International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Coden: IJARQG(USA)

Review Article

Biological properties and conservation of critically endangered plant *Withania coagulans* - Indian Rennet: A Review

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Abstract

Withania coagulans is one of the most privileged medicinal plant of ancient as well as modern medicine. It belongs to family Solonacea and is found to be distributed from East Mediterranean to regions of South Asia. In India it is commonly called as doda paneer (Indian Rennet) since it possesses milk coagulating properties. *W. coagulans* possesses several medicinal properties and is used in treatment of various diseases. Plant is known to possess many bioactive compounds responsible for its biological and pharmacological activities, withanolides being the main acitive biochemical constituent. The plant has become endangered due to unrestricted collection from wild stands for both traditional as well as medicinal purposes. Low germination rate and reproductive failure have also contributed towards the present endangered status of the plant. Considering the present status of the plant micropropagation studies have been conducted for conservation and mass propagation of the plant.

Keywords: Withanolides, conservation, micropropagation, genetic transformation.

Introduction

Withania, an important flowering plant of family Solanaceae consists of nearly 23 species occurring as native in parts of North Africa, Middle east of Mediterranean and Canary Islands and south west Asia. The genus is named after famous geologist and paleobotonist Henry Withania. *Withania coagulans* commonly called as Indian Rennet or Vegetable Rennet is an endangered medicinal plant possessing immense medicinal potential. In India it is locally called as Doda Paneer (Hindi), Khamjira (Punjabi), Punir Band (Sindi) or Spiubajja (Persian). The species is commercially important for its berries which are used as milk coagulating agent. The berries consist of substances having enzymatic property of coagulation of milk. Therefore, this plant is used traditionally for production of cheese since decades. *W. coagulans* possesses tremendous medicinal properties and is used in treatment of nervous exhaustion, disability, insomania, wasting diseases, impotence, Dyspepsia, flatulent colic and other intestinal infections. Berries of plants are used as blood purifiers and flowers have been reported to be antidiabetic. The twigs of plant are chewed for cleaning of teeth and smoke of plant is inhaled for relief in tooth ache. Beside these medicinal properties plant is known to possess various pharmaceutical activities like antimicrobial, anti-inflammatory, anti tumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immune suppressive, free radical scavenging and CNS depressant activities of the plant. (Mathur *et al* 2011, Khodaei *et al* 2012, Pezeshki *et al* 2011, Jaiswal *et al* 2009).

Distribution and Propagation

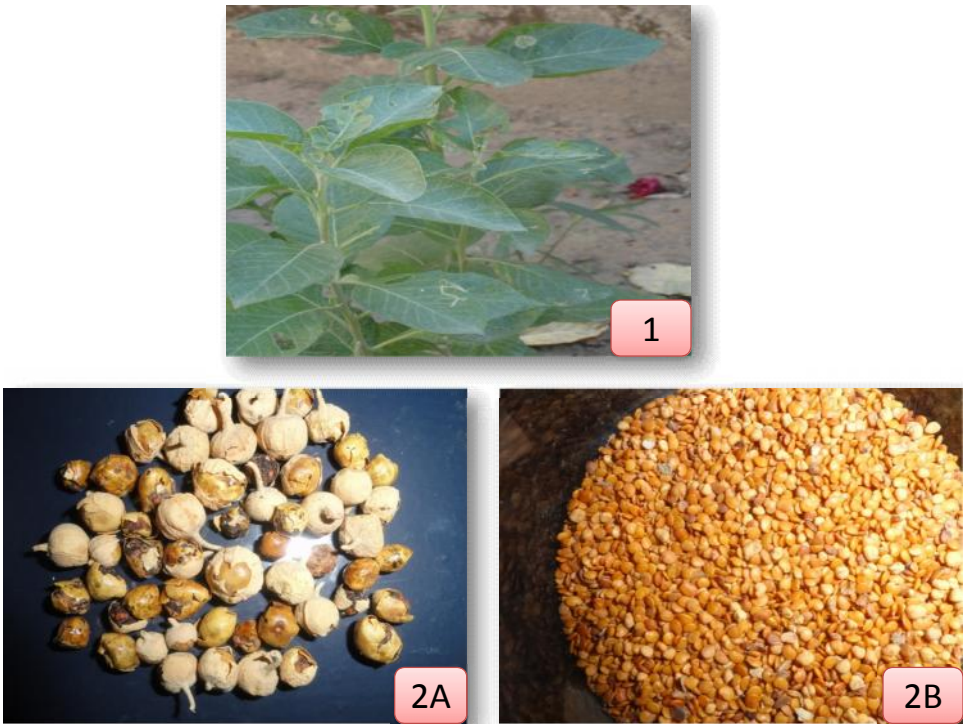
Withania coagulans is considered to be underutilized plant occurring scarcely as fragmented population in South Asia. It is found in Iran, Afghanistan, Pakistan, East India and Nepal. In India the plant is found in

drier part Rajasthan, Punjab, Gujrat, Simla and Kumaon region. It is distributed in dry hot stony places upto an altitude of 1700 m (Khodaei *et al* 2012, Pezeshki *et al* 2011, Gilani *et al* 2009, Negi *et al* 2006).

The plant is conventionally propagated through seeds and stem cutting. The plant has become endangered due to over exploitation of the plant either for medicinal purposes or for traditional uses such as fodder for animals, etc. Destruction of natural habitat has also contributed for the present endangered status of the plant. Low germination rate and reproductive failure due to dioceious nature of plant is also a hurdle in its mass propagation. (Valizadeh & Valizadeh 2009; 2011, Jain *et al* 2012, Sharma *et al* 2015).

Morphology

It is a rigid grey branched shrub of around 30 to 90 cm in height (Fig. 1). Branches are terete, clothed with dense grey or yellowish white tomentum. Leaves measure around 2.5-5.7 by 1.2-2 cm and are clothed with a persistant, grayish tomentum present on both sides which is not easily detachable. Petioles are long (6mm) but often indistinct. Flowers are unisexual present in axillary clusters. Pedicles (0.6mm) are long, deflexed and slender. Calyx (6mm) is long, campanulate clothed with tomentum. Corolla is 8mm long. In male flowers stamens are above level with top of the corolla tube with 2mm long filaments, anthers are 3-4 mm long. Ovary is ovoid without style or stigma. In female flowers stamens scarcely reaching half way up to corolla tube, anthers are smaller than in male flowers and sterile. Ovary is ovoid and glabrous. Berries are 6-8 mm in diameter globuse, smooth, closely frit by enlarged membranous calyx (Fig. 2A). Seeds are 2.5-3 mm in diameter somewhat ear shaped and globrous (Fig. 2B). The flowering period is from January to April (Dymock *et al* 1893, Khodaei *et al* 2012, Mathur *et al* 2011).



1- Young plant of *W. coagulans*; 2A- Fruits of *W. coagulans*; 2B- Seeds of *W. coagulans.*

Biological properties of *Withania coagulans*

Medicinal plants have been utilized for treatment of various diseases since ages. Moreover several advantages of medicinal drugs over synthetic drugs have enhanced the importance of traditional medicinal plants in modern medicine. *Withania coagulans* is one of the most privileged medicinal plant of ancient as well as modern medicine. The plant is known to possess diverse range of bioactive compounds which are crucial ingredient of various herbal formulations and other pharmaceutical products. Extracts of *Withania somnifera* and *Withania coagulans* are known to be present in well known herbal hepatoprotective medicine Liv-52.

Berries of the plant are used as blood purifier, twigs are chewed for cleaning of teeth and inhalation of smoke is found beneficial in toothache. The plant is used in treatment of insomnia, wasting disease, impotence, asthma, liver complaints. Flower of the plant has been found to be specifically useful in treatment of diabeties. Fruit of the plant are known to be sedative, emetic, alterative and diuretic and has been used in dyspepsia and other intestinal infections and are also applied to wounds.

The plant is known to have almost all known biological and pharmacological activity (Fig.3, Table 1) such as antimicrobial activity, anti inflammatory activity, anti tumour property, hepatoprotective property, anti hyperglycemic activity, cardiovascular activity, immunosuppressive activity, free radical scavenging and CNS depressant property. Fruit is applied to wounds, used in asthma bilsonsness and Stranjury. Seeds of *W. coagulans* are also diuretic and used to decrease inflammation of piles. The ripe fruits are also known to possess enodyne or sedative properties. Dried fruits (in Sind) are employed in dyspepsia, flatulent colic and other intestinal infections. Berries have also been blood purifiers. Although the herb has immense medicinal importance due to mentioned pharmacological properties but care should be taken hike utilizing the plant as medicine since it possess some level of toxicity.

Anticancerous or anti tumor activity is one of the most well established medicinal property of *Withania coagulans*. Many compounds such as withacoagulin A, withacoagulin C, withacoagulin D, withanolide J, withanolide E, withanolide F etc have been identified to possess antitumor activity. Anticancer activity of *Withania coagulans* has been associated with induction of apoptosis, inhibition of cell proliferation, caspase activation, anti-cytotoxic effect, DMSO inhibitory activity etc. Withanolides have been known to be antiproliferative, antimetastatic, antigiogenic, anti invasive and are also known to inhibit cell growth of various human cancer cell lines. (Khodaei *et al* 2012, Senthil *et al* 2007, Verma *et al* 1980, Budhiraja *et al* 1987). Mathur and Agarwal (2013) worked on anti carcinogenic potential of *W. coagulans*. In the work carried out aqueous and methanolic extract of fruit of *W. coagulans* were administered to DMBA induced skin papillonagenesis in rats and both the extracts were found to restrict the formation of tumor (cumulative number of papillomas) and decreased rate of tumor yield. Between the two methanolic extract was found to be more effective and the anti carcinogenic activity is attributed to presence of withanolides in the extract Conducted in vitro studies have revealed the diuretic potential of *W. coagulans* and these studies have also suggested utilization of the herb as diuretic agent. Dabheliya *et al* (2010) demonstrated diuretic potential of aqueous extract prepared from fruits of *W. coagulans*. It was observed that administration of the extract in divergent doses to Albino Wistar rats showed significant increase in urine volume as well as electrolytes. The study also indicated that the diuretic effect is attributed to presence of compounds of polar nature in the plant.

W. coagulans has been used in traditional Indian medicine system for treatment of diabetes mellitus. Fruits of *W. coagulans* are known to possess antihyperglycemic activity. With specific reference to Coagulin L, other alkaloids and steroids obtained from the plant possess antihyperglycemic activity. It has also been reported that presence of considerable amount of magnesium and calcium in *W. coagulans* has been found to be responsible for its role in Diabeties. In this context Jaiswal *et al* (2003) evaluated role of minerals in glycemic potential of aqueous extract of fruits of *W. coagulans.* In traditional system of medicine hypoglycemic plants have been used in their natural form, containing both inorganic and organic constituents. Contributions of minerals present in inorganic part of medicinal plants in enhancing hypoglycemic activity have been well established. The study concluded that the known hypoglycemic and antidiabetic potential of the plant can be attributed to the presence of magnesium and calcium ions in the extract. Beside the minerals, steroidal lactones were also found to contribute forward antidiabetic activity of *W. coagulans.* Work done by Yasir *et al* (2012) also confirmed hypoglycemic activity of *W. coagulans.* Administration of aqueous and hydroalcoholic extract of plant to test animals (Albino Wistar rats) showed significant decrease in blood glucose level, cholesterol and triglycerides.

Dyslipdemia is a well known complication associated with Diabetes mellitus, characterized by elevated risk of premature atherosclerosis. Drugs with antihyperlipidmic activity and hypoglycemic effect have been identified as potential anti htherosclerotic agents for management of diabetes. Saxena B (2010) investigated anti hyperlipidemic activity of *W. coagulans*. Aqueous extract of the herb was utilized to study its effect on level of lipid in diabetic induced rats and it was concluded that repeated oral administration of aqueous extract of *W. coagulans* possess significant hypolipidemic activity.

In a specific study conducted by Mathur and Agarwal (2011) extracts prepared from fruits of *W. coagulans* were shown to exhibit in vivo antimutagenic activity in a dose dependent manner. Cyclophosphamide was utilized as standard drug in the study. This also suggests the probable utilization of *W. coagulans* to formulate antimutagenic drugs.

W. coagulans is well known to possess antibacterial and antifungal properties mainly attributed to presence of withanolides, found to be present in almost entire plant. Both crude extract as well as essential oil extract of plant and have been analyzed to possess antimicrobial activity. *Staphyloccous aureus, Vibrio chlolerae, Micrococcus pyogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Enterobacter aerogenes* are some bacterial species against which antibacterial activity of *Withania coagulans* is well known (Sudhanshu *et al* 2012, Choudhary *et al* 1995, Khan *et al* 1993, Gaind and Budhiraja 1967, Khare 2007). The antifungal activity of herb has been determined effective against highly pathogenic fungi such as *Nigrospora oryzae, Aspergillus niger, Curvularia lanata, Microsporum canis* and *Epidermophyton floccosum.* In a study carried by Mughal *et al* (2011), antifungal activity of all extracts of *W. coagulans* have been reported to be effective against fungal strains such as *Trichoderma viride, Aspergillus flavus, Fusarium laterifum, Aspergillus fumigatus, Candida albicans, Trichophyton mentogrophytes* and *Microsporum canis*, however petroleum ether, methanolic and dichloromethane extract of *W. coagulans* has been reported to be most effective against tested fungal strains.

Table I: Summary of biological activities of *W. coagulans* identified by various authors

Author	Compound / Extract analyzed	Biological activity identified
Ruch *et al.*, 1989	Phenolic compounds	Anti oxidant
Choudhary *et al.*, 1995	Withanolides	Anti microbial
Budhiraja *et al.*, 1983		Cardiovascular Effect
Hasxlam *et al.*, 1989	Tannins	Protein Precipitating Activity
Gabor, 1979; Havsteen, 1983	Flavonoids and Flavones	Anti inflammatory
Crespy *et al.*, 2005		Anti oxidant Activity
Gaind and Budhiraja, 1967	Volatile oil from fruits	Anti bacterial Activity
Mathur *et al.*, 2011	Fruit Extract	*In vivo* antimutagenic and Anti oxidant potential
Hemlatha *et al.*, 2009	Aqueous Extract	Hypoglycemic activity
Budhiraja *et al.*, 1986		Hepato protective activity
Hemlatha *et al.*, 2000; Khan *et al.*, 1993		Hypolipidemic activity
Khan *et al.*, 1993		Free radical scavenging activity
Budhiraja *et al.*, 1977	Alcoholic Extract + Total Alkaloids	Anti inflammatory effect

Phytochemistry of *Withania coagulans*

The medicinal pharmacological and biological properties of *W. coagulans* are attributed to presence of a wide range of biochemical compounds in different parts of plant. Almost all parts of plants (leaves, roots, flower, fruit) have been reported to possess important biochemical compounds ranging from milk coagulating enzyme to free amino acids, essential oil, phenolic compounds, alkanoids, saponins, taxis, organic acids, carbohydrates etc. Phytochemically berries of the plant are the most important part and contain esterases, fatty acids, alkaloids and amino acids such as proline, valine, glycine, tyrosine, aspartic acid, glutamic acid, cystein,and aspargine. *W. coagulans* is also known as cheese maker or vegetable rennet because an enzyme called withanin which possess milk coagulating properties. The enzyme has been isolated from fruits and flowers and has been employed in parts of Pakistan, Afghanistan, India and Iran for production of cheese. (Mathur *et al* 2011, Dymock *et al* 1972, Wealth of India 1982).

Withanolides are main biochemical compound produced by the plant which is responsible for its ethanopharmacological activities. Withanolides is a common class of compound spread in various genera of family Solanaceae. Genus *Withania* has been a prominent source of withanolides. However other sources such as marine organisms (corals) and other plant families like *Taccaceae, Leguminoseae* has also been reported to produce withanolides.

Withanolides are C_{28} steroidal lactones. The basic structures of withanolide consist of six or five membered lactone ring formed on ergostane skeleton (Fig. 3B). The withanolide skeleton may be defined as 22-hydroxy ergostan-26oic-acid-26, 22olide. Different types of Withanolides have been identified based upon their chemical composition and structure (Mathur *et al* 2011). All the plants known to produce withanolides share two feature in common, one being the ability of incorporate oxygen at almost every position of carboxylic skeleton and side chain and the another characteristic feature of withanolide in presence of side chain of 9 C containing 6 or 5 membered lactone ring. The ring is often fused with carboxylic part of molecule through a C-C bond or through an oxygen bridge.

Biosynthesis of withanolides

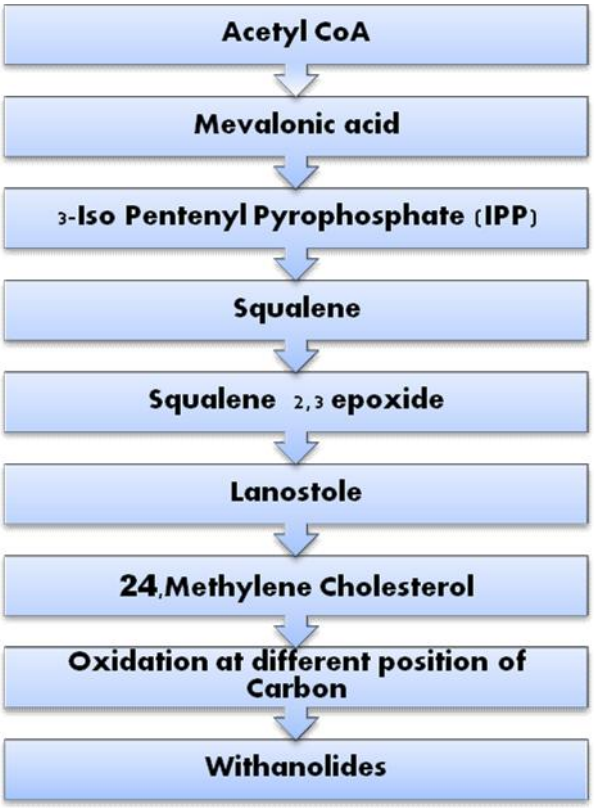


Fig.3A, Synthesis of withanolides

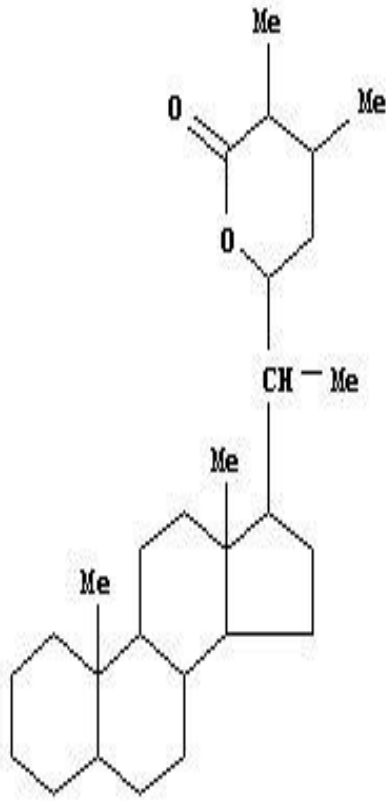


Fig.3B, Basic structure of Withanolide

The biosynthesis of withanoliodes (Fig.3A) begins with synthesis of cholesterol which is converted to 24methylene cholesterol which is believed to be the actual precursor of withanolides. Cholesterol synthesis occurs by condensation of two molecules of acetyl CoA to form acetoacetyl CoA which condenses with another molecule of acetyl CoA to form 3hydroxy-3-methylglutaryl CoA which on reduction forms mevalonic acid. Six molecules of mevalonic acid combine to form squalene. A series of phosphorylation reaction catalyzed by respective enzymes are involved in conversion of mevalonicacid to squalene. Incorporation of molecular oxygen leads to epoxidation of squalene and 2, 3- epoxysqualene is formed. As a result of cyclization of squalene chain and series of 1, 2 Trans migration of hydrogen atoms and methyl groups lanosterol is formed. Lanosterol is ultimately converted to 24 methylene cholesterol by removal of methyl group one ring of lanosterol and rearrangement of double bonds in another ring of lanosterol, followed by addition of methyl group at C-24, forming 24- methyl cholesterol which on reduction yields 24-methylenecholesterol which is biosynthetic precursor of steroidal lactones. (Khodaei *et al*. 2012, Kreis and Muller- Uri 2010).

Withaferin A is a crucial withanolide obtained from *W. coagulans*. Various reports have revealed biochemical and pharmacological properties of withaferin A. Withaferin A has been known to possess activities such as anti inflammatory, antioxidant. antitumor. etc. It is also associated with antimitotic activity and acts as mitotic poison and can arrest tumor cells at metaphase. Withaferin A diacetate and its derivative 4-dehydro- withaferin A possess cytotoxic activity. Antitumor property of withaferin A can be attributed to its ability to induce apoptosis by inhibiting topoisomerase –I DNA complex. Beside this withaferin A is also reported to promote formation of dendrites. Withaferin A is also associated with alteration of cytoskeletal architecture including actin microfilament aggregation mediated by covalent bonding of anexin11 (Mathur *et al* 2011).

Biotechnological Tools for Conservation for *Withania coagulans*

The entire technology of "Plant Tissue Culture" is based on this ability of plant cells to be influenced by their surroundings and differentiate to give rise to a range of organs dependent on the culture conditions (Bowles and Leyser, 1994). Plant Tissue Culture is the maintenance and propagation of plant parts excised as explants, under controlled environmental conditions.

In a significant study carried out by Valizadeh and Valizadeh (2011) a complete protocol for propagation of *Withania coagulans* was developed. Nodal segments were selected as explants and were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) (2-4mg/l) or indole butyric acid (IBA) alone and in combination of varying concentration. BA (2mg/l) + IBA (0.5mg/l) proved to be most suitable for in vitro multiple shooting (7.2 per explants). IBS (1-4 mg/l), IAA (0.25-1mg/l) and Kinetin (1-2mg/l) were analyzed for root induction in half strength MS media. IBA (2mg/l) was most efficient for in vitro root induction. In another study Valizadeh and Valizadeh (2009) obtained callus from in vitro culture of leaf and intermodal segments. Leaf segments were cultured on MS+ 2,4D (2-4mg/l) +BA (0.5-1mg/l) and MS + 2, 4D (2-4mg/l) +Kn (0.5-1mg/l). Callusing was observed in all combinations after interval of 14-16 days. Callusing occurred in internodal segments on MS medium fortified with 2,4D (2-4mg/l)+BA (0.25-0.5mg/l). Shoots were generated from the callus on MS +BA (2mg/l)+IBA(0.5mg/l) whereas ½ strength MS medium with IBA (2mg/l) was found to be most suitable for root induction.

Jain *et al* (2009) obtained prolific multiplication of axillary buds from nodal segments of *W. coagulans*. MS medium+ BA (0.5mg/l), Kn (0.5mg/l) were utilized in the study. Phloroglucinol (PG) (0.5mg/l) was found to improve induction and elongation of shoots buds. Root induction from *invitro* generated shoot was obtained on MS+ IBA (0.25mg/l) + Phenyl acetic acid (0.5mg/l) + Choline Chloride (2mg/l). A major problem concerned with mass propagation of *W. coagulans* is low germination rate in nature. In this respect work carried out by Sharma *et al* (2015) germination rate was greatly enhanced by pre treatment of seeds with HCl solution before culturing them onto media or germination in soil. IN the same study germinated seeds exhibited extensive growth onto MS medium supplemented with different adjuvants and resulted in invitro regeneration of large number of plants.

Beside micropropagation studies done with sole objective to conserve the endangered species, efforts have also been done to genetically modify or transform the plant for enhancement of production of crucial metabolites. Mirjalili *et al* (2011) introduced a transgene SSl encoding squalene synthase (which dimerises two molecules of farnesyl diphosphate to synthesize squalene) from *Arabidopsis thaliana* into *W. coagulans*. The gene was transferred in hairy root culture under a specific promoter and utilizing *Agrobacterium rhizogenes* A4. Higher level of phytosterols and withanolide production was obtained in transgenic roots as compared to control. In another study Mirjalili *et al* (2009) cultured leaf segments of *W. coagulans* with *Agrobacterium tumefaciens* (strain 58C1) which induced transformation of roots with increased capacity to produce withanolide A and witheferin A. The transformed roots showed the morphologies, one callus like root with high capacity to produce withanolides and another typical hairy root with fast growing capacity but lower withanolide production. Aux gene was found to be present in all transformed roots with callus like morphology indicating their prominent role in morphological transformation of roots. Mishra *et al* (2013) successfully achieved genetic transformation in regenerated plants from leaf explants of *W. coagulans* through *Agrobacterium tumefaciens* gene transfer. Agrobacterium strain LBA4404 having binary vector p1G121Hm containing B-glucoronidase gene (GusA) under control of suitable promoter. The obtained transgenic plants showed 100% frequency of transient GUS expression with 5% stable transformation

Epilogue

Medicinal plants have been the sole foundation of development of traditional as well as modern medicine. *Withania coagulans* is one such medicinal plant which possesses immense medicinal value and has been utilized in treatment of various diseases and disorders. Numerous pharmacological studies have been carried out which have confirmed the medicinal potential of the plant. Outcome of such studies suggest and support further research in development of more approved drugs by utilization of bioactive metabolites of W. *coagulans*. Due to overexploitation and unrestricted collection from wild stands, along with destruction of habitat and poor germination rate has rendered the plant endangered. Only few reports are available regarding *in vitro* micropropagation for conservation and mass propagation of the plant. Hence, more tissue culture studies are required for effective conservation of *Withania coagulans*.

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