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# Evaluation of *In Vitro* Antioxidant Properties of Methanolic and Aqueous Extracts of *Terminalia bellerica* Roxb Leaves

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**Abstract:** *The antioxidant activity of methanolic (MTBL) and aqueous (ATBL) extracts of Terminalia bellerica leaves (TBL) was evaluated by various in vitro techniques. $IC_{50}$ values of MTBL and ATBL were found to be 93 and 301 µg $mL^{-1}$ for FRSA, 42 and 252 µg $mL^{-1}$ for SARSA, 91 and 376 µg $mL^{-1}$ for LPO, 53 and 540 µg $mL^{-1}$ for HRSA, 142 and 544 µg $mL^{-1}$ for FTC, respectively. RP was detected to be 2.72 and 10.49 ASE $mL^{-1}$ for MTBL and ATBL. The MTBL showed better antioxidant activity than ATBL when compared with standard quercetin. TPC for MTBL and ATBL were 209.28 and 173.9 g $kg^{-1}$ of GAE, respectively. In conclusion, methanolic extract of TB leaf has higher antioxidant activity than the aqueous extract and it could be a good source of natural antioxidant.*

**Keywords:** *Terminalia bellerica*, Antioxidant, Total Phenolic Content.

## Introduction

Oxidative stress, defined as a disturbance in the balance between the production of Reactive Oxygen Species (ROS)/free radicals and antioxidant defenses. As a result, ROS/free radicals can attack on biomolecules such as lipid, protein and DNA in cells and tissues, thus inducing various cardiovascular and neurological disorders (Dhalla *et al.,* 2000; Sayre *et al.,* 2001). It not only causes lipid peroxidation, protein oxidation and oxidative DNA damage, but also creates interference in physiologic adaptation phenomenon and regulation of intracellular signal transduction mechanism. Two types of antioxidant (enzymatic and non-enzymatic) are present in living organisms that are usually effective in blocking harmful effects of ROS/free radicals. Many synthetic antioxidants are currently in use in various foods and pharmaceuticals industries but they may cause cellular toxicity; however, there is an increasing demand of consumers for consumption of natural antioxidants because of their good efficacy and lower side effects on health (Singh *et al.,* 2009a; 2009b).

*Terminalia bellerica* (Combrataceae) is one of an important constituent of a well-known ancient polyherbal formulation, Triphala. It is a large deciduous tree found throughout Indian forests and plains. The fruits of TB are commonly used for curing cough, diarrhea, fever, piles, oral thrush, and skin diseases and have analgesic, astringent, expectorant and laxative properties (Parekh and Chanda, 2007). The fruit also showed strong antimicrobial potential against different microbes such as bacteria, virus and protist (Gupta *et al.,* 2015, Singh *et al.,* 2016). Plant is a richest source of various bioactive phytocompounds such as polyphenols, terpenoids, tannins, flavonoids etc (Dhingra and Valecha, 2007). Various reported studies showed antioxidant potential of fruits of *T. bellerica* but there is lack on information about antioxidant potential of methanolic and aqueous extract of *T. bellerica* leaf. Present work focuses on evaluation of antioxidant potential of *T. bellerica* leaf extract and their correlation with the total phenolics.

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## Materials and Methods

### Plant Materials

Plant materials were collected from herbal garden of Narendra Dev University of Agriculture and Technology Kumarganj, Faizabad, U.P., India and identified with the help of Dr. M. N. Srivastava, Senior Scientist, Botany Division, CSIR-CDRI, Lucknow, India and the voucher specimens were submitted in CDRI herbarium.

### Chemicals and Reagents

Gallic acid, DPPH, TBA was purchased from sigma, USA and nitroblue tetrazolium, phenazine methosulphate, trichloroacetic acid, NADH, $FeCl_3$, SDS form SRL India. Rest of all chemicals used was of analytical grade.

### Extraction Procedure

Twenty grams of dried plant sample powder of *T. bellerica* leaf (TBL) was extracted with 70% methanol (MTBL) and double distilled water (ATBL) until decolouration, followed by evaporation in a vacuum rotary evaporator at 40°C and lyophilised till dryness.

### Antioxidant Studies

Total Phenolics Content (TPC) was estimated as described by the method of Ragazzi and Veronese (1973), Free radical, superoxide anion ($O_2^{\bullet-}$) radical and hydroxyl radical scavenging activity was measured by method of Yen and Duh (1994), Nishikimi *et al.,* (1972) and Halliwell *et al.,* (1987). Lipid peroxidation, Ferric thiocyanate, and reducing power were measured by method of Ohkaowa *et al.,* (1979), Tsuda *et al.,* (1994) and (Apati *et al.,* 2003).

### Statistical Analysis

Statistical analysis was done using prism software. Values of *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 of MS office excel 2007.

## Result and Discussion

### TPC

The phenolics have been characterized as phenolic acid, flavonoid and stilbenoids. Due to their useful antioxidant activity, phenolics have repeatedly been implicated as natural antioxidants in fruit, vegetable and other medicinal plants. The total phenolic content of MTBL and ATBL were found to be 209.28 and 173.9 g $kg^{-1}$ of GAE (Figure 1) which are in agreement with the earlier findings of Ayoob *et al.,* (2014). Plant extract containing high phenolic content showed better antioxidant activity than other. Various studies showed that the phenolic compounds are mainly responsible for antioxidant potential of medicinal plants; therefore the plant which contains high amount of phenolic compounds could be a natural source of antioxidant for various food and pharmaceutical industries (Gupta *et al.,* 2015; Singh *et al.,* 2009a; 2009b, Heim *et al.,* 2002).

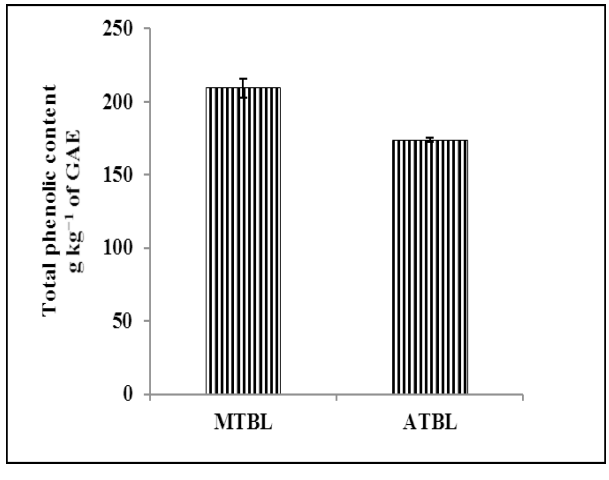



**Fig. 1** Amount of phenolics in MTBL and ATBL. Values are mean ± SD of three replications (n=3).

## Free Radical Scavenging Activity (FRSA)

DPPH radical scavenging activity of MTBL and ATBL is presented in Figure 2. The DPPH radical (DPPH*) scavenging effect of *T. bellerica* leaf increases with increasing concentration of extracts from 50-200 µg $mL^{-1}$. MTBL exhibited higher (79.99%) inhibition potential than ATBL (51.23%) in a concentration dependent manner from 50-200 µg $mL^{-1}$. Kathirvel and Sujatha (2012) showed that 70% methanolic leaf extracts of *T. chebula* inhibits DPPH* of inhibitory concentration ($IC_{50}$) of 143 µg $mL^{-1}$ which is lower than $IC_{50}$ value of 93 µg $mL^{-1}$ reported by us. DPPH is stable nitrogen centered, lipophilic free radical widely used in evaluating antioxidant potential of plant extracts in a relatively short time with high specificity compared to some other methods. The DPPH assay use to measure the ability of the plant extracts to donate electron to free radical resulting in decolorizing of the DPPH solution. Greater the decolorizing action, higher the antioxidant activity and this is reflected by higher anti radical power and lower value of $IC_{50}$.

## Superoxide Radical Scavenging Activity (SARSA)

MTBL showed higher superoxide scavenging activity (79.70%) than ATBL (62.09%) in a concentration dependent manner (Figure 3). $O_2^{\bullet-}$ is

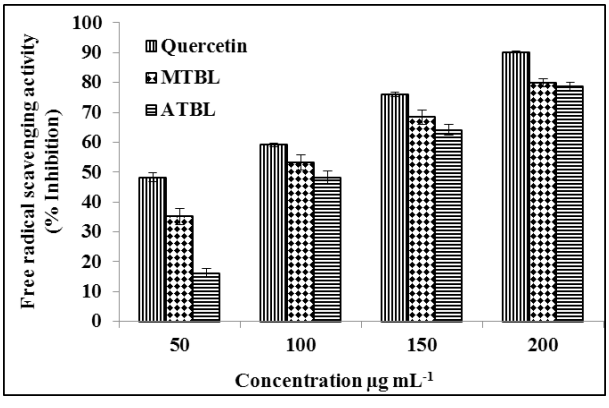



**Fig. 2** FRSA of MTBL and ATBL in comparison to standard quercetin. Values are mean ± SD of three replications (n=3).

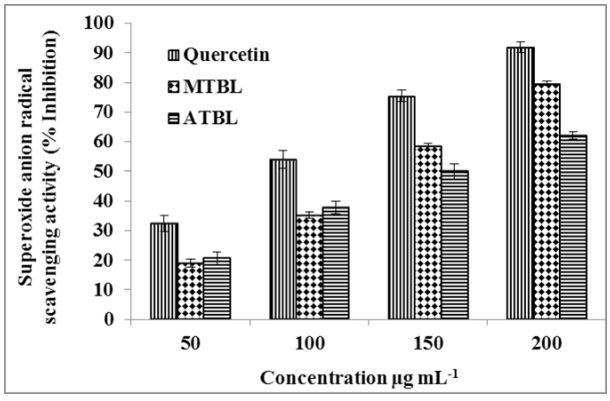



**Fig. 3** Inhibitory effects of MTBL and ATBL on superoxide anion radical at varying concentrations in comparison to standard quercetin. Values are mean ± SD of three replications (n=3).

a powerful free radical and have capability of creating auto-oxidizing chain reactions by oxidation of various biomolecules. It gives rise to generation of powerful and dangerous $OH^{\bullet}$ and $^1O_2$, both contribute to oxidative stress. The SARSA of *T. bellerica* leaf was 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 antioxidants present in the plant extracts scavenges the $O_2^{\bullet-}$ by inhibiting the formation of blue formazan complex. According to Alam *et al.,* (2011) the inhibitory potential of methanolic extract was 47.32% and aqueous extract did not show any $O_2^{\bullet-}$ scavenging activity which is different from our value 79.70 and 62.09% for MTBL and ATBL, respectively.

## Lipid Peroxidation (LPO)

The study was further carried out to determine the anti-LPO activity of plant extracts in presence of ferrous ion under *in vitro* conditions and was expressed in terms of percentage inhibition (Figure 4). MTBL showed higher anti-LPO activity (75.08%) than ATBL (54.82%) at 400 µg $mL^{-1}$

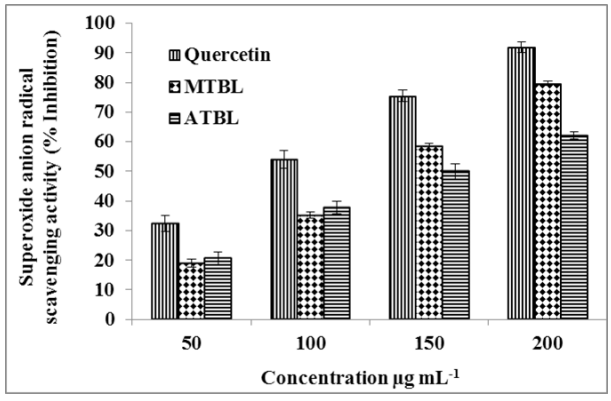



**Fig. 4** Inhibitory effects of MTBL and ATBL on LPO in comparison to standard quercetin by using egg homogenate as a lipid-rich source at varying concentrations. Values are mean ± SD of three replications (n = 3).

of plant sample was added to reaction mixture, respectively, and the result was in agreement with earlier finding (Sherin *et al*., 2015). LPO is a successive process of oxidative degradation of polyunsaturated fatty acids present in the biological membranes and production of a variety of secondary products including several aldehydes such as MDA. MDA reacts with TBA and produce pink colored products. MDA is the major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acid present in biological membranes (Vaca *et al.,* 1988). 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 LPO scavenging effects in *T. bellerica* leaf observed in our experiment may be due to the high contents of phenolic compounds or other radical scavengers present in the extracts which can terminate the peroxidation chain reaction easily and quench free radicals, thereby inhibiting the oxidation of lipid and other biological molecules.

## Hydroxyl Radical Scavenging Activity (HRSA)

In this experiment, protection of DNA by plant extracts against $OH^{\cdot}$ induced damage was determined in terms of the damage to its deoxyribose sugar moiety. The effect of *T. bellerica* leaf extract on hydroxyl radicals generated by $Fe^{3+}$ ion was measured by determining the degree of deoxyribose degradation, as indicated bythiobarbituric acid-malondialdehyde (TBA-MDA) adduct formation. HRSA of MTBL and ATBL are presented in Figure 5. MTBL showed high inhibition potential than ATBL in a concentration dependent manner. MTBL and ATBL extracts showed 42.06, 55.63, 65.22, 78.15% and 30.20, 37.72, 43.90, 53.86% inhibition at 50, 100, 150 and 200 µg $mL^{-1}$ concentrations, respectively. Hazra *et al.,* (2010) showed that 70% methanolic fruit extract of *T. bellerica* inhibits $OH^{\cdot}$ at $IC_{50}$ of 203 µg $mL^{-1}$ which is higher than $IC_{50}$ value of 53 µg $mL^{-1}$ reported by us.

## Ferric Thiocynate Assay (FTC)

Membrane lipids are the main targets of LPO by free radicals. The FTC method is used to assess the amount of peroxide produced during the initial stages of LPO, whereas TBA method is used to measure a later stage of LPO. 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 $Fe^{2+}$ thiocyanate complex with absorption maxima at 500 nm. Antioxidant/radical scavenge converts ferric iron back to ferrous iron, itself

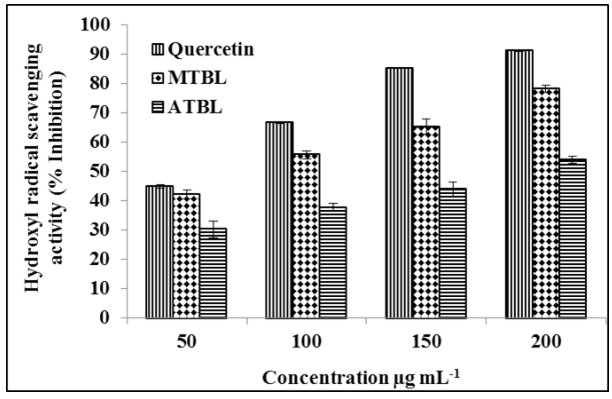



**Fig. 5** HRSA activity of MTBL and ATBL in comparison to standard quercetin. Values are mean ± SD of three replications (n = 3).

becoming oxidized, thus allowing another cycle of $OH^{\bullet}$ generation from renewed ferrous iron. MTBL and ATBL showed 36.19, 60.13, 65.96, 82.55% and 20.72, 26.34, 35.45, 51.91% ferric thiocynate activity at 50, 100, 150 and 200 µg $mL^{-1}$ concentrations, respectively (Figure 6) showing methanolic extract being more potent of peroxide generation. Result showed that the *T. bellerica* leaf extract is an active scavenger of $Fe^{3+}$ ion which is in agreement with the work done on known $Fe^{3+}$ scavengers (Singh *et al.,* 2013, 2014).

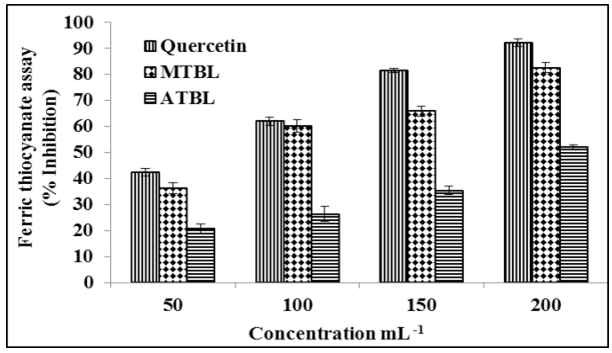



**Fig. 6** Inhibitory effects of MTBL and ATBL on ferric ion chelation by ferric thiocyanate assay method at varying concentrations in comparison with standard quercetin. Each value represents mean ± SD of three replications (n = 3).

## Reducing Power (RP)

RP of extracts was presented in terms of ascorbic acid equivalent per milliliter (ASE $mL^{-1}$). In tested extracts, MTBL showed high RP of 2.72 ASE $mL^{-1}$ than ATBL (10.49 ASE $mL^{-1}$), which is comparable to standard quercetin value 1.12 ASE $mL^{-1}$ (Figure 7). Refahy and Saad (2014) reported similar RP (2.66) of MTBL as showed by us (2.72 ASE $mL^{-1}$). With regard to RP, a higher reducing capacity might be attributed to higher amount of total phenolics (Singh *et al,* 2013).

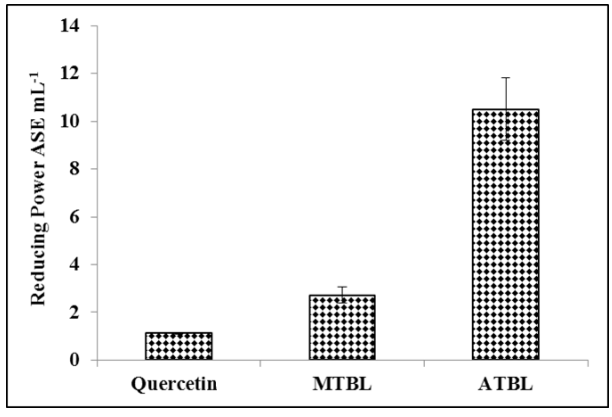
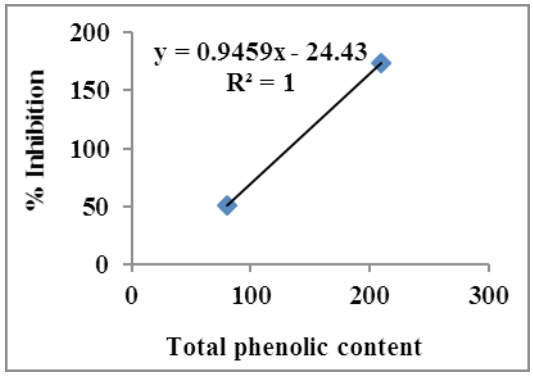



**Fig. 7** Reducing power (ASE $mL^{-1}$) of MTBL and ATBL in comparison with standard quercetin. Each value represents the mean ± SD of three replications (n=3).

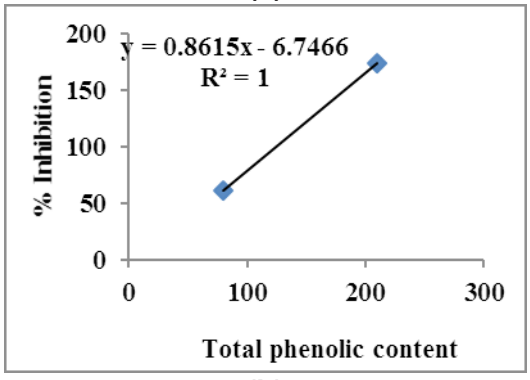
## Correlation between TPC and Antioxidant Activities

Correlation between TPC and antioxidant studies showed strong correlation coefficient of $R^2 = 1$ (Figure 8a to 8e). This indicates that the phenolic contents of *T. bellerica* leaf are responsible for its antioxidant activity. Many plants exhibit efficient antioxidant properties because of their phenolic constituents (Cook and Samman, 1996; Larson, 1988). These findings suggest that the phenolic contents of *T. bellerica* leaf are highly attributed to their antioxidant activity.

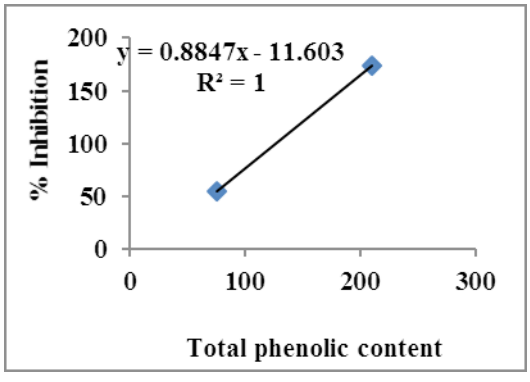
In conclusion, this study asserts the *in vitro* antioxidant potential of methanolic and aqueous extracts of leaves of *T. bellerica*. Results are compared with values of standard natural antioxidant quercetin. Out of the two extracts, methanolic extract have more potential antioxidant activity than aqueous extract. It is very clear that methanol can extract the antioxidant molecules better than water from *T. bellerica* leaf. In a nutshell, methanolic extract of *T. bellerica* leaf could be a potential source of natural antioxidants for food and pharmaceutical applications. Further studies are in progress to elucidate the mechanistic analysis of antioxidant, and anti-inflammatory potential of *T. bellerica* leaf.



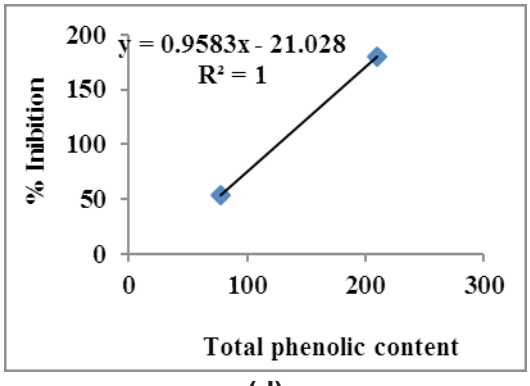

(a)



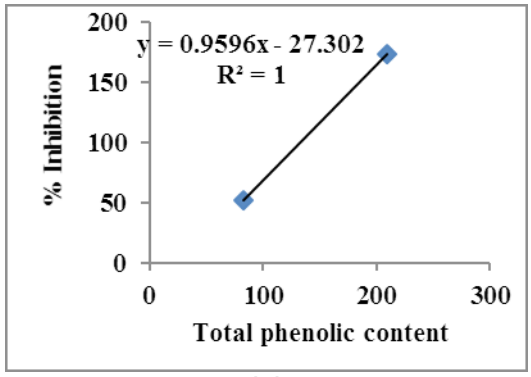

(b)




(c)




(d)




(e)

**Fig. 8** Linear Correlation between TPC (x axis) in the plant extracts in relation to their antioxidant activity (y axis). TPC versus FRSA (8a), TPC versus SARSA (8b), TPC versus LPO (8c), TPC versus HRSA (8d), TPC versus FTC assay (8e).

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