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IN VITRO ANTIOXIDANT EFFICACY OF *TERMINALIA BELLIRICA* SEED EXTRACT AGAINST FREE RADICALS

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ABSTRACT: In the present study *Terminalia bellirica* seeds, were evaluated for the *In vitro* anti-oxidant activity and phytochemical evaluation using different solvents such as methanol, ethylacetate, chloroform and aqueous. The *in vitro* antioxidant activity of *Terminalia. bellirica* is screened by standard antioxidant assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, hydroxyl radicals and Total antioxidant activity by ABTS assay. The ethylacetate extract of *T. bellirica* seed extract was found to be relatively high activity (84±0.6) than other extracts. Similarly the ethyl acetate exhibits high antioxidant status in ABTS and hydroxyl scavenging assays. In comparison to other extracts the ethyl acetate and methanolic extract found significant and prominent antioxidant activity due to the presence of high phenolic and steroid content. Finally this study concludes that all the seed extracts of *T. bellirica* exhibiting high antioxidant potential with respect to dose depending manner.

INTRODUCTION: Medicinal plants are emerged as folk medicines from very old times. Medicinal plant derived from natural products have good prophylactic properties and can be used to treat chronic and infectious diseases [1, 2]. Despite the vast progress promulgation in modern medicine, medicinal plants were still used to derive knock on free natural compounds in developing countries of recent decades [1]. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases [2]. Large number of medicinal plants has been scrutinized for their antioxidant properties.

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Natural antioxidants either in the form of raw extracts or their chemical constituents are extremely helpful to prevent the destructive processes caused by oxidative stress [3]. Even though the toxicity profile of most medicinal plants have not been scientifically evaluated, it is in general accepted that medicines derived from plant products are safer than their synthetic counterparts [4, 5]. Significant data has accumulated to prove, key roles for reactive oxygen species (ROS) and other oxidants in causing several disorders and diseases. The facts have brought the awareness of scientists to an appreciation of antioxidants for prevention and treatment of diseases, and maintenance of human health [6].

Human body has an inbuilt antioxidative mechanism and many of the biological functions such as the antimutagenic, anti-carcinogenic, and anti-aging responses originate from this property [7, 8]. Antioxidants stabilize or disable free radicals,

often before they attack targets in biological cells [9]. Recently curiosity in naturally occurring antioxidants has extensively increased for use in food, cosmetic and pharmaceutical products, because they have various activities and provide huge scope in correcting difference [10, 11]. The role of free radical reactions in disease pathology is well established and is known to be involved in many acute and chronic disorders in human beings, such as diabetes, atherosclerosis, aging, immune-suppression and neurodegeneration [12]. An imbalance between ROS and the inherent antioxidant capacity of the body, directed the use of dietary and /or medicinal supplements particularly during the disease attack.

Studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants such as phenolics, flavonoids, tannins, and proantho-cyanidins. The antioxidant contents of medicinal plants may contribute to the protection they offer from disease. The intake of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders [6]. Liver diseases remain a serious health problem. It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with consequent tissue injury.

Antioxidant agents of natural origin have attracted unique interest because of their free radical scavenging abilities [13]. The use of medicinal plants with high level of antioxidant constituents has been proposed as an effective therapeutic approach for hepatic damages [14].

T. belerica is also referred to as, Beleric Myrobalan in English, Bibhitaki in Sanskrit, Locally known as Bahera in India, has been used for centuries in the Ayurveda, a holistic system of medicine originating from India. The dried fruit used for medicinal purposes [15]. The fruits are ovoid grey drupes, obscurely 5 angled, narrowed into a very short stalk [16, 17]. The Phytoconstituents of seeds (*T.bellerica*) comprised of Glucoside (bellericanin) [18, 19], Gallo-tannic acid, Coloring matter, resins and a greenish yellow oil. Ellargic acid, gallic acid, lignans (termilignan and thanni lignan), 7-hydroxy 3'4' (methylene dioxy) flavone and anolignan B10. Tannins, ellargic acid, ethyl gallate, galloyl glucose and chebulaginic acid, phenyllemblin, β-sitosterol, mannitol, glucose, fructose and rhamnose [15, 16, 19].

The search for novel natural antioxidants of plant origin has ever since increased. It is not known which constituents of plant *T. belerica* are associated in reducing the risk of chronic diseases, but antioxidants appear to play a major role in the protective effect of herbal medicine. The present study was planned to investigate the antioxidant effect of different types of fruits of *T.belerica*

MATERIALS AND METHODS:

Methods Plant Collection: The seed of *T. bellirica* was collected from the local market and the same was botanically certified by plant anatomy research centre, west Tambaram. Further the 100 gm of *T. bellirica* seed was coarsely powdered using a electric blender. The powder was dried in an oven at 40 °C for 24 h.

Preparation of Different Solvent Extracts: The *T. bellirica* seed powder was extracted with the different solvents such as ethanol, acetone, chloroform and water. 10 gm of seed powder of *T. bellirica* was suspended in 200 ml of solvents. Extraction was done using soxhlet apparatus for 5 hours at a specific temperature of each solvents but not exceeding the boiling point [20]. The attained extract was filtered through syringe filter and the solvent was removed by evaporation using Buchi rota vapor under reduced pressure at 45 °C with 5 bars to get a constant mass and concentration of 1g. The resulting crude extract was then stored at 4 °C until use [21].

Determination of Phytochemical Constituents: The phytochemical assays were performed for the presence of secondary metabolites such as flavonoids, polyphenols, alkaloids, terpenoids, steroids, tannins, saponins, and glycosides. The above secondary metabolites were determined using standard phytochemical tests [22].

DPPH Radical Scavenging Activity Assay: The invitro free radical scavenging activity of (*T. bellirica* seeds) different extracts was evaluated by the standard method (2, 2′- diphenyl-1-picrylhydrazyl) DPPH assay [23, 24]. The 24 mg of DPPH (stock solution) was diluted with 100 ml of

methanol and stored at 20 °C until required. The stock solution was diluted with methanol to attain an absorbance of 0.98 ± 0.02 at 517 nm using the spectrophotometer for working solution (WS). 3 ml of WS was mixed with 100 µl of the sample at various concentrations (10 - 50 mg). The reaction mixture was vortexed and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared without sample. The scavenging activity was reckoned by the percentage of DPPH free radical scavenged as the following equation:

Scavenging effect (%) =
[(control absorbance – sample absorbance) / (control absorbance)]×100

ABTS Radical Scavenging Activity: The total free radical scavenging activity was calculated by the 2, 2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), ABTS cation scavenging activity [25, 26]. ABTS (7 mM) was dissolved with potassium persulfate (2.45 mM) and incubated overnight in the dark to generated a dark colored solution containing ABTS radical cations. Proceeding to use the ABTS radical cation it was further decepered with 50% methanol for an initial absorbance of 0.70±0.02 at 745 nm, at 30 °C. The *in vitro* free radical scavenging activity was determined by adding 300 µl of *Terminalia bellirica* extract with 3.0 ml of ABTS working standard in a cuvette. The decrease in absorbance was assessed by exactly one minute after adding the solution up to 6 min. The percentage of inhibition was calculated by the given below formula:

Scavenging effect (%) = [(control absorbance − sample absorbance)/ (control absorbance)] × 100

Hydroxyl Radical Scavenging Assay: Hydroxyl scavenging activity was determined by the efficacy of the different extracts (*T. bellarica* seed) to scavenge the hydroxyl radicals yielded by the Fe^{3+}-ascorbate-EDTA-H_2O_2 mixture (Fenton reaction) [27]. The reaction mixture was prepared by 500 µl of 2-deoxyribose (2.8 mM) dissolved in phosphate buffer (50 mM, pH 7.4), 200 µl of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 µl of H_2O_2 (200 mM) was added with or without (control) the extract solution (100 µl). The reaction was initiated by adding 100 µl of 300 mM ascorbate and incubated for 1 h at 37 °C. 0.5 ml of the reaction mixture was added to 1 ml of TCA (2.8%; w/v; aqueous solution), then 1 ml of 1% aqueous TBA were deciphered to the reaction mixture. The mixture was then incubated for 15 min on a boiling water bath. Then the mixture was cooled and the absorbance was taken at 532 nm against a blank (the same solution but without reagent). The scavenging activity on hydroxyl radical was calculated as follows:

Scavenging activity (%) = (1 - absorbance of sample/ absorbance of control) ×100

RESULTS: The phytochemical profile of seed (*Terminalia bellirica*) was screened for the presence of secondary metabolite such as alkaloids, carbohydrates, flavonoids, phenols, proteins, terpenoids, tannins, and sterols.The phytochemistry test on different extracts of *Terminalia bellirica* indicates the presence of above secondary metabolites in different concentration (**Table 1**). The alkaloid, phenols, steoids and tanin were notably found in the methanol and ethylacetate extract of *Terminalia bellirica* seeds. The Aqeuous and chloroform extract reveals that the presence of phytochemicals such as flavoids, saponon and tanins.

TABLE 1: PHYTOCHEMICAL PROFILE OF *TERMINALIA BELLIRICA* SEED EXTRACTS

Phytochemicals	*Termilnalia Bellarica* Extracts			
Secondary Metabolites	Methanol	Chloroform	Ethyl acetate	Aqueous
Alkaloids	++	-	++	-
Flavonoids	-	-	-	++
Steroids	+	+	+++	-
Glycosides	-	+	-	-
Saponins	-	-	-	+
Phenols	++	-	+++	+
Tannins	+	-	+	++

In vitro antioxidant capacity of methanolic, ethylacetate, chloroform and aqueous extracts of *T. bellrica* seed was examined using (2,2′- diphenyl-1-picrylhydrazyl) DPPH, 2,2′-azinobis (3-ethylbenz thiazoline - 6 - sulphonic acid) ABTS (Total antioxidant activity) and hydroxyl scavenging assays. The DPPH scavenging radicals was shown in the **Fig. 1** and found higher in the 50 mg of different extract *viz*., methanolic extract (73± 0.74), ethylacetate extract(84± 0.63), chloroform extract (51±0.82) and the aqueous extract (60.9 ±0.27). The antioxidant activity of standard ascorbic acid

was evaluated for determine the efficacy of *Terminalia bellirica* seed extracts.

The antioxidant potential was found to be significant ($P < 0.05$) when compared to ascorbic acid. This revealed that ethylacetate and methanolic extract have prominent antioxidant activity. The scavenging effects of DPPH radical and were in the following order: ethylacetate > methanolic > aqueous > chloroform.

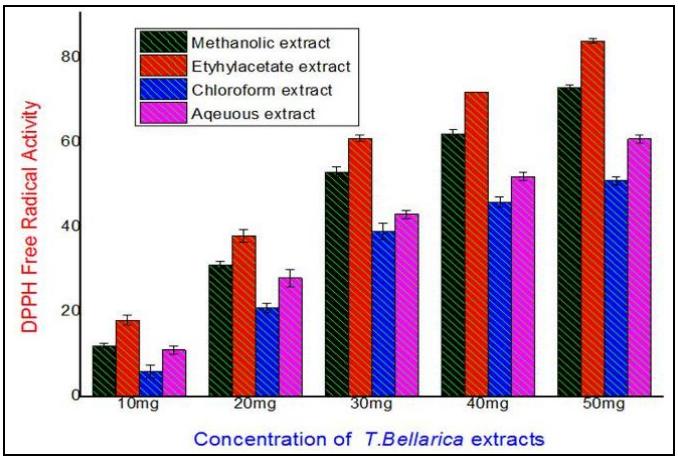

FIG. 1: DPPH FREE RADICAL SCAVENGING ACTIVITY OF (DIFFERENT EXTRACTS) *T. BELLIRICA* SEED

Similarly the hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals generated by the Fenton reaction. The total antioxidant activity was determined by ABTS radical scavenging activity all the fractions of *T. belarica* scavenged ABTS radical in a concentration dependent way (10 - 50mg) in the **Fig. 2**.

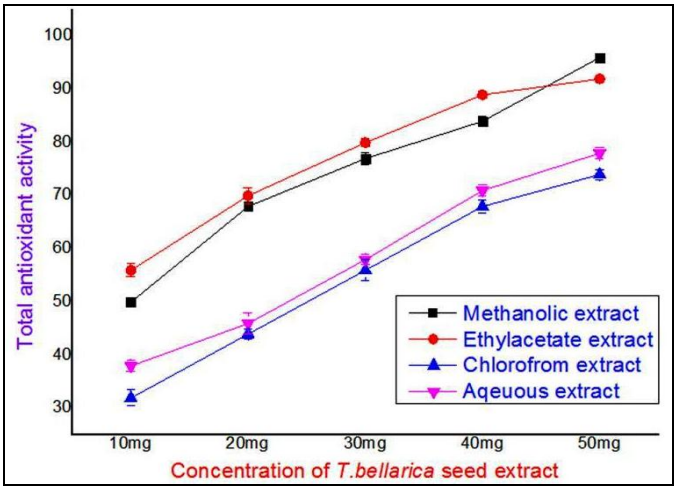

FIG. 2: ABTS (TOTAL ANTIOIDANT ACTIVITY) FREE RADICAL SCAVENGING PROPERTY OF (DIFFERENT EXTRACTS) *T. BELLIRICA* SEED

The hydroxyl radical scavenging activity of *T. bellarica* was shown in the **Fig. 3**. Higher activity was found in 50mg of different extract *viz.*, methanolic extract (86± 0.61), ethylacetate extract (81± 0.31), chloroform extract (64±0.42) and the aqueous extract (58.9 ±0.27).

The Present results showed that the antiABTS ability of *T. bellirica* seed extract showed similar effect to hydroxyl scavenging assay and notably it can be found in the order of ethylacetate > methanolic extract >aqueous extract > chloroform extract. The ethylacetate and methanolic extract exhibited prominent ABTS radical scavenging activities besides the aqeuous and chloroform extract.

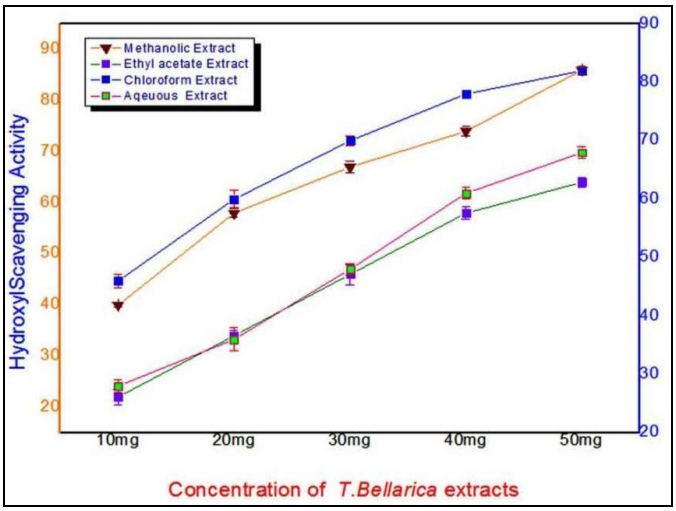

FIG. 3: HYDROXYL RADICAL SCAVENGING ACTIVITY OF (DIFFERENT EXTRACTS) *T. BELLIRICA* SEED

DISCUSSION: Several methods have been used to establish the antioxidant activity through *in vitro*. In order to allow rapid screening of compound which have low antioxidant activity *in vitro*, will probably show little activity *in vivo* [9]. But in the present study the *T. bellirica* seed extract demonstrated a good antioxidant property against the wide free radicals. Free radicals are known to play a crystal-clear role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and defend us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms [28]. The electron donation ability of natural products can be measured by 2, 20 - diphenyl - 1 – picryl hydrazyl radical (DPPH) purple-coloured solution bleaching [9].

The principle of this method is based on scavenging of DPPH through the addition of a radical species or antioxidant that lighten the DPPH solution.

The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [29]. In the present study among all the fractions tested, methanolic and ethyl acetate extract showed significantly higher inhibition percentage and positively linked with total phenolic content. Results of this study suggest that the plant extract of *T. bellrica* contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

Superoxide radical is well thought-out a major biological source of reactive oxygen species [30]. Although superoxide anion is a weak oxidant, it gives rise to generation of potent and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative strain [31]. The results of our study revealed that ethylacetate and methanolic extracts of *T. bellrica* had effective capacity of scavenging for superoxide radical and correlated with total flavonoid content thus suggesting its antioxidant potential.

Hydroxyl radical is one of the strong reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [6, 32]. The hydroxyl radical is considered as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and contributes to carcinogenesis, mutagenesis and cytotoxicity [41]. Hydroxyl radicals were produced by the reaction of H_2O_2 and the ferrous that would react with 2-deoxyribose.

The reaction was stopped by adding TBA reagent that would give a red colour if the malonaldehyde was formed as the result of the reaction between the radical and 2-deoxyribose. Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour [33]. All fractions of *T. bellrica* when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose.

ABTS radical scavenging assay involves a method that generates a blue/green ABTS+ chromophore *via* the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 745 nm. All the fractions possessed strong ABTS scavenging activity an observation that is supported by other researchers [34].

Plant products rich in phenolics are more and more used in the food industry since they hinder oxidative degradation of lipids and improve the quality and nutritional value of food [35]. Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur universally in plants [36]. The methanol extract and ethylacetate extract of *T. bellrica* exhibited the highest total phenolics content, than the chloroform and aqueous fraction [37]. Phenolic compounds of plants are also very important because their hydroxyl groups bestow scavenging ability. Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant activities [9]. Flavonoids are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti inflammatory, anticancer, and anti-allergic activities [38, 39].

Flavonoids have been shown to be highly valuable scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [40] implicated in several diseases.

So comparable with the findings in the literature for other extracts of plant products our results suggested that phenolic acids and flavonoids may be the major contributors for the antioxidant activity as the EC_{50} values of radical scavenging activity of various soluble fractions of *T. Bellrica* and the contents of phenolics or flavonoids exhibited significant correlation. In addition, there may be some interference rising from other

chemical components present in the extract, such as sugars or ascorbic acid [41].

CONCLUSION: The substitution of natural antioxidants instead of synthetic drugs (because of implications for human health) may be advantageous and human safety. In the present study, the free radical scavenging activity of *T. bellrica* seed extract demonstrated a high efficacy of antioxidant capacity due to the presence of high content of total phenolic, steroids, tanin and flavonoid content.

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