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ESTIMATION OF TOTAL ALKALOIDS IN WILD AND *IN-VITRO* REGENERATED *TINOSPORA CORDIFOLIA*

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ABSTRACT: *Tinospora cordifolia* (Willd) Miers (Synonym: *T. sinensis* (Lour.) Merr.) is a genetically diverse plant known for its immense medicinal properties due to the presence of bioactive compounds among which alkaloids are most vital. So, the present study aimed to estimate the total alkaloid content in wild and *in-vitro* regenerated plant & callus tissue of *Tinospora cordifolia.* Different solvents, *i.e.*, methanol, ethyl acetate, water, diethyl ether, and chloroform, have been tried and compared for their extraction efficiency. The presences of alkaloids were tested *via* Wagner's and Mayer's reagents. The quantitative estimation had been made by UV/Vis spectrophotometer using a dye-Bromocresol green. The methanol extracts of wild plant's stem showed the highest concentration (3.3%) followed by water (1.85%) and ethyl acetate (0.99%). The alkaloid content in chloroform and diethyl ether extracts of wild plant's stem was 0.49% and 0.28% respectively. The *in-vitro* regenerated plant extracts also showed the presence of alkaloids (though in minute amount) in all solvents *viz*. methanol (0.95%), diethyl ether (0.060%), chloroform (0.012%), water (0.80%) and ethyl acetate (0.67%). The methanol, water and ethyl acetate extracts of callus tissue hold alkaloids in the order- 0.79%, 0.65%, and 0.39% but the diethyl ether and chloroform extracts displayed the absence of alkaloids. So, methanol was found to be the most suitable solvent for extraction of alkaloids. This work further proves that *in-vitro* regenerated plant and also the callus tissue of *Tinospora cordifolia* retain their parental characteristics of alkaloid biosynthesis, which can be enhanced further by altering the physical and hormonal parameters of the cultural environment.

INTRODUCTION: Medicinal plants are extensively used by ancient people as folk remedies, but now these herbal drugs are widely used in the pharmaceutical preparations of modern medicines [1]. Plant-based herbal medicines are preferred for health care due to their lower cost, effectiveness, and lesser side effects on the human body.

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The use of traditional medicine and medicinal plants in most developing countries, on a normative basis for the maintenance of good health, has been widely observed [2]. In recent years, the research focus is increasing on wide spectrum of activities starting from *in-vitro* propagation to metabolomics of medicinal plants.

Among different medicinal plant species, *Tinospora cordifolia* (Willd) Miers (Synonym: *T. sinensis* (Lour.) Merr.) (Family Menispermaceae) is one of such genera which have valued ecological, economical and medicinal importance. *Tinospora cordifolia* (Willd.) Miers is an important medicinal climber, found in tropical regions of India, China, Sri Lanka, and Bangladesh [3].

The stem portion of *Tinospora* is bitter, stomachic, diuretic [4], stimulates bile secretion; causes constipation; allay thirst, burning sensation, and vomiting; enriches the blood and cures jaundice and also used as anti-diabetics, anti-inflammatory, anti-arthritic, antioxidant, anti-stress, hapato-protective and antineoplastic. The extract of the mature stem is useful in skin diseases [5, 6]. The root and stem of *T. cordifolia* are prescribed in combination with other drugs as an antidote to snakebite and scorpion sting [7, 8]. The plant is also used in Ayurvedic 'Rasayanas' to improve the immune system and the body resistance against infections. The listed medicinal properties of this plant are attributed due to the presence of different chemical constituents such as glycosides, steroids, diterpenoids lactone and alkaloids [9-11]. So, because of the presence of bioactive constituents medicinal plants show medicinal properties [12-15]. The therapeutic efficacy of *Tinospora* is mainly due to the presence of alkaloids, among which, Berberine chloride is most important [16-21]. This alkaloid also serve as precursors for the biochemical synthesis of several other known bioactive alkaloids [22].

Realizing the importance of this plant and its extract in clinical medicine, we attempted to initiate tissue culture of *T. cordifolia* and determine its biosynthetic potential to accumulate alkaloid. The main advantage of tissue culture technology is that it could give a continuous supply of alkaloids irrespective of the season. Tissue culture also helps in studying and understanding the regulatory mechanisms involved in the synthesis of these alkaloids, which is difficult to carry out in the intact plant. So, the present study aimed to estimate the total alkaloid content in *in-vitro* regenerated plant & callus culture of *Tinospora cordifolia* and compare it with its wild counterpart.

The literature review on the methods for alkaloid estimation offered many techniques for determination and quantification of alkaloids which include titrimetric methods, fluorimetry [23], colorimetry [24], chromatographic methods such as HPLC [25], ion chromatography [26], gas chromatography [27] and electrochromatography [28]. But, there were certain demerits associated with these methods. With the titrimetric method, there were chances of variation in values of samples due to dependency on the skills of the person performing the procedure. The chromatographic methods were not suitable for herbal materials where the therapeutic activity derives from a complex mixture of many closely related compounds.

Under computer-controlled instrumentation, ultraviolet-visible molecular absorption spectrophotometry [29] have been reported to be used for the estimation of alkaloids in plant samples and formulations. The determination of total alkaloids using the visible spectrophotometric method with Bromocresol Green (BCG) testified to be convenient, simple, rapid and efficient technique compared to other methods such as chromatography and electrophoresis that requires special equipment. BCG can react with certain alkaloids, *i.e.*, the ones that have nitrogen inside their structure, but not with amine and amide alkaloids [30]. So, in the present study, we select a spectrophotometric method for the estimation of total alkaloids from wild and *in-vitro* regenerated *Tinospora cordifolia* plant.

MATERIALS AND METHODS:

Plant Material and Tissue Culture: Plant material was collected from Botanical Garden of Kurukshetra University, Kurukshetra. The material was identified by Prof. B. D. Vashistha of Department of Botany, Kurukshetra University, Kurukshetra. A voucher specimen (Herbarium/ Bot.K.U./Biotech.-3-2017) of the plant was deposited at the herbarium, Department of Botany, Kurukshetra University Kurukshetra.

Leaf, Stem and Root segments from the wild *Tinospora cordifolia* plant (located in Botanical garden, Kurukshetra University, Kurukshetra, Haryana, India) were collected and brought to the laboratory in a sealed plastic bag and stored at 4 °C. The identifications and authentication of the plant were done from the Department of Botany, Kurukshetra University, Kurukshetra. The plant material was washed several times under running tap water followed by surface sterilization in a laminar air flow cabinet. The surface sterilized explants were cultured on MS medium supplemented with diverse combinations and concentrations of plant growth regulators and maintained at 25 ± 2 °C, with a photoperiod of 16/8-h (light/dark) for *in-vitro* regeneration studies.

The incubated explants were allowed to grow for a period of 4 weeks. The stem portion exhibited good regeneration capability but the leaf and root explants displayed poor regeneration response. So, for further regeneration studies, stem explants were preferred for supplementary propagation. After 5 to 6 weeks of culture, callus & multiple shoots were established from stem explants. The callus formed was taken and periodically sub-cultured for further proliferation & multiplication. The individual shoots were excised and cultured for rooting in MS medium supplemented with IAA (Indole-3-acetic acid). After differentiation and establishment of plantlets, the rooted, healthy plants were removed from culture vessels and transferred to culture tubes containing sterile distilled water.

Ten days later, plantlets were transferred to plastic pots. The potted plantlets were maintained inside the culture room at 25 ± 2 °C, with a photoperiod of 16/8- h (light/dark). Subsequently, after 30 days, the plantlets were transplanted to pots containing normal garden soil and kept under shade in greenhouse for another 2 weeks before transferring to outdoors under full sun. Subsequently, the established *in-vitro* regenerated plantlets and the proliferated callus tissue from the stem explants were used for alkaloid estimation studies and compared further for alkaloid content with their wild counterpart, *i.e.* the fresh stems from *Tinospora cordifolia* located in Botanical garden of Kurukshetra University, Kurukshetra, and Haryana, India.

Extraction of Alkaloids using Different Solvents: The fresh stems of wild *Tinospora cordifolia* plant were taken and washed under running tap water to remove soil and other contaminants followed by rinsing with sterilized distilled water. The washed material was dried at 37 °C for 72 h. After drying, it was ground to powdered form. The coarsely powdered stem was extracted for 8 h using different solvents (10g/100ml), *i.e.* diethyl ether, ethyl acetate, chloroform, methanol, and water by Soxhlet apparatus. The extract was filtered and allowed to evaporate. The extract residues were stored in the refrigerator and used further for qualitative and quantitative estimation of total alkaloids. The *in-vitro* propagated plants and callus tissues were dried and extracted using the same method.

Qualitative Determination of Alkaloids: The qualitative estimation of alkaloids was made using Wagner's and Mayer's reagents. In Wagner's test, 1ml of plant extract was treated with 2 ml of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and examined for the formation of a reddish-brown precipitate (or coloration). In Mayer's test, to 2 ml of extract, 2 ml of concentrated hydrochloric acid was added, followed by the addition of 3 drops of Mayer's reagent. The appearance of greenish cream colored precipitate indicated the presence of alkaloids.

Quantitative Estimation of Alkaloids Content in Wild and Tissue Culture Raised Plant Samples through Spectrophotometric Method: The extracts which confirmed the presence of alkaloid were subjected for quantitative estimation of total alkaloids through UV/Vis spectrophotometric method.

To determine alkaloids content in different samples, some part of extract residue was dissolved in 2N HCl and filtered. 1 ml of this solution was transferred to separating funnel and washed 3 times with 10 ml chloroform. The pH of this solution was adjusted to neutral by 0.1N NaOH. After that, 5 ml of BCG solution and 5 ml of phosphate buffer were added to this solution. The complex was extracted with 1, 2, 3 and 4 ml chloroform by vigorous shaking, the extracts were collected in a 10 ml volumetric flask and diluted with chloroform up to the mark and subsequently quantification was done spectrophotometrically.

Instruments, Reagents, and Solutions: Spectrophotometric measurements were made on a double beam UV-Visible spectrophotometer (Systronics (India) Limited) with 1 cm UV quartz cell. Bromocresol green was taken as acidic dye and alkaloid Berberine chloride as a standard solution. All chemicals used were of analytical grade, and double distilled water was used throughout the experiment. Standard Berberine chloride was taken from Natural Remedies Pvt. Ltd., Bangalore, India.

Bromocresol green solution (BCG) (10^{-4}M) was prepared by heating at 50-60 ºC, 10-15 min of 69.8 mg bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until completely dissolved and

the solution was diluted to 1000 ml using distilled water. Phosphate buffer solution (pH 4.7) was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na_2HPO_4 in 1000 ml distilled water) to 4.7 (4.5 to 4.9) with 0.2 M citric acid (42.02 g citric acid in 1000 ml distilled water).

The standard solution of Berberine chloride (100 µg/ml) was prepared by dissolving 1.0 mg of Berberine chloride into 10 ml of methanol within the volumetric flask

Wavelength Scanning: The standard solution, sample, and control check solution were scanned from 350-500 nm for wavelength with chloroform as controls. The results indicated that standard and sample solution all have maximum absorption at 415 ± 1 as shown in **Fig. 1**. So, wavelength (415) was chosen for measuring the wavelength of all standard and samples used in the experiment.

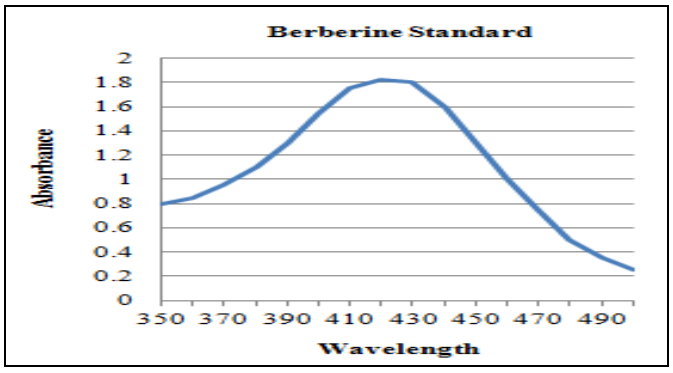

FIG. 1: THE SCANNING OF WAVELENGTH

Calibration Curve of Standard Berberine Chloride (20-100 µg/ml): The procedure [31] given by Patel *et al*., 2015 was followed to prepare calibration curve of Standard Berberine chloride. Suitable aliquots (0.2, 0.4, 0.6, 0.8 and 1 ml) of the standard stock solution were transferred to separating funnel to which 5 ml of phosphate buffer of pH 4.7 and 5 ml of bromocresol green (BCG) solution (10^{-4}M) was added. The mixture was shaken and the complex formed was extracted with 5 ml of chloroform. The chloroform layer was collected in 10 ml of volumetric flask, and more chloroform was added to make the volume up to mark. Absorbance was taken at 415 nm against the blank prepared as above but without Berberine.

The calibration curve **Fig. 2** was constructed by absorbance versus concentration of Berberine chloride as standard (µg/ml).

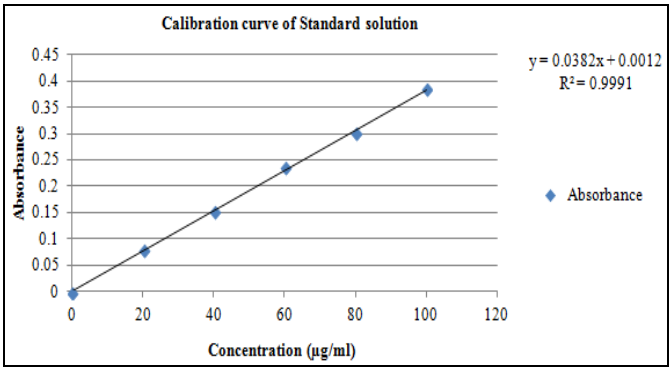

FIG. 2: CALIBRATION CURVE FOR STANDARD BERBERINE CHLORIDE

Method Validation: The method validation was according to International Conference on Harmonization guidelines for validation of analytical procedures [32, 33].

Linearity: The linear response was determined by analyzing six independent levels of the calibration curve in the range of 20-100 µg/ml for Berberine chloride. The results expressed in terms of correlation coefficient.

Precision: Intra-day precision and Inter-day precision were determined for a standard solution of Berberine chloride (40, 60, 80 µg/ml) for three times on the same day for intraday and on three different days for inter-day precision.

Limit of Detection and Limit of Quantization (LOD and LOQ): Calibration curve was repeated 3 times, and the standard deviation (σ) of the intercepts (response) was calculated. Then LOD and LOQ were measured by using mathematical expressions:

$$LOD = 3.3\sigma / S$$

$$LOQ = 10\ \sigma / S$$

Here, σ is the standard deviation of the response.
S is the slope of the calibration curve.
The slope may be estimated from the calibration curve of the analyte.

RESULTS AND DISCUSSION: The present study was conducted for the estimation of total alkaloids in different solvent extracts of plant *Tinospora cordifolia*. The results proved that the plant parts (Stem portion of wild plant, callus tissue, and *in-vitro* regenerated plant) used in this study contains alkaloids. The effectiveness of the extraction and estimation of total alkaloids in

different plant tissues was influenced by various factors, namely extraction procedure, solvent type, the particle size of the powder and the length of the extraction process.

The power of such factors for extraction of metabolites from Jembirit leaves was also reported by Salamah and Ningsih [34]. The significant influence of these parameters for alkaloid extraction in plant *Swietenia mahagoni* was presented by Ayuni and Nyoman [35]. In the processing of novel plant-based drugs, the first step is the screening of phytochemicals [36]. In the present work, we have taken five different solvents such as methanol, diethyl ether, chloroform, water, and ethyl acetate. The qualitative analysis of different plant extracts in different solvents showed the presence of alkaloids. The methanol, water and ethyl acetate extracts gave good results as compared to other solvents (Chloroform and Diethyl ether) extracts. The methanol extracts of wild plant's stem showed the highest concentration (3.3%) followed by water (1.85%) and ethyl acetate (0.99%). The alkaloid content in chloroform and diethyl ether extracts of wild plant's stem was 0.49% and 0.28% respectively. The *in-vitro* regenerated plant extracts also showed the presence of alkaloids (though in minute amount) in all solvents viz. methanol (0.95%), diethyl ether (0.060%), chloroform (0.012%), water (0.80%) and ethyl acetate (0.67%). The methanol, water and ethyl acetate extracts of callus tissue hold alkaloids in the order- 0.79%, 0.65%, and 0.39% but the diethyl ether and chloroform extracts displayed the absence of alkaloids. So, methanol was found to be the most suitable solvent for the extraction of alkaloids from wild and *in-vitro* regenerated *Tinospora cordifolia* **Table 3, 4** and **Fig. 3**.

The presence of alkaloids was evidenced by the cream-colored precipitate, *i.e.,* from the reaction of alkaloids with Mayer's reagent and the brown color precipitates from Wagner's reagent, as shown in **Table 1**. As chloroform and diethyl ether extracts of callus tissue showed the absence of alkaloids, so, these were not considered further for quantitative estimation of alkaloids.

TABLE 1: PRELIMINARY ANALYSIS OF ALKALOIDS IN DIFFERENT PLANT PARTS EXTRACTS

Tests for alkaloids/samples	Wild plant's stem extract					Callus tissue extract					*In-vitro* regenerated plant's extract				
	Met	Chl	DEE	EA	W	Met	Chl	DEE	EA	W	Met	Chl	DEE	EA	W
Wagner's Reagent	++	+	+	++	++	++	-	-	++	++	++	+	+	++	++
Mayer's Reagent	++	+	+	++	++	++	-	-	++	++	++	+	+	++	++

[The sign + represents the good results and the ++ represents the best results for the presence of alkaloids. Met- Methanol, Chl – Chloroform, DEE- Diethyl ether, EA- Ethyl acetate and W- Water]

When the presence of alkaloid was confirmed by preliminary methods, subsequently total alkaloids estimation was done *via* spectrophotometer method. The determination of total alkaloids using UV-Visible spectrophotometric method with Bromocresol Green (BCG) is a simple and sensitive technique that requires no special equipment. Bromocresol green react with alkaloids and form a yellow color complex, which can be extracted with chloroform and can be quantify using spectrophotometer [37].

The presence of total alkaloids in *T. cordifolia* plant's stem powder and its herbal formulations by spectrophotometer method was reported by Patel *et al*., in 2015 [24]. In our study, this method was exploited to extract total alkaloids from field grown *T. cordifolia* plant's stems and from *in-vitro* regenerated plant & callus tissue raised through tissue culture technology. The amount of total alkaloids from extracts of different plant tissue in diverse solvents, *i.e.* methanol, ethyl acetate, diethyl ether, aqueous and chloroform was calculated through spectrophotometer by comparing with the standard curve equation under same conditions.

The determination of operating time (OT) of the samples was performed because the exact alkaloid contents in plant material were not known. It was observed from the results that the standard operating time was 45-50 min while operating time of samples was 55-60 min. Furthermore, the maximum wavelength of BCG complex with Berberine was also observed **Fig. 1** and **2,** and it was found to be 415 nm. The curve for standard Berberine was measured at a maximum wavelength of 415 nm within an operating time of 58-60 min.

TABLE 2: RESULT OF VALIDATION OF PARAMETERS

Parameters	Berberine
Absorption Maxima (nm)	415
Slope	0.382
Intercept	0.001
Correlation coefficient (r^2)	0.999
Range (µg/ml)	20-100
Regression equation	y = 0.382x + 0.001
Intra-day Precision	0.408
Inter-day Precision	0.613
Limit of Detection (µg/ml)	0.017
Limit of Quantitation (µg/ml)	0.052

For standardization, alkaloid Berberine chloride was used. Berberine chloride reacted with BCG and the yellow color complex formed was extracted with chloroform at pH 4.7 and measured at λ_{max} of 415 nm. The UV-Visible spectra of standard Berberine chloride shown in **Fig. 2**. The calibration curve was constructed in the range of 20-100 µg/ml. It obeyed Lambert-Beer's law in this range.

The regression was calculated using the formula y = ax + b. The equation obtained from the standard curve is y = 0.382x + 0.001 where *x* is content (µg/mL), and *y* is absorbance. The *r*-value, calculated from the standard curve, is 0.9991. The quantitative analysis using spectrophotometer provided a dataset of the absorbance of each sample at different concentrations, as presented in graph **Fig. 2**.

The absorbance levels at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml (0.25 to 2 ml) of 0.1g/10ml (1000 µg/ml) solution of samples of the different plant's extracts (wild plant's stem part, *in-vitro* regenerated plant and callus tissue) in different solvents *i.e.,* methanol, diethyl ether, chloroform, ethyl acetate, and water were checked and found the best concentration of each solvent extracts, where the maximum amount of alkaloids were present. The best concentration for methanol, ethyl acetate, and water extracts was 0.150 µg/ml and for diethyl ether and chloroform extracts were 0.175 and 0.125 µg/ml as shown in **Table 3, 4** and **5** and **Fig. 3**.

TABLE 3: THE ABSORBANCE OF TOTAL ALKALOIDS IN DIFFERENT CONCENTRATIONS OF DIFFERENT SOLVENT EXTRACTS OF WILD PLANT'S STEM (*TINOSPORA CORDIFOLIA*)

S. no.	Samples	Concentration (µg/ml)	Absorbance	Amount of Total alkaloids in samples (µg) [x=y-0.001/0.382]	Total alkaloids Content (%)
1	Methanol	0.150	0.0136	0.0330 ± 0.00015	3.3
2	Diethyl ether	0.175	0.0028	0.0049 ± 0.00018	0.49
3	Chloroform	0.125	0.0020	0.0028 ± 0.00019	0.28
4	Water	0.150	0.0080	0.0185 ± 0.00010	1.85
5	Ethyl acetate	0.150	0.0047	0.0099 ± 0.00015	0.99

TABLE 4: THE ABSORBANCE OF TOTAL ALKALOIDS IN DIFFERENT CONCENTRATIONS OF DIFFERENT SOLVENT EXTRACTS *IN-VITRO* REGENERATED PLANT

S. no.	Samples	Concentration (µg/ml)	Absorbance	Amount of Total alkaloids in samples (µg) [x=y-0.001/0.382]	Total alkaloids Content (%)
1	Methanol	0.150	0.0374	0.00953 ± 0.0001	0.95
2	Diethyl ether	0.175	0.0012	0.00060 ± 0.00013	0.060
3	Chloroform	0.125	0.0055	0.0012 ± 0.0001	0.012
4	Water	0.150	0.0041	0.0083 ± 0.00017	0.80
5	Ethyl acetate	0.150	0.0035	0.0067 ± 0.00012	0.67

TABLE 5: THE ABSORBANCE OF TOTAL ALKALOIDS IN DIFFERENT CONCENTRATIONS OF DIFFERENT SOLVENT EXTRACTS OF CALLUS TISSUE

S. no.	Samples	Concentration (µg/ml)	Absorbance	Amount of Total alkaloids in samples (µg) [x=y-0.001/0.382]	Total alkaloids Content (%)
1	Methanol	0.150	0.0020	0.0079 ± 0.00017	0.79
2	Diethyl ether	0.175	0.0007	-	-
3	Chloroform	0.125	0.0009	-	-
4	Water	0.150	0.0038	0.0065 ± 0.00010	0.65
5	Ethyl acetate	0.150	0.0014	0.0039 ± 0.00019	0.39

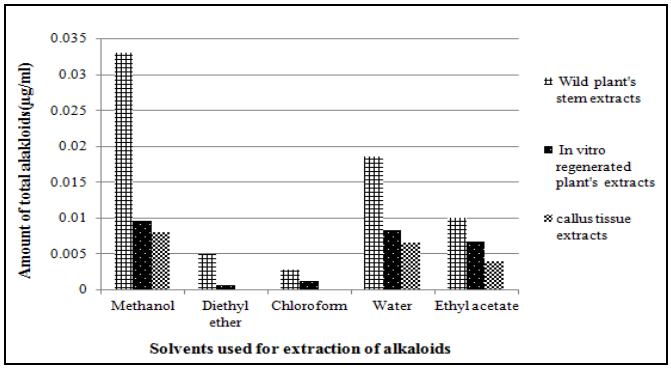

FIG. 3: CONCENTRATION OF TOTAL ALKALOIDS IN WILD PLANT'S STEM, *IN-VITRO* REGENERATED PLANT AND CALLUS TISSUE EXTRACTS OF DIFFERENT SOLVENTS

The alkaloids were estimated and characterized by comparing with spectral data (UV) with standard Berberine chloride. The standard values were in good agreement with the reported value for this compound [38]. Based on the above results, methanol was found to be the most suitable solvent followed by water and ethyl acetate. These solvents found to be more effective when used at a particular concentration. The best concentration for methanol, ethyl acetate, and water extracts was 0.150µg/ml and for diethyl ether and chloroform extracts were 0.175 and 0.125 µg/ml.

Results of alkaloid estimation of wild and tissue culture raised plant & callus tissue of *Tinospora cordifolia* showed the presence of alkaloid in parental explants (wild stem) as well as in their *in-vitro* raised counterparts (except for some quantitative differences) which proved that they retain their parental characteristics of alkaloid biosynthesis [39] which can be enhanced further by altering the physical and hormonal parameters of cultural environment. So, the utilization of tissue cultures technology for enhanced secondary metabolite production (alkaloids) of *Tinospora cordifolia* appears promising and beneficial as many of the curative properties of this plant depend on alkaloid contents.

These findings supported the use of *T. cordifolia* as anti-microbial, anti-inflammatory, anti-diabetic, antitumor, antioxidant, free radical scavenging agent, *etc*. Additionally, the spectrophotometer method used in this study was rapid, reliable, and sensitive and uses less dosage of sample. Thus, the estimation and quantification of total alkaloids by this method would be a promising approach.

CONCLUSION: The study has confirmed the presence of alkaloids in the wild and as well as in tissue culture raised *Tinospora cordifolia* by means of spectrophotometer analysis. So, the plant tissue culture technology can be exploited further to enhance and modify alkaloid & other metabolites of medicinal importance. Spectrophotometer method can be used for routine analysis of commercial plant samples dealing with standardization of alkaloids in pharmaceutical products because of its high sensitivity & simplicity.

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