A DISSERTATION ON

"CHARACTERIZING ASHWAGANDHA VARIETIES OF INDIA"

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES

INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILLMENT

FOR THE

DEGREE OF MASTER OF SCIENCE

IN BIOTECHNOLOGY

BY

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MSc. Biotechnology

Semester – IV-2022

Department of Biosciences,

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UNDER THE SUPERVISION OF

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CSIR CIMAP, LUCKNOW

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(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
पोस्ट आफिस - सीमैप, लखनऊ-226015, भारत

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TO WHOM IT MAY CONCERN

This is to certify that the dissertation entitled, "Characterizing Ashwagandha Varieties of India" is an original research work carried out by Ms. Alia Ansari, (Enroll.no: 2000102024), under the guidance of Dr Tripta Jhang, Principal Scientist, Division of Plant Breeding and Genetic Resource Conservation, CSIR-CIMAP, Lucknow during 1st February, 2022-31st May, 2022, for the partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology of the Integral University, Lucknow and that no part of this work has been presented earlier for any degree or diploma in this or any of the universities.

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This is to certify that the study conducted by Ms. **ALIA ANSARI** during the months Feb-May, 2022 reported in the present thesis was under my Co-supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is "**CHARACTERIZING ASHWAGANDHA VARIETIES OF INDIA"** is therefore, being forwarded for the acceptance in partial fulfillment of the requirements for the award of the degree of M. Sc Biotechnology, Department of Biosciences, Integral University, Lucknow, (U.P).

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TO WHOM IT MAY CONCERN

This is to certify that **Ms ALIA ANSARI** a student of M.Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled "**CHARACTERIZING ASHWAGANDHA VARIETIES OF INDIA"** successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. Tripta Jhang,** Principal Scientist, Plant Breeding and Genetic Resource Conservation Division at CSIR- Central Institute of Medicinal and Aromatic Plants, Lucknow. The dissertation was a compulsory part of her M.Sc. degree. I wish her good luck and bright future.

(**Dr. Snober S. Mir**)
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DECLARATION

I declare that this thesis entitled, “CHARACTERIZING ASHWAGANDHA VARIETIES OF INDIA" submitted to INTEGRAL UNIVERSITY for the partial fulfilment of the master’s degree in biotechnology is a presentation of my original research work and has been composed solely by myself. The work was done under the guidance of **Dr. Tripta Jhang,** Principal Scientist, Plant Breeding and Genetic Resource Conservation Division at CSIR- Central Institute of Medicinal and Aromatic Plants, Lucknow. It has not been submitted, in whole or in part, in any previous application for a degree. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of elaborative research and discussions.

Alia Ansari

Date

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ABSTRACT

Ashwagandha *[Withania somnifera* (L.) Dunal] known as Indian ginseng or winter cherry, has been an essential plant in Ayurvedic and indigenous medicine. In the present investigation nature and extent of genetic diversity were assessed among twelve genotypes of Ashwagandha through SSR markers.

Content

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
BPB	Bromo phenol blue
Conc.	Concentration
CTAB	Cetyl tri ammonium bromide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
Kb	Kilobase pair
M	Molar
Mg	Milligram
Min	Minute
ML	Milli liter
Mm	Millimolar
Ng	Nanogram
PCR	Polymerase chain reaction
PVP	Poly vinyl pyrrolidone
Rpm	Revolution per minute
TBE	Tris Borate EDTA
TE	Tris EDTA
U	Unit
UV	Ultraviolet
V	Volt

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CHAPTER 1

INTRODUCTION

Ashwagandha *[Withania somnifera* (L.) Dunal] known as Indian ginseng or winter cherry, has been an essential plant in Ayurvedic and indigenous medicine. It is a member of the Solanaceae family and has the chromosome number 2n=48. Only twospecies, *W. somnifera*(L.) Dunal and *W. coagulans*(L.)Dunal, are found primarily in India. Ashwagandha has been utilized as a home medicine for a variety of ailments. It can be found in various parts of India as well as North Africa's Mediterranean region. Madhya Pradesh, Rajasthan, Punjab, Himachal Pradesh, and Uttar Pradesh are among the states in India where it is grown. (B. Parita et al 2018).

The generic name Withania is derived from two Latin words "somnus" (meaning sleep) and "fero" (meaning to bear), and is in honour of Sir Henry Thomas Maire Witham (an English paleobotanist), with an orthographic variation of the last alphabet "m" into "n" with the addition of a commemorative termination "ia," whereas the specific epithet "somnifera" is derived from two Latin words "somnus" (meaning sleep) and "fer (Mir et al., 2012). Because of the resemblance of the fragrance of its roots to that of a sweating horse, its most frequent Indian name Ashwagandha was derived from two terms "ashva" (meaning horse) and "gandha" (meaning smelling) (Rajeswara Rao et al., 2012). It is one of the 36 plants already under cultivation, owing to their strong demand, which necessitates crop development activities to develop/provide premier cultivars to growers in order to boost farm profitability (Ved and Goraya, 2007). It has also been included in World Health Organization (WHO) monographs on selected medicinal plants due to its enormous therapeutic potential (Mirjalili et al., 2009). It is described as a herbal tonic and health food in Vedas and is considered as 'Indian ginseng' in the traditional Indian medicine.

Various withanolides have been isolated from *W. somnifera.* Withaferin A and 3-*β*-hydroxy-2,3-dihydrowithanolide F show promising antibacterial, antitumor, immunomodulating and anti-inflammatory properties (Budhiraja and Sudhir, 1987). Glycowithanolides withafurin-A and sitoindosides VII-X isolated from the roots of *W. somnifera* significantly reversed ibotenic acid induced cognitive defecits in Alzheimer's

disease model (Bhattacharya et al., 1995). The aerial parts of *W. somnifera* yielded 5-dehydroxywithanolide-R and withasomniferin-A (Atta-ur-Rahman et al., 1991).

Root and leaves are the main parts of the plants. Both leaves and roots of the plant are used as drugs. The dried roots are the primary economic part widely used as a sedative, general stimulant, tonic; for treating senile debility, inflammation, rheumatism, female disorders, dropsy, ulcers, scabies, dyspepsia, lumbar pains, abortion, and hiccups.

The major chemical constituents of the *Withania* genus, the Withanolides, are a group of naturally occurring C28-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. It possesses adaptogenic, tonic analgesic, antipyretic, anti-inflammatory, and abortifacient properties and is one of the most extensively used plants in various systems of medicine (Chopra *et al.* 1958).

Recently, various molecular marker techniques have been developed into powerful tools for diversity analysis and establishing relationship between cultivars, but very few reports are available on the genetic diversity of *W. somnifera*. Several PCR based markers have been used to provide information on genetic variation, evolutionary and phylogenic relatedness in plant species. PCR based approaches are in demand because of their simplicity and reliability along with low cost. Different genetic markers based on DNA polymorphism like Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and Single-Nucleotide Polymorphism (SNP) have been used for characterization of cultivars **(**Parita et al.,2018).

Among various classes of molecular markers, simple sequence repeats/microsatellites which are tandem repeats of one to six nucleotide long DNA motifs, have gained significant importance in plant breeding and genetics due to their multi-allelic nature, hypervariability, co-dominant inheritance, relative abundance, reproducibility, chromosome specific location, good genome coverage including organellar genomes, amenability to automation and high throughput genotyping**.**

TABLE 1: Synonyms of *Withania somnifera*

Sanskrit	Ashwagandha, Turangi-gandha
English	Winter Cherry
Hindi	Punir, Asgandh
Bengali	Ashwagandha
Gujrati	Ghodakun, Ghoda, Asoda, Asan
Telugu	Pulivendram,Panneru-gadda, panneru
Tamil	Amukkura, amkulang, amukkuram-kilangu, aswagandhi
Karnataka	Viremaddlinagadde,pannaeru, aswagandhi, kiremallinagid
Goa	Fatarfoda
Panjabi	Asgandh,Isgand
Bombay	Asgund, asvagandha
Rajasthani	Chirpotan

1.2TAXONOMY

- Kingdom: Plantae
- Subkingdom: Traheobionta
- Phylum: Spermatophyta
- Subphylum: Angiospermae
- Class: Dicotyledonous
- Order: Solanales
- Family: Solanaceae
- Genus: *Withania*
- Species: *somnifera*

1.3 species of Withania

Withania adpressa Cors.

Withania adunensis Vierh

Withania arborescens Dunal

Withania aristate Pauquy

Withania begoniifolia (Roxb.) Hunz. & Barboza

Withania breviflora Hunz

Withania chevalieri A. E. Gonc

Withania coagulens (Stocks) Dunal

Withania frutescens (L.) Pauquy

Withania grosii Pau

Withania holstii Dammer

Withania japonica (Franch. & Sav.) Hunz.

Withania macrocalyx (Chiov.) Chiov.

Withania mollis Dunal

Withania morisoni Dunal

Withania mucronate Chiov.

Withania novo Dunal

Withania novo-friburgensis Dunal

Withania obtusifolia Täckh.

Withania oocarpa Dunal

Withania origanifolia Paillieux&Bois

Withania pyrifolia Dunal

Withania qaraitica A.G. Mill. &Biagi

Withania reichenbachii Bitter

Withania reibeckii Sc hweinf. Ex Balf. f.

Withania sicula Lojac

Withania simonyaua R. Wagner ex Vierh.

Withania somnifera (L.) Dunal

Withania sphaerocarpa Hepper& Boulos

Withania suberosa Endl.

Withania villosa Pauquy

Objective:

1 To isolate DNA of 12 Ashwagandha varieties

2. To assess the genetic diversity among 12 varieties using Simple Sequence Repeat (SSR) markers

CHAPTER 2

2. REVIEW OF LITERATURE

Withania somnifera is an erect, evergreen (green in whole year), branching, tomentose shrub of 30 to 150 cm in height. Leaves are simple, petiolate with the leaf blade varying in shape from elliptic-ovate to broadly ovate, entire along margins, acute to obtuse at apex, oblique at base, clothed with a persistent grayish tomentum on sides, 4-10cm long and 2-7cm broad. Leaves on vegetative shoots are alternate and large and those on floral branches are opposite, arranged somewhat laterally in pairs of one small leaf and one large leaf, bearing in their axil a cymose cluster of 4-25 inconspicuous pale green monecious flowers. It produces flowers indeterminately round the year with a peak of flowering between March and July (Mirjalili et al 2009).

Traditional India has the most medicinal plant knowledge and is the largest producer of herbal medicines, accounting for nearly 40% of global production. Similar to another traditional system of medicines, the medicinal and aromatic plants constitute the backbone of Ayurveda. *Withania somnifera* and *W. coagulans* two such plants of genus *Withania* (Solanaceae) are widely described `as predominant medicinal plants in Indian and other traditional systems of medicine (Tuli and Sangwan 2010).

Recently, the medicinal plant has attracted more scientific and commercial attention due to the development of plant-based drug discovery and the popularization of drugs from plants. Important leads have been achieved against many pharmacological disorders like Alzheimer's, malaria, HIV/AIDS, and Cancer by implying natural products as drug molecules (Balunas and Kingdom 2005).Inthe plant kingdom, Solanaceae family is one of the largest families, it has 83 genera and more than 3000 species. It is very widely distributed throughout the world and the species diversity of this family has been reported highest in tropical South America which is also thought to be its center of origin for several Solanaceae species (Albuquerque et. al., 2006). This family is well-reputed for its economic importance, plants are known for their pharmacological values and genus *Withania* is one of them. *W. somnifera* is used in the traditional medical system dating back to more than 3000 years. The first indication of its use has been traced to Ayurveda which is the backbone of Traditional

Indian Medicine (TIM) is considered to be older than Traditional Chinese Medicines (TCM). The national medicinal plant board (NMPB) of India considers *W. somnifera* to be one of the top thirty-two most demanding medicinal plants, as evidenced by its high demand. It is estimated that the yearly global demand for withanolide-rich plants (an active constituent *of W. somnifera*) exceeds the annual supply, which is 5,905 tonnes. Approximately 12,120 tonnes are yearly demand, while 5,905 are annual supply (Sharada *et al.* 2007; Ramawat and Goyal 2008).

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W. somnifera L. Dunal*,* a member of the Solanaceae family, has been the most reputed Indian medicinal plant with immense therapeutic uses in Ayurveda, medicines system of Indian traditional, and several former conventional drug practices (Jaleel et al. 2009). It was recently reviewed that, in Sanskrit, Withaniais called Ashwagandha, meaning horse's smell due to its root odor resemblance with the sweaty horse. Other names for Withania are Vajigandha (means the smell of the strenagth), Varada (means granting wishes), Vajikari (means strengthening), Palashparni (poisoning leaves), and Vajiini (what promotes pregnancy). Ashwagandha is also known as Indian ginseng, Winter cherry, Amukkara in Sinhala, and Amukkrang Kilangu in Tamil (Fernandoania species, *W. somnifera* Dunal and W. coagulans Dunal are found in India. The specific epithet 'somnifera' originated from two Latin words 'Somnus' meaning sleep and 'fero'(free) meaning 'to bear' to indicate the sleep-inducing properties of the plant. In Bangladesh, Burma, and India, about 90%, 85%, and 80% of patients were treated with the medicinal drug by the practitioners of the traditional system (WHO, 1993). All parts of the plant contain chemical compounds called Withanolides/steroidal lactones to which most of the pharmacological activity is attributed (Asthana 1989; Bhattacharya et al. 1987; Devi et al. 1993; Davis et al. 2000). Although the presence of five distinct chemotypes of this species (three from Israel and one each from South Africa and India) has been reported (Abraham et al. 1968,

(Chakrabarti et al. 1974), the exact number of such chemotypes has yet to be determined through chemical profiling. Indian genetic resources (both wild and cultivated) exhibit a high level of morphological and phytochemical variability, which is largely unknown. Five morphotypes with morphological variability have been reported (Atal et al. 1975), as well as phytochemical variability in commercial products derived from ashwagandha preparations. Ashwagandha root texture determined by the starch and fiber content plays a significant role in the market price of the roots benefiting the farming community. Brittle roots because of their ease in making powder and are quoted to be characteristic root textural features of commercial ashwagandha (Atal and Schwarting, 1962). Studied several populations in the Indian region and reported 5 morphotypes exhibiting variability in morphology, especially in that of the root. Morphological, chemical, and molecular variability of different ashwagandha morphotypes has been studied by several researchers (Negi et al., 2000, 2006; Dhar et al., 2006; Arun Kumar et al., 2007).

Recently, a total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots have been identified, out of which 29 are common to both. It is also reported that the distribution of secondary metabolites varies significantly concerning different tissues, developmental stages, and chemotypes (Chatterjee et al., 2010; Dhar et al., 2013)

2.1. Different species of Withania

There are 26 species in the genus Withania, but only a few of them have been studied by modern pharmacologists for modern pharmacological research. *Withania somnifera*, *Withania coagulans*, *Withania frutescens*, *W.aristata, Withania obtusifolia*are some of the species of *Withania*. Species.*W. somnifera*is the most explored medicinal species in this genus.

A **B**

C **D**

FIGURE1. (A) Cultivated plant in the field at CSIR-CIMAP, **(B)** Fruits of *Withania somnifera,* immature fruits are green in colour (C) Ripened berries become deep red in colour (D) Root of *Withania somnifera*

2.2. DISTRIBUTION GEOGRAPHICAL:

The genus Withania is restricted and related to the old World; rather it closely belongs to the genus Physalis, the gooseberries. Withania possesses a natural occurrence, most probably in the drier and humid areas, spread from the Mediterranean region to throughout tropical region of Africa to South Africa and also from the Cape Verde Islands and Canary region to the Arabia and Middle East region like India, southern China and Sri Lanka. Ashwagandha is propagated and cultivated in gardens in the warmer and drier regions of Europe and became a natural herb in New South Wales and South Australia.

Generally, it is cultivated in India and in many other places as a medicinal crop (Govindaraju et al 2003), most probably for its fleshy roots. Ashwagandha is globally known but is not so common in all regions of South Africa, Botswana, Namibia, Lesotho and Swaziland. It is total absent in the western half of the Western and Northern Cape regions. It develops and cultivates in a wide range of vegetation types in dry and warm areas to areas with usually high humid region with high rainfall like coastal vegetation, savanna, grassland, scrubland, karoo, woodland, and mostly in margins of forests and thickets, besides water also, as on the river banks. Its presence is also observed in light shaded dark places as well as in full sun places, mostly among rocks where the roots are being kept cool.

2.3. HABITAT:

Ashwagandha grows in dry areas in India, on the Himalayas under 1600m, Baluchistan, Sri Lanka and in the Mediterranean area: spontaneous in Sicily and Sardinia (Kapoor 1990d; Kirtikar et al 1993). Used parts root, leaf, seed (Kapoor 1990d). *Withania somnifera* L. Dunal is a common herbaceous evergreen shrub of 30-150cm height. It grows as a weed along roadsides and in open waste places. It is distributed throughout the drier parts of India. It is now cultivated at different parts of the country due to its medicinal importance. The plant is usually clothed with minutely stellate tomentum. The leaves are 5-10 cm long, simple alternate, ovate, entire, thin with cuneate/connate base and are densely hairy with reticulate venation. However, near the inflorescence leaves are opposite with adnate.

2.4. Morphological characteristics:

Withania is a small or medium undershrub, 30-150cm height, erect, grayish, branching perennial, with strong disagreeable odor like horse's urine. Almost the complete plant is covered and surrounded with very short, small, fine, branched hairs and silver-grey in colour. The stems of Withania are brownish dark colour and erect, sometimes leaves are absent or less on lower part of stem. The leaves are in an alternate manner (opposite on flowering shoots), simple, possessing margins are slightly waved, narrowed into the 5–20mm long petioles, normally broadly ovate or oblong, 29–80mm long and 21–50mm broad. It is generally referred to as stellate tomentose, grayish, under shrub of 30-150cm high with long woody tuberous roots.

Flowers are generally small, greenish, axillary, monoecious or bisexual and solitary or in few-flowered cymes.

Seeds are normally many, discoid, reniform and yellow. The number of chromosome is 2n = 48 (Schonbeck 1972; Hepper 1991; Mozaffarian 2003). The corolla is 5-lobed, constrictly campanulate, 5–8mm long and light yellow to yellow-green in colour.

Ashwagandha fruit is usually a round hairless berry, 5– 8mm across, orange red to red in ripped condition and is enveloped by the enlarged calyx. Most of the seeds are very pale brown, 2.5mm across, sometimes kidney-shaped and squeezed with a rough surface and netted surface.

In Withania flowering time is generally from October to June, whereas the fruiting time is usually from October to July. *Withania somnifera* can be identified by the red fruit enclosed by the brownish, papery, turgid calyx. Collectors have mentioned it as a bad-smelling bush with generally strong-smelling roots and have also described that the leaves have a strong smell of green tomatoes (Mirjalili et al 2009).

Seeds: Numerous, yellow white, reniform, laterally compressed, poisonous (Kapoor 1990d; Kirtikar et al 1993).

STEM: Branches ligneous, tomentose at the apex covered with minute stellately hairs.

Leaves: Leaves simple, 2–6cm wide, 3–8cm long, alternate, petiole 1–2cm long. Leaves are ovate, glabrous, simple more than 10cm long.

Flowers: Flowers sessile, axillary clusters, 1cm long, it blossoms nearly throughout the year. Corolla greenish or yellow or white yellowish, 5mm long, lobes lancelet, acute and thin, calyx is visibly expanded around the fruit. The flowers appear in a bright yellow or greenish and it carries small berries which are orange red in color. Fruits are orange-red, carrying numerous seeds. Florescence appears and falls in spring season (Davis &Kuttan 2000). The stapet or filament base is appeared to the ovary and a groove between every stapet helps to allow the nectar to flow upward from the nectary at the ovary base (Kothari et al 2003). There are large numbers of trichomes present in stapet which also secrete some amount of nectar. Trichomes are also present on outer surface of calyx and corolla. Nectaries on different floral parts attract insects. There are 3200- 4000 pollen/anther and 16,250- 20,000 pollen/flower. Each ovary contains 26-32 ovules. Thus, the pollen ovule ratio is 625:1 indicating facultative xenogamy (Cruden 1977).

Roots: The roots of Ashwagandha are fleshy when dry, they are straight, cylindrical, tapering down, gradually unbranched of about 10-17.5cm long and 6-12milimeter diameter in thick. The main roots are brownish outer and creamy interior and bear fiber similar secondary roots having acrid taste and biter (Anonymous 1982). Roots are stout, fleshy and whitish brown in colour. Leaves simple, petiolate, elliptic-ovate to broadly ovate, entire, estipulate, cunate or oblique, glabrous, up to 10cm long, those in the floral region are smaller and opposite. Single layered epidermis presenting young root with 4-5 layers of cells of parenchymatous cortex whiles the endodermis being present as casparian stripes. Outer most layer of cortex consists of cork cambium. The endodermis always persists even after the secondary growth has taken place.

2.5. Chemical Constituents:

The major biochemical constituents of *W. somnifera* are steroidal alkaloids and lactones, a class of constituents together known as withanolides (steroidal lactones with ergostane skeleton) (Elsakka et al., 1990). The withanolides have the structural resemblance with the active constituents present in the plant Panax ginseng known as ginsenosides (Grandhi et al., 1994). The withanolides have C28 steroidal nucleus

with C9 side chain, having six membered lactone rings(Thakur et al., 1987; Puri, 2002). Therefore, because of this *W. somnifera* is named as an “Indian Ginseng” (Grandhi et al., 1994; Singh et al., 2001). So far 12 alkaloids, 35 withanoloids and several sitoindosides have been isolated and their structures have been elucidated (Mishra et al., 2000; Matsuda et al., 2001). The various alkaloids include withanine, somniferine, somnine, somniferinine, withananine, psuedo-withanine, tropine, psuedotropine, 3-α-gloyloxytropane, choline, cuscohygrine, isopelletierine, anaferine and anahydrine. Two acyl steryl glucoside viz. sitoindoside VII and sitoindoside VIII, two glycowithanoloids viz. sitoindoside IX or sitoindoside X have been isolated from the root. Withaferin A has been recently reported to be inhibitor of angiogenesis and thus protective in certain types of cancers (Mohan et al., 2004). Two glycowithanoloids (sitoindoside IX or sitoindoside X) possessed antistress activity and augmented learning acquisition and memory retention in both young and old rats (Ghosal et al., 1989). Recently, two new steroidal lactones of the withanolide-type have been isolated from the fruits of *W. somnifera* (Abou-Douh, 2002). The diverse active constituents present in different parts of the plant are believed to be responsible for the multiple medicinal properties of *W. somnifera*.

Table 2: Chemical constituents and pharmacological effects of different parts of *W. somnifera*

Plant parts	Chemical Constituents	Medicinal Uses Of The Plant
ROOTS	Withanolides and their derivatives such as sitoindosides, Ashwagandhanolides, and some tropane alkaloids	Rejuvenating tonic, alternative astringent, aphrodisiacal, phthisis, fever inflammation, asthma, bronchitis, leukoderma, arthritis, emmenagogue, abortifacient, cold
LEAVES	Several withanolides such as withaferin A, withanolide D, withanolide E, withanone, withanolide Z, withanolide B, 7-hydroxywithanolide, 27-deoxy withaferin A	Aphrodisiac, carbuncle, ulcer, sore painful swelling, fever, chest pain, anti-inflammatory, to cure eyesore, diuretic, narcotic, treatment of haemorrhoids

SEEDS	Some withanolides	Diuretic, narcotic, and hypnotic, to coagulate milk applied on wounds, relieving the poison of a serpent, rubbed on skin for ringworm
FRUITS	Withanamides	Anthelmintic, ulcers and tubercular glands

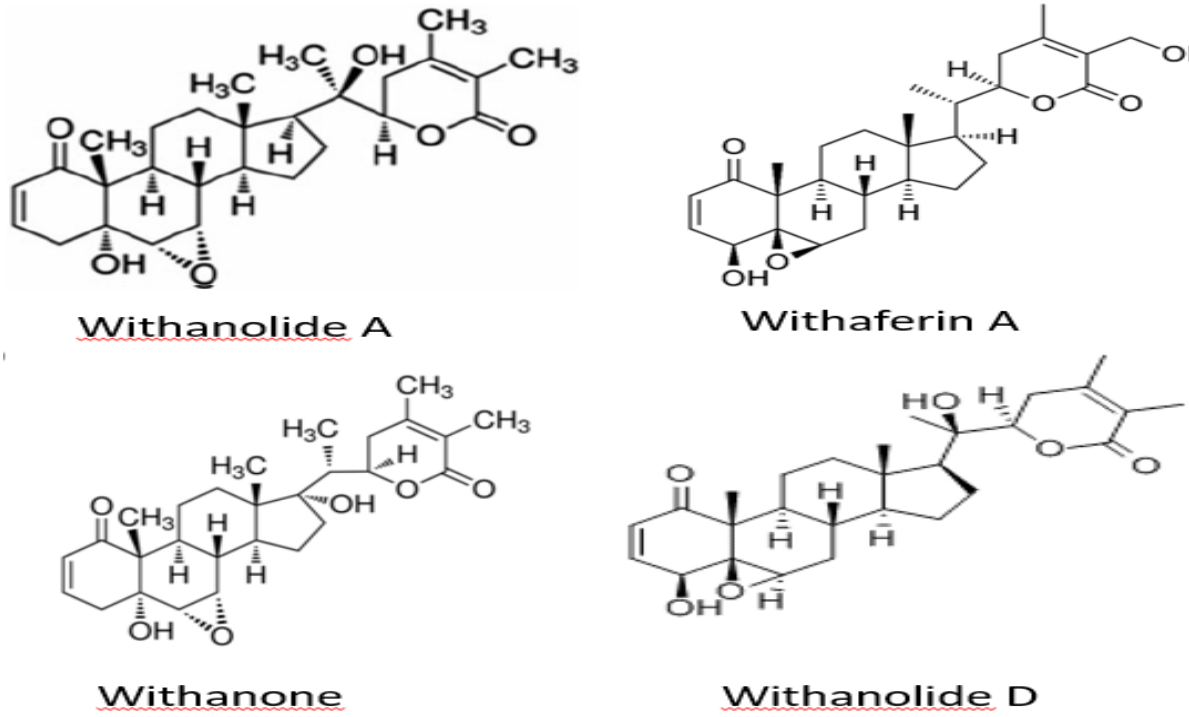

FIGURE 2: Withanolides of Ashwagandha (*Withania somnifera)*

2.5.1. Alcoholic compound

The earliest report available on the phytochemistry of the plant is by Power and Salway (1911) that studied the chemical principles of *W. somnifera and* reported the presence of several compounds from the roots and leaves of the plant. They reported two new monohydric alcohols, withaniol, $C_{25}H_{33}O_4OH$ and somnirol, $C_{33}H_{43}OOH$, new dihydric alcohol, somnitol, $C_{33}H_{44}O_5(OH)_2$, an acidic hydrolytic product, withanic acid,

$C_{29}H_{45}O_6COOH$, a nitrogen-containing component, $C_{12}H_{16}N_2$, phytosterol, $C_{27}H_{46}O$ and ipuranol. In addition, a mixture of fatty acids, consisting of stearic, cerotic, palmitic, oleic, and linoleic acids; an essential oil, and sugar wasobtained (Kalra and Kaushik *et al.* 2017).

2.5.2. Alkaloidal compounds

Later on, Majumdar (1952, 1955), examined the roots of Indian variety from Bengal and South African varieties and identified several nitrogenous bases, and partially characterized seven amorphous bases namely withanine, withananine, withananinine, pseudo-withanine, somniferine, somniferinine, somnine along with nicotine as eight components. The first six compounds were found to be alkaloids and the seventh one is a disintegrated product of withanine. Among these, withanine was found to be the main alkaloid, with 38% of the total alkaloid content. Schwarting*et al.* (1963) made the major breakthrough by isolating and characterizing eight bases present in the extract namely, tropine, pseudo tropine, 3a-tigloyloxytropane, choline, cuscohygrine, dl-isopelletierine, anaferine, and anahygrine the latter two being the new ones (Schwarting*et al.* 1963). Further, Schroter*et al.* (1966) isolated a pyrazole alkaloid withasomnine from the root of *W. somnifera*. Jayaprakasam *et al.* (2004) purified novel withanamides A, withanamide B, withanamide C, withanamide D, withanamide E, withanamide F, withanamide G, withanamide H, withanamide I from the methanolic extract of *W. somnifera* fruits. The structure of these compounds was determined by using serotonin, glucose, and long-chain hydroxyl fatty acid moieties (Jayaprakasam *et al.* 2004).Besides, withanolides and alkaloids, flavonoids, and phenolic compounds have also been reported from *W. somnifera* leaves. Kandil *et al.* (1994) have reported the presence of novel 6, 8-dihydroxykaempferol -3-*O*-rutinoside along with several known flavonoids, viz., quercetin, quercetin-3-*O*-rutinoside, Quinic acid, 4-*0*-caffeoylquinic acid, and 4,5-0-di caffeoylquinic acid from the aqueous ethanol extracts of the leaves. *W. somnifera* seeds have a total oil content of 4.14 to 11.23 percent, with quantitative differences in the number of seeds per fruit, seed weight, seed output, seed oil, and fatty acid composition of the seed oil (Khanna *et al.* 2007). Linoleic acid was found to be the most abundant fatty acid in all accessions' oil (38.7-60.1 %) in the oil of all accessions examined, along with moderate to low amounts of oleic acid (19.5-23.0%), palmitic acid (13.5-27.9%), stearic acid (2.7-5.7%), myristic acid (0.06-0.10%) and linolenic acid (0.90-1.50%) (Khanna *et al.* 2007).

2.6. Ethnobotany: In Ayurveda, *W. somnifera* is widely claimed to have aphrodisiac, sedative, rejuvenative and life prolonging properties. It is also used as a general energy-enhancing tonic known as Medharasayana (promotes learning and memory) and in geriatric problems (Nadkarni, 1976). The plant has traditionally been used to promote youthful vigor, endurance, strength, health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells (Williamson, 2002). It also helps counteract chronic fatigue, weakness, dehydration, weakness of bones and loose teeth, thirst, impotence, premature ageing, emaciation, debility and muscles tension. The leaves of the plant are bitter in taste and used as an anti-helminthic. The infusion is given in fever. Bruised leaves and fruits are locally applied to tumors and tubercular glands, carbuncles and ulcers (Nadkarni, 1976; Kapoor, 2001). The fruits of the plant have a milk-coagulating property attributed to the pulp and husk of the berry, which has been used in the preparation of vegetable rennet ferment for cheese (Atal & Sethi, 1963). The fruits are reported to be sedative, emetic and stomachic, blood-purifier and febrifuge, as an alternative, diuretic and bitter tonic in dyspepsia as well as a growth promoter in infants. The roots are also used in constipation, senile debility, rheumatism, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea (Watt, 1972; Singh & Kumar, 1998). The detailed uses of different parts of this plant are listed in Table 1. Besides its use as general tonic (Agarwal et al., 1999; Dhuley, 2000), several recent reports have demonstrated immunomodulator and antitumor effects of ashwagandha as well (Sharad et al., 1996; Budhiraja & Sudhir, 1987; Ziauddin et al.,1996; Agarwal et al.,1999; Devi, 1999; Mirjalili, 2009). Moreover, extracts of various parts of the plant have been reported to possess antioxidant, anti-serotogenic, anticancer and anabolic properties and has beneficial effects in the treatment of arthritis, stress and geriatric problems (Asthana & Raina, 1989; Gandhi et al., 1994; Davis and Kuttan, 2000; Singh et al., 2001; Prakash et al., 2001; Mishra et al., 2000; Mirjalali et al., 2009). The plant extracts are also used in folk, ayuvedic, Unani and Sidha systems of medicine and the biological activities associated with different extracts are summarized in Table 3. The plant was found to be active against a number of pathogenic bacteria (Kurup, 1956) and possess a strong antibacterial and antifungal activity against various pathogens including Salmonella typhimurium and in the treatment of murine aspergillosis (Dhuley, 1998; Ziauddin et al., 1996; Owais et al., 2005).

2.7. Toxicity profile of *W. somnifera*

W. somnifera is considered to be a safe drug. In one of the studies, a 2% suspension of ashwagandholine (total alkaloids from the roots of *W. somnifera*) prepared in ten-percent glycol using two percent gum acacia as suspending agent was used to determine acute toxicity. The acute LD50 value was found to be 465 mg/kg (332–651 mg/kg) in rats and 432 mg/kg (229– 626 mg/kg) in mice (Malhotra et al., 1965). The extract had no profound effect on central nervous system or autonomic nervous system in doses of up to 250 mg/100 g of mice in toxicity studies. However, it affected spontaneous motor activity in still higher doses. In another long-term study, *W. somnifera* was boiled in water and administered to rats in their daily drinking water for eight months while monitoring body weight, general toxicity, wellbeing, number of pregnancies, litter size, and progeny weight (Sharma et al., 1986). The estimated dose received by the animal was 100 mg/kg/day. The liver, spleen, lungs, kidneys, thymus, adrenals, and stomach were examined histopathologically and were all found to be normal. The rats treated with *W. somnifera* showed weight gain as compared to the control group. The off springs of the group receiving *W. somnifera* were found to be healthier compared to control group (Sharma et al., 1986). The different doses of the extract (30, 75 and 150 mg/kg) potentiated pentobarbitoneinduced sleeping time in a dose-dependent fashion (Prabh et al., 1990). Aphale et al. (1998) carried out the subacute toxicity studies with *W. somnifera* and ginseng. Both the drugs were administered for a period of 90 days and various safety parameters such as food consumption, body weight, hematological, biochemical and histopathological studies on various organs like brain, heart, lung, liver, spleen, kidney, stomach, testis and ovaries were recorded. The results revealed that both the plant preparations did not show any toxicity pattern (Aphale et al., 1998). But, in one study, when the entire plant extract was administered to mice as 25% of the diet, microscopic lesions were found in the liver and lungs along with vascular and tubular congestions of the kidneys. The leaf extract of *W. somnifera* was reported to possess antigenotoxic potential (Rani et al., 2005; Russo et al., 2001). These extensive toxicological studies demonstrated that the plant is nontoxic in wide range of reasonable doses and it can be assumed that the doses in which its preparations are indicated in humans are expected to be very safe. As of today, no herb-herb or herb–drug interactions have been reported in the literature with *W. somnifera* (Arseculeratne et al., 1985).

2.8. Pharmacological Profile of Withania

The pharmacological activity of numerous extracts from different sections of the plant has been widely researched. Antitumor activity (Devi et al., 1992), anti-inflammatory activity (Al Hindawi et al., 1989), immunomodulatory activity (Ziauddin et al., 1995), and analgesic action (Ziauddin et al., 1995) have all been reported for *W. somnifera* (Twaij et al., 1989). Studies on its antistress activity found that when mice were subjected to swimming stress, their swimming time increased and their stomach ulcers decreased (Grandhi et al., 1994). Stress-induced increases in dopaminegic receptor population in the corpus striatum were inhibited by *W. somnifera*'s antistress action (Sakena et al., 1988). The goal of this study was to see how effective *W. somnifera* is at reducing stress caused by cold swimming.

Anticancer Activity

Significant antitumor and radio-sensitizing properties of withaferin A and withanolide D are well documented (Devi *et al.* 1992). Withaferin A was poison (Davis and Kuttan 1998). It is also reported to retard the growth of Ehrlich ascites carcinoma, sarcoma 180, and sarcoma Black and E 0771 mammary adenocarcinoma Mathur *et al.* (2004) reported the inhibitory action of -oxo-5B, 6B-epoxy-with a-2-enolide on skin carcinoma induced by UV radiations. Methanolic extract of *W. somnifera*has been shown to induce the proliferation of stem cells. Jayaprakashan *et al.* (2003) reported the inhibition of growth of breast, lung, central nervous system, and colon cancer cell lines by decreasing their viability in a dose-dependent manner. Widodo *et al.* (2010) stated that selective killing of cancer cells by Ashwagandha leaf extract and its component withanone involves ROS signaling

Anti-inflammatory Properties

W. somnifera was reported to reduce the glycosaminoglycans content, ADP/O ratio in mitochondria in the granuloma tissue, subsequent change in the Mg^{2+} dependent-ATPase and succinate dehydrogenase enzyme activity, thereby uncoupling the oxidative phosphorylation (Begum *et al.* 1988) leaf extract of *W. somnifera and* its major constituent withaferin A.*W. somnifera* derived steroidal lactones, such as withanolide A is far less effective (Kaileh *et al.* 2007). The extract of *W. somnifera* has shown anti-inflammatory effects by inhibiting the formation of granuloma tissues in a

variety of rheumatological conditions (Al- Hindawi *et al.* 1992). Aqueous suspension of *W. somnifera* root exhibited anti-inflammatory properties through inhibition of complement system, lymphocyte proliferation, and delayed-type hypersensitivity (Rasool and Varalakshmi 2006). Similarly, hydro-alcoholic extract of *W. somnifera also* possessed a marked anti-inflammatory effect against denaturation of protein in vitro. The effect was plausibly due to the alkaloid and withanolide contents of *W. somnifera* (Chandra *et al.* 2012). The anti-inflammatory activity of the plant was further supported by a study conducted by Khan *et al.* (2011) in the assessment of cholinesterase and lipoxygenase inhibitors activity of the plant.

Antioxidant and Free Radical Scavenging Activities

The brain and nervous system are relatively rich in lipids and iron, this is more to free radical damage than other tissues because both are known to promote the generation of reactive oxygen species (Halliwell and Gutteridge 1989). Free radical damage of the nervous system may lead to a neural loss in cerebral ischemia, aging, and neurodegenerative disease, e.g., epilepsy, schizophrenia, Parkinson's, Alzheimer's, and other diseases (Jesberger and Richardson 1991; Sehgal *et al.*2012).

Antimicrobial Activities

The antibiotic potential of the leaf extract of *W. somnifera* was reported for the first time by Kurup (1956) against *Salmonella aureus.* In recent times, antimicrobial properties of *W. somnifera* against a range of bacteria and fungi were documented (Ziauddin *et al.* 1996; Dhuley 1998; Mishra *et al.* 2000). Arora *et al.* (2004) reported the antibacterial/ synergistic activity of the methanol, hexane, and diethyl ether extracts from both leaves and roots of *W. somnifera* against *S. Typhimurium* and *E. coli.* Owais *et al.* (2005) showed the antibacterial activity of Ashwagandha extracts against pathogenic bacteria by in vitro Agar Well Diffusion method and in experimental salmonellosis in Balb/C mice. Ghosh (2009) purified a 30 KDa monomeric acidic lectin-like protein from the leaves of *W. somnifera* showing inhibitory action against major phytopathogens in *vitro*. The peptide sequence showed similarity to concanavalin A-like lectin from *Canavalia ensiformis* and caused distinct cell wall adhesion of the protein-treated hyphae under SEM (Ghosh 2009).

Anti-tumor

As anti-inflammatory, cardioactive, and central nervous system effects of *W. somnifera* involve angiogenic processes thus it was hypothesized that the *W. somnifera* extracts might contain angiogenesis inhibitors. It was found that withaferin A inhibited cell proliferation in human umbilical vein endothelial cells (HUVECs) with an IC50 value of 12 nM through a process associated with inhibition of cyclin D1 expression (Mohan *et al.* 2004). Withaferin A was found to have anti-angiogenic activity in vivo at doses that are 500-fold lower than those previously reported to exert anti-tumor activity in vivo thus holding a promise for a potent anti-tumor drug. Studies at the molecular level revealed that withaferin A inhibits binding of Sp1 transcription factor to VEGF (vascular endothelial cell growth factor) gene promoter, to exert its antiangiogenic activity (Prasanna *et al.* 2009).

Anti-stress and Aphrodisiac Properties

Bhattacharya reported anti-stress activity associated with glycosides (sitoindosides VII and VIII) found in this plant (1987,2000,2003). Other research (Dubey *et al.* 2000; Singh *et al.* 2001) has backed up Ashwagandha's effectiveness as an anti-stress adaptogen. The higher concentrations of inorganic elements like Fe, Mg, K, and Ni in the roots of this plant were said to play a key role in the drug's diuretic and aphrodisiac properties (Lohar*et al.* 1992).

Immunomodulatory activity and hematopoiesis

The role of *W. somnifera* as an immunomodulator has been extensively studied. Sitoindoside IX and sitoindoside X, isolated from *W. somnifera* Dunal, have immunomodulatory and CNS effects (anti-stress, memory, and learning) in doses of 100–400 g/mouse and produced *statistically significant mobilization and activation of peritoneal macrophages, phagocytosis,* and increased activity of the lysosomal enzymes secreted by the activated macrophages. Thus *W. somnifera* attenuates cerebral function deficits in the geriatric population and provides non-specific host defense (Rahman *et al.* 1999).The root extract of *W. somnifera* has immunomodulatory effects in three myelosuppression models in mice: cyclophosphamide, azathioprine, or prednisolone, and significant increases in hemoglobin concentration, red blood cell count, white blood cell count, platelet count,

and body weight were observed in *W. somnifera* treated mice compared to untreated control mice (Ziauddin *et al.* 1996).

Anxiety and depression

W. somnifera has been used to stabilize mood in patients with behavioral disturbances. Bioactive glycowithanolides (WSG), isolated from *W. somnifera* roots, have been assessed at the dose of 20 and 50 mg/ kg, orally once daily for 5 days for anxiolytic and antidepressant actions in rats. It was found that WSG gives results compared to standard benzodiazepine lorazepam in the dose of 0.5 mg/kg, IP for anxiolytic studies, and standard tricyclic anti-depressant, imipramine in the dose of 10 mg/kg, IP for the antidepressant investigations (Bhattacharya *et al.* 2000b).

Antidote activity

The peptides found in snake venom belong to a wide family of peptides that comprises proteins of various origins, neurotoxins, mycotoxins, cardiotoxins/cytotoxins, and enzymatic toxins (Girish *et al.* 2004). WSG, a glycoprotein isolated from *W. somnifera*, inhibits hyaluronidase in *Najanaja* and *Daboshia russelii* venom, with IC50 values of 52 and 36 g for *N. naja* and *D. russelii*, respectively. It also helps to reduce the rapid diffusion of poisons by inhibiting phospholipase A2 of poisonous cobra venom (Machiah *et al.* 2006). Kumar *et al.* (2015) recently reported that *W. somnifera* has an antidote effect against arsenic-induced toxicity.

Cardiovascular Protection

Malhotra and co-workers (1981) reported the hypotensive effect due to autonomic ganglion blocking action as well as a depressant action on the higher cerebral centers of the extracts of *W. somnifera.* Further studies by numerous workers confirmed *W. somnifera as* a cardio-protective agent that provides a scientific reason for the use of this medicinal plant in Ayurveda as Maharasayana (Gupta *et al.* 2004; Mohanty *et al.* 2004; Sehgal et al.2012).

Effect on Nervous System

Ashwagandha is a superior medicine for exhaustion with nervous irritability due to its sedative effect on the central nervous system (Mir *et al.* 2012). The drug-induced

enhancement of cortical muscarinic acetylcholine receptor capacity, according to Schliebs and colleagues (1997), explains the cognition and memory-enhancing effects of *W. somnifera* extracts. This plant's extracts were linked to improvements in scopolamine-induced memory deficits in mice, according to Dhuley (2001). *W. somnifera* extracts were also found to have an anti-Parkinsonian effect on neuroleptic-induced catalepsy, inhibiting haloperidol or reserpine-induced catalepsy. This was attributed to the potent antioxidant, antiperoxidative, and free-radical quenching properties of *W. somnifera extracts*.

Rejuvenating Effect

W. somnifera was found to have growth-promoting properties, which were attributed to withanolides (Budhiraja and Sudhir 1987). In children and elderly people, Kuppurajan *et al.* (1996) found significant improvements in hemoglobin, packed cell volume, mean corpuscular volume, serum, iron, body weight, handgrip, and total protein. The hypoglycemic and diuretic effects of Ashwagandha roots in humans were studied by Andallu & Radhika (2000). A decrease in blood glucose levels, as well as significant increases in urine sodium and volume, as well as a decrease in serum cholesterol and triglyceride levels.

2.9. *Withania somnifera* in COVID-19

The coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus2 (SARSCoV-2), is currently posing a high global health threat and challenges in containing the pandemic situation. This virus has infected millions of people and killed hundreds of thousands of people around the world. The rapid spread and rising number of SARS-CoV-2 cases necessitate the development of early therapeutic and preventive measures to combat COVID-19. As of today, no effective therapeutic drugs or vaccine candidates are available for COVID-19 treatment, despite significant efforts in this direction, and only a few of these have reached the final stages of clinical trials. Adequate prevention and control measures, such as prompt COVID-19 diagnosis, contact tracing, immediate quarantine, and biosafety measures, as well as the discovery of effective vaccines, drugs, and therapies, could prevent the devastating consequences of this pandemic. Developing a vaccine against COVID-19 may take too long before it is available to protect the health of millions of people

worldwide. As a result, currently available antiviral drugs are being investigated against COVID-19 through drug repurposing in order to identify new therapeutic uses for old and existing drugs, which appears to be a promising approach to curing COVID-19. A combination of antiviral drugs (favipiravir, ritonavir, lopinavir) and antimalarial drugs(hydroxychloroquine and chloroquine) are currently being used as promising COVID-19 therapeutic agents. Aside from these medications, corticosteroids such as dexamethasone have been shown to be among the most effective. The Science of Life is an ancient traditional medicinal system that originated and is still practiced in India. It has been used to reduce SARS-CoV-2 infection and treat COVID-19-associated patients. It describes many medicinal plants and herbs that have a wide range of therapeutic usefulness in curing various ailments, diseases, and disorders, such as Allium sativum (Garlic), *Withania somnifera*(Ashwagandha), *Zingiber officinale* Roscoe (Ginger), *Tinospora cordifolia*(Giloy), *Ocimum sanctum* (Tulsi), *Curcuma longa* (Turmeric, Haldi) as a result, researchers are focusing on developing medicines and drugs based on medicinal, aromatic herbs and plants that contain active phytochemical constituents that could aid in the treatment of COVID-19 patients. Herbal bioproducts and purified bioactive substances derived from them may have anti-SARS-CoV-2 activities by directly impeding virus entry or replication. Surprisingly, some natural products are said to be involved in the inhibition of the receptor angiotensin-converting enzyme 2 (ACE2) or the Transmembrane protease serine 2-encoded serine protease enzyme, both of which are required by coronavirus to infect host cells. Furthermore, plant-based products, such as chymotrypsin and papain-like proteases, have been shown to inhibit the life cycle-associated proteins of SARSCoV-2. *Withania somnifera* L. (Solanaceae), also known as Ashwagandha or Indian ginseng, is used in Ayurveda for vitality, cardioprotective action, and the treatment of a variety of disorders such as respiratory diseases, skin disorders, and neurological disorders. *Withania somnifera* has antiviral, anti-inflammatory, immunomodulatory, antioxidant, antimicrobial, anti-diabetic, neuroprotective, analgesic, anti-tumor, anti-aging, anti-arthritic, anti-stress, and immunomodulatory properties. It is a rich source of phytochemicals such as Withaferin A (WFA), steroidal alkaloids and lactones, as well as many other chemical compounds. *Withania somnifera* aids in the maintenance of a healthy mental and physical state, the rejuvenation of the body in cases of poor health, and the improvement of immunity. *W. somnifera* phytochemicals have been shown to have potent antiviral activity against a

variety of viral infections, including chikungunya, human papillomavirus (HPV), hemagglutinin type 1 and neuraminidase type 1, herpes simplex, and hepatitis C virus. Currently, the Indian government, the Indian Medical Research, and the Council of Industrial and Scientific Research have recommended the utilization of *W. somnifera* as a therapy against COVID-19. Withanolides, the active ingredients of *W. somnifera*, have shown promising potential to manageCOVID-19, with Withanolide D, Withaferin-A, Withanoside X, and Withanoside I–VII providing significant biological action. Several studies have shown that withanolides such as WFA, Withanoside V and X, andwithanone (Wi-N) have the potential to reduce the severity of SARSCoV-2 and to be useful in treatingCOVID-19 patients (Chikhale et al., 2020; Kumar et al., 2020a; Straughn and Kakar, 2020).

Table.3. Traditional uses of *Withania somnifera* (Ashwagandha).

PLANT PART	SYSTEM OF MEDICINE	USES	REFERENCES
ROOTS	Ayurveda	Rejuvenating drug, tonic, Alternative pungent, astringent, Aphrodisiac, Phthisis	Dutta (1877), Kumar et al., (1980), Sen Gupta (1984)
	Siddha	Aphrodisiac, fever, inflammation	SPC (1992)
	Unani	Asthma, bronchitis, leukoderma, Arthritis, emmenagogue	Stewart (1869), Mathani (1973)
	Folklare	Abortificiant, cold, asthma, Tuberculosis, fever	Dutta (1877), Kumar et al., (1980), Singh and Kumar (1998)
LEAVES	Ayurveda	Aphrodisiac, carbuncle, Ulcers, painful swelling	Dutta (1877), Kumar et al., (1980), Singh and Kumar (1998), Mhaskar et al., (2000)
	Siddha	Fever, chest pain, sores, swelling	SPC (1992)
	Unani	External pains, anti-inflammatory	UPC (1993)
	Folklare	Cure eyesores, boils, diuretic Narcotic, treatment of syphilis and hemorrhoids	Shah and Gopal (1985), Sharma et al., (1992)
SEEDS	Ayurveda	Diuretic, narcotic and hypnotic	Dalzell and Gibson (1861)
	Siddha	Siddha	--
	Unani	Unani	--
	Folklare	To coagulate milk, Applied on open wounds, Relieving the poison of a serpent rubbed on skin for ringworm in human beings and animals	Dalzell and Gibson (1861), Rao (1977), Sahu (1982), Shah and Gopal (1985), Dafni and Yaniv (1994)
FRUITS		anthelmintic, ulcers and tubercular glands	Nadkarni (1976), Kapoor (200

Table.4.Biological activity of root extracts of *Withania somnifera*(Ashwagandha*).*

ROOT EXTRACT	BIOLOGCAL ACTIVITY
Alcoholic extract	Neurological, Radiosensitizer, Anticonvulsant, Anti-inflammatory, Sedative, Anti-tumour, Antibacterial.
Methanolic extract	GABA mimetic activity GABA receptor mediates anti-convulsant activity, Protective effect as amygdaloid kidlling Anti-inflammatory (70% extract), Antistress.
Chloroform-Methanol extract	Prevention of Alzheimer's disease (Sehgal, et al., 2012), Immunomodulatory, Anti-inflammatory, Nematocidal, Hepatoprotective,
Water extract	Nephroprotective, Antistress, Antianxiety, Hypothyroidism. Anti-convulsant, Anti-inflammatory, Antiarthritic, Hepatoprotective,
Root powder	Antiulcerogenic, Antistress, Anticancer & Radiosensitizer, Psychophysiological, Pulmonary tuberculosis, Epilepsy, Nervine tonic, Easy abortion, General tonic in seminal disease, Glandular swellings in bubonic plague, Hypoglycaemic diuretic.
Decoction	Anticonvulsant, Cold & Chills, Health restorative to old & pregnant.
Petroleum ether extract	Insecticidal

Table 5. Chemical constituents of pharmaceutical importance identified in *Withania somnifera.*

Plants Parts	Chemical Constituent	Reference
Roots	Sitoindosides VII (Acylsteryl-glucoside)	Bhattacharya et al., (1987)
	Sitoindosides VIII (Acylsteryl-glucoside)	Bhattacharya et al., (1987)
	Sitoindosides IX (Glycowithanolide)	Ghosal et al., (1988)
	Sitoindosides X (Glycowithanolide)	Ghosal et al., (1988)
	Withanine (Alkaloid)	Majumdar (1955)
	Withananine (Alkaloid)	Majumdar (1955)
	Ashwagandhanolide	Subaraju et al (2006), Mirjalili et al., 2009
Leaves	Withaferin (Steroidal lactone)	Anjaneyulu and Satyanarayana Rao (1997)
	Withaferin A (Steroidal lactone)	Kirson et al., (1970),Lavie et al., (1965) Lavie et al., (1966)
	Withanolide D (Steroidal lactone)	Kirson et al., (1970), Lavie et al., (1968)
	Withanolide E (Steroidal lactone)	Glotter et al., (1977)
	Withanone (Steroidal lactone)	Dhalla et al., (1961b), Kirson et al., (1971)
	Withanolide Z (Novel)	Pramanick et al (2008)
	Withanolide B	Pramanick et al (2008)
	7-hydroxywithanolide	Pramanick et al (2008)
	3α-methoxy-2, 3-dihydro-	Anjaneyulu and Satyanarayana Rao (1997)
	27-deoxywithaferin A (Steroidal lactone)	Kirson et al., (1970)
	4β, 17α-dihydroxy-1-1oxo-	Lavie et al., (1965)
	5β, 6β-epoxy-22R-witha-	Lavie et al., (1965)
	2, 24-dienolide (steroidal lactone)	Kirson et al., (1970), Lavie et al., (1968)
	4β-dihydroxy-5β, 6β-epoxy-	Glotter et al., (1977)
	1-oxo-22R-witha-2, 14-24-	Dhalla et al., (1961b),
	Trienolide (steroidal lactone)	Kirson et al., (1971)
	5, 20α (R)-dihydroxy-6α, 7α-epoxy-1-oxo-(5α) - Witha-2, 24-dienolide (steroidal lactone) 2, 3-dihydrowithaferin A-3beta-O-sulfate	Menben Von and Stapel (1973)
Seeds	Withanolide –WS 2 (aliphatic ester) Withanolide –WS 1 (aliphatic ketone)	Xu et al (2009), Kundu et al., (1976a, b), Khan et al (1993

2.10. MOLECULAR MARKERS

Molecular markers are identifiable DNA sequences, found at specific locations on the chromosome, transmitted by the laws of inheritance from one generation to the next. Molecular markers can be useful tools to both facilitate the *Withania* breeding program (Marker Assisted Selection, MAS) and to aid in the characterization of the *Withania* germplasm(varieties) Different kinds of molecular markers are in use, such as AFLPs,

RAPDs, RFLPs, microsatellites, and SNPs. They may differ in a variety of ways such as their technical requirements, the amount of time, money, and labor needed, the number of genetic markers each marker in a given population. The information provided by the markers for the breeder will vary depending on the type of marker system used. Molecular markers are routinely employed in various aspects of plant genome analysis such as taxonomy, phylogeny, genetics, and plant breeding. Molecular markers include biochemical constituents (e.g., secondary metabolites in plants) and macromolecules for, example proteins and deoxyribonucleic acids (DNA). Analysis of secondary metabolites is, however, restricted to those plants that produce a suitable range of metabolites that can be easily analyze and which can distinguish between varieties. Hence, amongst the molecular markers used, DNA markers are more suitable and ubiquitous to most living organisms.

2.10.1.DNA Based Markers

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. A wide variety of techniques have, therefore, been developed in the past few years for visualizing DNA sequence polymorphism. The term DNA- fingerprinting was introduced by Alec Jeffrey in 1985 to describe bar-code-like DNA fragment patterns generated by multi locus probes after electrophoretic separation of genomic DNA fragments.

Properties of Ideal DNA Marker

- Highly polymorphic nature
- Codominant inheritance (determination of homozygous and heterozygous states of diploid organisms)
- Frequent occurrence in the genome
- Selective neutral behavior (the DNA sequences of any organism are neutral to environmental conditions or management practices)
- Easy access (availability)
- Easy and fast assay
- High reproducibility

- Easy exchange of data between laboratories

Molecular markers are used to examine DNA polymorphism and are divided into two types: hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are seen by hybridizing restriction enzyme-digested DNA to a labeled probe, which is a DNA fragment of known origin, sequences, or loci, using particularly or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated by electrophoresis, and banding patterns are recognized using various methods such as staining and autoradiography.

PCR is a flexible technology that was developed in the mid-1980s. Since the introduction of thermostable DNA polymerase in 1988. The primer sequences are chosen so that base-specific binding to the template can occur in reverse orientation. PCR is incredibly sensitive and runs at a high rate. Its use for a variety of purposes has opened up a multitude of new possibilities in the field of molecular biology.

2.10.2 Microsatellites

Microsatellites have become one of the most prominent molecular markers in recent years, having applications in a wide range of sectors. The two key characteristics that make microsatellites of great relevance for much genetic research are their high polymorphism and a relative case of scoring. Microsatellites, also known as simple sequence repeats (SSRs), are tandemly repeated motifs of 1-6 bases that have been identified in all bacterial and eukaryotic genomes studied to date. They can be found in both coding and non-coding regions and are usually distinguished by a high degree of length polymorphism. The cause of such polymorphism is unknown; however, it appears to be the result of slippage events during DNA replication (Schltterer&Tautz et. al., 1992). Even though the mechanism of microsatellite evolution is still unknown, SSRs have been widely used in various domains since their original description (Litt&Luty 1989; Tautz 1989; Weber & May 1989) because of their high survivability, which makes them particularly effective genetic markers. Microsatellites have proven to be an incredibly helpful tool for genome mapping in many organisms (Schuleret al., (1996); Knapik*et al.* (1998), but its applications range from ancient and forensic DNA

research to population genetics and conservation/management of biological resources (Jarne & Lagoda et al., 1996).

2.10.3. DNA polymorphism using microsatellites

A variety of methodologies (both hybridization- and PCR-based) have been developed to examine DNA polymorphism in eukaryotes using microsatellite sequences. The first such effort, which used in-gel hybridization of digested and electrophoresed genomic DNA with end-labelled oligonucleotides corresponding to microsatellites, revealed multi locus RFLP fingerprinting in humans (Ali et al., 1986). Later, multiple PCR-based techniques were employed to develop microsatellite-based markers in a range of plant systems.

2.9.4. PCR based approaches

PCR is used to detect DNA polymorphisms at individual loci using locus-specific primers flanking the microsatellites (Tautz et al., (1989); Weber & May et al., (1989)) or using synthetic oligonucleotides as primers, each complementary to a microsatellite motif randomly distributed throughout the genome (Meyer et al., (1993); Wu et al., (1994).

2.10.5. Sequence tagged microsatellite sites (STMS)

The sequence flanking specific microsatellite loci in the genome is believed to be conserved within a particular species, across species within a genus, and rarely even across related genera. These flanking sequences, therefore, can be used for designing primers to amplify individual microsatellite loci and the technique is described as sequence-tagged microsatellite sites (STMs) analysis (Beckman & Soller et al., (1990)). The STMS markers loci. Since the development of STMS markers required cloning and sequencing, initially it is very cost and labor intensive, but once the locus-specific primers become available, the approach becomes cost effective.

2.10.6. Single Nucleotide Polymorphism (SNP)

As suggested by the acronym, an SNP (single nucleotide polymorphism) marker is just a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. For such base position with sequence alternatives in genomic DNA to be considered as an SNP, it is considered that the least frequent

allele should have a frequency of 1% or greater. Although in principle, at each position of a sequence stretch, any of the four possible nucleotide bases can be present, SNPs are usually biallelic in practice.

2.10.7. Chemotypic variability:

A considerable degree of chemotypic variability exists in the *W. somnifera* germplasm. There is a substantial variability not only in the profile of the different bioactive constituents and other phytochemicals but also in their concentration/ accumulation in the plant. The identification of the elite genotypes rich in the phytochemicals having desired bioactive properties may be brought under cultivation and could be used as a breeding material for the crop improvement programs. Based on the variability in the profile of the different bioactive constituents, Ashwagandha has been classified into various chemotypes. Three distinct chemotypes were recorded from Israel and one each from India and South Africa (Abraham et al., 1968, 1975; Eastwood et al., 1980; Kirson et al., 1971), although the later reports suggest the presence of more than one chemotype in India (Chaurasiya et al., 2009; Kaul et al., 2009; Singh et al., 2017b). The major bioactive of Israel chemotypes I, II, and III were recorded as withaferin A, withanolide D, and withanolide E, respectively (Abraham et al., 1968, 1975) and the major bioactive in Indian chemo type were recorded as withanone and withaferin A (Dhalla et al., 1961; Kirson et al., 1977). Whereas the major bio actives present in the South African chemotype were recorded as withaferin A and withaferin D (Kaul et al., 2009; Kirson et al., 1970). Some new withanolides have also been reported from the hybrids of these chemotypes (discussed in Section 15.8.1). Further, in an attempt to estimate the accumulation of a bioactive, i.e., withanolide A in 25 diverse genotypes of Ashwagandha, grown in India, it was found that withanolide A was maximum in the genotype UWS 59, which was possessing the highest concentration of the alkaloids too (Chauhan et al., 2019). Besides the chemotypic variability, the variability in the production of bioactives in *W. somnifera*, the leaf oncogenic phase related variabilities have also been suggested. The biogenesis of withanolide may start as early as in primordial stage, reaches maximum in the young leaves, and declines afterward along the maturity (Chaurasiya et al., 2007)

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Material

12 different genotypes of *Withania somnifera,* including released varieties from CSIR-CIMAP having considerable genetic and geographical diversity have been taken to study phenotypic variation. The genotypes were grown in an Augmented Block Design at the experimental research farm of the CSIR-CIMAP, Lucknow, India, in the year 2022 under normal fertility conditions. The experimental farm is located at 26.5ºN latitude and longitude of 80.50ºE with above mean sea level which has a semi-arid subtropical climate with an annual rainfall of 900 nm.

Table 6: List of plant material used in this study

S. No.	Genotype	Ecotype
1	RV 100	Nagouri
2	PRATAP	Kashmiri
3	CIM-PUSHTI	Kashmiri x Nagouri
4	NMITLI-101	Kashmiri
5	JAWAHAR ASHGANDH-20	Nagouri
6	POSHITA	Kashmiri
7	JAWAHAR ASHGANDH-134	Nagouri
8	VA-I	Nagouri
9	ARKA	Nagouri
10	AWS-I	Nagouri
11	NMITLI-118	Kashmiri
12	CHETAK	Nagouri

TABLE.7. PREPARATION OF BUFFER AND REAGENTS

- **CTAB DNA extraction buffer**

ReagentStock	**WorkingBuffer**	**(100 ml)**
Tris-Cl	1M	100 Mm	10 ml
EDTA	0.5M	20Mm	4ml
NaCl	5 M	1.5 M	30 ml
CTAB	20%	2.5%	12.5 ml
B-mercaptoethanol	0.3%	0.3%	200ul
PVP	1%	1%	
Double distilled water	makes up accordingly		

- **1M Tris-Cl (pH 8.0; 1L)**

Component

Tris Buffer	121.1 g
Double distilled water	1000 ml

12.1 g of Tris base was mixed with 700ml of ddH_2O by stirring and the pH was adjusted to 8 by adding HCl. The final volume was made up to 1L, autoclaved, and stored at room temperature.

0.5 M EDTA (pH 8.0; 500ml)

Components

EDTA	93.5 g
Double distilled water	500ml

93.5g of EDTA was dissolved in 200 ml of ddH_2O and pH was adjusted to 8 by adding NaOH pellets. The final volume was made up to 500 ml, autoclaved, and stored at room temperature.

- **5 M NaCl (500 ml)**

Components

NaCl	146.1 g
Double distilled water	500ml

146.1 g of NaCl was mixed with 500 dd H_2O by stirring, autoclaved, and stored at room temperature.

- **20% CTAB**

Components

CTAB	20g
Double distilled water	100 ml

20% of CTAB was added to 100 ml 0f dd H_2O and heated in a water bath to dissolve completely.

High salt T.E. Buffer

Reagents	Stock	Final Conc.	Volume (100 ml)
NaC	15M	100 Mm	20 ml
Tris-Cl	1 M	20 mM	1 ml
EDTA	0.5 M	0.1 mM	0.2 ml

The contents are mixed and the final volume was made up to 100 ml, autoclaved, and stored at room temperature.

- **10 X TBE Buffer (1L)**

Components

Tris base 108g

Boric acid 55g

0.5 M EDTA (pH 8.0) 40 ml

Double distilled water 1000ml

108g Tris base and 55 g Boric acid was dissolved in 800 ml H_2O and 40 ml of 0.5 M EDTA was added to it. Now the final volume was made up to 1 L, autoclaved andstored at room temperature. The buffer was diluted to 0.5X and used accordingly when needed.

- **70% Ethanol**

Components

Absolute ethanol 70 ml

Distilled water 30 ml

Chloroform: Isoamyl alcohol (24:1)

Reagents

Chloroform 96 ml

Isoamyl alcohol 04 m

3.3. GENOMIC DNA ISOLATION

Genomic DNA was isolated from the leaves of 12 genotype under study by using a protocol developed by Khanuja et al., (1999) specifically for the medicinal and aromatic plant as follows.

Healthy and young leaves were taken, and blot dried using sterile filter papers. Fresh plant tissue was placed into a mortar and pestle and the tissue was ground finely using liquid nitrogen (3g fresh tissue or 0.5g dry tissue). Freeze-dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA. The material was transferred in a polypropylene tube and 3 ml of freshly prepared extraction buffer was added and mixed by inversion to the slurry. The material with extraction buffer was incubated at 60ºC in a shaking water bath for 1-2 hr. 3ml of Chloroform: Isoamyl alcohol (24:1) was added and mixed by slurry. This was centrifuged at 10,000rpm for 10-15 min. The upper aqueous layer was carefully transferred to another 10 ml polypropylene tube 0.7 volume of chilled isopropanol was added and the mixture was incubated at -20ºC for 15 minutes to precipitate the DNA. After 1 h, the mixture was mixed slowly and carefully producing fibrous nucleic acid which was scooped and transferred to a 1.5 ml microfuge tube and centrifuged at 10,000rpm for 10 min. decant the supernatant without disturbing the pellet and subsequently wash twice with 500µl ice-cold 70% ethanol. Decant the ethanol remove the residual ethanol by drying in a Speed Vac. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve the DNA pellet in 20µl TE buffer (10mM Tris, pH 8, 1 mM EDTA).

3.4. QUANTIFICATION OF DNA

Using a Nanodrop spectrophotometer, the concentration and purity of extracted genomic DNA were determined (ND-1000 Spectrophotometer). Light with a wavelength of 260 nm is absorbed by nucleic acid. The absorbance ratio at 260 nm and 280 nm is used to determine the purity of DNA. A ratio of 1.8 is widely regarded as "pure" for DNA. An optical density of 1 at 260 nm corresponds to a DNA concentration of 50ng/l for double-stranded DNA The absorbance at 260 nm is used to calculate the concentration of nucleic acid for quantification. The purified DNA was used after being diluted in 50µl MQ water.

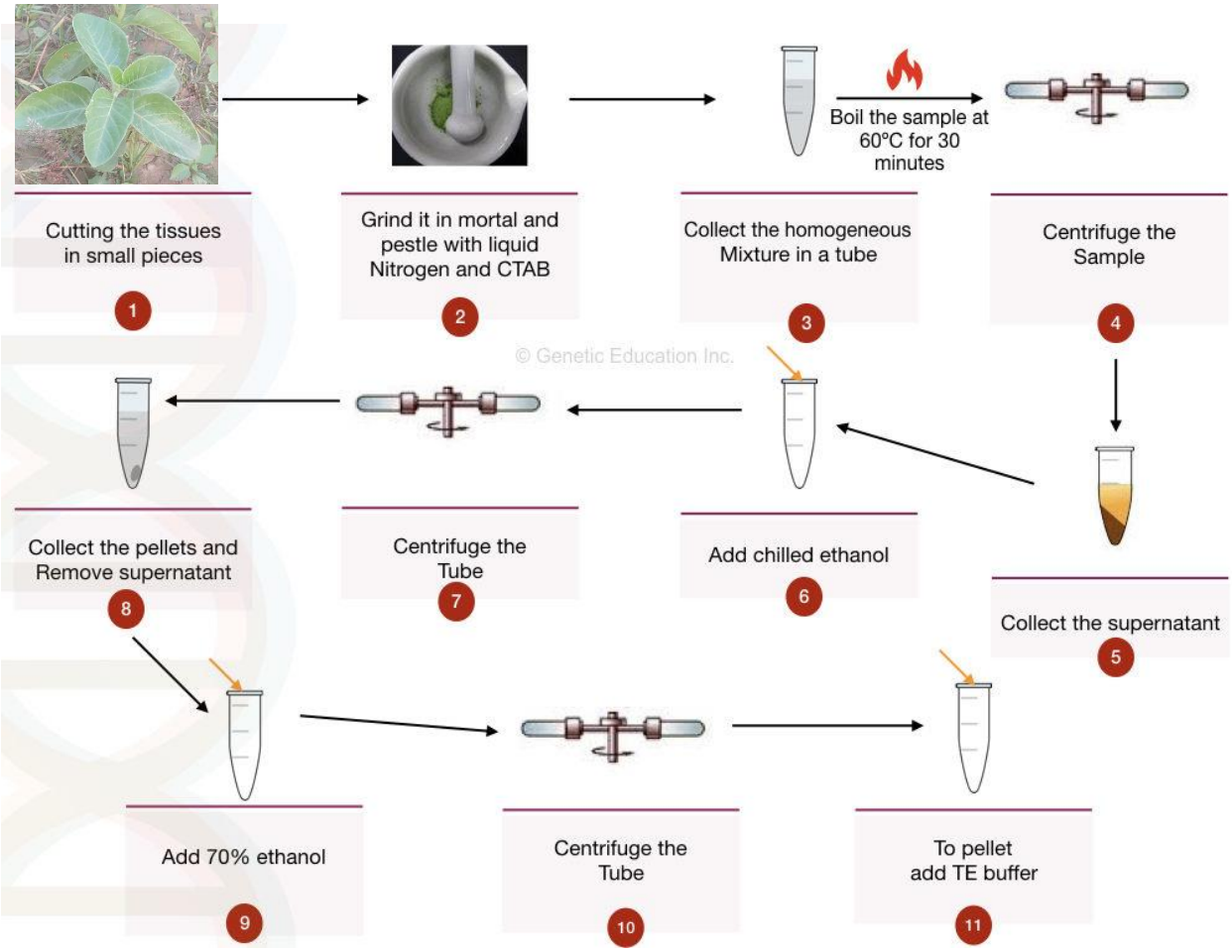

FIGURE 3. GENOMIC DNA EXTRACTION BY CTAB METHOD

3.5. GEL ELECTROPHORESIS

The purity of purified DNA was determined by running 2µl of each DNA sample through a 0.8 % agarose gel. 2.4g of agarose was weighed and dissolved in 300ml of 0.5X TBE Buffer by boiling until a clear solution was obtained. After the gel had cooled, Ethidium Bromide (3g/ml) was added to it. After placing the combs, the gel was poured into the casting tray. After solidification of gel, the combs were removed very carefully without breaking the wells formed. The gel tray was placed in the electrophoretic tank containing 0.5X TBE buffer. Aliquots (2µl) of DNA from each sample were loaded with 1x loading dye (5µl). Electrophoresis was performed at 120V/cm for 1 hours, and the results were photographed and analyzed using a gel documentation system **(SyngeneGBox).**

3.6. PCRAMPLIFICATION

Polymerase chain reactions (PCR) for amplification of DNA preparation were performed in a 10µl volume. The taq DNA polymerase, buffer, DNTP, mg+2, MQ, and primer are used for PCR reaction mixture. Simply added 1µl buffer, 0.5µl DNTP, 0.5µl Mg+2, 1µl primer, 0.2µl taq, 1µl DNA(25ng) and 5.8µl MQ in10µl of reaction volume. The DNA amplification was carried out using the **Bio-Rad C1000 Touch Thermal Cycler** using the following thermal profile: Initial denaturation at 94ºC for 4 minutes; followed by 32 cycles of denaturation at 94ºC for 1 minute; followed by annealing at respective temperature for 1 minute; elongation at 72ºC for 1:30 minutes and final extension at 72ºC for 7 minutes.

3.7. Screening of PCR products

For the detection of PCR amplicons, they were resolved on 3% agarose SFR gel (Amresco Inc., Solon, OH, USA), (9 g of agarose was weighed and dissolved in 300 ml of 0.5X TBE Buffer by boiling, till the transparent solution was obtained) containing Ethidium bromide (2.5µg/ ml). After pre-run of the gel for half an hour, the PCR products were loaded on the gel by mixing 6x of loading dye along with a 50 bp DNA ladder (New England Biolabs**)** at 120V for 1:30 h in 0.5X TBE Buffer. The resolved amplicons were observed and photographed under gel documentation.

Table8: Thermal profile of molecular markers

	Temperature	Time duration	
Step 1	95ºC	4 minutes	Initial Denaturation
Step 2: No of Cycles 32	94ºC 50ºC- 55ºC 72ºC	1 minute 1 minute 1:30 minutes	Denaturation Annealing Extension
Step 3	72ºC	7 minutes	Final Extension

3.8. MOLECULAR ANALYSIS

3.8.1.SSR ANALYSIS

SSR is a simple sequence repeat. SSR marker series were developed for *Withania somnifera.* Primers were screened initially by Gradient PCR providing suitable annealing temperature and 16 primers were selected to screen on the entire population to detect polymorphism. TG series was developed by Jhang *et al.* (unpublished) for the study. A total of 6 primers were used in the present study for the assessment of genetic diversity among Ashwagandha genotypes. The taq DNA polymerase, buffer, DNTP, mg+2, MQ, and primer are used for PCR reaction mixture. Simply added 1µl buffer, 0.5µl DNTP,0.5µl Mg+2, 1µl primer, 0.2µl taq, 1µl DNA(25ng) and 5.8µl MQ in10µl of reaction volume. The DNA amplification was carried out using the **Bio-Rad C1000 Touch Thermal Cycler.** Using the following thermal profile: 4 min denaturation at 94ºC; followed by 32 cycles of denaturation at 94ºC for 1 minute; followed by annealing at (50ºC - 55ºC) for 1 minute; elongation at 72ºC for 1:30 minutes and final extension at 72ºC for 7 minutes PCR products were amplified on 3% agarose gel using 1X TAE buffer, stained with ethidium bromide and photographed on a gel documentation system. The 50bp DNA ladder was used to determine the size of amplified fragments.

Table 9: Novel in house developed SSR primer sequences used in the study with their

annealing temperatures.

SR NO.	PRIMER	SEQUENCE	T_m
1	WATG 122	Jhang *et al.* 2009 (unpublished)	50°C
2	WATG 651	Jhang *et al.* 2009 (unpublished)	51°C
3	WATG 297	Jhang *et al.* 2009 (unpublished)	51°C
4	WATG 426	Jhang *et al.* 2009 (unpublished)	51ºC
5	WATG 71	Jhang *et al.* 2009 (unpublished)	52ºC
6	WATG 367	Jhang *et al.* 2009 (unpublished)	52ºC

CHAPTER 4

RESULT AND DISCUSSION

12 Genotypes were developed in Rabi session of 2021-2022. The material used in this study was planted at the CSIR-CIMAP Lucknow experimental farm in October 2021.

4.1.DNA Quantification

DNA of plant samples was isolated using modified CTAB method (Khanuja et al. 1999). The quality was determined by running it on 0.8% agarose gel while the quality was determined with the help of gel documentation.

TABLE10: DNA quantification of *Withania somnifera* plant leaf sample

S. NO.	GENOTYPE	YEILD (ng/ µl)
1.	RV 100	50
2.	PRATAP	50
3.	CIM-PUSHTI	50
4.	NMITLI-101	50
5.	JAWAHAR ASHGANDH-20	50
6.	POSHITA	100
7.	JAWAHAR ASHGANDH-134	50
8.	VA-I	50
9.	ARKA	100
10.	AWS-I	45
11.	NMITLI-118	30
12.	CHETAK	30

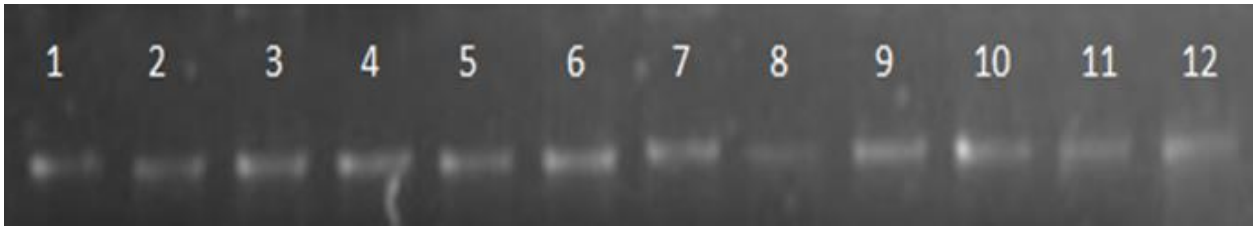

FIGURE 4: Quality and quantity of diluted DNA as observe on 0.8% agarose gel

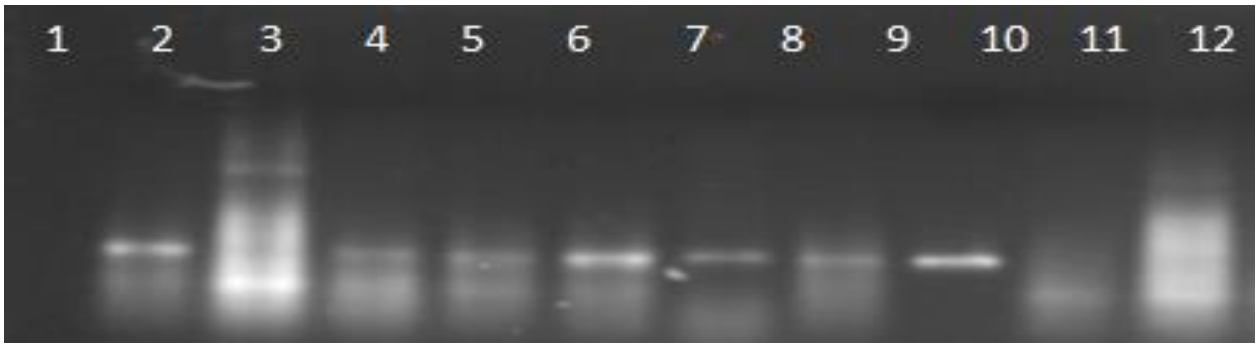

Figure 5: PCR amplification profile of SSR marker ATG 367 showing the polymorphism among 12 *Withania* genotypes.

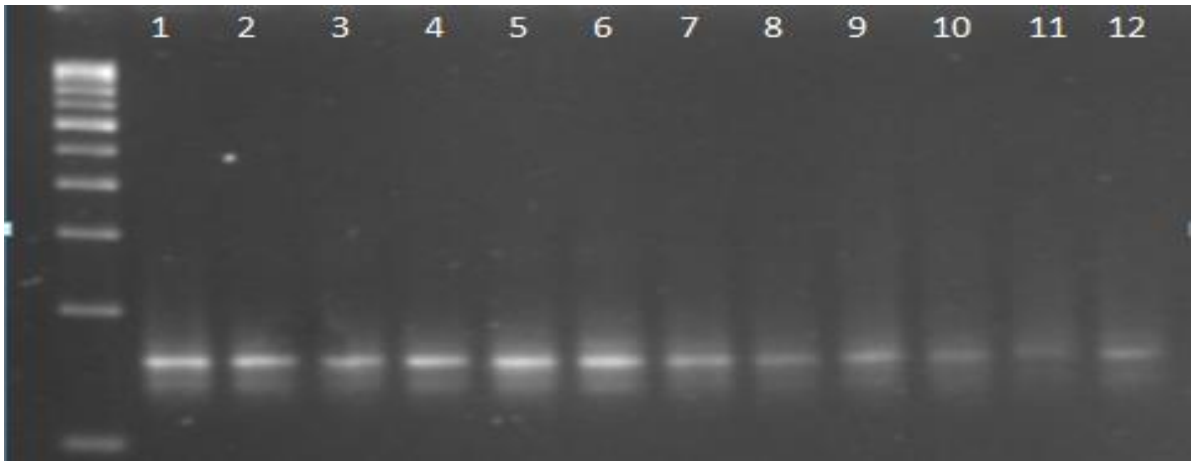

Figure 6: PCR amplification profile of SSR marker ATG 71 showing the polymorphism among 12 *Withania* genotypes.

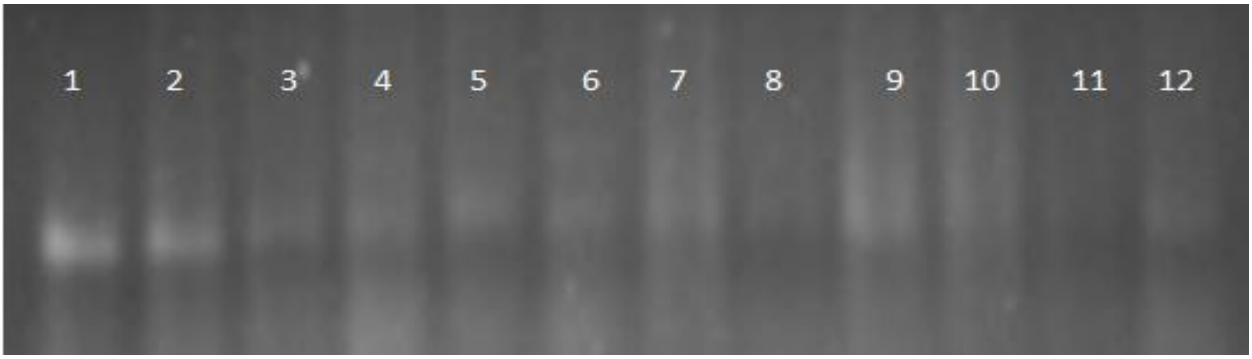

Figure 7: PCR amplification profile of SSR marker WATG 122 showing the polymorphism among 12 *Withania* genotypes.

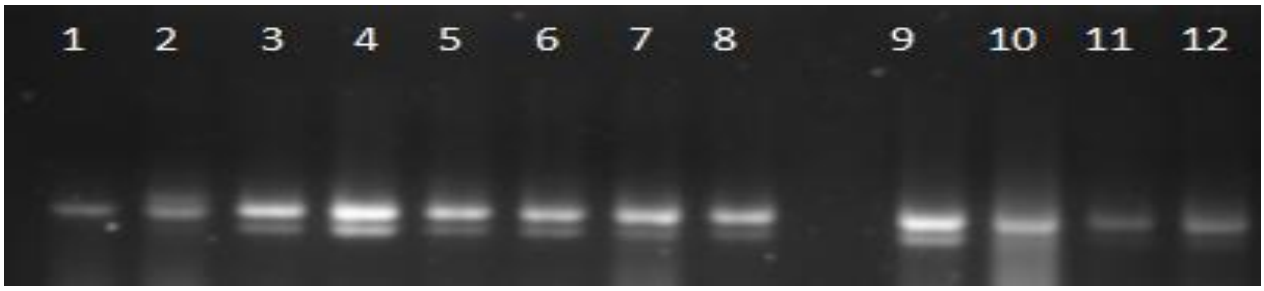

Figure 8: PCR amplification profile of SSR marker TG 651 showing the polymorphism among 12 *Withania* genotypes.

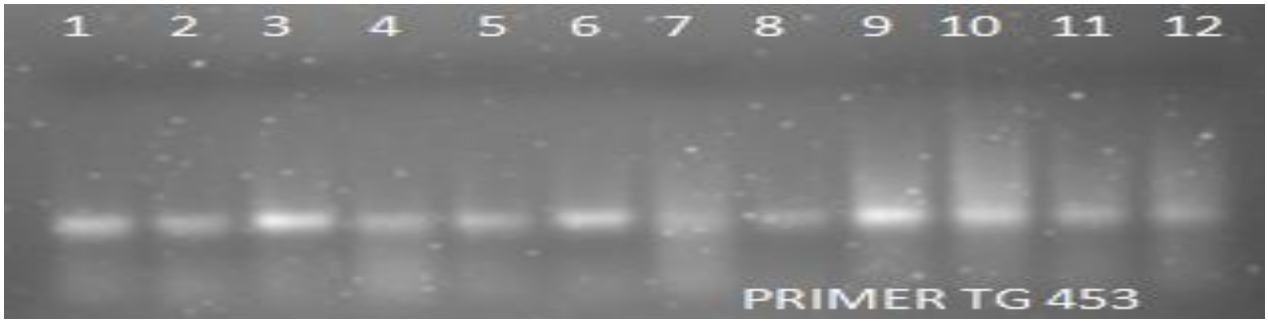

Figure 9: PCR amplification profile of SSR marker TG 453 showing the polymorphism among 12 *Withania* genotypes.

CONCLUSION

Present study entitled “Characterizing Ashwagandha varieties of India” has been undertaken for the assessment of the diversity of Ashwagandha genotypes using "SSR markers”. The genomic DNA extracted from each of the 12 genotypes was subjected to a polymerase chain reaction with 16 SSR primers. Among these tested primers, 5 primers showed polymorphism. Varieties under study could be distinctly resolved by 5 of these polymorphic markers. This will help to check seed purity and adulteration in the export.

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APPENDIX-1

Sr. No.	Name
1.	Autoclave
2.	Centrifuge
3.	DNA Electrophoresis Unit
4.	Freezer (-20
5.	Microwave
6.	Ice Flake Machine
7.	Gel Documentation System
8.	PCR Machine
9.	pH Meter
10.	Spinwin(Microcentrifuge)
11.	Vortex
12.	Water bath
13.	Weighing Machine
14.	Nanodrop (1000)
15.	Water Purification System
16.	Sonicator
17.	Buchi Rotavapour R-300

2. Plastic and Glassware used in this study

1.	Beaker
2.	Tips(1ml, 200µl, 10µl)
3.	Conical Flask
4.	Measuring Cylinder
5.	Microcentrifuge Tube
6.	PCR Tubes
7.	Pipettes
8.	Mortar and Pestle
9.	Polypropylene Oakridge tube
10.	Graduated Bottle
11.	Round bottom flask
12.	Muslin cloth
13.	Crucible
14.	Conical bottom centrifuge tube
15.	Mini centrifuge tubes

APPENDIX- II

Reagents for Agarose gel electrophoresis

1. 6x loading dye

Reagents	Final conc.	Volume (for150ml)
Bromophenol blue	0.25	10mg
Xylene cyanol	0.25	10mg
Glycerol	30	1.2ml

2. 0.8% Agarose gel

Reagent	Stock	Volume for (300 ml)
TBE	10x	15ml
		2.4ml
		270ml

3. 3% Agarose gel

Reagent	Stock	Volume for (300 ml)
TBE	10x	15ml
Agarose		2.4ml
Double distilled water		270ml