Scientific Validation and Authentication of Genuine and Market samples of *Tinospora cordifolia*

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**ABSTRACT**

Guduchi (*Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms) is one of the most versatile rejuvenate herbs. The Sanskrit name guduchi means the one, which protects the body. It is also called as amrta or nectar, as it is extremely useful in strengthening the immune system of the body and keeping the functions of its various organs in harmony. Therefore, in the present research work attempts have been made to provide a complete study on Guduchi Satva where its authentication and validation was worked out in a series of manner. In standardization, *Tinospora cordifolia* Genuine and market sample were screened for various pharmacognostic characterization, Phytochemical screening and antibacterial assay. Studies revealed that market sample was full of adulterations and cannot be use safe and effective drug. This identification was used to form various markers for standardization of guduchi satva. These characters can further be used in Ayurvedic Pharmacopoeia as Key markers. Hence these markers can scientifically used to check the adulteration and retain the quality of drug.

**Key words:** *Tinospora cordifolia, Adulteration, Validation, Markers*

**INTRODUCTION**

Guduchi (*Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms) is also known as *Amrita* in Ayurvedic System of medicine. *Amrita* means divine nectar is attributed to the drug in recognition of its capacity to impart youthfulness, vitality, and longevity to the patient (1).

Drug consists of the dried stem with bark intact. The plant is mainly known for its medicinal properties in Ayurvedic Medicine (2). Guduchi satva was used in ancient time as general tonic and now experimentally established as anticancer (3), antiulcer (4), antipyretic (5), antihepatitis (6), immunomodulatory (7), antioxidant (8), hypoglycemic (9), antineoplastic (10), cardiotonic, antibacterial, antimicrobial, anti-inflammatory, antiarthritic, analgesic and diuretic (11) agent. A reverse phase HPLC method was also developed to quantitatively estimate the berberine content in the stem of *T. cordifolia* and *T. sinensis* (12).

Though it is a multipurpose drug still its Ayurvedic authentication is so poorly studied that various parameters to identify the guduchi satva from genuine sample are still not available. Various drugs having sufficient clinical data are guggul, brahmi, ashwagandha, amlaki, guduchi, kutki, shatavari and shunthi (13) Pharmacopoeia of India (1996) (14) covers few botanical monographs like clove, guggul, opium, mentha etc. The Ayurvedic Pharmacopoeia of India gives monographs for 258 different Ayurvedic drugs. The standards mentioned are quite inadequate to build quality of the botanical materials (15). As increasing demand of the authentic drug lead to adulteration of various formulations is a common phenomenon. Even micropropagation of multipurpose drug - *Tinospora cordifolia* was also attempted (16). Therefore, in the present research work attempts have been made to complete study on Guduchi Satva where its authentication and validation was worked out in a series of manner. In standardization of Guduchi satva, authentic stem of *Tinospora cordifolia* and Market sample of Giloy Satva were compared and microscopic, tlc and bio- markers were generated to prove the authentication of the drug. These markers can be used to procure safe, efficacious and quality product of guduchi at quarantines.

**MATERIALS AND METHODS**

**Plant Material**

**Collection and Identification**

Authentic samples: *Tinospora cordifolia* (Genuine sample) was procured from wild region and authenticated from Herbarium, Department of Botany, Mahatma Gandhi Institute of Applied Sciences, JECRC Campus, Jaipur, India and authenticate as Voucher specimen No. AMP 102. Market samples of Guduchi Satva (Baidyanath ltd.) was procured from Chunnilal Attar Ayurvedic Store, Ghat Gate, Jaipur in the month of October, 2008.

**Pharmacognostic Characterization:**

**Morphological Properties**
Microscopical evaluation deals with the identification of various characters of tissues, cells and cell contents by microscope. Methods of preparing specimens of crude materials for microscopical studies vary depending on the part used like leaf, stem, root, bark, flower, fruit and also on the nature of the material i.e. entire, cut or powdered.

**Preparation of sections:** Drugs which are hard to cut, are boiled for 20 to 30 min in water. Cross section or transverse section with a razor blade was taken. Thin materials such as leaves, slender stems or flat seeds are placed in a potato slit and sections were taken with ordinary blade. The section was placed on a slide, clear with chloral hydrate, covered with a cover glass and observed under microscope.

**Powder microscopy**
Likewise, powder of the selected species and their adulterants were subjected to microscopic analysis and the structures of various evaluations were drawn.

For examination of powder characteristics, sufficient amount of powdered drug (fruit or leaves) in chloral hydrate on a slide was warmed over a low flame or on a hot-plate for a short time, covered it with a cover glass and observed under microscope (4, 10, 20 or 45 X). Disintegration of hard and woody tissues: The material was cut into pieces and few pieces transferred to a test tube containing 4 ml of nitric acid and heated to boiling. Later, powdered potassium chloride was gently added, warmed and allowed to react leading to disintegration of the tissue. When completely bleached, pressure was applied with glass rod for complete disintegration of the tissues. The material was allowed to settle down, decant the liquid and the settled material washed repeatedly with water until the acidity is removed. The material was transferred on to a slide, a drop of glycerol added, covered with a cover glass and observed under microscope.

**Microscopic test**
Starch – For examining the presence of starch, the specimen was taken in I₂ wherein starch turns blue. Aleurone grains – For examining the presence of aleurone grains a specimen was prepared in I₂ and aleurone grains stained yellow. Fixed oil -For examining the presence of fixed oil, specimen was stained with sudan red resulting in the droplet of fixed oil to become pink coloured.

**Phytochemical Screening**

**Thin Layer Chromatography (TLC)**

**Extraction procedure**
Preparation of guduchi satva as per the recommendation of API, India (9). For TLC profile of selected species each of the dried and powdered (100 gm.) test samples of Guduchi stem and Giloy satva were Soxhlet extracted successively in petroleum ether, benzene, chloroform, ethyl acetate and methanol for 6 h. These extracts were filtered, evaporated to dryness and weighed. Each extract (10 mg) was dissolved in 10 ml to make a concentration of 1mg/ml used for further studies.

**TLC plates**
Each extract was applied on silica gel G Thin Layer Chromatography (TLC) coated plates (Merck: 20x20 cm; with thickness 0.2-0.3mm) which were activated at 100°C for 30 minutes and brought to room temperature, just before use. Each extract of various species was applied 1cm above the edge of the chromatographic plates along with the reference compounds and developed in air-tight chamber already saturated with ~200 ml of various solvent systems.

**TLC spraying reagents**
Various extracts of test samples were subjected to different solvent systems for identification of any significant biomolecules. After having used different solvent systems, on the basis of better resolution of spots for generating “Thin Layer Chromatography [TLC] fingerprints” for chemical libraries of the test drugs following solvent systems were used in the present study- hexane :acetone (3:1) for petroleum ether extracts, benzene :ethyl acetate (1:2) for benzene extracts, chloroform : methanol (1:2) for chloroform extracts, benzene : ethyl acetate (2:1) for ethyl acetate extracts, and chloroform :methanol (2:1) for methanol extracts.

**TLC spraying reagents**
During the work of present studies, different visualizing reagents i.e. 10% sulphuric acid [10 ml conc. sulphuric acid dissolved in 100ml absolute alcohol], I₂ vapour (Saturated iodine chamber) and Dragendorff reagent were used.

**Qualitative TLC**
Thin glass plates were coated (0.2-0.3 mm) with silica gel G (30 g/60 ml distilled water) and dried at room temperature. The coated plates were activated in an oven at 100°C for 30 minutes and cooled. The plates were then placed in developing tanks having ~ 150 ml of an organic solvent mixture of pet ether: benzene (1:3). The lid of the developing tanks was sealed with vacuum grease. The plates were removed after making the solvent front and were air-dried. The dried plates were sprayed with Dragendorff reagent (8 g bismuth subnitrate dissolved in 25 ml 30% HNO₃ and further addition of 28 g KI and 1 ml of 6 N HCl) and alkaloid positive spot (Rf value) was calculated.

**Antibacterial Assay**

**Sources of test organisms**
Bacteria – Pure culture of all test organisms, namely
Escherichia coli (Gram negative), Pseudomonas aeruginosa (Gram negative) and Bacillus subtilis (Gram positive), Staphylococcus aureus (Gram positive), were obtained through the courtesy of Microbiology Lab, Mahatma Gandhi Institute of applied Science (MGiaS), Jaipur, which were maintained on Nutrient broth media.

**Culture of test microbes**

For the cultivation of bacteria, Nutrient Agar Medium (NAM) was prepared using 20 gm Agar, 5 gm Peptone, 3 gm beef extract and 3 gm NaCl in one liter distilled water and sterilized at 15 lbs pressure and 121°C temperature for 25-30 minutes. Agar test plates were prepared by pouring approximately 15 ml of NAM into the Petri dishes (10 mm) under aseptic conditions. A saline solution was prepared (by mixing 0.8 % NaCl) in distilled water, followed by autoclaving and the bacterial cultures were maintained on this medium by regular sub-culturing and incubation at 37°C for 24 hours.

To prepare the test plates, in bacteria, 10-15 ml of the respective medium was poured into the Petri plates and used for screening. For assessing the bactericidal efficacy, a fresh suspension of the test bacteria was prepared in saline solution from a freshly grown Agar slant.

**Preparation of test extracts**

Crushed powder (50 g) of all the species were successively soxhlet extracted with petroleum ether (60°-80°C), Benzene, Chloroform, Ethyl acetate, Methanol and Distilled water. Later, each of the homogenates was filtered and the residue was re-extracted twice for complete exhaustion, the extracts were pooled individually. Each filtrate was concentrated to dryness in vitro and re dissolved in respective solvents, out of which 80 mg/10 disc i.e. 8 mg/disc concentration were stored at 4°C in a refrigerator, until screened for antibacterial activity.

**Bactericidal assay**

For both, bactericidal in vitro Disc diffusion method was adopted (19), because of reproducibility and precision. The different test organisms were proceeded separately using a sterile swab over previously sterilized culture medium plates and the zone of inhibition were measured around sterilized dried discs of Whatman No.1 paper (6 mm in diameter), which were containing 8 mg of the test extracts, its control (of the respective solvent) and tetracycline as reference drugs separately. Such treated discs were air-dried at room temperature to remove any residual solvent, which might interfere with the determination, sterilized and inoculated. These plates were initially placed at low temperature for 1 h so as to allow the maximum diffusion of the compounds from the test disc into the agar plate and later, incubated at 37°C for 24 h in case of bacteria, after which the zones of inhibition could be easily observed. Five replicates of each test extract were examined and the mean values were then referred. The inhibition zones in each case were recorded and the activity index (AI) was calculated as compared with those of their respective standard reference drugs (AI = Inhibition Zone of test sample / Inhibition zone of standard).

**RESULTS AND DISCUSSION**

**Microscopic Identification:**

In microscopic studies, powdered drugs was studied for its starch grain at 10 and 40 x. resultant showed that *Tinospora cordifolia* (genuine) and Giloy satva (market sample) have different starch grains, simultaneously various other starch grains were studied for comparison. The structure of starch grain present in stem powder of *Tinospora cordifolia* are somewhat oval with concentric rings and hilum eccentric but it was surprising to see that slides of market sample resembled to the starch grain of maize powder (*Zea mays*) (Fig. 1 and 2).

Therefore it was proved that the market sample of guduchi satva is adulterated by powder of maize, a cheap adulterant and hence proved that microscopic character can safely be used in the screening and identification of *Tinospora cordifolia* in guduchi satva. Further, this identification character can be safely used in Ayurvedic Pharmacopoeia and check the quality of the drug and enhance its therapeutic potentials.

**TLC Chromatograms as fingerprints**

Thin Layer Chromatography has been regarded as a simple, rapid and inexpensive method for the separation, identification and semi-quantification of a wide variety of substances. An attempt was made to compare various extracts of *Tinospora cordifolia* and Giloy satva by running the chromo plates in different solvents so as to generate various fingerprints which can later be useful in its standardization.

**Tinospora cordifolia** (Genuine) TLC

In *Tinospora cordifolia* petroleum ether extract in solvent system – petroleum ether: benzene (1:3) showed three spots on exposure to 10% sulphuric acid were seen at Rf value of 0.09 (purple), 0.21(light pink) and 0.34 (pink); benzene extract in solvent system – benzene : ethyl acetate (1:2) showed seven spots on exposure to 10% sulphuric acid were seen at Rf value of 0.06 (greenish brown), 0.14 (pinkish brown), 0.20 and 0.30 (dark pink), 0.40 (dark pinkish brown), 0.57 (pinkish green) and 0.99(dark green); chloroform extract in solvent system – chloroform: methanol (1:2) showed nine spots on exposure to 10% sulphuric acid were seen at Rf value of 0.04 (dark green), 0.11 (yellowish green), 0.22 (Light yellowish green), 0.36 (parrot green), 0.45 (light yellowish green), 0.52 (parrot green), 0.73 (light pink), 0.79 (dark pinkish green) and 0.88 (greenish brown).
parrot green), 0.59 (light green), 0.63 (very light green), 0.82 (light brownish green) and 0.91 (dark brownish green); ethyl acetate extract in solvent system - benzene: ethyl acetate (2:1) showed two spots on exposure to 10% sulphuric acid were seen at Rf of 0.09 (very light yellow) & 0.93 (light yellow); methanol extract in solvent system - chloroform: methanol (2:1) showed four spots on exposure to Dragendroff reagent were seen at Rf value of 0.09 (buff orange), 0.60 (light buff orange), 0.85 (buff orange) & 0.97 (dark buff orange) (Fig. 3).

**Giloy satva (Market Sample) TLC**

In Giloy satva petroleum ether extract in solvent system - petroleum ether: benzene (1:3); benzene extract in solvent system - benzene: ethyl acetate (1:2); ethyl acetate extract in solvent system - benzene: ethyl acetate (2:1); methanol extract in solvent system - chloroform: methanol (2:1) showed no spot on exposure to 10% sulphuric acid; chloroform extract in solvent system - chloroform: methanol (1:2) showed one spot on exposure to 10% sulphuric acid was seen at Rf value of 0.95 (light yellow) (Fig. 4).

These results showed that the market sample not possessing bioactives that were present in authentic sample. Therefore, these TLC fingerprints of genuine sample can be safely used in future for generation of chemical libraries.

**Antibacterial Efficacy**

Various sequential extracts of Guduchi (*Tinospora cordifolia*) and Giloy satva (market sample) were screened for various test microbes and their inhibition zones and activity indexes were calculated.

**Antibacterial efficacy of *Tinospora cordifolia***

Antibacterial activity of *Tinospora cordifolia* was performed against *Escherichia coli*, maximum efficacy was exhibited by distilled water extract (IZ – 24mm); *Bacillus subtilis*, maximum efficacy was exhibited by methanol extract (IZ – 11mm), mediocre efficacies were shown by pet ether, benzene, ethyl acetate and distilled water extracts having inhibition zone-10mm, 9mm, 8mm & 7mm respectively; *Pseudomonas aeruginosa*, maximum efficacy was exhibited by methanol extract (IZ – 12mm); *Staphylococcus aureus*, maximum efficacy was exhibited by distilled water extract (IZ – 17 mm). While, mediocre efficacies were shown by pet ether, benzene, chloroform and ethyl acetate extracts having inhibition zone-12mm, 9mm, 8mm & 12mm respectively (Fig. 5).

**Antibacterial efficacy of Giloy satva** market sample

Antibacterial activity of Giloy satva was performed against *Escherichia coli*, maximum efficacy was exhibited by distilled water extract (IZ – 22 mm) and no efficacies were shown by pet ether, benzene, chloroform and ethyl acetate; *Bacillus subtilis*, maximum efficacy was exhibited by methanol extract (IZ – 14 mm) and no efficacies were shown by pet ether, benzene, chloroform and ethyl acetate; *Pseudomonas aeruginosa*, maximum efficacy was exhibited by methanol extract (IZ – 11mm) and no efficacies were shown by pet ether, benzene, chloroform and ethyl acetate; *Staphylococcus aureus*, the maximum efficacy was exhibited by methanol and distilled water (IZ – 13 mm) and minimum efficacy was shown by pet ether, benzene, chloroform and ethyl acetate (Table 1).
Standardization of ayurvedic drugs and plant materials is the need of the day. Several Pharmacopoeias containing monographs on plant materials describe only the physico-chemical parameters. Hence, modern methods describing the identification and quantification of active compounds in the plant material may be useful for proper standardization of herbs and their formulation. *Tinospora cordifolia* (Menispermaceae) is one such plant which is widely used in indigenous system of medicine. Among the complex mixture of biologically active compounds in the plant, microscopic identification, TLC, and bio efficacies can be used as an analytical characterization to determine the quality of plant material of different sources. It is very surprising that the simple tests which are not useful in identification sometimes become very beneficial for the characterization and validation of important Ayurvedic drugs. Hence, the Pharmacognostic markers are simply an authentic compounds in the plant material may be useful for one such plant which is widely used in indigenous which are not useful in identification sometimes become very beneficial for the characterization and validation of important Ayurvedic drugs. Hence, the Pharmacognostic markers are simply an authentic...
if we use the correct parameters for the identification of the drug it will be very useful for Ayurvedic drugs to not only enhance their quality and efficacy but also their therapeutic potentials as drugs.

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