DISCUSSION
Phytochemical Studies

The alcoholic extract of *Withania somnifera* has been reported to contain alkaloids, withanolides, flavonoids, sitoindosides VII, VIII, IX & X, amino acids and inorganic constituents. These components have been reported to be present both in roots and leaves of the plant. Withanolides are steroidal lactone ring containing compounds characterized by C$_{28}$ basic skeleton with a nine carbon atoms side chain in which C$_{22}$ and C$_{26}$ are appropriately oxidized to form a six membered δ lactone ring. However, withanolides have been reported to be present more in the leaves. Therefore, in the present investigations leaves of the plant were used for isolation of withanolide fraction (W-1) and preparing the alcoholic extract. The results of the present phytochemical studies are in conformity with earlier findings. The alcoholic extract is known to contain withanolides, alkaloids and other constituents. The withanolide fraction was isolated and was purified by column chromatography and crystallization. The withanolide fraction was assessed for pharmacodynamic activity and the effects were compared with alcoholic extract of *Withania somnifera*. The purified withanolide fraction was assessed for its identity and structure by physical, chemical and spectroscopic examinations such as IR, UV and NMR. The purity was assessed by spectrophotometric analysis and was confirmed that the withanolide fraction contains no other component other than withanolides.
**Pharmacodynamic Studies**

*Withania somnifera* is a medicinal plant of repute, widely used in Ayurvedic system of medicine as health tonic, haematinic, for treatment of debility, premature ageing and impotency. Its dry powder, crude extract, alkaloids and some of the withanolides especially withaferin A have been studied pharmacologically by various workers with divergent results. Investigations have proved that the plant is useful in most of the above conditions.

Recently the alcoholic extract of *Withania somnifera* and some of the withanolides especially withaferin A present in *Withania somnifera* have been reported to have antitumor and radiosensitizing effects.\(^{116-125}\) However most of the chemotherapeutic agents used in malignancy exhibit toxicity to normal tissues resulting in undesirable side effects. Withaferin A has also been reported to be toxic.\(^{118}\) On one hand *W. somnifera* is being used as a tonic, antiageing, haematinic and has been included in a large number of Ayurvedic patent preparations for these conditions and on the other hand some of its constituents such as withaferin A has been reported to have anticancer activity. Moreover, total withanolide fraction has not been evaluated pharmacologically so far. Therefore, a comparative study of pharmacodynamic effects of alcoholic extract of *Withania somnifera* and purified crystalline withanolide fraction has been carried out. The results on various organs /organ systems have been discussed as under.

**Haemopoietic system**

The results of the present study on haemopoietic system indicate that both WS and W-1 treatments produced a significant increase in red blood corpuscles count, haemoglobin content and bone marrow cellularity.
with an increase in erythroid series. The rise in RBC count and haemoglobin levels in the WS treated group is in accordance with the report of Kuppurajan et al.\textsuperscript{150} and Sharada et al.\textsuperscript{157} The increase in RBC count and haemoglobin levels supports the traditional use of \textit{W. somnifera} as haematinic and growth promoter. This study also indicates that haemopoeitic effect of WS can mainly be attributed to withanolide fraction (W-1) of \textit{W. somnifera}.

Sharada and co-workers have also observed an increase in RBCs, haemoglobin and WBC and reported in their studies that the haematinic effect of WS is due to the high iron content of \textit{W. somnifera}. However, in the present study the withanolide fraction which is devoid of iron as confirmed by British Pharmacopoeia method\textsuperscript{152} has also produced an increase in RBC count and haemoglobin levels. It indicates that increase in RBC and haemoglobin due to WS is not solely due to the presence of iron alone in \textit{W. somnifera} but some other mechanism is also involved.

Davis and Kuttan have reported that WS treatment prevented the lowering of haemoglobin, RBC count, TLC and platelets caused by cyclophosphahamide treatment. They found a significant increase in the number of bone marrow cells and $\alpha$ - esterase positive cells in \textit{W. somnifera} and cyclophosphahamide treated group as compared to cyclophosphahamide alone treated animals. In an other study by Shohat et al Withaferin A was found to be less toxic to bone marrow as compared to the cytotoxic drug cyclophosphamide, used in cancer chemotherapy.\textsuperscript{118} The increase in bone marrow cellularity by WS in the present study is supported by the report of Ziauddin et al.\textsuperscript{99} and Davis and Kuttan\textsuperscript{140} who
have observed that WS treatment enhances the differentiation of bone marrow cells.

WS treatment lowered the formation of micronucleated polychromatric erythrocytes formed after radiation, indicating that it reduces the chromosomal damage caused by radiation.\textsuperscript{194}

The increase in RBC count, haemoglobin levels and the decrease in WBC count observed with WS and W-1 are similar to the effects exerted by glucocorticoids.\textsuperscript{195} The results could possibly be explained on the basis of sharing of steriodal nucleus, by withanolides with glucocorticoids. Quereshi et al have reported that the increased red cell production observed may be attributed to some constituents in the plant such as steroids, which may influence androgen levels in the body.\textsuperscript{196} Further Rao et al have proposed that testesterone and related androgenic derivatives are most potent hormones capable of stimulating erythropoiesis.\textsuperscript{197} Moreover, \textit{W. somnifera} is reported to increase testesterone levels,\textsuperscript{198} thereby indicating that probably the steroidal components i.e. the withanolides which also have produced haematinic effects in the present study, may be responsible for producing erythropoietic effect. It justifies the use of Ashwagandha as haematinic, tonic and growth promoter.

\textbf{Antiinflammatory Activity}

Inflammation is a response of vascularised tissue of body to injury involving the infiltration of cells, production of mediators and release of hydrolytic enzymes. The inflammatory response is a polyphasic tissue reaction and different chemical mediators have different mechanism of producing inflammation. Various anti-inflammatory agents may act at only one component of the reaction or on a single phase of the
component. Determination of paw oedema is apparently simple, sensitive and quick procedure for evaluating the response to anti-inflammatory agents.\textsuperscript{199} Hence various phlogestic agents, viz. carrageenan, histamine and formalin, which produce inflammation by different mechanisms/mediators were used to produce inflammation. Carrageenan-induced inflammation is useful in detecting orally active anti-inflammatory agents.\textsuperscript{200} Di Rosa \textit{et al} and Flower \textit{et al} have reported that carrageenan produces inflammation in the rat paw by release of various mediators.\textsuperscript{201, 202} The initial phase is due to the release of histamine and serotonin, the kinins play a role in the middle phase and prostaglandins could be the most important mediator in the final phase (3-5hr) in post carrageenan response.\textsuperscript{203, 204} Histamine by itself produces acute inflammation in the rat paw. Formalin produces oedema by release of histamine, serotonin, prostaglandins and bradykinin.\textsuperscript{205, 206}

Both WS (1gm/kg) and W-1 (50mg/kg) administration prior to phlogestic challenge did not produce any effect on inflammation produced by various phlogestic agents in acute conditions. It indicates that both WS and W-1 lack anti-inflammatory activity in models of acute inflammation.

These results are supported by the studies of Sudhir \textit{et al} who have reported that \textit{W. somnifera} was ineffective in acute inflammation,\textsuperscript{40} though Al-Hindawi \textit{et al} and Sahni and Srivastava found the plant to be effective against carrageenan induced inflammation.\textsuperscript{37, 93}

It is well known that oxygen derived free radicals are one of the causes of inflammation, which cause peroxidation of membrane lipids and ageing.\textsuperscript{207} Sen \textit{et al} & Blake \textit{et al} reported that oxygen derived free radicals (ODFR’s) have been implicated in the causation of rheumatoid
In rheumatoid arthritis, physical movement induces hypoxia-reperfusion in the synovial joint and triggers generation of reactive oxygen species and ODFR's. Chaturvedi et al have reported that the levels of TBARS in rheumatoid arthritis have been observed to be increased when compared to healthy controls.

Superoxide free radical (O$_2^-$) produced by leukocytes, found in an inflamed rheumatoid joint, degrade various high molecular polymers in the synovial fluid and thus may play an important role in initiating an inflammatory response. Superoxide dismutase, which catalyses the dismutation of the O$_2$ radical to molecular oxygen and hydrogen peroxide, has been implicated in protection against degradation of synovial fluid. McCord et al and Petrone et al have proposed that the anti-inflammatory activity of superoxide dismutase is due to its scavenging of phagocyte-produced superoxide and preventing of the migration of neutrophils and production of neutrophils chemotactic factor.

For subacute oedema, formalin induced arthritis and cotton pellet granuloma methods were used. Formalin produced oedema over a period of 10 days, with simultaneous increase in levels of TBARS and decrease in levels of GSH, catalase, superoxide dismutase and glutathione-S-transferase in paw tissue. It indicates that the increased generation of oxygen free radicals and free radical mediated membrane lipid peroxidation are also involved in inflammatory process.

Both WS (1g/kg) and W-1 (50mg/kg) treatments produced significant reduction in oedema volume indicating anti-inflammatory activity in subacute model of inflammation. WS and W-1 treatments also decreased the levels of TBARS and increased the levels of glutathione-S-
transferase, indicating a significant role of antioxidant property of WS and W-1. The anti-inflammatory activity was further confirmed by histopathological examination of the paw tissue.

The inflammatory granuloma is a typical feature of established subacute inflammatory reaction.\textsuperscript{199, 216} The cotton pellet granuloma method is widely employed to evaluate the transudative, exudative and proliferative components of subacute inflammation.\textsuperscript{217} Dorfmann and Dorfmann have reported that commonly granuloma formation is very responsive to corticosteroids and rather insensitive to non-steroidal anti-inflammatory drugs.\textsuperscript{218}

The anti-inflammatory activity of WS & W-1 observed in this study is supported by the studies of Kulkarni \textit{et al} and Sudhir \textit{et al}, who have also reported similar observations. Kulkarni and co-workers reported the efficacy of \textit{W.somnifera} in patients with osteoarthritis. In addition they also observed that WS possesses immunomodulatory activity which could alter the immunopathological process rapidly and it may also contribute towards the beneficial effects.\textsuperscript{219} Ahmad \textit{et al} coworkers have reported that anti-inflammatory activity of many plants have been attributed to their high steroid contents.\textsuperscript{220} Withanolides are steroidal in nature and thus indicate that anti-inflammatory activity of WS is mainly due to W-1 and could be due to the antioxidant property of WS and W-1 observed in the present study. This could probably justify the use of \textit{W.somnifera} in rheumatoid arthritis.

Implantation of cotton pellets produced formation of granulation tissue. As is evident from results, both WS and W-1 significantly reduced the weight of granulation tissue formation as compared to control. It
reveals that both WS and W-1 are effective as anti-inflammatory agent in subacute models of inflammation. The findings of this study are in conformity with the studies of Al-Hindawi and co-workers who reported similar observations and in addition they also reported that the animals treated with extract of the plant did not show the reduction in spleen and body weight, which usually accompanies steroid therapy, thereby indicating that the plant has a safe profile. Sahni and Srivastava and Nashine et al also reported significant anti-granuloma activity of *W. somnifera*.

Bector *et al* and Kulkarni and co-workers have proposed that there is a close co-relation between the degree of anti-inflammatory activity found by cotton pellet granuloma method and the clinical usefulness of WS in rheumatoid arthritis and other chronic inflammatory diseases.¹⁴₈,²¹⁹

The effectiveness of WS in subacute inflammation can partly be attributed to the presence of withanolides in the plant. Though other constituents may also be playing an important role in the anti-inflammatory activity of the plant.

**Hepatoprotective activity**

The preventive action in liver damage induced by CCl₄ has been widely used as an indicator of the hepatoprotective activity of drugs in general.²²¹ Padma and Shetty have reported that CCl₄ produces liver damage by causing lipid peroxidation in membrane, altering lipid metabolism and decreased protein synthesis in the injured hepatocytes.²²² The hepatic damage is marked by various enzymatic and non-enzymatic parameters in CCl₄ treated rats. Thus SGOT, SGPT and SALP are the
most sensitive tests, which are considered as the index for diagnosis of liver diseases.\textsuperscript{223,224}

In the present study rats treated with CCl\textsubscript{4}: Olive oil regimen developed significant hepatic damage which was observed by a substantial increase in the levels of SGOT, SGPT and SALP. Treatment of rats with WS, W-1 and hydrocortisone prior to and concomitant with the CCl\textsubscript{4} challenge produced an alleviation of the hepatic injury to a considerable extent which was reflected by the ability of the WS and W-1 to lower the elevated serum enzyme levels, resulting from administration of CCl\textsubscript{4} alone. The increased levels of SGOT, SGPT and SALP in serum are indicative of cellular leakage of enzymes into blood stream and loss of functional integrity of cell membrane in liver.\textsuperscript{225,226} In view of this, the WS and W-1 mediated reduction in levels of SGOT, SGPT and SALP towards the respective normal values is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl\textsubscript{4}. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes.\textsuperscript{227} In the present study carbon tetrachloride produced hepatic damage by lipid peroxidation as observed by an increase in TBARS and decrease in GSH levels. This increase in TBARS and decrease in GSH levels was prevented by WS and W-1 treatments. Also WS and W-1 treatments restored the levels of antioxidant enzymes catalase, superoxidedismutase and glutathione-s-transferase towards normal levels, thereby confirming the efficacy of WS and W-1 in counteracting the CCl\textsubscript{4} induced liver damage.
An increase in lipid peroxidation has already been reported after CCl₄ exposure. Recnagel have suggested that CCl₄ produces hepatotoxicity through conversion by P-450 to a highly reactive toxic free radical CCl₃.²²² This enhanced generation of CCl₃ radical initiates peroxidative changes in polyunsaturated fatty acid constituents of various biomembranes.²²⁹ The CCl₄ induced liver cell injury is both severe and rapid in onset, producing with in less than 30 minutes a decline in hepatic protein synthesis and within 2 hours, there is swelling of smooth endoplasmic reticulum and dissociation of ribosomes from the rough endoplasmic reticulum.²³⁰ The lipid export from the hepatocytes is reduced owing to their ability to synthesise apoproteins to complex with triglycerides and thereby facilitate lipoprotein secretion, resulting in fatty liver.²³¹ The ability of WS and W-L to protect the liver from CCl₄ induced damage might be attributed to its direct anti peroxidative effect or may be due to its ability to restore the activity of antioxidant glutathione and SOD.

Subramoniam et al have reported that CCl₄ treatment also reduces the levels of drug metabolizing enzymes in liver, therefore metabolism of pentobarbaritone is reduced, thereby resulting in prolongation of pentobarbaritone induced sleeping time.²³² WS and W-L treatments decreased the pentobarbaritone induced sleeping time as compared to that of treated control animal indicating the hepatoprotective action against CCl₄ toxicity via improving the status of drug metabolising enzymes in liver. These observations are also consistent with the results of the histopathological studies, which show that WS and W-L treatments were efficient in preserving the structural integrity of the liver.
The results of the study are supported by the observations made by Singh et al and Sudhir and Budhiraja.\textsuperscript{130, 131} Who have reported that WS and Withaferin A are effective against CCl\textsubscript{4} induced hepatotoxicity. Recently, Bhattacharya and co-workers observed that the glycowithanolides from \textit{W. somnifera} were effective in attenuating the iron-induced hepatotoxicity.\textsuperscript{133} They have reported that iron produces liver damage by lipid peroxidation and glycowithanolides possess antioxidant action which is responsible for the hepatoprotective activity against hepatic damage caused by iron.

The results of the present study thus indicate that WS and W-I protect the liver from CCl\textsubscript{4} induced damage by preventing the peroxidation of membrane lipids. The hepatoprotective effect of \textit{W. somnifera} can probably be attributed to the withanolides present in the plant. It indicates that the clinical use of \textit{W. somnifera} as a hepatoprotective agent in traditional system of medicine is justified and may be due to its lipid peroxidation attenuating property.

\textbf{Antioxidant activity}

\textit{W. somnifera} is widely reported to possess antioxidant activity. Withanolides being the major chemical constituent of the plant were assessed for the antioxidant potential in the brain and liver. Brain was selected as the brain and nervous system are relatively more susceptible to free radical damage than other tissues because they are rich in lipids and iron, both of which are known to be important mediators in generating reactive oxygen species. The brain also uses nearly 20 percent of the total oxygen supply.\textsuperscript{135, 136} Liver being a versatile organ of metabolism and detoxification and plays a pivotal role in regulation of physiological
processes. It is involved in several vital functions such as metabolism, secretion and storage. Taking these facts into consideration and the fact that *W. somnifera* is reported to possess hepatoprotective activity, hence parameters of antioxidant activity were assessed in liver also.

The improvement in the antioxidant status as observed by increase in glutathione and catalase levels due to WS and W-1 treatments indicate that both the alcoholic extract and withanolide fraction possesses antioxidant activity. The results are supported by the studies of Bhattacharya *et al* and Panda and Kar who observed the antioxidant property of the *W. somnifera*. The antioxidant activity of withanolides observed in the present study could possibly be responsible for the antioxidant activity of the plant. In addition, the flavonoids present in the plant may also be contributing towards the antioxidant activity of *W. somnifera*.

**Central Nervous System**

In the studies involving CNS, the effect of WS and W-1 were assessed for general behaviour, analgesic, sedative-hypnotic and anticonvulsant effects.

The studies on general behaviour revealed that WS produced a moderate degree of decrease in spontaneous activity, sound response and touch response. However the pain response was not affected by WS. These findings indicate the depressant effect of WS, which is in accordance with the reports of Malhotra *et al* and Singh *et al*. However, W-1 produced only mild degree of CNS depression as compared to WS, thereby indicating that sedative properties of WS cannot be attributed to withanolides. These effects are probably due to various
other components, like alkaloids which have been reported to possess sedative property. Chlorpromazine also produced a moderate to severe degree of CNS depression as observed by a significant reduction in spontaneous activity, sound response and touch response. The depressant effect produced by WS may probably be because of decreased neuronal excitability by modulating neurotransmitters in the CNS.

Pentobarbitone (30mg/kg) administration induced sleep lasting for 64.1 ± 2.63 min. WS (1g/Kg) administration significantly potentiated the pentobarbitone induced hypnosis by prolonging it to more than that of control group. The increase in sleeping time by WS is indicative of the central depressant effect of *W. somnifera*. The increase in pentobarbitone induced sleeping time by WS is similar to the earlier observations reported by Malhotra *et al*, Vaidya Prabha *et al* and Singh *et al*.69, 70, 72 These authors demonstrated that extract of *W. somnifera* was able to prolong the sleeping time induced by pentobarbital in rats. Dey and Chatterjee have also reported that the extract of *W. somnifera* potentiated the sleeping time induced by thiopental.233

W-1 (50mg/Kg) administration, however, did not prolong the pentobarbitone induced hypnosis, indicating that W-1 lacks any significant influence on central nervous system. A study by Schliebs *et al* indicated that WS administration produces a reduction in catecholamine levels in brain tissue.80 Further, it is also reported that catecholaminergic drugs increase the waking time, probably by increasing the availability of norepinephrine.234 The potentiation of pentobarbitone induced sleeping time with WS could probably be due to the presence of alkaloids, which might exert antiadrenergic effect at various sites in the adrenergic
neuraxis. Bernard et al have proposed that the mechanism by which the plant extract acts, appears to involve stimulation of the inhibitory synapse in the brain.\textsuperscript{235}

The analgesic activity of WS and W-1 was assessed by Tail flick and Caudal compression method. Both WS and W-1 pretreatments were found to be ineffective in producing analgesia as tested by these methods, indicating that both components are devoid of any analgesic activity. The results are consistent with the reports of Kulkarni et al and Malhotra et al who have observed that administration of alcoholic extract of \textit{W. somnifera per se} did not produce any analgesia.\textsuperscript{69,75} However, Singh et al in their studies, reported that the alcoholic extract of the plant possesses analgesic activity.\textsuperscript{70}

The results of assessment for anticonvulsant activity indicate that WS pretreatment in dose of 1gm/Kg protected against maximal electroshock induced seizures as observed by decrease in extensor phase compared to that of control. But WS administration in low dose of 500mg/kg did not offer protection against MES Seizures. Phenytoin pretreatment in dose of 25mg/kg also protected against MES induced seizures by almost completely abolishing the extensor phase. However, phenytoin in sub therapeutic dose of 12.5mg/kg did not completely abolish the extensor phase, but only decreased the duration of extensor phase. Administration of WS in low dose (500mg/kg) together with subtherapeutic dose of phenytoin (12.5mg/kg), potentiuated the anticonvulsant effect of phenytoin, leading to almost complete abolition of extensor phase of MES induced Seizures, compared to control group. The results of the present study are consistent with and supported by the
studies of Kulkarni et al and Rai et al who have reported the anticonvulsant activity of alcoholic extract of *W. somnifera* in MES induced convulsions.\(^74,76\) W-1 (50mg/Kg) pretreatment was unable to protect against MES induced Seizures indicating lack of anticonvulsant activity. It is possible that the anticonvulsant activity of WS is due to presence of components other than withanolides. Although the exact mechanism of anticonvulsant activity of WS is not clear, it may be speculated that the mechanism of anticonvulsant activity of WS could be like that of phenytoin. WS possibly exerts synergistic effect on stabilization of neuronal membrane thereby slowing the rate of recovery of voltage-activated sodium channels from inactivation.

In the PTZ induced model of epilepsy the onset of convulsions was at 76.4 ± 5.92 seconds. WS (1gm/Kg) pretreatment delayed the onset of clonic convulsions produced by PTZ to 118.9 ± 6.4 seconds and also decreased the percent of mortality. W-1 (50mg/Kg) pretreatment did not produce any delay in onset of convulsions by PTZ, indicating that W-1 lacks significant antiseizure activity. Clonazepam (1mg/Kg) rendered total protection against PTZ induced convulsions by completely abolishing the onset of clonic convulsions. It is well reported that the aniconvulsant action of clonazepam is due to its ability to enhance GABA - induced increase in conductance of chloride.\(^236\) Flumazenil (4mg/Kg), a benzodiazepine antagonist when administered prior to clonazepam, blocked its antiseizure activity against PTZ induced convulsions. Flumazenil treatment also blocked the anti-convulsant effect of WS against PTZ induced convulsions, thereby indicating that anticonvulsant property of WS probably is mediated through GABAergic pathway.
Studies by Kulkarni et al also indicate that GABA receptors are involved in mediation of anticonvulsant property of *W. somnifera* extract.\(^{75}\) The extract of *W. somnifera* has been reported by Kulkarni et al to abolish in a dose dependent manner the tonic extensor phase of PTZ induced convulsions. Further it has been reported by Mehta et al that the administration of methanolic extract of *W. somnifera* simultaneously with GABA produced significant potentiation of the protective effect of GABA.\(^{82}\) Mehta et al have also reported using radioligand binding studies that the methanolic extract of WS inhibited the specific binding of \(^3\text{H}\) GABA and \(^{35}\text{S}\) t-butylbicyclo phosphorothionate (TBPS), enhanced the binding of benzodiazepine agonists such as \(^3\text{H}\) flunitrazepam to their putative receptor sites, suggesting that the extract may be acting by modulating GABAergic pathways at various levels. It further indicates that WS might contain a component which has a GABAmimetic activity (presumably this component is able to pass the blood brain barrier) that could be responsible for its observed anticonvulsant effect. The withanolide fraction lacks most of the central effects.

The present study indicates that effects of *W. somnifera* on CNS are probably due to the components other than withanolides, such as glycowithanolides & alkaloids. Since, the withanolides have not exhibited any effect on CNS.

**Cardiovascular System**

The alcoholic extract of *Withania somnifera* produced a dose dependent fall in blood pressure in dogs. The possible site for the hypotensive response could be cholinergic, histaminergic, dopaminergic, gabaergic pathways and / or β-adrenergic receptors. Since the hypotensive
response was blocked by atropine, it indicates that the *Withania* extract acts like a cholinergic agonist. Subsequent administration of WS after atropine produced a rise in blood pressure, which could be due to its action on autonomic ganglia – a nicotinic response. The rise in blood pressure was not blocked by tolazoline and propranolol but was blocked by pentolinium tartarate (a gangliolytic agent) which further leads to the possibility of cholinergic agonistic action of WS on autonomic ganglia. Both these effects indicate towards the presence of cholinomimetic like agents in the *Withania* extract, which might have great affinity for the cholinergic receptors, at various sites in the cholinergic pathway. The stimulation of cholinergic receptors induces bradycardia and a drop in peripheral resistance, resulting in hypotension. It is pertinent to consider the fact that the extract contains several pharmacologically active constituents which might act on a variety of central and peripheral receptors. The rise in blood pressure by WS after atropine can be attributed to the presence of nicotine in the extract, as has been reported by Majumdar.44

The withanolide fraction produced a fall in blood pressure, which was blocked by atropine, but not by mepyramine. It indicates that the hypotensive effect of W-1 is due to its action on muscarinic cholinergic receptors. Subsequent administration of W-1 did not produce a rise in blood pressure indicating thereby that W-1 lacks nicotinic effects. It is possible that W-1 which is a major constituent of WS could be acting at various peripheral sites of cholinergic pathway. These results are in accordance with the observations made by Budhiraja *et al* and Malhotra *et al*.144, 237
WS and W-1 produced a negative inotropic and chronotropic effect in the Langendorff's heart preparation which was blocked by atropine. The cholinergic effect observed is similar to the response observed in dog blood pressure. The withanolides have close structural similarity to aglycones of the cardiac glycosides in possessing hydroxyl group at C\textsubscript{14} and a 6-membered unsaturated lactone attached to a steroidal ring at C\textsubscript{20} instead of C\textsubscript{17} as given in Fig 75. Though the withanolides resemble cardiac glycosides in their chemical structure, their myocardial effects observed are opposite to each other. As the pharmacological activity of the glycosides resides in aglycone,\textsuperscript{238,239} the withanolide was anticipated to have cardiotonic effect like the aglycones but in the present study it was found to have negative inotropic and chronotropic effects. Even the hypotensive response is contrary to the effects of cardiac aglycones, which cause an increase in arterial blood pressure in man and dog.

![Structural resemblance of withanolide to digitoxigenin.](image)

**Fig. 75**: Structural resemblance of withanolide to digitoxigenin.

A = Withanolide, B = Digitoxigenin
Although the effects of the withanolide fraction are not similar to those of cardiac glycosides, the possibility of cardiotonic response with some withanolides cannot be ruled out at present. Screening of a large number of cardiac glycosides and aglycones has revealed that the position of lactone ring attached to ring D and other moieties present in the steroid ring affect the efficacy of cardiac glycosides considerably. Structure of various rings and spatial arrangement in the steroid ring system also affect the potency of glycosides.\(^{238-245}\) Another reason for the absence of positive cardiotonic response could be due to the fact that lactone ring at C\(_{20}\) does not give the same type of response when at C\(_{17}\).

Positive inotropic and chronotropic effect was observed in frog heart with the withanolide fraction, which was not blocked by propranolol. The qualitative difference in response in rabbit and frog heart could be due to species difference as many other lactones and glycosides exert different responses in amphibians and mammals.\(^{240,245}\) The positive inotrophic and chronotropic effects appear to be due to direct action of W-1 on the frog heart as these persisted even after prior administration of propranolol - a \(\beta\) adrenergic receptor blocking drug.

A large number of commercial preparations containing *Withania somnifera* are available in the market which are being used for the treatment of hypertension and relief from mental stress. The results of the present study support the utility of *Withania somnifera* as an antistress and antihypertensive agent.

**Wound Healing Activity**

The alcoholic extract and withanolide fraction of *W. somnifera* indicated the absence of wound healing activity as assessed by resutured
incision wound and dead space wound models. It is known that withanolides are steroidal lactones, which structurally resemble corticosteroids. Further, it is very well known that corticosteroids retard the wound healing process. Various authors have reported that anti-inflammatory steroids decrease the tensile strength of healing wound, slow the rate of epithelization and neovascularization.\textsuperscript{246-248} Moreover, inflammation is said to be an essential prelude and vital aspect of wound healing. No inflammation / no repair is a valid dictum.\textsuperscript{249} As the alcoholic extract and withanolide fraction have been found to possess anti-inflammatory activity, the lack of wound healing activity of WS & W-1 is explainable. Though the plant is used traditionally for healing of wounds, possibly it could be due to the antimicrobial and antiseptic properties of the plant.

**Reproductive System**

In the studies on male reproductive system WS and W-1 treatments produced a significant increase in sperm count and motility in rats. The improved sperm count and motility suggests a stimulatory effect of the treatments on testicular sperm production and epididymal sperm maturation. The increase in organ weights observed could, in part be, due to increased spermatogenesis.

The biochemical analysis of testes revealed an increase in protein content and decrease in cholesterol level, which could be attributed to the improved testicular growth and increased steroidogenesis respectively.\textsuperscript{250, 251} These results are also confirmed by histopathological studies which indicate that WS and W-1 treatments produced increased cellularity of spermatocytes and spermatids with large and closely arranged
semiferous tubules. Banks et al have proposed that the increased presence of spermatocytes and spermatids in the seminiferous tubules are signs of increased spermatogenesis.\textsuperscript{252}

Abdel-Magied et al have also observed the similar findings, using extract of \textit{W. somnifera}, in testes of rats.\textsuperscript{198} The spermatogenic effect of WS and W-I observed in this study may be due to a direct or indirect effect via hypothalamic pituitary axis. The stimulation of the hypothalamus, pituitary gonadotroph or Leydig cells may indirectly elicit spermatogenesis. But Abdel-Magied et al simultaneously, have also observed a drop in testesterone and FSH levels by treatment with \textit{W. somnifera}, indicating that the plant has no stimulatory effect on Leydig cells, pituitary gonadotrophs and the FSH.\textsuperscript{198} Quereshi et al have reported that increase in weight of the testes is attributed to various chemical constituents of herbal drugs possessing androgenic activity.\textsuperscript{196}

Aitken proposed another theory that reactive oxygen species (ROS) such as superoxide anion (\textit{O}_2^-), the hydroxyl radical (\textit{OH}) and hypochlorite radical (\textit{OHCl}) produced by spermatozoa and contaminating leucocytes damage the plasma membrane, which loses fluidity and integrity, vital for their survival and sperm-oocyte fusion.\textsuperscript{253,254} Verma and Kanwar\textsuperscript{255} have also observed that the poor sperm motility is related to poor antioxidant potential with concomitant rise in the rate of MDA generation in the spermatozoa suspended in seminal plasma vis a vis those in BWW (Biggers, Whitten and Whittinghom) medium, which has significant antioxidant potential.

Alvarez et al and Mennella et al have also reported that poor sperm motility is related to the deficient antioxidant potential viz. Catalase and
superoxide dismutase of spermatozoa, that protect them from the adverse effects of lipid peroxidation and other manifestations of oxygen toxicity.\textsuperscript{256-257}

Therefore, it is pertinent to assume that the withanolides present in \textit{Withania somnifera}, in addition to their steroidal structure resembling with testosterone, might also be eliciting the improved spermatogenesis, due to the antioxidant properties as observed in other effects and studies reported earlier.

In the study on female reproductive system in rats, significant changes were noted after WS and W-1 treatments. Both WS and W-1 produced a significant increase in weight of ovary and uterus. The significant gain in ovarian weight noted in the animals suggests profound folliculogenesis. The increase in uterine weight indicates that WS and W-1 possess estrogenic activity also. WS and W-1 treatments produced an increase in protein content and a simultaneous decrease in cholesterol levels in the ovaries and uterus as compared to that of control rats. Means \textit{et al} have proposed that the increase in protein content of the ovary indicates a increased ovarian growth as FSH is essential for protein synthesis in gonads.\textsuperscript{250} On the other hand, it has also been reported that a decrease in the cholesterol level in the ovaries indicates the increased availability of pituitary gonadotrophs for steroidogenesis.\textsuperscript{249}

The results are further supported by the findings of histopathological studies, which indicate increased folliculogenesis as observed by increase in number of growing follicles with well developed granulosa. The WS and W-1 treatments increased proliferation of
endometrial stroma, increase in thickness of endometrium and myometrium.

The results of the present study with WS are in accordance with the observations of Al Qarawi et al who reported similar observations along with a simultaneous increase in FSH levels in the rats. They also observed that WS treatment produced an increase in body weight and peri-ovarian accumulation of adipose tissue in the animals and suggested that adipogenesis may have been the cause of the significant increase in the body weight noted in the WS treated rats.

The significant changes observed in the W-1 treated group indicate that probably the activity of *W. somnifera* is due to presence of withanolides, which structurally resemble oestrogens. These properties justify the traditional use of the plant in infertility without any toxic effect.

**Toxicity Studies**

In the acute toxicity study, all the mice survived with doses upto 300mg/kg i.p. of withanolide fraction. With an increase in the dose, the mortality was increased. All the animals died when W-1 was administered at a dose of 700-mg/kg i.p. The LD$_{50}$ of the withanolide fraction was found to be 489.7mg/kg. Hence about 1/10$^{th}$ of the LD$_{50}$ i.e., 50mg/kg i.p was used for various pharmacological studies. No animal died with W-1 (50mg/kg) and WS (1gm/kg) during the course of study, which indicate that the withanolide fraction has no lethal effect.

Sharada *et al* in their studies for the toxicity of *Withania somnifera* reported that mice were able to tolerate high dose of *W. somnifera* extract and found the LD$_{50}$ of the extract to be 1260mg/kg.\textsuperscript{147}
In the acute toxicity study of withaferin A on mice by Sharada et al, the LD$_{50}$ of withaferin A was found to be 87mg/kg with no mortality being observed at 50mg/kg.\textsuperscript{118}

During the present study the food and water intake by the animals with these doses were normal. An increase in body weight gain was observed during subacute toxicity studies, indicates good health and well being of the animal. Darcy et al have reported that body weight loss is usually an indicator of ill health.\textsuperscript{259} Winder et al also have reported that change in body weight has been used to assess the course of the disease and the response to therapy of to drugs.\textsuperscript{260} Further Begum and Sadique have observed that \textit{W. somnifera} treatment produced an increase in body weight, which they attributed to increased absorption capacity of intestine. It is also reported by Walz et al, that there is a loss of body weight during arthritic conditions, due to alterations in metabolic activities.\textsuperscript{261}

Considering the above facts, and the observations of the present study, it indicates that WS and W-1 are effective in subacute inflammation, resembling arthritis. The plant is observed to possess androgenic and anabolic effects. The various biochemical parameters were unaffected by subacute treatment with WS and W-1. This was further confirmed by changes in organ weight and histopathologic studies.

The present study indicates that both WS and W-1 have no toxic effect at the doses and duration used in the study. It also supports the wide use of the plant traditionally, without any specific toxic effect thereby indicating it to be safe for human use.

Iwai \textit{et al} have reported that a strong association between chronic inflammation and development of cancer is well established.\textsuperscript{262}
Substantial experimental evidence so far has strongly implied that free radicals are involved in both the initiation and promotion process of carcinogenesis.\textsuperscript{263} More over, the role of free radicals in the multistep process of carcinogenesis is well established and the antioxidants are reported to offer a protective effect against cancer.\textsuperscript{264,265} The proven chemopreventive action of few antioxidants in experimental models have further strengthened this theory.\textsuperscript{266,267}

Recently Prakash \textit{et al} have studied the chemopreventive activity of \textit{Withania somnifera} in experimentally induced fibrosarcoma tumors in albino mice and observed that treatment of animals with \textit{W. somnifera} extract significantly reduced the tumor incidence, tumor volume and enhanced the survival of mice, with simultaneous improvement in antioxidant status. They proposed the chemopreventive activity of \textit{W. somnifera} in experimental tumors to be due to antioxidant and detoxifying properties.\textsuperscript{268} Moreover, \textit{W. somnifera} is known to possess free radical scavenging activities. It has also been shown to induce catalase and superoxide dismutase activities in mice livers (Panda and Kar).

In the traditional system of medicine, there is a class of herbs, including \textit{Withania somnifera}, known as adaptogens. Adaptogens cause adaptive reactions to disease, are useful in many unrelated illnesses and appear to produce a state of non-specific increased resistance (SNIR) to adverse effects of physical, chemical and biological agents. They are usually glycosides (Steroidal components).\textsuperscript{67}

The presence of steroidal structure resembling corticosteroids and the antioxidant activity of withanolides observed in the present study may, at least in part, be responsible for the anti-inflammatory, hepatoprotective,
antiaging, immunomodulatory, anti-stress and antitumor activities produced with *W. somnifera* in experimental animals and in clinical situations.

It has been observed that the withanolides lacked the central effects. The effects on the CNS observed with the extract, could be due to other constituents in the plant such as alkaloids.⁶⁹⁻⁷²⁷⁴ Therefore, the various fractions i.e. alkaloids and withanolides can be isolated and utilized in formulations for specific purposes. In addition the withanolides can be further fractionated and utilized for various specific activities.

The present comparative pharmacodynamic study of alcoholic extract of *W. somnifera* and withanolide fraction reveal that withanolides contribute to most of the activities of the plant *W. Somnifera*. The present study with alcoholic extract of *W. somnifera* and withanolide fraction did not show any toxicity, even on rapidly multiplying cell systems such as haemopoietic and reproductive system. In the ayurvedic system of medicine the combination of drug is used. The rationale for this type of treatment is that toxicity of active components may be counteracted by other components, which may not be having the desired therapeutic property. Further studies are warranted to find out the components that are responsible for counteracting the toxic effects of alcoholic extract and withaferin A.