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EFFECT OF SOLVENT SYSTEMS AND EXTRACTION TECHNIQUES ON TOTAL PHENOL, TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY OF A POTENTIAL MEDICINAL PLANT *TINOSPORA SINENSIS* (LOUR.) MERR.

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ABSTRACT: *Tinospora sinensis* (Lour.) Merr. belongs to the family Menispermaceae is, an important medicinal plant. Ayurveda, an Indian medicinal system, strongly advocates use of *T. sinensis* as a rejuvenator and several other therapeutic activities. The study's objectives were to assess the effect of the extraction procedure and different solvent systems on antioxidant, total phenol content and total flavonoid content of *T. sinensis*. Two conventional extraction methods, cold maceration and Soxhlet extraction, were used to extract four parts of the plant and six different solvent systems *viz*., petroleum ether, ethyl acetate, chloroform, acetone, ethanol and methanol were used according to their polarity gradient. The total phenolic content was determined using the Folin-Ciocalteau method, and total flavonoid content was determined by aluminium chloride. The antioxidant activity was performed following two *in-vitro* technique assays, DPPH and ABTS radical scavenging assay. All the extracts of six solvents have potent antioxidant activity and methanol and ethanol extracts showed higher levels. The assimilatory root, stem, root and leaf of *T. sinensis* possess total phenol and flavonoid content. The polar solvent extracts showed higher activities and maceration is a more efficient extraction technique for this plant.

INTRODUCTION: Natural products are important sources for drug development. Plants are well-known to retain bio-active compounds with significant folkloric health benefits [1, 2]. In recent years, natural antioxidants explored in plants have devoted serious interest due to their extensively acclaimed nutritious and remedial values. The antioxidant properties of plant-derived compounds/ extracts are relevant for analyzing the biochemical mechanisms of certain traditional remedies and other medical applications [3]. Antioxidant is "any substance that delays prevents or removes oxidative damage to a target molecule" [4].

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Antioxidant activity mechanisms are the most effective path to eradicate and reduce the action of free radicals, which cause oxidative stress. Oxidative stress is a main causative aspect in the stimulation of several severe diseases, including atherosclerosis, diabetes mellitus, cancer, Parkinson's disease, immune dysfunction and is involved in premature aging [5]. Plant-derived antioxidants, especially, phenolics have gained considerable importance due to their potential health benefits [6].

Tinospora sinensis (Lour.) Merr. (Menispermaceae) is one such plant that is widely used in the Indian System of Medicine (ISM) [7]. The plant is frequently used in traditional Ayurvedic medicine. It has various remedial properties such as jaundice, rheumatism, urinary disorder, skin diseases, diabetes, anaemia, inflammation, allergic condition, anti-periodic, radioprotective properties, *etc*. [8]. The antioxidant and immunomodulatory

potential of *T. sinensis* have been extensively investigated both *in-vitro* and *in-vivo*. This plant's therapeutic activity is due to its phytoconstituents like diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, essential oils, a mixture of fatty acids and polysaccharides. These phytoconstituents are present in different parts of the plant body, including the root, stem, and whole part [9].

The identification, isolation, and characterization of the bioactive compounds from medicinal plants matrix need cautious selection of appropriate extraction methods and solvents [10]. An extraction technique is that which can acquire extracts with high yield and with the least changes to the functional properties of the extract required because it is affected by many variables determining the appropriate conditions of a plant extract, including several extractions, the extraction time and ratio of solvent to raw material, pressure and extraction temperature [11].

So, selecting the appropriate extraction method and solvent is necessary based on sample matrix properties, chemical properties of the analytes, matrix-analyte interaction, efficiency, and desired properties [12]. Besides the extraction method, the used solvent is an important factor that can significantly affect the extracts extraction yield and antioxidant activity [13, 14]. This communication reports the efficacy of different solvents and extraction techniques on extract's yield in different parts of the *T. sinensis* plant. The extract's yield was determined by determining the amount of certain bioactive substances like total phenol content and total flavonoid content present in the extract and evaluating of antioxidant activities of the extracts.

MATERIALS AND METHODS:

Collection of Material and Preparation of Sample: Stem, leaf, assimilatory root and root of *T. sinensis* were collected from the Botanical Garden of Dibrugarh University, Dibrugarh, Assam. The taxonomic identification and authentication of the plant was done in the Department of Life Sciences, Dibrugarh University. A voucher specimen of *T. sinensis* (Lour.) Merr. (No. DU/L. Sc/SR-I/493) was deposited in the Herbarium of the Department of Life Sciences. Collected plant materials were separately washed in tap water and allowed to surface dry at room temperature. Materials were then sliced and finally shade dried. Dried materials were ground in a mixture grinder and stored in airtight containers and labelling properly as root, stem, leaf and assimilatory root sample.

Extract Preparation: Sample extracts were prepared in 1:10 using six different solvents according to their polarity gradient. Two extraction techniques *viz.* Maceration and Soxhlet extraction were used.

Maceration Technique: Powdered samples were successively extracted in Petroleum ether, ethyl acetate, chloroform, acetone, ethanol and methanol. The contents were left at room temperature for 72 hrs with frequent shaking.

Soxhlet Extraction Technique: The Soxhlet extraction technique was carried out by successive extraction of samples in Petroleum ether, ethyl acetate, chloroform, acetone, ethanol and methanol. The extraction process continued until the solvent in the siphon tube became colourless.

Filtration and Evaporation of Extracts: The extracts were filtered using Whatman No. 1 filter paper, a water bath concentrated the filtered extracts and the residual extracts were dried. The extracts were kept and stored in a refrigerator at 4 °C until use.

Determination of Total Phenolic Content (TPC): TPC was determined using the Folin-Ciocalteau method [15] with slight modifications in sodium carbonate concentration. As described by Polu *et al.* the concentration of sodium carbonate was 6% (w/v) but in our study, we have used 7.5% (w/v) because it gives better results. The absorbance was measured at 740 nm and converted to the phenolic content according to a calibration curve made with catechol (mg/ml).

Determination of Total Flavonoid Content (TFC): TFC of each extract was determined using aluminum chloride method [16] with slight modifications. In our study, the absorbance was taken at 517 nm instead of 415 nm because it gives better results. The calibration curve was prepared using quercetin (mg/ml).

***In-vitro* Antioxidant Activity Assay of the Extracts:**

DPPH Radical Scavenging Assay: 2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacities of the extracts were estimated by reducing the reaction colour between DPPH solution and sample extracts, as described previously by [17]. The standard calibration curve was prepared using Ascorbic acid. DPPH radical inhibition percentage was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = (A°\text{-}A_1) / A° \times 100$$

Where, A° is the absorbance of the control sample, and A^1 is the absorbance of a sample. All tests were performed in triplicate, and the average of three determinations was used to plot in the graphs.

ABTS Radical Scavenging Assay: 2, 2-azinobis-(3 - ethylbenzothiazoline - 6 -s ulphonate) (ABTS) assay, was determined using the method described by [18, 19]. The standard was calculated from the plotted graph of scavenging activity against the concentrations of ascorbic acid, and the inhibition percentage was calculated using the following formula:

$$\text{ABTS scavenging activity (\%)} = (A^0 - A_1) / A^0 \times 100$$

Where, A^0 is the absorbance of the control sample, and A_1 is the absorbance of the sample.

Results are presented as means ± S.D from the mean. Assays results are analyzed statistically using the Microsoft Excel program 2019.

RESULTS AND DISCUSSION: The results on solvent efficacy and extraction techniques for the extraction of bioactive substances are presented in **Table 1** to **Table 4**. Methanolic extract of assimilatory root in cold maceration recorded higher TPC (14.65±0.03 µg/ml) followed by methanolic extract of root (12.43±0.12µg/ml) and Soxhlet methanolic extract of assimilatory root (10.72±0.07µg/ml). The lowest TPC was recorded in petroleum ether leaf extract (0.76±0.02µg/ml). The cold maceration revealed better extraction technique for obtaining TPC in *T. sinensis* **Fig. 1**. TPC was recorded highest in methanolic extract, indicating better extraction of *T. sinensis* phenolics in more polar solvents. Phenolics are often extracted in higher amounts in more polar solvents such as methanol/ethanol than Hexane/ Petroleum ether [20].

TABLE 1: TOTAL PHENOLIC CONTENT (TPC) OF *TINOSPORA SINENSIS*

Sample	Total Phenol Content (µg/ml)					
	Cold Maceration					
	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Acetone extract	Ethanol extract	Methanol extract
Stem	1.03±0.02	3.21±0.07	3.91±0.03	4.76±0.12	7.56±0.05	10.21±0.11
Leaf	0.98±0.07	2.8±0.11	3.43±0.02	3.98±0.05	6.43±0.01	9.97±0.07
Assimilatory root	1.89±0.11	4.1±0.05	5.82±0.04	8.74±0.01	10.32±0.04	14.65±0.03
Root	1.02±0.04	3.9±0.07	4.83±0.11	6.78±0.02	9.87±0.08	12.43±0.12
	Soxhlet Extraction					
Stem	0.91±0.12	1.27±0.04	2.13±0.11	3.89±0.04	5.13±0.06	7.64±0.11
Leaf	0.76±0.02	0.97±0.12	1.48±0.03	2.67±0.08	3.89±0.01	5.12±0.03
Assimilatory root	0.98±0.07	2.02±0.02	3.67±0.07	5.32±0.13	7.39±0.04	10.72±0.07
Root	0.94±0.11	1.78±0.05	2.45±0.12	3.54±0.02	6.12±0.06	8.23±0.01

Data are expressed as the mean of triplicate (n=3) ± (SD).

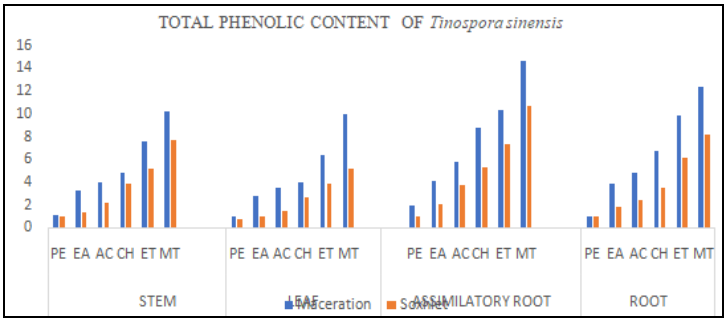

FIG. 1: COMPARATIVE ANALYSIS OF TOTAL PHENOL CONTENT OF DIFFERENT PARTS OF *TINOSPORA SINENSIS*. "PE- Petroleum ether, EA- Ethyl acetate, AC- Acetone, CH- Chloroform, ET- Ethanol, MT- Methanol"

TFC of *T. sinensis*, extracted with six different solvents using cold maceration and Soxhlet extracting techniques, are given in **Table 2**. Methanolic extract of root in maceration technique recorded higher TFC (15.32±0.02µg/ml) followed by methanolic extracts of assimilatory root (13.23±0.12µg/ml), stem (12.01±0.03µg/ml). On the other hand, in Soxhlet extraction technique methanolic extract of assimilatory root showed higher TFC (9.23±0.02µg/ml). The results indicated that the maceration technique is superior in the case of TFC than Soxhlet extraction **Fig. 2**. This may be due to high temperature and elaborate extraction period in the Soxhlet extraction may enhance the possibilities of thermal degradation of bioactive components.

TABLE 2: TOTAL FLAVONOID CONTENT OF FOUR DIFFERENT PARTS OF *TINOSPORA SINENSIS*

Sample	Total Flavonoid Content (µg/ml)					
	Cold Maceration					
	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Acetone extract	Ethanol extract	Methanol extract
Stem	0.98 ±0.01	4.23±0.11	3.43±0.08	5.78±0.12	10.52 ±0.02	12.01±0.03
Leaf	0.19±0.04	3.99±0.14	4.29±0.05	7.42±0.17	9.32±0.02	11.42±0.11
Assimilatory root	2.12±0.12	7.13±0.04	6.57±0.15	8.47±0.05	11.54±0.02	13.23±0.12
Root	1.56±0.07	3.78±0.11	7.45±0.14	8.12±0.07	10.67±0.04	15.32±0.02
	Soxhlet Extraction					
Stem	0.77±0.02	2.89±0.04	2.12±0.12	4.73±0.06	6.27±0.03	8.71±0.04
Leaf	0.12±0.11	1.56±0.09	3.31±0.05	4.12±0.02	5.91±0.11	7.83±0.05
Assimilatory root	1.59±0.07	4.72±0.11	3.79±0.02	5.68±0.13	7.14±0.07	9.23±0.02
Root	0.97±0.02	3.45±0.08	4.11±0.11	5.16±0.03	6.57±0.07	8.91±0.11

Data are expressed as the mean of triplicate (n=3) ± (SD).

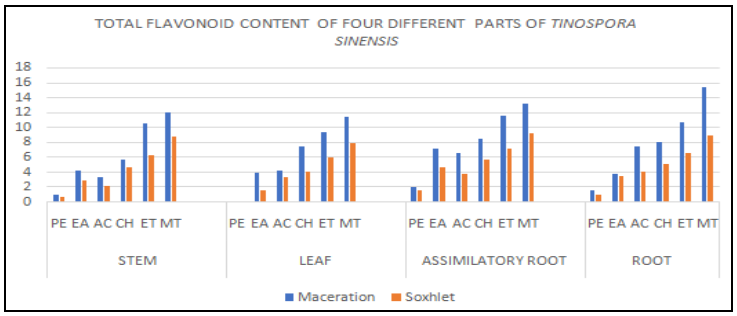

FIG. 2: COMPARATIVE ANALYSIS OF TOTAL FLAVONOID CONTENT OF DIFFERENT PARTS OF *TINOSPORA SINENSIS*. "PE- Petroleum ether, EA- Ethyl acetate, AC- Acetone, CH- Chloroform, ET- Ethanol, MT- Methanol"

In **Table 3**, DPPH scavenging activity of four parts of *T. sinensis* using two extraction techniques were presented. The methanolic extract of assimilatory root using maceration technique shows higher scavenging activity (72.23±0.02%), followed by methanolic extract of root (71.22±0.11%), methanolic extract of stem (69.56±0.02%) and Soxhlet methanolic extract of assimilatory root (65.34±0.20%).

TABLE 3: DPPH RADICAL SCAVENGING ACTIVITY OF *TINOSPORA SINENSIS*

Sample	DPPH radical scavenging activity (% Inhibition in mg/ml)					
	Cold Maceration					
	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Acetone extract	Ethanol extract	Methanol extract
Stem	38.72±0.11	42.51±0.17	48.32±0.02	53.91±0.02	62.69±0.21	69.56±0.02
Leaf	42.70±0.21	51.53±0.04	50.17±0.03	51.88±0.01	56.60±0.02	61.15±1.52
Assimilatory root	45.80±0.12	48.84±0.26	60.74±0.09	66.4 ±0.02	64.86±0.02	72.23±0.02
Root	43.92±0.62	39.77±0.24	47.12±0.13	54.33±0.07	63.12±0.43	71.22±0.11
	Soxhlet Extraction					
Stem	26.15±0.32	34.32±0.91	41.21±0.42	47.56±0.81	53.32±0.57	60.15±0.74
Leaf	24.32±0.21	32.55±0.48	44.71±0.23	49.51±0.11	54.12±0.19	59.41±0.24
Assimilatory root	36.52±0.45	42.63±0.22	54.21±0.18	60.72±0.31	62.61±0.07	65.34±0.20
Root	32.40±0.32	37.45±0.07	42.34±0.14	51.67±0.04	57.89±0.41	63.76±0.16

Data are expressed as the mean of triplicate (n=3) ± (SD).

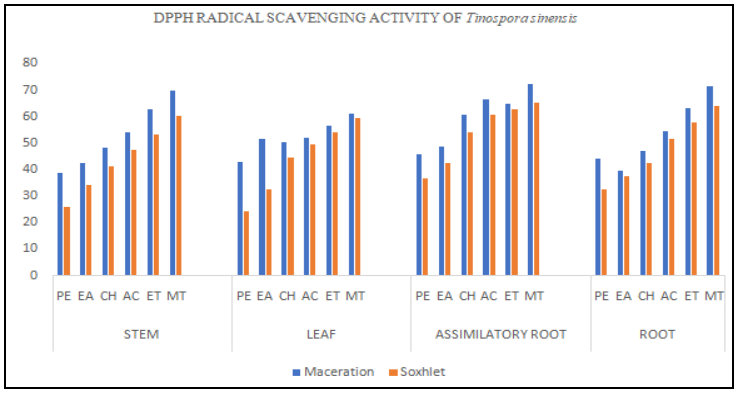

FIG. 3: COMPARATIVE ANALYSIS OF DPPH ACTIVITY OF DIFFERENT PARTS OF *TINOSPORA SINENSIS*. "PE- Petroleum ether, EA- Ethyl acetate, AC- Acetone, CH- Chloroform, ET- Ethanol, MT- Methanol"

The DPPH and ABTS scavenging assays are widely used to evaluate various samples' free radical scavenging ability, including plant extracts. DPPH is a stable nitrogen-centered organic free radical with an absorption peak at 517 nm. It loses absorption as accepting electrons or free radical species, observed by noticeable discoloration from purple to yellow. DPPH can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations. The ABTS assay was based on the reduction of ABTS radicals by antioxidants in the samples. **Tables 3 and 4** show that all the extracts revealed radical scavenging capability in the ABTS and DPPH assays. The methanolic extract of the assimilatory root through maceration technique showed higher activity (92.50±0.02mg/ml), followed by ethanolic extract of the stem (91.49±0.15mg/ml), methanolic extract of root (91.23±0.07mg/ml) and Soxhlet methanolic extract of assimilatory root (82.57±0.05 mg/ml).

TABLE 4: ABTS ANTIOXIDANT ACTIVITY OF *TINOSPORA SINENSIS*

Sample	ABTS radical scavenging activity (% Inhibition in mg/ml)					
	Cold Maceration					
	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Acetone extract	Ethanol extract	Methanol extract
Stem	62.08±0.07	77.54±0.12	84.87±0.09	89.91±0.11	91.49±0.15	90.67±0.05
Leaf	58.95±0.12	72.04±0.06	84.16±0.13	69.20±0.08	82.2±0.03	86.82±0.11
Assimilatory root	61.85±0.20	69.76±0.11	86.45±0.04	82.60±0.03	84.92±0.11	92.50±0.02
Root	54.74±0.14	78.79±0.21	82.75±0.23	89.90±0.14	90.18±0.09	91.23±0.07
Soxhlet Extraction						
Stem	56.23±0.11	64.81±0.02	69.52±0.16	73.79±0.12	81.34±0.03	79.32±0.12
Leaf	49.71±0.05	57.45±0.06	61.36±0.04	69.51±0.14	71.57±0.07	75.45±0.11
Assimilatory root	58.65±0.09	67.34±0.02	65.43±0.11	71.56±0.08	79.65±0.11	82.57±0.05
Root	54.37±0.02	59.66±0.13	62.25±0.09	69.27±0.11	75.41±0.07	80.95±0.02

Data are expressed as the mean of triplicate (n=3) ± (SD)

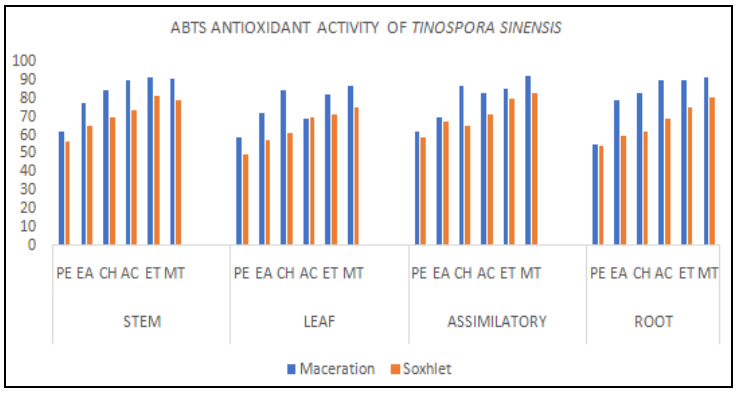

FIG. 4: COMPARATIVE ANALYSIS OF ABTS ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF *TINOSPORA SINENSIS*. "PE- Petroleum ether, EA- Ethyl acetate, AC- Acetone, CH- Chloroform, ET- Ethanol, MT- Methanol"

It is noted that the maceration technique performed better than Soxhlet extraction, and the polar solvents performed better radical scavenging activity in both methods **Fig. 3** & **4**. It might be due to the thermal processing conditions in the loss of natural antioxidants because heat may accelerate their oxidation and other degenerative reactions. The previous investigations support the results reports [21]. Thus, heating temperature be in consideration during the extraction of plant materials. Based on these results, it can be said that *T. sinensis* possesses bioactive substance (s) having good important antioxidant activity. Moreover, the maceration seems to be a better extraction technique than the Soxhlet extraction technique.

In the correlation analysis, the greater correlation was noted between TPC and DPPH (0.92) followed by TFC and DPPH (0.90) and moderate correlation was detected between TPC and ABTS (0.78) followed by TFC and ABTS (0.76). On the other hand, a moderate correlation was noted between DPPH and ABTS (0.72). The correlation analysis revealed that the quantity of phenolic and flavonoid compounds is strongly correlated with antioxidant activities of the plant extracts of *T. sinensis*.

CONCLUSION: The present study revealed that different parts of *T. sinensis* exhibit significant phenolic content, total flavonoid content and antioxidant activity. Based on their above activities, the polar solvents and maceration technique showed a more efficient extraction method for this plant. Further investigations are required to identify the active components and their mechanism responsible for these antioxidant and other properties.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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