Research Article

Quantification of Phytochemicals and Evaluation of Antioxidant Potential of Ethanolic Leaf Extract of *Terminalia bellerica, Terminalia chebula* and *Emblica officinalis* vis-a-vis *Triphala*

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ABSTRACT

The present study was carried out to find out the concentrations of bioactive phytochemicals (ascorbic acid, carotenoid, total phenolic contents, protein and carbohydrate) and evaluation of antioxidant activities of ethanolic leaf extract of *Terminalia bellerica* (TB), *Terminalia chebula* (TC), *Emblica officinalis* (EO) and their formulation *Triphala*. Among the tested leaf extracts, highest ascorbic acid content was present in EO (118.36 µg $100g^{-1}$ of fresh weight) followed by *Triphala* (115.57), TB (99.18) and TC (94.33). Highest carotenoid content was present in *Triphala* (6.53 µgg^{-1} of FW) followed by TB (6.09), TC (5.80) and EO (4.15) whereas TB leaf extract had highest TPC (215.66 mgg^{-1} of GAE of dry weight) followed by *Triphala* (213.33), TC (213.05) and EO (177.37). Out of three ethanolic leaf extracts, TB showed minimum IC_{50} for FRSA (58 µgml^{-1}), SARSA (38 µgml^{-1}), lipid peroxidation (103 µgml^{-1}), hydroxyl radical scavenging activity (35 µgml^{-1}), FTC activity (117 µgml^{-1}) as well as high reducing power (1.77 ASEml^{-1}). On the basis of our results, it may be concluded that high concentration of phenolic compounds and other bioactive phytochemicals in *Triphala* and leaf extract of its three constituents are potential source of natural antioxidants.

Keywords: Antioxidant, *Triphala,* Total phenolic content, Reducing power, Phytochemicals.

INTRODUCTION

Free radicals are molecules possessing unpaired electrons and thus, they are reactive and short lived in a biological system. Depending upon the rate and site of production, free radicals can mediate both harmful modifications to biomolecules and/or participate in useful cellular signal transduction processes.[1] ROS comprise both free radical and non free radical oxygen intermediate such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet}$), singlet oxygen ($^1O_2$) and Hydroxyl radical ($OH^{\bullet}$). Reactive oxygen and nitrogen species (ROS/RNS) are produced as byproduct of normal physiological processes which require reductive internal environment in cells living in an oxidizing atmosphere. ROS are produced in response to pathogens, hormones, alcohol, UV radiation, cigarette smoking, nonsteroidal anti-inflammatory drugs and inflammatory response. Disruption of normal cellular homeostasis by redox signaling may result in cardiovascular[2,3], neurodegenerative diseases[4] and cancer.[5] ROS are produced within the gastrointestinal (GI) tract and results in pathogenesis of gastrointestinal mucosal diseases.[6] When the balance between ROS/RNS and these defenses is disturbed, the result is oxidative stress. Living organism evolved mechanisms to maintain this overall reductive potential, commonly known as antioxidant defenses.

Antioxidants are reducing agents which can safely interact with free radicals and terminate the chain reaction initiated by free radicals before vital molecules are damaged, by removing free radical intermediates.[7] Experimental and epidemiological studies have shown that many natural and synthetic drugs act as antioxidants and are involved in reduction of oxidative stress developed due to free radicals.[8] Natural antioxidants have the potential to neutralize free radical to overcome oxidative stress.[9,10] Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last 2 decades.[11] In recent years, the use of natural antioxidants has been promoted because of apprehensions on the safety of synthetic drugs.[12]

Triphala, is one of the most commonly used herbal formulation in Ayurveda. It is widely accepted phytomedicine because of its exclusive capability to gently cleanse and detoxify the body while at the same time strengthen and nourish it due to rich source of phytochemicals. The formulation consists of equal proportions of fruits of three plants *T. bellerica, T. chebula* and *E. officinalis.*[13]

E. officinalis (EO), commonly known as Indian gooseberry or Amla, belongs to family Euphorbiaceae. The fruit is used either alone or in combination with other plants to treat many complaints such as common cold, fever and stomachic. It is also used as antipyretic, anti-inflammatory, hair tonic, dyspepsia and digestive agent. The dried fruits of EO contain many active phytochemicals[14] and flavonoids[15] that may be responsible for its antioxidant activity.

T. chebula (TC), generally known as Harad belongs to family Combretaceace. It is also called as "*King of Medicines*" because of its miraculous power of healing a wide spectrum of health problems. The fruit of TC is being used for the treatment of different diseases and disorders since times immemorial. The phytochemical analysis showed that its dry fruit is a rich source of various

phenolic and flavonoid compounds which are well known for their free radical scavenging and iron chelation property.[16]

T. bellerica (TB) Roxb, is a large deciduous tree generally known as Baheda, belongs to family Combretaceace. TB is commonly used in treatment of various gastrointestinal complaints and a variety of throat disorders, including cough, hoarseness, as well as eye disorders. The dried fruits of TB possess antimicrobial,[17] anti-diabetic,[18] anti-atherosclerotic[19] and hepatoprotective activities.[20]

On the basis of a number of studies it is clear that fruits of all these three plants have many pharmacological properties. But the leaf of these plants has not yet been explored for antioxidant activities. Present study focused on the evaluation of antioxidant potential of ethanolic leaf extract of TB, TC and EO and it was also compared with their formulation, *Triphala*.

MATERIALS AND METHODS

Plant materials

Plant materials collected from Herbal Garden of Narendra Dev (ND) University of Agriculture and Technology Kumarganj, Faizabad, Uttar Pradesh, were chopped, dried, powdered and stored in polythene bags at 4°C till further analysis. Identification of different plant samples was carried out and confirmed with the help of Dr. M. N. Srivastava, Senior Scientist, Botany Division, CSIR-Central Drug Research Institute, Lucknow, India and the voucher specimens were submitted in CDRI herbarium.

Chemicals and reagents

Gallic acid, quercetin and bovine serum albumin (BSA) were procured from Sigma-Aldrich, St. Louis, USA. β-Carotene, ascorbic acid, Folin Ciocalteau's phenol reagents were the product of E. Merk, Mumbai, India. Nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), potassium ferricyanide, trichloroacetic acid, ferric chloride and sodium dodecyl sulphate were purchased from SRL India. All other reagents and chemicals used were of analytical grade.

Estimation of phytochemicals

Ascorbic acid content of plants was estimated by the method of Arlington,[21] and reported as mg $100g^{-1}$ of fresh weight (FW) of tissues. Carotenoids were estimated by the method of Jensen[22] and reported as µg g^{-1} of FW. Total phenolic content (TPC) was measured using the method of Ragazzi & Veronese[23] and reported in terms of mg of gallic acid equivalent (GAE) g^{-1} of dry weight (DW). Protein content was estimated by the method of Lowry et al.[24] and reported as mg g^{-1} of DW. Carbohydrate content was estimated by method of Anthrone[25] and reported as mg g^{-1} of DW.

Extraction procedure for antioxidant assay

Twenty grams of dried and powdered plant samples were extracted with 70% ethanolic solvent (in water) until decoloration. The extracted solvent was evaporated at 40°C in a vacuum rotary evaporator and lyophilized till dryness. The powdered form of plant extract was stored at -4°C and used for the antioxidant activity determination.

Antioxidant studies

Free radical scavenging activity (FRSA)

FRSA of the extracts was measured by using DPPH stable radical according to Yen and Duh 1994 method.[26] Each extract (0.1 ml) was added to freshly prepared DPPH solution (6×10^{-5} M in HPLC grade 2.9 ml methanol) and mixed vigorously. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 515 nm until a stable value was obtained.

Inhibition (%) = [(Blank absorbance-sample absorbance)/blank absorbance] × 100

The inhibitory concentration (IC_{50}) which represents the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, representing a parameter widely used to measure antioxidant activity, was calculated from a calibration curve by linear regression. EC_{50} was calculated as IC_{50} (µg ml^{-1})/concentration of DPPH ml^{-1} and expressed as µg mg^{-1} DPPH. For rational reasons of clarity, the ARP was determined as the reciprocal value of the EC_{50}, representing a comparable term for the effectiveness of antioxidant and radical scavenging capacity:

ARP = $1/EC_{50} \times 100$, the larger the ARP, the more efficient the antioxidant.

Superoxide anion radical scavenging activity (SARSA)

This assay was based on the capacity of the extract to inhibit the reduction of NBT by the method of Nishikimi et al.[27] Three milliliters of reaction mixture containing different aliquot of plant extracts (50, 100, 150, 200 µl) with 0.1 M phosphate buffer (pH 7.8), 60 µM PMS, 468 µM nicotinamide adenine dinucleotide reduced (NADH) and 150 µM NBT was incubated for 5 min at ambient temperature. Absorbance was read after 6 min at 560 nm using UV-Vis spectrophotometer. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample.

Reducing power (RP)

RP of the extracts was determined by using slightly modified method of ferric reducing-antioxidant power assay.[28] Each extract (1.0 ml) was mixed with 2.5 ml of phosphate buffer (0.1 M, pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide and was incubated at 50°C for 20 min. After completion of incubation period, 2.5 ml of 10% (w/v) TCA was added to terminate the reaction. The

upper layer (2.5 ml) was diluted with equal volume of deionized water. Finally, 0.5 ml of 0.1% (w/v) $FeCl_3$ was added and after 10 min the absorbance was measured at 700 nm against a blank. RP was expressed as ascorbic acid equivalents (1 ASE = 1 mM ascorbic acid). The ASE value is inversely proportional to RP.

Lipid Peroxidation (LPO)

A modified thiobarbituric acid-reactive species (TBARS) assay method of Ohkawa et al. was applied to measure the LPO formation, using egg homogenate as lipid rich media.[29] Egg homogenate (10% in 0.2 M PBS, 0.5 ml), test extract (0.1 ml) and deionized water (0.85 ml) were mixed in a test tube. Finally, FeSO4 (0.07 M, 0.05 ml) was added to the reaction mixture and incubated at 37°C temperature for 30 min to induce LPO. Thereafter, acetic acid (20%, 1.5 ml), TBA (0.8% prepared in 1.1% sodium dodecyl sulphate, 1.5 ml) and TCA (20%, 0.05 ml) were added, vortexed and then heated in a boiling water bath for 60 min. After cooling, butanol (5 ml) was added to each tube and centrifuged for 10 min at 3000 rpm. The absorbance of the organic upper layer was measured at 532 nm by UV-Vis Spectrophotometer.

Ferric thiocynate assay (FTC)

The reaction mixture containing 400 µl of different concentration of ethanolic plant extracts, 200 µl of diluted linoleic acid (25 mg ml^{-1} in 99% ethanol) and 400 µl of 50 mM phosphate buffer (pH 7.4) was incubated for 15 min at 40°C. A 100 µl aliquot of this was then mixed with a reaction mixture containing 3 ml of 70% ethanol, 100 µl of ammonium thiocyanate (300 mg ml^{-1} in DW) and 100 µl of ferrous sulphate. Red color developed was measured at 535 nm.[30]

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by a mixture of Fe^{3+}-EDTA, H_2O_2 and ascorbic acid and were assessed by monitoring the degraded fragments of deoxyribose, through malondialdehyde (MDA) formation.[31] The reaction mixtures contained ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2 mM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM) with different concentrations of the plant extracts in a final volume of 1 ml. It was incubated at 37^0C for 1 h and then 1 ml of 2.8% TCA (w/v in water) and 1 ml of 1% thiobarbituric acid (TBA) (w/v) were added. The mixture was heated in a boiling water bath for 30 min. It was cooled and absorbance was taken at 532 nm.

Statistical analysis

Statistical analysis was done using prism software. Values from *in vitro* antioxidant activities were reported as mean ± standard deviation (SD) of three determinations. The r^2 value and regression equation were calculated through plotting graph between TPC on x-axis and antioxidant deciding parameters on y axis with the help MS office excel 2007.

RESULTS

Phytochemical estimations

In order to find out the concentration of phytochemicals in the plants which impart antioxidant activity, ethanolic extracts of TB, TC, EO leaf and *Triphala* were examined for their ascorbic acid, carotenoids, total phenolics, protein and carbohydrate contents (Table 1).

Table 1: Phytochemical contents in TB, TC, EO and *Triphala*

Plant name	Plant's Part	Ascorbic acid (mg $100g^{-1}$ of FW)	Carotenoid (µg g^{-1} of FW)	TPC (mg g^{-1} of GAE of DW)	Protein (mg g^{-1} of DW)	Carbohydrate (mg g^{-1} of DW)
TB	Leaf	99.18±2.44	6.09±0.32	215.66±8.08	386.17±11.01	230.9±1.90
TC	Leaf	94.33±2.44	5.80±0.69	213.05±2.70	370.51±13.02	221.87±4.75
EO	Leaf	118.36±4.76	4.15±0.25	177.37±6.49	272.28±6.70	197.37±6.49
Triphala	-	115.57±2.00	6.53±0.41	213.33±1.52	413.88±10.99	251.00±6.24

Values are mean ± SD of three replications, TPC: Total phenolic content, GAE: Gallic acid equivalent, FW: Fresh weight, DW: Dry weight

Ascorbic Acid

Tested plants showed varying level of ascorbic acid ranging from 94.33 to 118.36 mg $100g^{-1}$ of FW (Table 1). Among the plant samples, the highest value of ascorbic acid was present in EO leaves (118.36 mg $100g^{-1}$ of FW) making them a good source of ascorbic acid, which is almost similar to their formulation *Triphala* (115.57 mg $100g^{-1}$ of FW). The moderate levels of ascorbic acid were present in TB (99.18 mg $100g^{-1}$ of FW) and TC (94.33 mg $100g^{-1}$ of FW) leaves.

Carotenoids

Carotenoid content of the tested plants are presented in Table 1. Among the tested extracts, *Triphala* had the highest concentration of carotenoids *i.e.* 6.53 µg g^{-1} of FW, whereas the lowest concentration of carotenoids was noticed in EO leaves (4.15 µg g^{-1} of FW). The leaf extracts of TB and TC showed moderate and almost equal amount of carotenoids.

Total Phenolic content

The TPC in the tested medicinal plants ranged between 177.37 to 215.66 mg g^{-1} of GAE of DW, which has been summarized in Table 1. Results showed that TB leaf had highest value of TPC followed by *Triphala* (213.33), TC (213.05) and EO (177.37) mg g^{-1} of GAE of DW.

Protein

Protein contents of plant samples ranged from 272.28 to 413.88 mg g^{-1} of DW (Table 1). The highest value of protein was present in *Triphala* (413.88) fallowed by TB (386.17), TC (370.51) and EO (272.28 mg g^{-1} of DW).

Carbohydrate

The carbohydrate content in different parts of plants ranged between 197.37 to 251.00 mg g^{-1} of DW (Table 1). The highest carbohydrate content was observed in *Triphala* (251.00) followed by TB (230.90), TC (221.87) and EO Leaf (197.37 mg g^{-1} of DW).

Table 2: Free radical scavenging activity (FRSA) and superoxide anion radical scavenging activity (SARSA) of ethanolic leaf extract of TB, TC, EO, *Triphala* and standard Quercetine

Plant name	$^{1}IC_{50}$ (µg ml^{-1}) FARSA	EC_{50} (µg mg^{-1} of DPPH)	ARP	$^{2}IC_{50}$ (µg ml^{-1}) SARSA
TB leaf	58±0.004	3956	0.025	38±0.0005
TC leaf	74±0.001	4869	0.020	84±0.001
EO leaf	77±0.001	5086	0.019	120±0.001
Triphala	76±0.002	5043	0.019	44±0.0005
Quercetine	40±0.002	1739	0.057	30±0.001

Values are mean ± SD of three replications (n=3), IC_{50}: Inhibitory concentration, EC_{50}: Efficiency concentration, ARP: Antiradical power

Antioxidant studies

Free Radical Scavenging Assay (FRSA)

FRSA in form of IC_{50} values among the tested ethanolic leaf extracts ranged from 58 to 76 µg ml^{-1}. TB leaf showed minimum and EO leaf showed maximum IC_{50}. IC_{50} value of above extracts when compared with their formulation *Triphala*, showed the decreasing order of antioxidant activity as follows: TB> TC> *Triphala*> EO (Table 2). Leaf extracts of all three plants scavenge the free radicals by 85.02 (TB), 83.04 (TC) and 80.91% (EO) at concentration of 200 µg ml^{-1}. Highest inhibition of DPPH radical was observed by the TB leaf. TB leaf showed concentration dependent increase in scavenging activity by 33.71, 57.22, 70.87 and 85.02%, respectively when 50, 100, 150, 200 µg ml^{-1} of extract were used (Figure 1). These inhibitions were more than *Triphala* which showed 24.88, 44.76, 61.72 and 81.89%, scavenging activity, respectively.

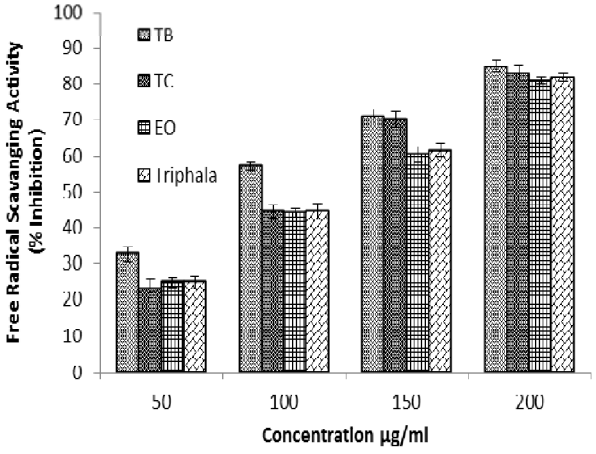

Figure 1: Free radical scavenging activity of ethanolic extract of TB, TC, EO leaf and *Triphala* against DPPH radicals. Values are mean ± SD of three replications (n=3).

Superoxide Anion Radical Scavenging Activity (SARSA)

SARSA values in form of IC_{50} among the tested ethanolic leaf extracts ranged between from 38 to 120 µg ml^{-1}. Tested TB extract showed minimum and EO showed maximum IC_{50} value. The IC_{50} value of above extracts when compared with *Triphala*, showed decreasing trend in antioxidant activity as follows: TB> *Triphala*> TC> EO (Table 2). Leaf extract of all three plants scavenge the superoxide radical by 84.40 (TB), 82.18 (TC) and 78.78% (EO) at concentration of 200 µg ml^{-1}. Highest inhibition of NBT was observed by the TB leaf. TB leaf showed concentration dependent increase of SARSA by 23.80, 41.92, 64.91 and 84.40%, respectively when 50, 100, 150, 200 µg ml^{-1} of extract were used. These inhibitions were more than *Triphala* which showed 18.80, 34.40, 56.24 and 74.04%, respectively (Figure 2).

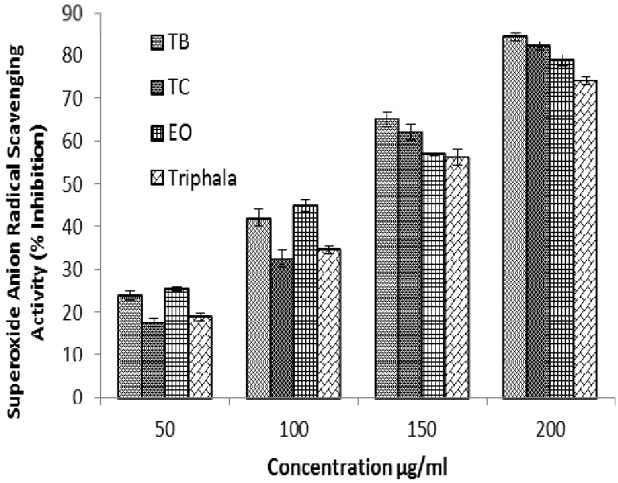

Figure 2: Inhibitory effects of ethanolic extract of TB, TC, EO leaf and *Triphala* on superoxide anion radical. Values are mean ± SD of three replications (n=3).

Reducing Power (RP)

RP is determined to measure reductive ability of antioxidant, which is evaluated by transformation of Fe (III) to Fe (II) in presence of the plant extract. Among leaf extracts of TB, TC and EO, TB leaf extract showed maximum RP which is comparable to standard Quercetin value (1.12 ASE ml^{-1}) (Table 3). Increasing order of RP was TB> *Triphala*> TC> EO (Table 3).

Table 3: Reducing power (RP), lipid peroxidation (LPO), ferric thiocynate assay (FTC) and hydroxyl radical ($OH^{\bullet}$) scavenging activity of ethanolic extract of TB, TC, EO leaf, *Triphala* and standard Quercetin

Plant name	Reducing Power ($ASEml^{-1}$)	$^{3}IC_{50}$ (LPO) µg ml^{-1}	$^{4}IC_{50}$ (FTC) µg ml^{-1}	$^{5}IC_{50}$ ($OH^{\bullet}$) µg ml^{-1}
TB leaf	1.77±0.18	102±0.007	117±0.003	35±0.002
TC leaf	2.06±0.02	133±0.004	162±0.012	36±0.001
EO leaf	2.12±0.08	134±0.006	186±0.005	33±0.003
Triphala	1.98±0.087	104±0.003	181±0.54	33±0.002
Quercetin	1.12±0.02	98±0.002	45±0.01	17±0.001

Values are mean±SD of three replications (n=3), ASE: Ascorbic Acid Equivalent, IC_{50}: Inhibitory Concentration, EC_{50}: Efficiency Concentration

Lipid Peroxidation (LPO)

Results of LPO of tested extracts showed that TB (103 µg ml^{-1}) and *Triphala* (104 µg ml^{-1}) have almost similar IC_{50}. Decreasing order of anti-lipid peroxidation activity was similar to that of SARSA *i.e.* TB> *Triphala*> TC> EO (Table 3). Among the tested leaf extracts of TB, TC and EO, TB leaf had high potential to inhibit the LPO, whereas EO leaf extract showed minimum LPO inhibition. TB leaf extract showed 43.67, 63.21, 83.90 and 89.35% inhibition when 250, 500, 750 and 1000 µg ml^{-1} plant extracts were added to reaction mixture, whereas *Triphala* had 25.85, 49.13, 73.27, 83.61% inhibition (Figure 3).

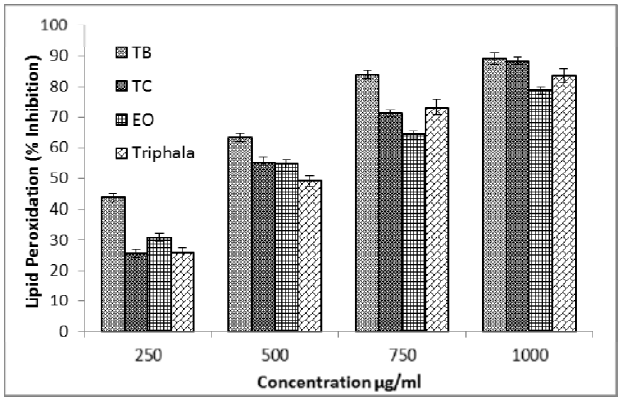

Figure 3: Inhibitory effects of ethanolic extract of TB, TC, EO leaf and *Triphala* on lipid peroxidation using egg homogenate as a lipid rich source. Values are mean ± SD of three replications (n=3).

Ferric Thiocynate Assay (FTC)

FTC value in form of IC_{50} among the tested ethanolic leaf extracts, TB showed minimum (117 µg ml^{-1}) IC_{50} followed by TC (162), *Triphala* (181) and EO (186 µg ml^{-1}) (Table 3). In tested leaf extracts of TB, TC and EO, TB leaf had high potential to inhibition, whereas EO leaf extract showed minimum FTC inhibition (Figure 4). Ethanol extract of TB leaf showed 33.47, 56.91, 73.58 and 90.25% inhibition when 50, 100, 150 and 200 µg ml^{-1} plant sample were added to reaction mixture, whereas ethanolic extract of *Triphala* showed 26.26, 46.18, 64.81, 86.01**%** (Figure 4).

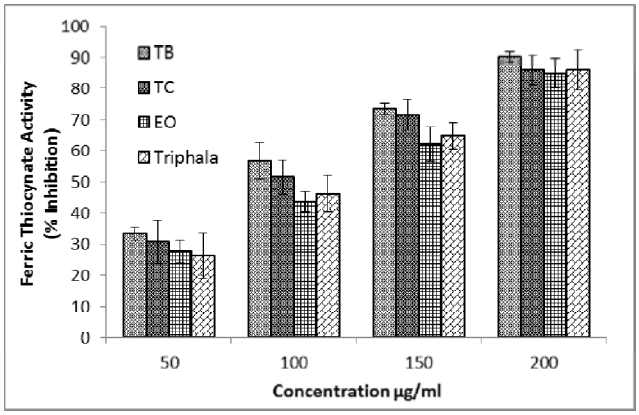

Figure 4: Inhibitory effects of ethanolic extract of TB, TC, EO leaf and *Triphala* on ferric ion chelation by ferric thiocyanate assay method. Values are mean ± SD of three replications (n=3).

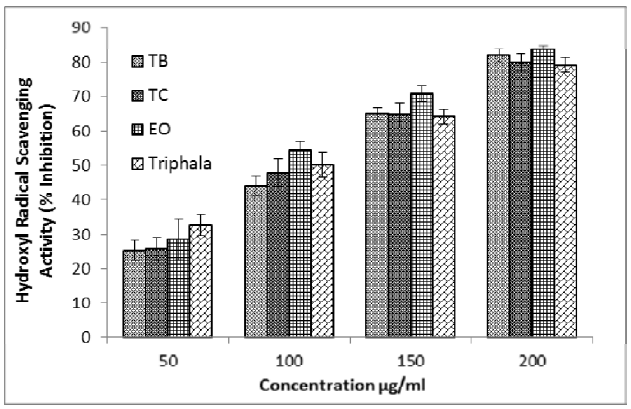

Figure 5: Inhibitory effects of ethanolic extract of TB, TC and EO leaf and *Triphala* on hydroxyl radical mediated deoxyribose degradation. Values are mean ± SD; n=3**.**

Hydroxyl radical ($OH^{\bullet}$) scavenging activity

In this experiment, protection of DNA by plant extracts against $OH^{\bullet}$ induced damage was determined in terms of

the damage to its deoxyribose sugar moiety. The effect of TB, TC, EO plant leaf extract and *Triphala* on $OH^{\bullet}$ generated by Fe^{3+} ion was measured by determining the degree of deoxyribose degradation. The IC_{50} values of TB (35 µg ml^{-1}) and TC (36 µg ml^{-1}) were almost similar, whereas EO and *Triphala* have shown similar (33 µg ml^{-1}) values (Table 3). EO leaf and *Triphala* showed almost similar $OH^{\bullet}$ scavenging activity at concentration of 200 µgml^{-1} (Figure 5).

Correlation between TPC in the plant extract in relation to their antioxidant activity

The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that these compounds contribute to the strong antioxidant activity.[32] The correlation between TPC and FRSA of TB leaf extract had a correlation coefficient of R^2=0.977 (y=17.058+18.81), TC leaf R^2=0.996 (y=19.033+4.68), EO leaf R^2=0.998 (y=18.458+6.52) and *Triphala* had R^2=0.999 (y=18.81+6.23). This suggests that in TB leaf extract, 97.7%, TC leaf 99.6%, EO leaf 99.8% and *Triphala* 99.9% antioxidant activity is contributed by phenolics compound. The remaining antioxidant activity is due to non phenolics compounds. Activity may also come from the presence of other secondary metabolites such as volatiles oils, flavonoids, metalloprotein, vitamins, etc.

DISCUSSION

Ascorbic acid is the primary antioxidant in plasma and cells.[33] It has the ability to enhance body's antioxidant defense and is important healing of ulcers and delays the onset of other diseases.[34] The many relevant species reduced by ascorbic acid include various ROS, RNS, sulfur radicals, O_3 and HOCl. Ascorbic acid reduces heavy metal ions (Fe, Cu) that can generate free radicals via the Fenton reaction and thus, it can have pro-oxidant activity although its main function is as an antioxidant.[35] Supplements of vitamin C provide a constant supply of new reduced ascorbic acid, thus turning a sole cycle of iron dependent $OH^{\bullet}$ generation, in situations of localized iron overload, into a series of cycles, i.e., ascorbate-driven repetitive free radical generation by iron.[36, 37]

Carotenoids are powerful free radical scavenger and provide support to the body's immune system against infections. Epidemiological studies have shown that a high intake of carotenoid rich diet and amount of β-carotene in plasma is associated with decreased incidence of cancers, cardiovascular diseases, age related muscular degeneration and cataract formation. The protective role of carotenoids in the body is to diminish the degradation of antioxidant enzymes due to deactivation of singlet oxygen.[38] Antioxidant properties of biological carotenoids depend on retinol-binding proteins and other endogenous antioxidants.[39] Alpha and beta carotene, lycopene and cryptoxanthin are the main carotenoids in food as well as in the body.[40] Beta-carotene has been shown to have peroxy radical scavenging activity and suppress lipid peroxidation.[41, 42] Antioxidant properties can be reversed to pro-oxidant behavior depending on O_2 tension or carotenoid concentration.[43]

Extraction of phenolics with ethanol is most suitable for evaluating antioxidant activities.[44] It has been observed that TPC is mainly responsible for the antioxidant activity of plants. High antioxidant activity is associated with high phenolics content, a finding reported previously many times.[45, 46]

According to Singh et al. (2008b) proteins have excellent potential to scavenge free radicals. Proteins can inhibits lipid peroxidation through inactivation of ROS and chelation of peroxidative transition metals.[47, 48] Protein isolate from the herb, *Phyllanthus niruri* L., plays hepatoprotective role against carbon tetrachloride induced liver damage via its antioxidant properties.[49]

Among various natural antioxidants, polysaccharides in general have strong antioxidant activities and can be explored as novel potential antioxidants.[50] As reported recently, polysaccharides isolated from fungal, bacterial and plant sources were found to exhibit antioxidant activity, and were proposed as useful therapeutic agents.[51, 52] Polysaccharide rich pulp fraction of litchi showed good antioxidant activity.[53] It has also been found that consumption of complex carbohydrates in combination with different antioxidant micronutrients may enhance the antioxidant defenses and improve lipid metabolism.[38]

Our results showed that TB leaf extract significantly reduced the DPPH radicals in dose dependent manner in comparison to the leaf extract of TC and EO leaf and *Triphala* (Figure 1). DPPH has advantage being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition[54] than other laboratory generated free radicals such as hydroxyl radical and superoxide anion. A freshly prepared DPPH solution reveals a deep purple color with absorption maximum at 517 nm.[55] This color generally fades when an antioxidant is present in the medium.[56] Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a light yellow color product *i.e.* 2,2-diphenyl-1-hydrazine resulting in a decrease in absorbance at 517 nm.[57] Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time period.

The SARSA of *Triphala* and leaf extracts of TB, TC and EO were monitored by a non-enzymatic method known as PMS-NADH-NBT reduction system. In this method, $O_2^{\bullet}$-derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the

blue formazan, which is measured spectrophotometrically at 560 nm. The decrease in color intensity showed that antioxidant present in the plant extracts scavenges the $O_2^{\bullet}$. Superoxide anion is a weak oxidant which gives rise to generation of powerful and dangerous $OH^{\bullet}$ and $^1O_2$, both contribute to oxidative stress. Antioxidants present in the plant extracts reacts with superoxide radicals and decreases their color absorption intensity. The plant extract reduce the superoxide anion and inhibit the formation of blue formazan complex.[58] TB leaf extract showed good potential to scavenge superoxide at lower IC_{50} (Table 3).

Antioxidant activity and RP value are related to each other.[59] The RP of a compound may acts as a significant indicator of its potential antioxidant activity.[60] The RP of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction and radical scavenging activities.[61] In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration. With regards to RP, higher reducing capacity might be attributed to higher amount of TPC and flavonoids.[62] I0t was observed in this study that TB leaf showed maximum RP followed by *Triphala*, TC and EO leaf extract showing increase in phenolic contents (Table 3). This is well correlated with the studies mentioned earlier.

Lipid peroxidation products such as malondialdehyde (MDA) are considered useful and reliable indicators of oxidative damage, due to the susceptibility of membranes to attack by ROS.[63, 64] MDA is a secondary end-product of the oxidation of polyunsaturated fatty acids and reacts with thiobarbituric acid (TBA) to yield a pinkish-red colored MDA-TBA adduct, with maximal absorbance at 532nm.[65, 64] LPO has been shown to be potential endogenous source of cardiovascular risk.[66] Bartsch (1996) reported that MDA forms adducts with DNA adenine and cytosine which contribute to carcinogenicity and mutagenicity.[67] So, the plant parts having better protection against free radical induced LPO may be used as anti-LPO as well as anticarcinogenic/antimutagenic substances. The high percentage of LPO scavenging effects in TB leaf observed in our experiment (Figure 3) may be due to the high contents of phenolics (Table 1) or other radical scavengers present in the extract.

FTC is used to measure the production of peroxides at the initial stage of oxidation where as TBA method is used to measure a later stage of LPO that results in production of aldehydes and ketones.[68] FTC assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of ammonium thiocyanate, forming an intense red Fe^{2+} thiocynate complex with absorption maxima at 500 nm. Antioxidant/radical scavenge converts ferric iron back to ferrous iron, itself becoming oxidized, thus allowing another cycle of $OH^{\bullet}$ generation from renewed ferrous iron.[69] Our result with ammonium thiocyanate experiments showed that the TB leaf extract is an active scavenger of Fe^{3+} ion which is in agreement with the work done on known Fe^{3+} scavengers.[70] Chelating agent present in plant extracts may inactivate metal ions and potentially inhibit the metal dependent generation of free radicals.

CONCLUSION

It is well-known that ROS have significant positive correlation with several diseases such as ageing, atherosclerosis, inflammatory injury, cancer and cardiovascular diseases. The results obtained by us are with respect to the antioxidant activities of the ethanolic extract of TB, TC, EO leaf and *Triphala*. Plant extract containing higher phenolic compound showed maximum antioxidant activity. The antioxidant activities of extracts of *Triphala* and its constituent plant (TB, TC and EO) leaves may be attributed to their strong hydrogen donating and metal chelating ability, reducing potential, effective hydroxyl and free radical scavenging activity and high levels of phenols that might be responsible for its efficacy as pharmaceuticals.

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