2. REVIEW OF LITERATURE
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Various species of the genus *Withania* have been found to contain alkaloids and withanolides. Pharmacologically the different crude extracts of some of these plants have been reported to possess sedative and hypotensive properties. Out of various species of this genus, detailed studies have been made on the roots and leaves of *Withania somnifera* and its roots are now official in Indian Pharmacopoeia, 1966. However, very few reports are available about the work on other species of this genus except that some interest has been recently shown for the purification and exploration of proteolytic enzymes of *Withania coagulans* for the preparation of cheese and dahi. In order to co-relate various investigations, a brief description of the work done on the various species of the genus is described here.

*Withania somnifera* Dunal:

*Withania somnifera*, commonly known as Ashwagandha, has been described in early and modern Indian literature as a curative for many ailments mainly as a sedative, tonic, aphrodisiac, in senile debility, spermatorrhea,
and applied to carbuncles, obstinate ulcers, rheumatic swellings and used as abortifacient (Kirtikar and Basu, 1933; Nadkarni, 1954; Chopra et al, 1958). Various parts of the plant especially its roots and leaves have been investigated chemically, pharmacologically, bacteriologically and clinically. Early investigators (Power and Salway, 1911; Majumdar and Guha, 1933), isolated hentriacontane, ipuranol, Withanol, somniol, somnirol, withanic acid, an essential oil and fatty acids such as palmitic, stearic, ocetic, oleic and linoleic from the roots or leaves of this plant. Presence of several nitrogenous bases was also established. Later chemical investigations of the roots resulted in the isolation, naming and partial characterization of seven amorphous bases and nicotine (Majumdar, 1955). Schwarting et al (1963) isolated and characterised eight alkaloids namely cuscohygrine, choline, anahydrine, tropine, pseudotropine, anaferine, isopelletierine and 3-tropyl tigloate. Schroter and Neumann (1966) isolated a pyrazole alkaloid.

Since 1962 a series of C_{28} steroidal 22, 26β-lactones built on an ergostane type skeleton with various substitution patterns have been isolated from leaves of
Withania somnifera. These are called withanolides. First compound of this group was isolated by Yarden and Lavie (1962). Structure of this compound and many other compounds isolated of this series has been ingeniously elucidated by Lavie and coworkers (1965 onwards) by the use of $^{13}$C NMR, mass spectrometry and on their behaviour in the presence of trichloroacetyl isocyanate. The first compound isolated and called Withaferin A has been identified as 4β, 27-dihydroxy-1-oxo-5β, 6β-epoxy-witha-2, 24-dienolide and a large number (about 40) of such compounds of this series have been isolated from this plant so far and their structure elucidated. Several other compounds, such as 22, 26-$\alpha$-lactols, 23,26- and 28,26-$\gamma$ -lactones, ring D aromatic compounds and 13,14-seco-derivatives are also biogenetically related to the withanolides. Kirson and Glotter (1981) have classified the various withanolides according to the following structural features: 1β(-hydroxywithanolides, 14-hydroxy withanolides, 15-hydroxy withanolides, 16-hydroxy withanolides, 17-hydroxy withanolides and compounds with modified side chain such as epoxylactols, epexylatones, acnistins and physalins.

On the basis of substitution pattern of the
steroidal lactones, different chemotypes of *Withania somnifera* plants have been recognised and, three of these designated as chemotypes I, II and III have been found to occur in Israel (Abraham et al, 1968). Isolation and structure elucidation of twelve steroidal compounds from Indian chemotype have been reported by Kirson et al (1971). Similar compounds from chemotype I and II were isolated by Abraham et al (1975) and from chemotype III by Kirson et al (1977). Eastwood et al (1980) reported the isolation of Withanolides from African chemotype. The Withanolides have been isolated from seeds (Kundu et al, 1976 a) and roots of the plant (Menssen & Stapel, 1973).

In addition to genetic studies at the chemical level, a number of compounds considered to be intermediate steps in the biogenetic sequence of withanolides have been isolated from *Withania somnifera* (Nittala and Lavie, 1981; Vande Velde and Lavie, 1981; Vande Velde and Lavie, 1982).

Isolation of chlorinated withanolides from *Withania somnifera* has also been reported (Nittala et al, 1981). Chemistry of the withanolides and their related compounds has been reviewed by Kundu et al, 1976 b, Tursunova et al, 1977; Kamernitskii et al, 1977; Glotter
et al, 1978; Kirson and Glotter, 1981, which indicate that so far withanolides have been isolated from the plants of family Solanaceae only belonging to the genera *Withania*, *Aconitum* (*Dunalia*), *Physalis*, *Nicandia*, *Datura* etc., where they occur mainly in the leaves.

Sedative, hypnotic and antiseptic activities have been attributed to the different crude extracts of this plant. The first unsaturated lactone isolated from *Withania somnifera* leaves by Kurup (1956) was shown to possess remarkable antibacterial properties (Kurup, 1956; Kurup, 1958; Kurup and Das, 1963) and similar effect with crude extract was observed by Bhatnagar et al (1961). *Withaferin A* isolated from *Withania somnifera* leaves is identical to the lactone of Kurup. In addition to the remarkable antibacterial properties of lactones described by Kurup and confirmed by others (Ben-Efrain and Yarden, 1962; Lavie, 1965; Sethi et al, 1974) *withaferin A* has been found to possess antifungal (Kupchan et al, 1965; Dasgupta et al, 1970); and antitumor activity (Shohat et al, 1967; Shohat et al, 1970; Shohat and Joshua, 1971a; Shohat, 1972). Shohat and Joshua (1971b) reported that *withaferin A* in 0.01-0.5% concentration inhibited growth of *Allium cepa* by arresting metaphase. Choudhry
and Neology (1975) found that withaferin A and D (40 μg/ml) inhibit RNA formation by sarcoma isotumor cells and inhibit protein formation. Withaferin A and E have been found to have immunosuppressive effect on human B and T lymphocytes (Shohat et al, 1978). Antibacterial effects of some other C_{28} withanolides have also been reported by Sethi et al (1974). Withaferin A inhibits adjuvant arthritis in rats and graft versus host reaction in chicken (Füger, 1973). Another withanolide 5,20α(R) dihydroxy-6α−7α-epoxy-1-oxo (5α')-witha-2,24-dienolide has also been found to have immunomodulating properties (Bähr and Hansel, 1982).

Several withanolides were recently found to be antifeedants when offered to larvae of *Spodoptera littoralis* (Ascher et al, 1980).

Sedative effects of the drug have been reported by Trebut (1886), Pitini (1924), Wsiki (1948), Trutneva (1971), Fontaine and Erdos (1976), Singh et al (1978, 1979). Garg and Prasar (1965); Jawwal and Anand (1962) found that the drug has antifertility effect. Gupta et al (1977) found it rejuvenator. Bector et al, 1968 and Lahkar, 1981 found
the roots to be effective in acute rheumatoid arthritis. Anti-inflammatory effects of the roots have been studied by Sharma and Singh, 1980; Anbalagan and Sadique, 1981.

Trutnev (1971) found that visamine, a new alkaloid from W. somnifera prolonged hexenal induced sleeping time and had hypothermic and nicotinolytic effect but produced no effect on mice exposed to electroshock or treated with corazole, arecoline or strychnine. The alkaloid had no effect on cardiac vascular system or autonomic nervous system and smooth muscles. LD$_{50}$ was 450 mg/kg S/C and 125 mg/kg i.p.

Hewett et al (1971) found that withasomine, an alkaloid, had narcotic, slight analgesic, spasmolytic and depressant effect on CNS as well as ANS. However, it is pertinent to mention that none of the alkaloidal constituents of W. somnifera isolated so far by various workers possesses marked physiological properties (Abram et al, 1965; Kundu et al, 1976). Mitali (1984) has reported that the alcoholic extract of the leaves of W. somnifera possess CNS depressant, hypotensive, and anti-inflammatory effects.
Withania ashwagandha, Kaul:

It is a closely related species to Withania somnifera. Comparative histological and chemical studies of Withania somnifera Dunal and Withania ashwagandha Kaul have been reported by various workers (Sastry et al, 1960; Dhalla et al, 1961 and Khafagy et al, 1962). Detailed pharmacological studies of total alcoholic extract, total alkaloids named as ashwagandholine and various fractions of alkaloids have been reported (Malhotra et al, 1960 a,b; Malhotra et al, 1965 a,b; Prasad and Malhotra, 1968). It has been found that alcoholic extract, total alkaloids and acetone soluble alkaloidal fraction have sedative effect in different species of animals. It also has biphasic action on various smooth muscles, prolonged hypotensive, brady-cardiac and respiratory stimulant effects. Malhotra et al (1969) have further reported that alcohol soluble and water soluble alkaloids have anti-inflammatory effect. Subramanian and Sethi (1971 a) reported the isolation of two steroidal lactones from this plant, one of which has been identified as \(5,20\alpha(R)\)-dihydroxy-6\(\alpha\),7\(\alpha\) \(-\)epoxy-1-oxo(5 \(\alpha\)) witha-2,24-dienolide.
Withania frutescens

Presence of chlorogenic acid has been reported by Politis (1948). Goenzalez et al (1972, 1974 a) have isolated and characterised withaferin A, dihydrowithaferin A; 4β, 27-dihydroxy-1-oxo-5β, 6β-epoxy-22R-witha-2, 14, 24-trienolide; 4β, 17β, 27-trihydroxy-1-oxo-22R-witha-2, 5, 24-trienolide; its 2, 3 dihydro derivative; 4β-hydroxy-5β, 6β-epoxy-1-oxo(22R)-witha-2, 14, 24 trienolide and a chlorine containing withanolide 6α-chloro-5β-hydroxy-withaferin A.

Withania aristata

Goenzalez et al (1972, 1974 b) have isolated and identified a number of withanolides from the aerial parts of Withania aristata such as Withaferin A, dihydrowithaferin A; 4β, 27 dihydroxy-5β, 6β-epoxy-1-oxo(22R)-witha-2, 14, 24 trienolide (m.p. 256-259 o); 4β-hydroxy-5β, 6β-epoxy-1-oxo(22R)-witha-2, 14, 24-trienolide, 22-hydroxy derivative and 1α, 14α-dihydroxy-22R-witha-5, 24-dienolide.

Withania obtusifolia Tack

It is less pubescent, leaves: thin, delicate,
narrow elliptic, berries: larger and dark. No reference about the phytochemical investigation of this plant could be traced (Tackholm, 1956) except that it contains amorphous alkaloid in leaves which did not show atropine like action (Fahmy, 1963).

**Withania coagulans** Dunal

Morphological characteristics and uses of *W. coagulans* plant have been described in the introductory part (see page 3). Review about the work on the other aspects is as under:

**Esterase activity**

It was suggested by Lea (1883) that the extract obtained from the berries of *Withania coagulans* Dunal could be used for the preparation of cheese. Since then a number of attempts have been made for the extraction and use of vegetable rennet for cheese making (Kothavalla and Khubchandani, 1940; Yeshoda, 1941; Narain and Singh, 1942; Dastur, 1949). Yeshoda isolated the enzyme from aqueous extract of the berries by precipitating it with 65% saturation of ammonium sulphate which could coagulate
milk but had no other proteolytic activity. It was most active at 48°C. Dastur et al (1948) obtained the enzyme from aqueous extract of the berries by precipitation with 2 vol. of acetone. The optimum temperature of the enzyme was found between 45°-65° and the enzyme isolated compared favourably with that of Hansen's liquid (a source of the animal rennet by Hansen's Laboratory, Denmark) and powered rennet (Yeshoda, 1941). It has been further reported that extracts of seeds, leaves and stems had no coagulating activity. Dastur et al (1948) found that the most convenient way of preparing the enzyme is to extract the berries with water and precipitate the enzyme with 2 vol. of acetone and dry the enzyme at low temperature.

Atal and Sethi (1961) reported evidence for the presence of proteolytic enzyme in the aqueous extract of the fruits but the properties of this enzyme were not investigated any further. Sethi (1968) reported the aminoacid composition of the proteolytic enzyme.

Qureshi et al (1963) isolated the enzyme from aqueous extract of the fruits by precipitation with alcohol, one part of which could coagulate about 5000 parts of milk.
It was reported that curd and cheese made with crude enzyme from *Withania Coagulans* develop bitter taste (Krishnamurthi and Subramanyam, 1948; Qadri and Wahid, 1968; Qadri et al, 1970). Further attempts were, therefore, made by various workers to purify the enzyme (Qadri and Wahid, 1968; Amir and Sultana, 1970; Qadri et al, 1970; Singh et al, 1973). It has been found that the pulp is the richest source of milk coagulating enzyme (Qadri et al, 1970) and there are at least 2 esterases; one extracted at pH 5.0 (Esterase I) and the other extracted at pH 10 (esterase II) (Qadri and Wahid, 1968). Esterase I was isolated by extraction with 0.01M sodium acetate buffer pH 5.0 and dialysed against the same buffer for 24 hours. Its kinetic and inhibition studies were made using p-nitrophenyl acetate and metal ions and other potential inhibitors. The Michaelis and Menten constant of the enzyme for p-nitrophenyl acetate is $85 \times 10^{-5}$M while the optimum pH and temperature are 8.5 and 55°, respectively. Al$$^{+++}$, Zn$$^{++}$, Cu$$^{++}$, Hg$$^{++}$, iodate and fluoride ions and EDTA inactivated the enzyme.

Qadri et al (1970) extracted the enzyme at pH 5.5 and compared the qualities of dahi (yogurt) and cheese prepared with this enzyme with one prepared with
vegetable rennet compared favourably with that of animal rennet.

Amir et al (1970) isolated the enzyme from the aqueous extract of the berries with 20% saturation of sodium chloride. Proteins precipitated with 10% sodium chloride concentration were discarded. In addition to milk clotting activity, it was found to have proteolytic activity when tested against casein. The optimum temperature for proteolysis was found to be 55° as compared to 45-65° reported for milk clotting activity (Dastur et al, 1968). Metals ions like Ag++, Hg++, Cu++, Al+++ and Zn++ strongly inhibited the proteolytic activity. It was also found that storage of the enzyme in the dried state or in phosphate buffer at pH 7 stored in frozen state caused considerable loss of activity.

Recently the esterase II has also been purified and its characteristics studied (Qadri and Wahid, 1975). Esterase II contains S-S bonds whereas esterase I contains-SH group which are integral part of the active centres of the enzymes.
Other constituents

In addition to the enzymes, the fruits of *Withania coagulans* have been found to contain a number of other constituents. Atal and Sethi (1963) isolated triacontane and β-sitosterol from the nonsaponifiable portion of the oil. They also reported the presence of proline, hydroxyproline, valine, tyrosine, aspartic acid, glycine, asparagine, cysteine and glutamic acid in alcoholic extract. Using butanol: HCl (98:2) as solvent system for chromatography, they reported the presence of 3 alkaloids in alcoholic extract. Two alkaloids in crude form, m.p. 118-122° and 145-147° were also isolated.

Atal et al (1964) reported the presence of linoleic acid in *Withania coagulans*. Israili and Siddiqui (1965) reported the physical constants of the oil. Further, using paper chromatography they reported the presence of 14 fractions in crude alkaloidal extract. They also isolated some alkaloids in crude form and prepared their picrates. Melting points of the alkaloids with the m.p. of their picrates (in parenthesis) are: 125-128° (187-188°), 90-92° (62-63°), 81-82° (151-152°) and 128-130° (261-262°). Isolation
of alkaloids from the fruits has been reported by Nazer-ud-din and Zaidi (1965) also.

Galnd and Budhiraja (1967) isolated a volatile oil from petroleum ether extract and a petroleum ether soluble alkaloid, m.p. 232-233°. Sethi and Subramanian (1970) reported the pharmacognostical studies on roots and leaves of the plant. The composition of seed oil has been reported by Salam et al (1970) and reported the presence of palmitic, stearic, oleic, arachidic and linoleic acids respectively as 11.64, 2.66, 3.49, 12.56 and 69.65%.

Salam and Wahid (1969) have reported the presence of D-galactose, D-arabinose (1:1 ratio) and maltose in traces as free sugars in the seeds of Withania coagulans. Polysaccharide of the seed was composed of D-galactose and D-arabinose in the ratio of 3:5.

**Constituents of roots and leaves**

From the roots of Withania coagulans, Subramanian and Sethi (1969) isolated withaferin A and a withanolide, m.p. 272-273°, the structure of which was found to be $5\alpha, 20\alpha (R)$-dihydroxy-6$\alpha, 7\alpha$ -epoxy-1-oxo-witha,2,24-
dienolide (Subramanian et al, 1971b). From the same source, Sethi and Subramanian (1975, 1976) isolated another withanolide, m.p. 215-217° and identified as 5β, 27α-dihydroxy-1-oxo-6α, 7α-epoxy-witha-2,24-dienolide. Another withanolide, m.p. 275-276° has been isolated from roots by Kirson et al (1969) and has been identified as 5α, 17α-dihydroxy-1-oxo, 6α, 7α-epoxy-witha-2,24-dienolide.

Subramanian and Sethi (1971c) isolated 4 withanolides from Withania coagulans leaves and identified as 5,20α(R)-dihydroxy-6α-7α-epoxy-1-oxo-witha-2,24-dienolide, withaferin A and 2 minor withanolides one of which is probably withanone.

Pharmacological studies

Siddiqui et al (1963) assessed the pharmacological activity of water soluble portion of alcoholic extract of the fruits and found that it had depressant effect on central nervous system. It caused fall of blood pressure but stimulated respiration. It also showed smooth muscle relaxant effects. The extract was found to be non-toxic up to a dose of 8 gm/kg when injected i.p. in mice.

Gaind and Budhiraja (1967) investigated the
various extracts of the whole fruit for antimicrobial activity and found that alcoholic extract containing the alkaloids was most active. An essential oil isolated from the petroleum ether extract of the fruits was found to have antibacterial and anthelmintic activity. Hasnavimarsh effects have been studied by Sharma (1978).

**Scope of present investigations**

The fruits of *Withania coagulans* are reported to be useful for a number of ailments. Survey of literature has revealed that chemically the fruits of *W. coagulans* contain alkaloids, proteolytic enzymes, oils and sugars. Four steroidal lactones have been isolated from roots and four from leaves of this plant. The water soluble portion of alcoholic extract of the fruits has been reported to possess depressant effect on CNS, CVS and respiratory stimulating properties. It has been found to cause relaxation of the smooth muscles and antispasmodic effects. Total alkaloids and volatile oil possess anthelmintic and antibacterial activity.

In view of the usefulness of the fruits in
various ailments and important pharmacological actions of *W. coagulans* revealed with crude extracts, it is proposed to undertake further phytochemical and pharmacological studies so as to establish the identity and assess the pharmacological effects of the constituents of *W. coagulans* fruits. The fruits will also be investigated for actions based on the claims made in Ayurvedic and Unani systems of medicine.

**PLAN OF WORK**

The study will be carried out as under:

1. **Phytochemical studies**: Isolation, purification and characterization (as far as possible) of chemical constituents of the fruits.

2. **Pharmacological studies**: Major constituents of the fruits of *Withania coagulans* will be used to study their pharmacological effects.

**MATERIAL AND METHODS**

For the present investigations, the fruits of *Withania coagulans* will be studied as under:
1) For isolation of the constituents, usual phytochemical methods as reported by Paech and Tracey (1955) will be followed.

2) Pharmacological activity of the various extracts/major constituents will be assessed as follows:

   a) Effects on central nervous system.
   b) Effects on cardiovascular system.
   c) Effects on smooth/skeletal muscles of the common laboratory animals.
   d) Liver function tests.
   e) Anti-inflammatory actions.
   f) Toxicological studies.
   g) Any other possible effect observed during investigations.