |
| | *Bacillus subtilis* | - | - | - | - | - | - | - | - | - | - |
| | *Staphylococcus aureus* | 14 | 13.5 | 14 | 12 | 10.5 | - | 10 | 12.5 | 11.5 | 10 |
| | *Klebsiella pneumoniae* | - | - | - | - | - | - | - | - | - | - |
| **Methanol** | *Escherichia coli* | 13 | - | 15 | - | - | - | - | - | - | - |
| | *Bacillus subtilis* | - | - | 15 | - | - | - | - | - | - | - |
| | *Staphylococcus aureus* | - | - | 15 | - | - | - | - | - | - | - |
| | *Klebsiella pneumoniae* | 15 | - | - | - | - | - | - | - | - | - |

### Fractionation of selected samples by column chromatography and antioxidant activity

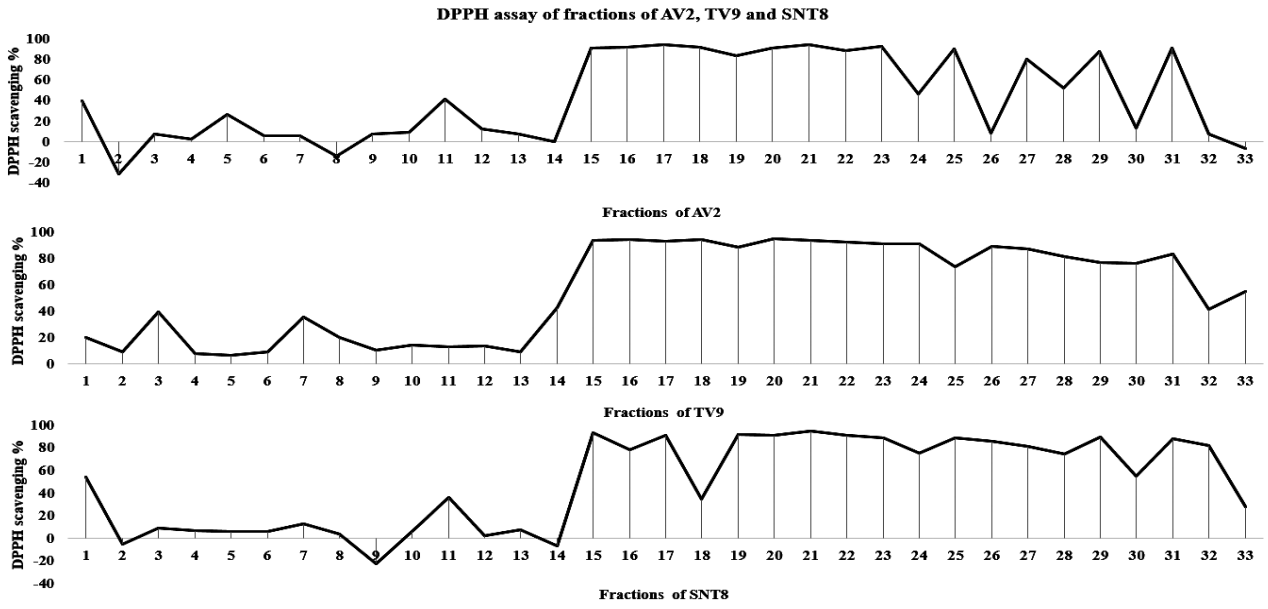
Fractionation of samples by column chromatography was done with three samples (AV2, TV9 and SNT8) because all responded well in *in vitro* assay: AV2 showed the best DPPH scavenging activity; TV9 showed the highest flavonoid content and antibacterial activity; SNT8 and AV2 both recorded the best results in total phenol quantification. Preliminarily, DPPH scavenging activity was assessed with the fraction at this stage. According to results, antioxidant activity was found to be high from the 15th solvent fraction i.e., diethyl ether and ethyl acetate (1:1). Moreover, further solvent fractions showed high antioxidant activity for all the clones. **Fig. 4** has been provided to depict DPPH free radical scavenging activity shown of different fractions (expressed as 1–33 in **Fig. 4**).

## Discussion

Phenols, flavonoids, terpenoids and alkaloids are the major compounds of tea leaves known to be soluble in acetone and methanol, while their affinity to non-polar solvents like petroleum benzene is very low [15]. This was reflected in the results of experiments carried out as part of this research. As a China variety of clone (mainly cultivated in Darjeeling hills), the volatile flavour compound (VFC) of AV2 is higher than the other TV clones and Garden-series clones used in this research [16]. Petroleum benzene performs better as a solvent to extract various volatile oils when compared with the other two solvents used in this research (acetone and methanol). Volatile oils may possess good antioxidant properties, [17] which may be the probable reason behind the high antioxidant activity detected in the petroleum benzene extract of AV2 leaf, when compared with other samples. Tea is praised for its antioxidant activities [18].

Many literatures have quantified or assessed the antioxidant activity of tea with numerous assays *in vitro* and *in vivo*. The objective of this research was to show antioxidant activity by assessing DPPH assay. DPPH free radical scavenging activity was considered as a parameter to compare antioxidant activity

**Figure 4** DPPH free radical scavenging activity shown by different fractions (1–33) of AV2, TV9 and SNT8



among samples of different tea clones used in this research and to evaluate the best tea clones reported for their promising biological activities. AV2 tea clone is famous for its flavour [8] and it also possesses antioxidants and NO reducing activity [9]. It is also reported that clone TV23 contains higher polyphenol content, which is confirmed in the results of this research (see **Table 2**) [10]. Different solvents were chosen from non-polar to polar (petroleum benzene, acetone and methanol) in this experiment in order to differentiate the activity exhibited by different groups of antioxidant molecules depending on their affinity towards solvents of different polarities. Plant-derived phenolic compounds are reported to exhibit anticancer, antioxidant, antimicrobial, anti-inflammatory properties [19, 20]. They are also reported to prevent diseases such as cardiovascular diseases, obesity, neurodegenerative diseases and diabetes [19, 20]. Total phenolic content is a major group of molecules found in tea leaves and is responsible for the quality of the tea. The total phenolic content of tea consists of polyphenols, phenolic acids and flavonoids [21]. Flavonoids are a group of polyphenolic compounds of which tea is an important natural source. [22]. Flavonoids represent antioxidant activity by scavenging free radicals inside the body [14]. Kumar *et al.*, 2013 [23] have reported that these compounds can prevent coronary heart disease, liver diseases and have anti-inflammatory, anticancer and antiviral activities. Flavonoid biosynthesis of *C. sinensis* has been reported by He *et al.*, 2018 [24]. The Cambod variety of tea can produce special flavonoids named anthocyanin pigment [25]. Flavonoid is known to be soluble in acetone and methanol [15], therefore acetone and methanol extracts showed maximum total phenol content and flavonoids, unlike petroleum benzene extracts.

Like DPPH, FRP assay is another important parameter to understand antioxidant activity [26]. Except for AV2, petroleum benzene extracts did not respond well. According to Ghosh *et al.*, 2020, [12] samples which show increasing absorbance with increasing concentration are considered as samples with FRP activity. From results of this FRP assay, clones TV2, TV9, TV23, and TV29 were found to exhibit promising FRP activity for acetone extracts as steady and proportional absorbance spectra were found with increasing concentration for these samples. Similarly, absorbance spectra for TV2 showed comparatively better FRP activity for acetone extracts among all samples and for methanolic extracts; results of TV2, TV9, TV20, SNT8 and AV2 clones were remarkable (**Fig. 1**). However, a reverse tendency with proportional decrease in reducing power with increasing concentration has been observed for TV9, TV29 and TEEN ALI- 17/1/54 clones. This extremely variable result may be due to the presence of a wide range of biomolecules in different tea clones. Exploration of the phytochemistry of each clone is important to ascertain the real cause behind such variable activity.

The NO reducing ability is an important characteristic of a potent antioxidant [27]. NO reducing ability is also linked with important biological activities such as anti-inflammation, anticancer, antitumor activity etc. [28]. It has also been implicated in different physiological roles in the cardiovascular system, i.e. regulation of vascular tone, myocardial contractility, antithrombotic effects in the vasculature, endothelial-leukocyte interactions, endothelial integrity and permeability, etc. [29]. Interestingly, the best result (increasing absorbance with increasing concentration) was found in the Cambod variety tea clones. Of the petroleum benzene leaves extracts, TV22, TV23 and AV2 samples were found to be the best as a prominent upward curve for absorbance was clearly seen with increasing concentration (see **Fig. 2**). Regarding methanolic extracts, TV20 and AV2 leaves were found to show significant NO reducing activity unlike the other samples.

All samples extracted in all the three solvents were found to have NO reducing activity. Overall, acetone solvent was found to be the best to extract components responsible for NO reducing activity.

Different compounds have different affinities towards different solvents depending on polarity and other physicochemical properties [15]. In our results, petroleum benzene extracts did not show any antibacterial activity against EC. This may have been due to absence of antibacterial molecules in the samples extracted with this solvent. Significantly, antibacterial activities were found less in petroleum benzene extracts than in acetone or methanol extracts. Future chromatography studies on all samples dissolved in wide range of solvents may help to uncover the reasons for this. Acetone has the polarity for dissolving polar compounds as well as some non-polar compounds. Thus, acetone extract showed good antibacterial activity against EC and SA bacteria for all the samples except TV23. Interestingly, TV9 clone responded well in methanol extraction, showing antibacterial activity against three of the bacteria (*Escherichia coli, Bacillus subtilis, and Staphylococcus aureus*). Results of this assay clearly revealed TV9 as the most promising sample for antibacterial properties.

Regarding solvent fractions, good results were found in diethyl ether and ethyl acetate (1:1) fraction and further polar solvent fractions. In the non-polar range, AV2 exhibited comparatively better scavenging activity for hexane, benzene and chloroform: diethyl ether (1:1) fractions. Interestingly, petroleum benzene extract of AV2 exhibited good results in previous *in vitro* antioxidant assays. Thus, assessment of fractionation and further DPPH assay on fractions validated the antioxidant potential of selected samples and potentials of solvent for extraction for further sophisticated chromatographic analysis. Polar solvent fractions exhibited good antioxidant properties for all the samples, however, in the AV2 clone, some non-polar solvent fractions also exhibited good antioxidant properties. This result suggested that AV2 may contain exclusive bioactive components soluble in non-polar solvents that are probably non-polar volatile aroma components. However, further research is needed to examine this result. Moreover, the good antioxidant properties of solvent fractions shown in this research validates results of DPPH free radical scavenging activity, quantification of total phenol, flavonoid, FRP, NO reducing activity, antibacterial assay etc. done on samples extracted with three different solvents.

# Conclusion

Antioxidants play an important biological role in the human body to destroy free radicals and protect the human body against many major diseases and disorders [14]. Antioxidants are found in our diet in fruit and vegetables, tea and wine. Tea, rich in bioactive components, is considered a healthy beverage with antioxidant activities and stimulating properties for the human body. The aim of this research was to study the biochemical characters, antioxidant and antibacterial properties of different tea clones used for cultivation by small tea growers of the Terai region. The outcomes of this research have provided comparative preliminary data on the biochemical characters of studied tea clones, which will help inform future research in this area.

**Conflicts of interest:** None

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