



ANTIOXIDANT ACTIVITY OF SUBCRITICAL WATER EXTRACT (SWE) OF TAMARIND (*Tamarindus indica* L) LEAVES

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ABSTRACT

In the present work, a novel environmentally friendly technique, subcritical water extraction (SWE), is employed for the extraction of antioxidant compounds from *Tamarindus indica* leaves. For comparison, *Tamarindus indica* was also extracted by maceration method. Antioxidant activity of the extracts was evaluated using commonly accepted chemical 2, 2-Diphenyl-2-picrylhydrazyl radical scavenging; nitric oxide radical scavenging and total reducing power assays. In general antioxidant activity of subcritical water extract was found to be significantly higher than the extract obtained by maceration method.

Chemical composition of the extracts was determined in terms of total phenol content and total flavonoid content by colorimetric methods. Total phenolic content of *Tamarindus indica* leaves extracts estimated as gallic acid equivalent was found to be in the range 10.88– 19.57 mg/g of extract and flavonoid content estimated as rutin equivalent was found to be in the range 10.29 – 50.55 mg/g of extract.

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INTRODUCTION

The use of plant extracts as natural antioxidants has received increased interest due to the concerns on the negative health effects by the use of synthetic antioxidants (Abramovic and Abram, 2006; Kowalski, 2007; Azizkhani and Zandi, 2009). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most commonly used synthetic antioxidants in the food samples. Although the synthetic antioxidants are very effective they are cause of concern due to the associated side effects such as their possible toxicity and as promoters of carcinogenesis (Rodríguez-Meizoso, I. et al., 2006; Anbudhasan et al., 2014). In this context there is a wide interest in finding natural antioxidant compounds that could replace synthetic antioxidants. Some studies have reported the use of herbs and spices, commonly used as food ingredients to flavor different types of food preparations, since they contain a wide variety of bioactive compounds that are beneficial for health. Also, several studies have reported the presence of potent antioxidants in the plants as vitamins, flavonoids, and other phenolic compounds that act as scavengers of free radicals and inhibitors of lipid peroxidation (Chang et al., 2002; Upendra, K, Sharma, et al., 2008).

Among the various plants reported for antioxidant activity, Tamarind (*Tamarindus indica*) has gained much importance as

a versatile nutraceutical crop with diverse uses and it has been used for centuries as a medicinal plant. Tamarind is a leguminous tree belonging to the family Fabaceae. The Tamarind is a long-lived, medium-growth tree, which attains a maximum crown height of 39 to 59 ft (12 to 18 metres). Tamarind is probably indigenous to tropical Africa (Diallo et al., 2007) and has been cultivated for long in the Indian subcontinent also. It is widely distributed throughout the tropical belt, from Africa to South Asia, northern Australia, and throughout Oceania, Southeast Asia, Taiwan and China. Different parts of this plant are used in traditional medicine for the treatment of infectious diseases (Julio César Escalona-Arranz et al., 2010). Various studies have confirmed medicinal value of Tamarind (Jouyex et al., 1995; Ushanandini et al., 2006; Cheryl Lans 2007). All parts of this plant are considered to be a good source of a large number of bioactive compounds, including flavonoids, tannins, polyphenols, anthocyanidin, riboflavin, niacin, ascorbic acid, β-carotene, etc. (Komutarin et al., 2004; Ushanandini et al., 2006; El-Siddig et al., 2006; Julio César Escalona-Arranz et al., 2010) which contribute to its wide usage as a natural antioxidant.

Extraction of antioxidants from plant tissues has usually been accomplished by conventional extraction processes such as solid-liquid extraction. Nowadays, there has been a huge upsurge for developing rapid, reliable, and reproducible methods for the efficient extraction of bioactive compounds from plants to increase their therapeutic functionality. Different extraction techniques are reported in the literature,

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such as pressurized liquid extraction, subcritical extraction (SCE), maceration, ultrasound-assisted extraction (UAE), and Soxhlet (Rodríguez-Meizoso, I. *et al.*, 2006; Upendra, K, Sharma *et al.*, 2008; Yogendra Kumar *et al.*, 2011). Recently, there has been an increasing interest in the use of environmentally clean technologies able to provide high quality and high activity extracts while precluding any toxicity associated to the solvents. In this sense, both, supercritical fluid extraction (SFE) with carbon dioxide (CO₂) as a solvent and subcritical water extraction (SWE) meet the requirements to be considered clean and safe processes (King, 2000; I. Rodríguez-Meizoso *et al.*, 2006).

The goal of the present investigation was to study the selectivity of SWE at several temperatures to extract antioxidant compounds from *Tamarindusindica* leaves. Nevertheless, to the best of our knowledge, there is no report that could illustrate the feasibility of SWE as a rapid and efficient extraction tool for the determination of antioxidant activity of *Tamarindus indica* leaves in comparing it with maceration.

MATERIALS AND METHODS

Plant Material

Tamarindus Indica leaves were collected from the area Bengaluru, India in the month of June 2017 under natural condition. Voucher specimen is preserved in Defence Bio-Engineering and Electromedical Laboratory, Bengaluru after ethanobotanical identification of species.

Apparatus

Accelerated Solvent Extractor, Model No. ASE 350, Dionex Thermo and UV- VIS Spectrophotometer, Model No. Lambda 950, Perkin Elmer.

Reagents

Butylated hydroxytoluene (BHT) & Rutin (Sigma Aldrich Chemicals, USA), Folin-Ciocalteu reagent & Ascorbic Acid [Vitamin-C] (Sisco Research Laboratories, India), 1, 1'-diphenyl-2-picrylhydrazyl [DPPH], 3, 4, 5-trihydroxybenzoic acid [Gallic Acid], and 2, 4, 6-tripyridyl-s-triazine [TPTZ].

Extraction Procedure

Maceration

10g of powdered *Tamarindus Indica* leaf sample was soaked at room temperature in 200ml of distilled water. Supernatant solution was decanted after 24 h, filtered through muslin cloth and the filtered solution was centrifuged at 8000rpm for 10 min. Finally, the supernatant solution was dried and obtained extract was stored at 5 °C for the further studies.

Subcritical water Extraction

Extractions were carried out in 33mL extraction cells, from 2 g of sample using water as solvent. Individual extractions were carried out at different temperature (25, 50, 100, 150 and 175°C), at constant pressure of 1500psi for 15 min extraction time at each temperature. Previous to each experiment an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature (the heat-up time is automatically fixed by the equipment). Namely 5 min heat-up was used when extraction temperature was set at 50°C and 100°C, 7 min at 150°C and 9 min at 175°C.

The extraction procedure was as follows: (i) sample is loaded into cell, (ii) cell is filled with solvent up to a pressure of 1500 psi, (iii) initial heat-up time is applied, (iv) static extraction with all system valves closed is performed for 15 min, (v) cell is rinsed (with 60% cell volume using extraction solvent), (vi) solvent is purged from cell with N₂ gas and (vii) depressurization takes place. Between extractions, a rinse of the complete system was made in order to overcome any extract carry-over (Rodríguez-Meizoso, I. *et al.*, 2006). The collected extracts were stored at 5 °C for the further studies.

Determination of total Phenol Content (K. Thaipong et al. 2006)

Total phenol content of extracts the obtained by Maceration and ASE methods were determined by the Folin-Ciocalteu method (K. Thaipong *et al.* 2006) and the results were expressed in milligram of gallic acid equivalents (GAE) per 1 gram of extract.

Determination of Total Flavonoid Content (Yanping Zou et al., 2004)

Total flavonoid content of extracts the obtained by Maceration and ASE methods were determined by the reported method (Yanping Zou *et al.*, 2004). Rutin was used as standard compound for the quantification of total flavonoid. All values were expressed as milligram of rutin equivalents per gram of extract.

Determination of Antioxidant Activity

DPPH radical Scavenging Activity

The DPPH radical scavenging activity of the test samples were evaluated by Blois method (1958) with minor modifications. Initially, 0.1 ml of the samples at a concentration of 0.01, 0.05, 0.10 and 0.20 mg/ml was mixed with 1 ml of 0.2 mM DPPH (dissolved in methanol). The reaction mixture was incubated for 20 min at 28°C under dark. The control contained all reagents without the sample while methanol was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer. The scavenging DPPH radical activity (%) of the tested sample was calculated as $(1 - \text{absorbance of sample} / \text{absorbance of control}) \times 100$. The DPPH radical scavenging activity of vitamin-C was also assayed for comparison.

Nitric Oxide (NO) Scavenging Activity

Nitric oxide (NO) radical scavenging activity of the *Tamarindus Indica* leaf extracts was determined by incubating sodium nitroprusside (5Mm, in PBS) with different concentrations (0.01 – 0.20 mg/ml) at 25°C for 2h. After incubation, 0.5ml of incubation solution was mixed with 0.5ml of Griess reagent (Green *et al.*, 1981) and absorbance was measured at 550nm. The percentage inhibition of nitric oxide was calculated using the following equation as $(1 - \text{absorbance of sample} / \text{absorbance of control}) \times 100$. The NO radical scavenging activity of BHT was also assayed for comparison.

Determination of Total Reducing Power (Yanping Zou et al., 2004)

1.0 mL of leaves extract solution (0.2-1.0mg/ mL) was mixed with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50 °C for 20 min. afterward,

2.5 mL of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 3000 rpm for 10 min. A 2.5 mL aliquot of the upper layer was combined with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) solution of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. Vitamin C is used for the standard graph.

Statistical Analysis

Each analysis was done three times from the same extract in order to determine their reproducibility. Results are expressed as mean±SD.

RESULTS AND DISCUSSION

Subcritical water Extraction

Subcritical water extraction (SWE) is an environmentally-clean technique that, in addition, provides higher extraction yields to extract solid samples (Rodríguez-Meizoso *et al.*, 2006). Therefore, it is recently emerged as a useful tool to replace the conventional/traditional extraction methods.

SWE is carried out using water starting from 100 °C under high pressure (1500psi) to maintain water in the liquid state. Table 1 provides the extraction yield of *Tamarindus indica* leaves in different extraction conditions. The most important factor to take into account in this type of extraction procedures is the dielectric constant (ϵ). This parameter can be modulated easily, within a wide range of values, by only tuning the extraction temperature. Water at room temperature is a polar solvent, with a dielectric constant close to 80. However, this level can be significantly decreased to 27 when water is heated up to 250 °C under pressure. This dielectric constant value is similar to that of ethanol. Therefore, it is appropriate to solubilize less-polar compounds (Miller & Hawthorne, 2000; Rodríguez -Meizoso *et al.*, 2006) from the sample.

In this study, the effect of sub critical water extraction method, for the efficient extraction of antioxidant compounds from *Tamarindus indica* leaf was investigated. Until now, to the best of our knowledge, there is no such report available that could highlight the feasibility of sub critical water extraction procedure as an efficient method for the extraction of antioxidant compounds from *Tamarindus indica* leaf. For comparison, *Tamarindus indica* leaf was also extracted by maceration method. During extraction, it was seen that maximum extraction yield (45.13%) was achieved with sub critical water extraction followed by maceration (20.01%) method (Table 1). However, taking into consideration the solvent consumption and time needed for extraction, SWE was found to be the best feasible approach for the rapid and efficient extraction of antioxidant compounds.

Total Phenol and Flavonoid Contents

As a part of chemical composition analysis, total flavonoid and total phenol content of *Tamarindus indica* leaf extracts were determined by colorimetric method. The results as given in the Table 1 indicate the presence of higher total phenolic and flavonoid content in the sub critical water extract obtained at 150°C in comparison with the extract obtained by maceration method. Phenolics are the major plant compounds with antioxidant activity. This activity is believed to be mainly due to redox properties, which play important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen,

or decomposing peroxides (Costantino *et al.*, 1992). Results obtained in the present study revealed that the level of these phenolic compounds in the *Tamarindus indica* leaf extracts were considerable. The results strongly suggest that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

Table 1 Extraction of *Tamarindus indica* leaves, determination of total phenol and flavonoid content.

Method of extraction	Extraction temperature °C	% of Yield*	Total phenol mg/g ± SD*of extract	Total flavonoid mg/g ± SD*of extract
Maceration	25±5	20.01	9.98 ± 0.31	08.55 ± 0.85
	LT	29.07	10.88 ± 0.18	18.03 ± 0.49
	50	30.51	11.64 ± 0.12	19.19 ± 1.02
Subcritical water	100	32.31	13.09 ± 0.11	30.72 ± 1.75
	150	43.76	19.57 ± 0.13	50.75 ± 2.53
	175	45.13	18.90 ± 0.24	46.35 ± 2.12

* Average of three determinations

Flavonoids are benzo- γ -pyrone derivatives which includes flavanes, flavones, flavonols catechins and anthocyanidines. These possess a wide spectrum of biological activities such as antifungal, anticancer, hypoglycaemic, antibacterial, antiviral, antihistaminic, spasmolytic and radioprotection properties (Jagtap S *et al.*, 2009; Perry & Foster, 1994; Ertan *et al.*, 1989; Amella *et al.*, 1985; Cazarolli *et al.*, 2008; Londhe *et al.*, 2009). Some of these properties derive from the free radical-scavenging activities of flavonoids and there are many reports relating to the flavonoids reactivities with active oxygen species. Recently, interest in these substances has been stimulated by the potential health benefits arising from their antioxidant activity. Antioxidant activity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. It has recently been recommended to employ at least two different in vitro models because of the differences between various free-radical scavenging assay systems (Schlesier *et al.*, 2002; Shiva Kumar and Yogendra 2018).

Antioxidant Activity Evaluation of *Tamarindus Indica* Leaves extracts

DPPH assay

Subcritical water extract of *Tamarindus indica* leaf showed stronger DPPH free radical scavenging activity than the extract prepared by Maceration method at all the concentrations tested from 0.05 to 0.50 mg/ml (Fig. 1). At 0.5 mg/ml, the highest percentage of DPPH radical scavenging activity of 42.90% was observed in the SWE, significantly higher than that of extract prepared by Maceration method (21.36%). However, as anticipated, their radical scavenging activity was inferior to BHT, which is known to be one of the standard reducing agent. From 0.01 to 0.20 mg/ml, the DPPH radical scavenging activity of *Tamarindus indica* leaf extracts increased but increased slowly when the concentration exceeded 0.20 mg/ml probably because the reaction of the scavenging radical activity gradually tended to stabilize at 0.20 – 0.50 mg/ml. A similar observation is reported in the literature (Sun *et al.*, 2007; Yogendra *et al.*, 2013). Flavonoids with free hydroxyl groups have a potent antioxidant activity. The results revealed that the DPPH radical scavenging activity of *Tamarindus indica* leaf extracts might be attributed to the electron donating ability (Chang *et al.*, 2002; Shivakumar & Yogendra, 2018).

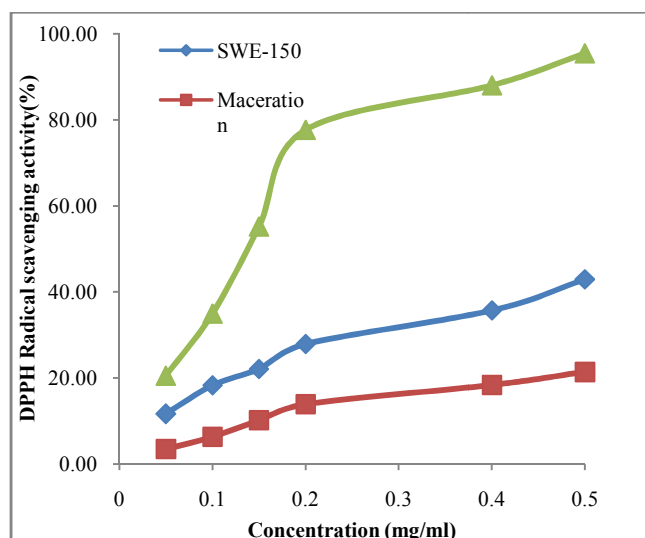


Fig 1 DPPH radical scavenging activity of SWE-150 extract, Maceration extract and BHT.

Nitric oxide Scavenging Activity

Nitric oxide (NO) scavenging activities of SWE-150 extract, maceration extract and BHT were analysed. The NO scavenging activity of the extracts was found to be concentration dependent as shown in Fig. 2. The SWE-150 extract exhibited higher nitric oxide scavenging activity than maceration extract in a dose-dependent manner. However, as anticipated, their nitric oxide scavenging activity was inferior to BHT, which is known to be one of the standard reducing agent. At 0.4 mg/ml, the NO radical scavenging activity of the BHT, SWE-150 extract and maceration extract were found to be 87.68 ± 2.67 , 51.82 ± 1.91 and 36.15 ± 1.47 %, respectively.

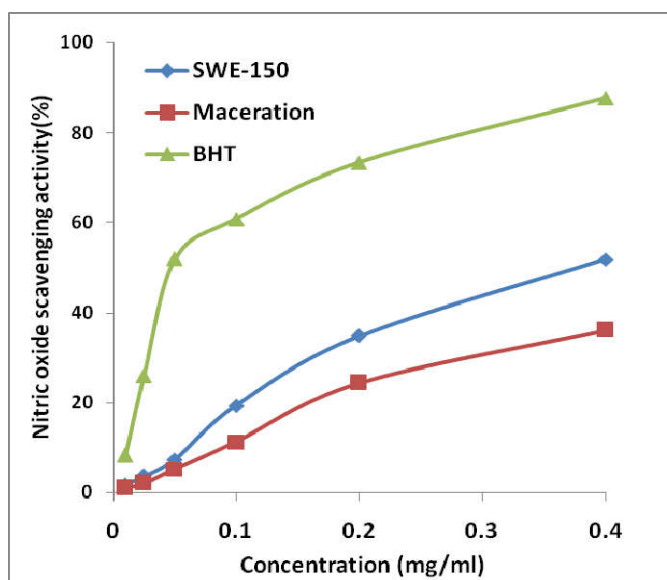


Fig 2 Nitric oxide radical scavenging activity of SWE-150 extract, Maceration extract and BHT.

Reducing Power

The reducing power of *Tamarindus indica* extracts, which may serve as a significant reflection of the antioxidant activity, was determined using a modified iron (III) to iron (II) reduction assay (Joseph M. Awika, *et al.*, 2003). In this assay, the yellow color of the test solution changes to various shades of green

and blue depending on the reducing power of extracts or compounds. The presence of reductants in the solution causes the reduction of the Fe^{3+} /Ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm (Yanping Zou *et al.*, 2004).

Total reducing power of *Tamarindus indica* extracts and ascorbic acid (Vit-C) is shown in Fig. 3 for comparison. All the extracts have showed some degree of reducing power; however, as anticipated, their reducing power was inferior to Vit-C, which is known to be a strong reducing agent. Reducing power of the extracts increased with increasing amount of extract. The equation of reducing power (y) and amount of extract (x) for the extract prepared by SWE and maceration methods were $y = 4.3x + 0.001$ ($r^2 = 0.996$) and $y = 2.421x - 0.009$ ($r^2 = 0.992$) respectively. Indicating that reducing ability correlated well with amount of the extracts.

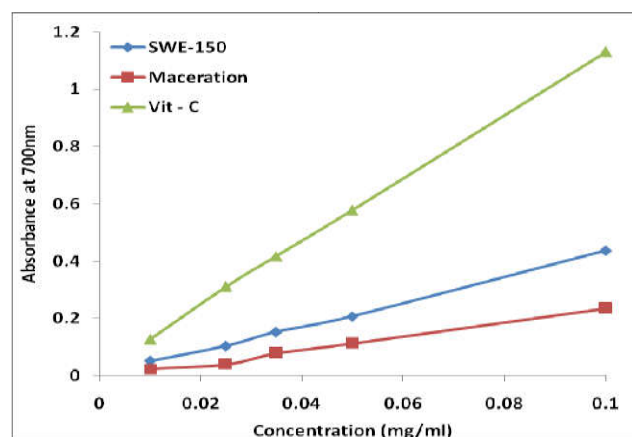


Fig 3 Total reducing power of SWE-150 extract, Maceration extract and BHT. Higher absorbance values indicate higher reducing activity.

It will be relevant to mention here that earlier reports have demonstrated the correlation between the phenolic content of plants to their antioxidant power (Costantino *et al.*, 1992; Nitin *et al.*, 2010; Yogendra *et al.*, 2013). In this study also, a good correlation has been indicated between the phenolic content and the antioxidant power of extracts. The reducing power of ascorbic acid, and *Tamarindus indica* extracts followed the following order: ascorbic acid > SWE 150 > Maceration.

CONCLUSION

Subcritical water extraction of *Tamarindus indica* leaf was found to be a better approach than maceration because the use of subcritical water imparted higher antioxidant activity to the extracts besides ensuring low solvent consumption, ease, and rapidity of the over the maceration extraction method. Antioxidant activity of the extracts was evaluated using DPPH & NO scavenging assays.

Total phenolic and flavonoid content present in the extracts of *Tamarindus indica* leaves demonstrate the increased antioxidant power. The obtained results demonstrate the practical feasibility of eco-friendly subcritical water extraction method to substitute the traditional time-consuming methods for efficient extraction of antioxidative compounds to provide nutraceutical-rich formulations.

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