Assessment of antimicrobial, antioxidant and cytotoxicity properties of *Camellia sinensis* L.

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Abstract: The phytochemical screening, antimicrobial, antioxidant and cytotoxic properties of *Camellia sinensis* were evaluated in the present study. The phytochemical screening revealed the presence of an applicable amount of lycopene, *β*-carotenes, flavonoids and tannins in *C. sinensis*. Among the phytochemicals, tannin was found to be significantly higher in tea plant. The antimicrobial activity of plant extracts against selected bacterial strains namely, *Escherichia coli, Staphylococcus aurous*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, Marginella morganii* and *Haemophilus influenzae* was investigated. The results showed that the stem part of *C. sinensis* presented greater antimicrobial potential than the leaf and root. Antioxidant activity (assessed through % inhibition of linoleic acid per oxidation test) was the highest (89.22%) in *n*-hexane extract of root part as compared to other extracts. Finally, the cytotoxicity analysis (haemolytic activity against human erythrocytes) of plant extract showed the negligible (%) lysis of RBCs ranging from 1.73 to 4.01%. In conclusion, it can be suggested that *C. sinensis* is the potential source to obtain bioactive phenolic compounds with high antimicrobial and antioxidant properties, which could possibly be exploited for the treatment of various infectious diseases.

Keywords: *Camellia sinensis,* phytochemicals, antimicrobial, antioxidant, cytotoxicity.

INTRODUCTION

Camellia sinensis L. a cultivated evergreen plant, has long been appreciated by human beings around the globe for its medicinal and therapeutic potential (Sharangi, 2009). It is the second most consumable beverages on earth after water (El Sheikh *et al*., 2015). Tea plant contains an array of bioactive components such as polyphenols, flavonoids, catechins, xanthones, caffeine, tannins, fats, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C) and some inorganic elements (Kavanagh *et al*., 2001; Koo *et al*., 2007). The healthful beneficial properties, however, are predominantly attributed to the presence of polyphenols (Sharangi, 2009). Due to their vicinal dihydroxy or tri-hydroxy structural property, polyphenols exhibit ability to quench free radicals i.e. reactive oxygen species (ROS) by allowing electron delocalization, and thus prevents damage to macromolecules (Khan and Mukhtar, 2007).

Tea catechins are water soluble compounds that possess remarkable antimicrobial effect on Gram-positive bacteria as compared to Gram-negative bacteria. It also shows strong antioxidant property that may protect the body from oxidative damages caused by free radicals. Other characteristic features like taste, color and fragrance are also associated directly and/or indirectly to these catechins (Wang and Goodman, 1999). Chiefly, six types of catechin compounds are present in green tea namely, catechin, gallocatechin (GC), epicatechin (EC), epigallocatechin (EGC), epicatechingallate (ECG) and epigallocatechingallate (EGCG). EGCG is the most potent chemo preventive agents among all the catechin compounds acting as apoptosis inducers and anti-cancerous by suppressing the growth of human transformed cells (Farhoosh *et al*., 2007; Sharangi, 2009). The flavonoids have antimicrobial, antioxidant, anti-allergic and anti-inflammatory effects (El-Sheikh *et al*., 2015).

The emergence of the multiple drug resistant pathogens has fascinated the Pharmacologists interest to discover new compounds having strong antimicrobial potential with novel mechanism of action (Zakir *et al*., 2015). Literature survey revealed that about 50,000 people die in the world per day due to infectious diseases (IDs). The fungus *Candida albicans* and bacteria like *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* are thought to be the leading source of various IDs (Chanda and Baravalia, 2012). According to World-Health Organization (WHO), about 80% of the world's population in the developing countries (like Pakistan) relies on plant derived medicines/drugs that play a promising role in the treatment of IDS (Ahmad and Beg, 2001). On the basis of a number of epidemiological and clinical studies, the American Medicinal Association (AMA) suggests that regular consumption of green tea can minimize cholesterol level, maintain blood pressure,

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and decreases the threat of atherosclerosis, particularly coronary artery diseases (CADs). The National Cancer Institute reported that green tea may decrease the frequency of various types of cancers because of the presence of highly effective antioxidants. Extensive studies have confirmed the potential efficacy of plant extracts against various bacterial and fungal pathogens. It is reported that more than 20,000 plant species have been shown therapeutic relevance as classified by WHO (Ashraf *et al*., 2015). The bioactive compounds present in plants also have fundamental role towards modern drug development (Zakir *et al*., 2015).

Keeping in mind the strong medicinal background, *Camellia sinensis* was investigated in the present study to determine phytochemicals and to explore its antimicrobial, antioxidant and cytotoxic properties by using various extracts.

MATERIALS AND METHODS

Collection and preparation of sample

Camellia sinensis whole plant was collected from Mansehra, Khyber Pakhtunkhwa, Pakistan during the month of September, 2012. The plant was further identified by Department of Botany, Abdul Wali Khan University, Mardan; K.P.K. After identification, the plant leaves and stems were detached, washed with distilled water and air dried followed by oven drying. The dried plant parts were grounded to fine powder form with the help of pestle mortar and electric blender. Samples were preserved in fine plastic bags and stored at 4ºC for further analysis. Both polar and non-polar organic solvents including *n*-hexane, aceton, chloroform, ethanol, methanol, and water were used for the preparation of different extracts of tea plant.

Test microorganisms

All the bacterial strains i.e. *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acenetobacter baumannii, Marginella morganii* and *Haemophilus influenzae* were kindly provided by National Institute of Health (NIH) Islamabad, Pakistan. All microbial homogenous inocula were prepared in sterile Lauria Bertani (LB) broth medium (Sigma Aldrich Company, USA). Optical density (OD_{660}) of each culture in the range of 0.5-1.0 was considered optimal for antimicrobial activity assessment.

Determination of bioactive compounds

Determination of total flavonoid contents

Total flavonoid contents determination was carried out by spectrophotometric assay as reported (Lillian *et al.*, 2007). Briefly, 5g of samples were dissolved in 50mL of aqueous ethanol (80% v/v), and mixtures were placed in shaking incubator for 24h. After 24h, the extracts were centrifuged (at 3,000g for 15min), pellets were discarded and supernatants were kept in 50mL falcon tube at 4ºC. A 250µl extract containing flavonoid was mixed in 1.25mL of distilled water and 75µl of $NaNO_2$ solution (5.0% w/v). After 5 min, 150µl of 10% $AlCl_3.H_2O$ was added and incubated for 6 min. After this, 500µl of 1M NaOH and 275µl of distilled water were added to the mixture and absorbance of the solution was measured at OD_{415}.

Determination of β-carotene and lycopene

Previously reported method of Rosales, (2002) was adopted for *β*-carotene and lycopene determination. Ten gram of the sample was dissolved in 100mL of methanol and solution was kept in temperature controlled shaker for 24h. The extract was centrifuged, filtered (Whatman filter paper No. 1) and supernatant was kept in hot water-bath for solvent evaporation. The dried evaporated sample was extracted with acetone: *n*-hexane mixture (4: 6). The reaction mixture was analyzed spectrophotometrically for *β*-carotene and lycopene at 453, 505, 645 and 663 nm. *β*-carotene and lycopene were calculated by using the following equations:

Lycopene (mg/50ml) = $0.0458A_{663} + 0.372A_{505} - 0.0806A_{453}$

β-carotene (mg/50mL) =$0.216\ A_{663} – 0.304A_{505} + 0.452\ A_{453}$

Determination of tannins

Tannins were estimated by the methodology described by Makkar *et al*. (1993). Different concentrations of tannic acid (3-50 mg) were made by diluting it serially from stock solution (50 mg/100mL 70% acetone). The tannin extract (50µl) was mixed with 950µl of distilled water followed by addition of 0.5ml of Foline Ciocalteu's phenol and 2.5ml of 20% $NaCO_3$ solution with continuous agitation. Before measuring the absorbance at 725 nm, the solution was incubated at room temperature for 40 min. 70% acetone was used as blank and treated in the same way as that of positive control.

Antimicrobial assay

The agar disc diffusion method (NCCLS, 1997) was followed for the determination of antimicrobial activity of fresh tea plant extract against selected bacterial strains such as *E. coli, S. aurous*, *P. aeruginosa, K. pneumoniae, A. baumannii, M. morganii* and *H. influenzae*. A 100mL suspension of the test bacteria was inoculated in to sterile nutrient agar (Darmstadt, Germany) and molten aliquots were then poured into sterilized Petri plates separately. Sterilized filter discs impregnated with the sample solution (50µL) were employed in inoculated Petri plates with the help of disinfected forceps. The plates were incubated at 37ºC for 24h and antibacterial activity was determined in terms of inhibition zones (mm) using a zone reader.

Antioxidant assay

The ferric ion reducing power capabilities of the extracts of fresh tea plant was assessed by adopting the modified

Table 1: Phytochemicals (lycopene, *β*-carotene, flavonoids and tannins) in *C. sinensis*

Phytochemicals	Concentration (mg/50 ml) (mean ± S.D)		
	Leaf	Stem	Root
Lycopene	0.9 ±0.16	0.7 ±0.01	0.4 ±0.07
β-carotene	2.5±0.07	1.0±0.06	2.0 ± 0.01
Flavonoids	10.0±0.33	4.1±1.16	10.0±0.44
Tannin	135.4±2.84	47.9±1.02	87.0±2.50

All the values are the average of triplicate samples (n = 3) ± S.D. ($p \leq 0.05$).

Table 2: Antimicrobial activity of different extracts of *Camellia sinensis* plant (Leaf, stem and root) against selected bacterial strains

Leaf						
Tested microorganisms	*n*-Hexane	Acetone	Chloroform	Ethanol	Methanol	Water
	Diameter of inhibition zone (mm)					
E. coli	N.D.	17.5±0.12	N.D.	16.0±0.13	16.1±0.23	N.D.
S. aureus	N.D.	17.1±0.09	10.5±0.09	14.2±0.17	18.0±0.13	11.8±1.00
k. pneumonia	N.D.	20.2±0.24	11.2±0.18	15.5±0.12	13.2±0.06	N.D.
H. influenzae	N.D.	14.2±1.00	N.D.	14.3±0.27	N.D.	13.5±1.00
A. baumannii	N.D.	13.3±0.06	N.D.	14.6±1.00	12.4±1.00	12.7±1.00
P. aeruginosa	N.D.	16.1±0.14	N.D.	19.3±0.21	14.1±0.08	12.2±1.00
M. morganii	N.D.	14.7±0.08	N.D.	15.2±1.00	13.2±0.16	10.4±1.00
Stem						
E. coli	17.2±0.43	17.2±0.21	N.D.	18.7±0.11	14.6±0.11	N.D.
S. aureus	N.D.	19.6±0.14	N.D.	17.1±0.12	15.6±0.18	N.D.
k. pneumonia	N.D.	23.2±0.17	N.D.	15.0±0.12	16.3±0.16	N.D.
H. influenza	N.D.	19.3±0.18	N.D.	20.1±0.11	15.1±0.18	12.6±0.09
A. baumannii	N.D.	16.4±0.23	N.D.	14.6±0.17	16.4±1.00	12.2±0.16
P. aeruginosa	N.D.	23.4±0.23	N.D.	22.0±0.21	18.0±0.23	12.1±0.15
M. morganii	N.D.	17.1±1.00	N.D.	16.3±1.00	13.7±1.00	N.D.
Root						
E. coli	17.4±0.28	15.4±0.18	N.D.	16.3±0.24	16±0.23	12.5±0.18
S. aureus	N.D.	14.2±0.14	N.D.	16.1±1.00	18±0.57	12.0±0.22
k. pneumoniae	N.D.	14.3±0.11	N.D.	15.2±0.14	15±0.18	12.1±0.07
H. influenzae	N.D.	15.3±0.21	N.D.	14.1±0.09	15.2±0.15	12.6±0.31
A. baumannii	N.D.	15.4±0.14	N.D.	14.3±0.14	14.3±0.13	N.D.
P. aeruginosa	N.D.	14.2±0.12	N.D.	13.2±0.08	15.1±0.47	N.D.
M. morgannii	N.D.	14.3±1.00	N.D.	14.6±1.00	14.7±0.24	N.D.

N.D. = Zone of inhibition not detected. All values are mean ± SD of triplicate samples.

method of Yen and Chen, (1995). The extract (750 µL) of each sample was mixed with an equal amount of phosphate buffer (200mM, pH 6.6) and potassium ferrciyanide (1% w/v). After incubating the mixture at 50ºC for 20 min, an equal volume of trichloroacetic acid (TCA, 10% w/v) was added as reaction terminator. Then 1.5mL supernatant of this mixture was mixed with equal volume of distilled water and 0.1mL of $FeCl_3$ solution (0.1% w/v). Blanks were also prepared in parallel following the same procedure except the sample. Absorbance of each sample was measured spectrophotometrically at OD_{700} after 30 min. Ascorbic acid (Vitamin C) was used as the standard positive control.

Cytotoxicity studies

The method of Powell *et al.* (2000) was followed for the cytotoxicity analysis of various extracts of tea plant. Briefly, freshly collected human blood (3 mL) was gently mixed in heparinized tubes to avoid coagulation,

transferred into a sterile Falcon tube (15 mL) and centrifuged (5min at 850×*g*). Supernatant was decanted and RBCs were washed three times with chilled sterile isotonic phosphate buffered saline (PBS) solution. Various extracts of tea plant (20 µL) were taken in 2mL Eppendorf tubes; diluted 10 times with RBCs suspension (7.068 × 10^8 cell/mL) and incubated at 37 °C for half an hour. After brief centrifugation, collected supernatants (100µL) were diluted with chilled PBS (900µL) and added into 96 well plates. Triton X-100 (0.1%) and PBS were taken as positive and negative controls, respectively, for each assay.

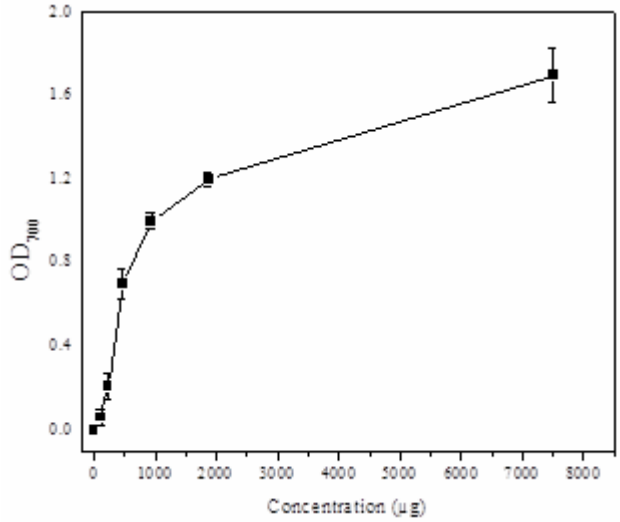

Fig. 1: Antioxidant activity of ascorbic acid at different concentrations of plant (*C. sinensis*) extract

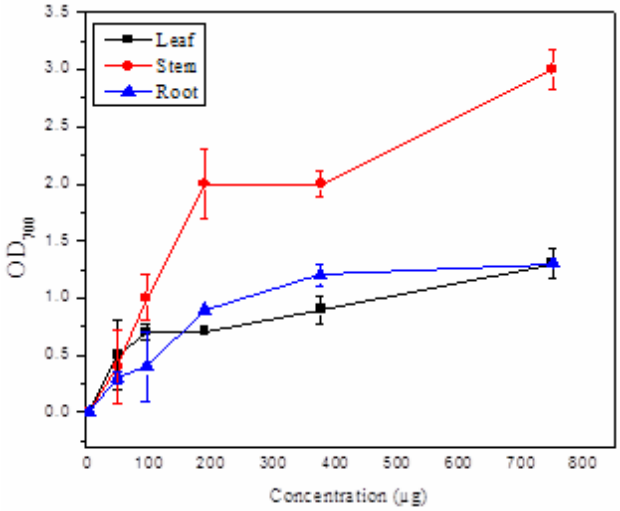

Fig. 2: Antioxidant activity of flavonoids of different parts of tea plant using different concentrations

RESULTS

Bioactive compounds determination

In the present study, the bioactive compounds such as lycopene, *β*-carotenes, flavonoids and tannins from *C. sinensis* were determined and results are given in table 1. Among the compounds investigated, the concentration of tannins was found to be significantly higher in tea plant. Further, the leaf part exhibited the highest tannin concentration followed by stem and root. The recorded concentrations were 135.4, 47.9 and 87mg/50 ml in leaf, stem and root, respectively. Other constituents i.e. flavonoids, *β*-carotene and lycopene were comparatively lower than that of tannins. Overall, the concentrations of all phytochemicals were found to be the higher in leaf (part) as compared to the root and stem.

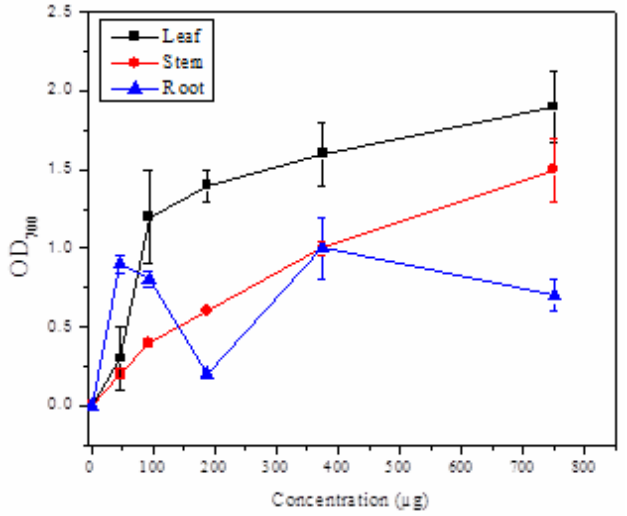

Fig. 3: Antioxidant activity of tannins in different parts of tea plant using different concentrations

Antimicrobial activity

The antimicrobial activities of different parts of *C. sinensis* plant were tested against seven different bacterial strains including *E. coli, S. aureus, K. pneumoniae, H. influenzae, A. baumannii, P. aeruginosa and M. morgani* (table 2). It was observed that the investigated extracts of tea plant except *n*-hexane and chloroform exhibited considerable antimicrobial effects against all the tested microorganisms showing different zone of inhibition. The results revealed that the stem part of *C. sinensis* presented relatively greater antimicrobial potential than the leaf and root tissues. Aceton, ethanol and methanolic extracts of stem part of tea plant showed excellent inhibitory activities against *P. aeruginosa* with inhibition zones of 23.4±0.23, 22.0±0.21 and 18.0±0.23 mm, respectively. The highest antimicrobial activity of aqueous extract was recorded against *H. influenzae* with inhibition zone of 13.5±1.00 mm. Chloroform fraction showed activity only against *S. aureus* and *K. pneumoniae*, whereas *n*-hexane extract was found to be almost completely ineffective against all the tested bacterial strains. The extent of inhibition zones depends on the strain and the kind and concentration of the extract. Comparing the seven bacterial strains investigated, it is evident that *P. aeruginosa* is the most sensitive, exhibiting the largest zone inhibition diameter in the presence of aceton extracts

of stem part of the tea plant. All the bacterial strains were resistant to inhibitory action of the *n*-hexane fraction. The antibacterial potential may be attributed to bioactive constituents present in the plant. Generally, the acetone extracts showed better inhibition in stem part and showed moderate inhibition in leaf and root tissues.

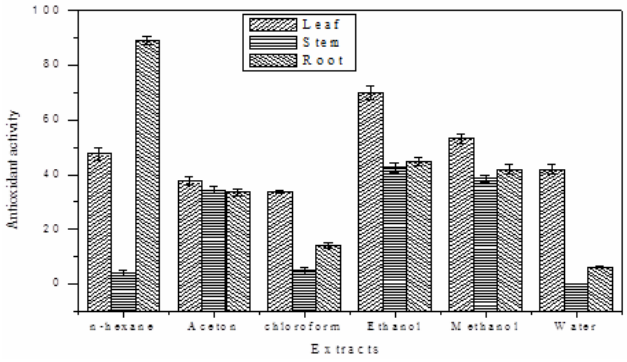

Fig. 4: Antioxidant activity of various extracts of *C. sinensis* exhibited as % inhibition of linoleic acid.

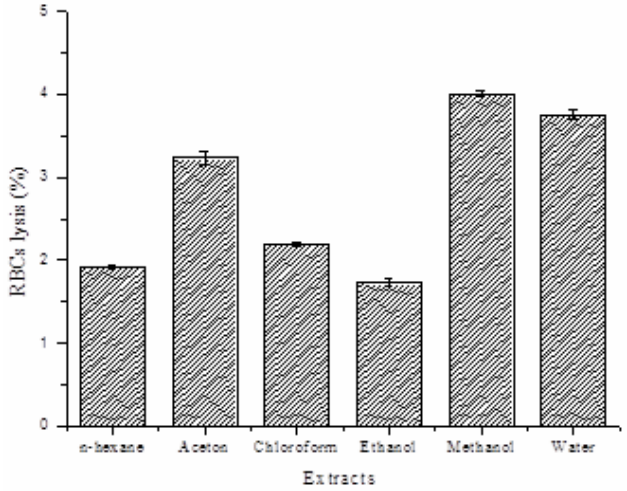

Fig. 5: Cytotoxicity assay by heamolytic activity of various extracts of *Camellia sinensis*

Antioxidant activity

Antioxidant activity of phytochemicals

In vitro antioxidant activity of phytochemicals i.e. tannins and flavonoid of *C. sinensis* was evaluated using ascorbic acid as a standard. Results of antioxidant activity of phytochemicals are displayed in fig. 1 (standard; ascorbic acid), fig. 2 (flavonoids) and fig. 3 (tannins) in terms of absorbance at OD_{700}. It was observed that phytochemicals demonstrated different antioxidant activities at different concentrations of tea plant extract. The antioxidant activity was enhanced with increasing concentrations of the sample. As compared to tannin which showed the moderate activity, the flavonoid exhibited the greater antioxidant activity. The highest activity of flavonoid (A_{700}=3.0) was found in stem followed by root and leaf at 750 µg concentration which is even more active than the standard ascorbic acid. As the tannin showed absorbance of 1.9, 1.5 and 0.5 in leaf, stem and root, respectively, it is considered less active than ascorbic acid.

Antioxidant activity of various extracts of C. sinensis tissues

The in-vitro antioxidant activity of various extracts of leaf, stem and root parts of *C. sinensis* were determined, and results are illustrated as % inhibition of linoleic acid per oxidation in the fig. 4. All parts of tea plant exhibited (%) inhibition of linoleic acid peroxidation activity ranging from 0.1 to 89.22%. All extracts showed the greater activity in leaf part, while moderate activity in root and stem. The highest % inhibition in linoleic acid per oxidation was recorded in the *n*-hexane extract of root tissue of tea plant.

Cytotoxicity studies

The cytotoxic potential of various extracts of *C. sinensis* was assessed by carrying out the haemolytic activity against human red blood cells (RBCs). The results are shown in fig. 3 as percentage lysis of RBCs by comparing the absorbance of samples with the positive control (Triton X-100). The positive control showed almost100% lysis, whereas the negative control (phosphate buffer saline (PBS)) displayed no lysis of RBCs. With reference to controls, various extracts of *C. sinensis* exhibited different (%) lysis of RBCs, i.e. *n*-hexane extract (1.92±0.03), acetone (3.24±0.08), chloroform (2.19±0.02), ethanol (1.73±0.05), absolute methanol extract (4.01±0.03) and aqueous extract (3.76±0.06).

DISCUSSION

Due to beneficial effects on human health, many epidemiological studies were done on bio-pharmacological activities of Pakistani medicinal plants including tea during the last few years (Khan *et al*., 2012, 2013; Ashraf *et al*., 2014a, b; Zakir *et al*., 2015). Tea (*C. sinensis L.*) plant contains a variety of compounds that play a key role for tea quality and its accompanying health benefits. In the present study, an appreciable amount of phytochemicals i.e. lycopene, *β*-carotene, flavonoids and tannins were determined in *C. sinensis* collected from Mansehra, Khyber Pakhtunkhwa, Pakistan. It has been reported that significant amount of these phytochemicals may be responsible for their healing effect against various pathogenic microbes (Din *et al*., 2015). The strong antimicrobial potential exhibited by the various extracts of *C. sinensis* in the current study predominantly attributed to the presence of different antioxidant polyphenols (better known as catechins) in tea plant. The main active components responsible for antibacterial activity in tea plant appear to be epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), gallocatechin-3-gallate (GCG) and epicatechin (EC). The gallated catechins (EGCg and ECg) disengage proteins and rupture the bacterial lipid bilayer cell membrane by shifting the membrane fluidity and morphology, that ultimately inhibits the bacterial growth (Isogai *et al.,* 2001). The

results suggested that many pathogenic bacteria such as such as Gram-positive *S. aureus* could possibly be killed by the regular consumption of green tea. Similar types of studies were undertaken by Peter *et al*., (2005) reporting that tea exerted significant efficacy as an antimicrobial agent against antibiotic resistant strains and clinical isolates of bacteria and fungi. Recently, Zakir *et al*. (2015) evaluated the antimicrobial activities of various extracts of green and black tea (*C. sinensis*) against different bacterial strains and demonstrated that green tea extracts carried superior activities than the black tea. However, both the tea extracts showed great potential against these bacterial strains.

Flavonoids and tannins are polyphenolic compounds which act as antioxidant agent and may protect the body from oxidative damage. Oxidative stress in the body can cause serious damage to biological macromolecules including protein, carbohydrate, lipid and DNA which may lead to various degenerative diseases including cardiovascular diseases (CVDs), cancer, immune system degeneration and cataracts. These compounds interact with free radicals and inactivate them by quenching singlet and triplet oxygen hydrolyzes hydrogen peroxide (H_2O_2) and inhibits enzyme activities (Johns and Eyzaguirre, 2006). From the results, it may be concluded that ethanol extract possesses significant antioxidant activity.

The cytotoxicity test (haemolytic test) was employed to evaluate the cytotoxic effect of various extracts of fresh *C. sinensis* plant. The percent haemolysis remained in the range of 1.73-4.01% that was lower than the previously reported values of percent hemolysis of human erythrocytes by various other plant extracts (Powell *et al*., 2000; Sharma and Sharma, 2001; Riaz *et al*., 2012). The mechanical-stability of RBCs membrane is considered as good parameter to assess the in vitro toxicity screening of different compounds and/or environmental pollutants. When human cells are treated with a cytotoxic compound, the cells may undergo loss of membrane integrity and perish quickly as a result of cell lysis that ultimately causes different problems to human health (Riaz *et al*., 2012).

In view of the above-mentioned facts, we can suggest that the tea plant (commonly available in Pakistan) is a natural source of biologically active constituents of great value that could possibly be used as potential raw material in pharmaceutical industries for the effective control of infectious diseases.

CONCLUSION

In conclusion, tea plant extract showed applicable antimicrobial and antioxidant activities against the tested microorganisms. The cytotoxicity of various plant extracts assessed through haemolytic test revealed that the tea plant shows only negligible cytotoxicity compared to the positive control. Therefore, the results of the present study highlight the therapeutic significance of tea plant in traditional medicines for the treatment of various infectious diseases. Further, the consumption of natural products like tea may neutralize the side-effects of chemical drugs.

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