# *Analgesic activity of Withania somnifera*

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**Abstract**— Pain on the basis of duration can be classified as acute and chronic pain. Acute pain is easy to cure as compared to chronic pain. Pain perception threshold depends on the type of disease and psychological condition of patient. Despite of advances in the medical sciences, an effective remedy for pain is still not available. Many laboratories have active research programs on pain management. Plants have provided many drugs to the modern medicine. Opium obtained from Papaver somnifera latex is the most effective treatment of pain. Salicylic acid obtained from Salix bark is a starting point for development of non-steroidal anti-inflammatory drugs. The structure activity studies on opium and salicylic acid have increased our understanding on structural requirements of analgesic drugs and also their adverse effects. There are several plants which possess analgesic principles. The fractions obtained from various plants relieve pain by acting through central and peripheral mechanisms.

Preliminary studies have indicated antinociceptive activity of *Withania somnifera* using hot plate analgesiometer and acetic acid induced writhing (Husni et al., 1989). However, there are no reports on the mechanism of antinociceptive activity. In the present study we have studied antinociceptive activity of withanolide rich fraction of W. somnifera fraction using formalin induced pain test and sodium chloride induced eye wiping in mice.

Various pain modulators like atropine, naloxone, mecamylamine, pentazocine, pCPA, sertraline have been used to study their effect of antinociceptive potential of withanolide rich extract. Atropine is muscarnic antagonist, naloxone is narcotic antagonist, mecamylamine is nicotinic antagonist and pentazocine is a widely used mixed agonist-antagonist opioid, sertraline is SSRI, pCPA is serotonine depletor.

**Index Terms**— *Withania somnifera*, Eye wiping test, formalin induced pain.

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## 1 Introduction

Pain is a mixture of noxious stimuli with sensation. Pain is commonly observed to be associated with toothache, migraine, inflammation, infection, increased ocular pressure, burn, wound, reduced blood supply to heart, intestinal colic, renal colic, parturition, and various diseases like renal calculi, cancer etc. Pain is an important warning sign indicating deviation in the internal environment of body. Perception of pain is very much subjective and its intensity varies from person to person. Young children and old people are more sensitive than adults. Generally it is observed that men are more tolerant than women. Pain perception increases in presence of inflammation. The somatic pain arising due to physical injury can be located easily but if pain is due to inflammation of any visceral organ, it is referred on specific areas of skin like pain of intestinal or renal colic or pain of cardiac ischemia which is referred on the left side of upper body and travelling to the left thumb. Pain due to infected teeth is pulsating, continuous, and disturbing. Pain can be considered as a protective reflex as it helps avoid further damage. Pain can be induced by several types of noxious stimuli such as thermal, chemical and mechanical origin. Pain is associated with many ailments and unless the underlying causes subside, pain is not relieved.

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Pain and pleasure both are primary motivators of action (Melzak, 1965). The International association for the study of pain (IASP) defined pain as "An unpleasant sensory or emotional experience associated with actual or potential damage". Pain is not only sensation but also hunger and thurst (Wall, 1999). Phenomenon of pain is multidimensional which is described as pain location, quality, intensity (National institute of Health, 1987). Human beings affected in physical, mental and social aspects due to pain (Koleva et al., 2005). Pain is varying with the experience of preamputation pain (Melzak, 1973). Pain is nothing but nociception which means emotional experience associated with actual or potential damage, pain is one type of stimulus to brain which may cause or prevent damage. Nociception is the process in which pain stimulus is transferred from site of stimulation to central nervous system (Fein, 2012). In large number of diseases they found pain as most common suffering. Each individual is having different pain perception threshold. Threshold of pain is not fundamental quantity as pathological examinations of urine, blood etc. (Rang et al., 1988). There is no relationship between amount of pain and the extent of tissue damage (Turk and Melzak, 2001). Davis (1996) described pain characteristics as given in Table 1.

### *1.1 Withania somnifera*

Biological source - Withania somnifera Linn.
Common Name- Winter cherry, Withania root
Family- Solanaceae

Withania somnifera is also known as Ashwagandha, Indian ginseng, winter cherry is an important ancient plant. The roots of Ashwagandha have been employed in Indian traditional systems of medicine, Ayurveda and Unani. It grows in dry parts in sub-tropical

regions, Rajasthan, Punjab, Haryana, Uttar Pradesh, Gujarat, Maharashtra and Madhya Pradesh. The pharmacological activity of the root is attributed to the alkaloids and steroidals lactones. Among the alkaloids, withanine, pseudo-withanine, tropine, pseudo-tropine, somniferine, somnine are mainly present. The plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, and more recently to treat asthma, ulcers, insomnia, and senile dementia. Clinical trials and animal's research support the use of Ashwagandha for anxiety, neurological disorders, inflammation, and Parkinson's disease. Oral administration of Withania somnifera Linn, root powder showed the anti-arthritic effect in adjuvant induced arthritic rats (Kaur et al., 2012). Sharma et al., (2000): reported that Withania somnifera produced significant increase in neutrophil count. Paclitaxel administration showed fall in WBC count. Withania somnifera when administered for 4 days before paclitaxel treatment and continued for 12 days caused significant reversal of neutropenia of paclitaxel. Ws root powder decreased the total lipids, cholesterol and triglycerides in hypercholesteremic animals. Ws also showed strong cardioprotective effect in experimental model of isoprenaline-induced myonecrosis in rat. Ws used as adjuvant during cancer chemotherapy for prevention of bone marrow depression associated with anticancer drug.

Aggarawal et al., (2006): They reported that *Ws* is used in asthma, hypertention, to treat tumors.

| Characteristics | Potential Elements |
|---|---|
| **Temporal** | Acute or persistent onset and duration course<br>, including breakthrough pain |
| **Intensity**<br>**(0-10 numeric scale)** | Pain "on average" last day or week, pain<br>"at its worst" last day or week, pain "at<br>its least" last day or week pain right now |
| **Topography** | Focal or multifocal, focal or referred, superficial<br>or deep |
| **Quality** | Any descriptor (e.g., aching, throbbing,<br>stabbing or burning) |
| **Relieving factors** | Volitional or non-volitional pain |

## 2. MATERIALS AND METHODS

The herbal extract of *Withania somnifera* was purchased from Natural Remedies Bangalore. The extract contained 5% of Withanolides. All the chemicals used for the study were of analytical grade. The drug and chemicals were purchased from local vendors of Pune. Eye wiping test and formalin induced pain animal models used for antinociceptive activity.

### 2.1 Eye wiping test

Rationale: Sodium chloride induced eye wiping is the type of trigeminal neuralgia. It's also called as prospalgia, its characterised by intense pain. Trigeminal nerve is paired cranial nerve which has three major branches ophthalmic nerve, maxillary nerve and mandibular nerve, all the three branches of the nerve may be affected, pain may be left in the eye, ear cheeks, scalp, forehead, teeth or jaw and side of the face. (UF Shands, 2012). TN is not easily controlled but can be managed with a variety of treatment options (Sarmah, 2008).

Procedure: Experiments were performed on adult Swiss Albino mice (20-22 gm.) of either sex. Animals were housed in a standard condition of 12-hr light/dark cycle and 22 ± 1 °C room temperature and had freely access to food and water. Animals were treated and cared according to the ethical guidelines.

The animals were placed on a 50 × 50 cm table for 10-min habituation period. One drop of 5 M NaCl at room temperature was put into the right or left eye of animal. Immediately after instilling NaCl solution, the animals began to wipe the eye with ipsilateral forepaw, and the number of eye wipes was counted during 30 s. Mice were injected with Vehicle, Withanolide (30 mg/kg) and standard drugs like naloxone (1.5mg/kg), mecamylamine (2mg/kg), atropine (5mg/kg) and pentazocine (10mg/kg), sertraline (10mg/kg), pCPA (150mg/kg) for 3 days were given. Each animal was injected only once (Farazifard et al., 2005).

### 2.2 Formalin induced pain test:

Rationale: In this method formalin injected in subplantar area of the hindleg exhibits both neurogenic and inflammatory phases of pain. In the first phase which extends from 0-5 minutes after formalin injection, the pain is due to the neurogenic phase. The centrally acting analgesics inhibit both phases of formalin-induced pain. Inflammatory pain associated with the second phase (15-30 minutes) is accompanied by release of several inflammatory mediators and is inhibited by drugs known to inhibit cyclo-oxygenase metabolites derived from the arachidonic acid pathway (Dubuisson and Dennis, 1977).

Procedure:

Experimental group of mice was injected 0.02ml of

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1% formalin into sub-plantar space of hind paw. The number of paw licking was noted between 0-5 min (phase 1st) and phase (2nd) after formalin injection. Animals were pretreated with vehicle (5ml/kg), Ws (30mg/kg) 30 minutes before formalin injection, naloxone (1.5mg/kg), mecamylamine (2mg/kg), atropine (5mg/kg), pCPA (150mg/kg), pentazocine (10mg/kg), sertraline (10mg/kg) was given 15 minutes before Ws. The paw licking time of treated animals was compared to control group and represented as % inhibition.

$$\% \text{ inhibition} = \frac{N - Nt}{N} \times 100$$

Where, N = Average number of paw licking of control group

Nt = Average number of paw licking of test group

(Seigmund et al.,1957; Kulkarni, 1999; Vogel et al., 2002).

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## 3. Pharmacological Results

Eye wiping test and Formalin test of chloroform and ethanol fraction of *Withania somnifera*:

**Effect of chloroform, ethanol, ethyl acetate fraction and crude extract of *Withania somnifera* on sodium chloride induced eye wiping in mice**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg), ethanol fraction of *Withania somnifera* (30mg/kg), and ethyl acetate fraction of *Withania somnifera* (30mg/kg) showed significant (***p < 0.001) decrease in number of eye wipings as compared to vehicle control group. Treatment with crude extract showed significant (***p < 0.001) decrease in no. of eye wipings.

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml / kg i.p | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg / kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30mg / kg oral | 9.6 ± 0.24*** |
| Ethyl acetate fraction of Ws | 30 mg / kg oral | 9.6 ± 0.24*** |
| Crude extract of Ws | 30 mg/kg oral | 13± 0*** |

**Effect of atropine and chloroform, ethanol fractions of *Withania somnifera* on sodium chloride induced eye wiping in mice**

*Treatment with chloroform fractions of Withania somnifera (30mg/kg) and atropine showed significant (*p < 0.05) decrease in number of eye wiping as compared to vehicle control group. Treatment with ethanol fraction of Withania somnifera (30mg/kg) and atropine showed significant (**p<0.01) change as compared to vehicle and ethanol extract of Withania somnifera.*

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml/kg i.p | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg/kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30 mg/kg oral | 9.6 ± 0.24*** |
| Atropine + CHCl3 fraction | 5 mg/kg i.p | 7.6 ± 0.37* |
| Atropine + Ethanol fraction | 5mg/kg i.p | 7.4 ± 0.37** |

**Effect of mecamylamine, chloroform and ethanol fractions of *Withania somnifera* on sodium chloride induced eye wiping in mice**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and mecamylamine showed significant (*p < 0.05) decrease in number of eye wiping as compared to vehicle control group and chloroform group. Treatment with ethanol fraction of *Withania somnifera* (30mg/kg) and mecamylamine showed significant (***p<0.001) decrease in number of eye wipings as compare to vehicle and ethanol fraction of *Withania somnifera*.

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml/kg oral | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg/kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30mg/kg oral | 9.6 ± 0.24*** |
| Mec + $CHCl_3$ fraction | 2mg/kg i.p | 7.4 ± 0.50* |
| Mec + Ethanol fraction | 2mg/kg i.p | 7.2 ± 0.31*** |

**Effect of naloxone, pentazocine, chloroform and ethanol fractions of Withania somnifera on sodium chloride induced eye wiping in mice**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and naloxone showed significant (***p < 0.001)

increase in number of eye wipings as compared to chloroform group and vehicle group. Treatment with ethanol fraction of *Withania somnifera* (30mg/kg) and naloxone showed increase in number of eye wiping. In this naloxone reverses the antinociceptive activity. Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and pentazocine showed significant (**p < 0.01) decrease in number of eye wiping as compared to vehicle control group and chloroform group. Treatment with ethanol fraction of Withania somnifera (30mg/kg) and pentazocine showed significant (**p<0.01) as compare to vehicle and ethanol fraction of *Withania somnifera*.

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml/kg i.p | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg/kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30mg/kg oral | 9.6 ± 0.24*** |
| Naloxone+ CHCl3 fraction | 1.5 mg/kg sc | 12.2 ± 0.48*** |
| Naloxone+ Ethanol fraction | 1.5 mg/kg sc | 13 ± 0.31*** |
| pentazocine + CHCl3 fraction | 10 mg/kg i.p | 7 ± 0.31** |
| pentazocine + Ethanol fraction | 10 mg/kg i.p | 7.2 ± 0.34** |

**Effect of pCPA, Sertraline, chloroform and ethanol fractions of *Withania somnifera* on sodium chloride induced eye wiping in mice**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and pCPA showed significant (***p < 0.001) decrease in number of eye wiping as compared to vehicle control group and chloroform group. Treatment with ethanol fraction of Withania somnifera (30mg/kg) and pCPA showed significant (***p<0.001) change as compare to vehicle and ethanol fraction of *Withania somnifera*. Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and sertraline showed significant (***p < 0.001) decrease in number of eye wiping as compared to vehicle control group and chloroform group. Treatment with ethanol fraction of *Withania somnifera* (30mg/kg) and sertraline showed significant (***p<0.001) change as compare to vehicle and ethanol fraction of *Withania somnifera*.

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml/kg oral | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg/kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30mg/kg oral | 9.6 ± 0.24*** |
| pCPA+ CHCl3 fraction | 150 mg/kg i.p for 3 days | 5.2 ± 0.37*** |
| pCPA+ Ethanol fraction | 150 mg/kg i.p for 3 days | 7.4 ± 0.50*** |
| Sertraline + CHCl3 fraction | 10 mg/kg i.p | 3.4 ± 0.24*** |
| Sertraline + Ethanol fraction | 10 mg/kg i.p | 6.2 ± 0.37*** |

**Effect of 15 days treatment of chloroform and ethanol fractions of Withania somnifera on sodium chloride induced eye wiping in mice**

Fifteen days treatment with chloroform fraction of Withania somnifera (30mg/kg) and ethanol fraction of Withania somnifera (30mg/kg) showed significant (*p<0.05) change as compared to vehicle and chloroform, ethanol fraction of Withania somnifera that is decrease in number of eye wiping in mice

| Treatment | Dose(mg/kg) | No. of eye wiping (mean ± SEM) |
|---|---|---|
| Vehicle | 5ml/kg i.p | 22.8 ± 1.39 |
| Chloroform fraction of Ws | 30mg/kg oral | 9.2 ± 0.37*** |
| Ethanol fraction of Ws | 30 mg/kg oral | 9.6 ± 0.24*** |
| CHCl3 fraction 15 days | 30 mg/kg oral | 4.6 ± 0.24* |
| Ethanol fraction 15 days | 30 mg/kg oral | 4.4 ± 0.24* |

**Effect of mixed fractions (ethanol + chloroform + ethyl acetate) of *Withania somnifera* on sodium chloride induced eye wiping in mice**

Three mixed fraction of Withania somnifera group showed significant decrease in number of eye wipings as compared to vehicle group.

| **Treatment** | **Dose (mg / kg)** | **No. of eye wiping (mean ± SEM)** |
|---|---|---|
| Vehicle | 5ml/kg i.p | 22.8 ± 1.39 |
| Chloroform fraction of *Ws* | 30mg/kg oral | 9.2 ± 0.37*** |

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| | | |
|---|---|---|
| Ethanol fraction of *Ws* | 30 mg/kg oral | 9.6 ± 0.24*** |
| Mixed fraction (ethanol + $CHCl_3$ + ethyl acetate) | 30 mg/kg oral | 11 ± 0.5* |

**Effect of chloroform, ethanol fraction and crude extract of *Withania somnifera* on formalin-induced pain in mice during 1st phase (0-5 min) and 2nd phase (15-30 min).**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg), ethanol fraction of Withania somnifera (30mg/kg), showed significant (***p < 0.001) decrease in number of paw lickings during first phase and second phase as compared to vehicle control group. Treatment with crude extract showed significant (***p < 0.001) change that is decrease in number of paw licking in mice during 1st and 2nd phase of inflammation as compared with vehicle.

| Treatment | Dose (mg/kg) | No. of paw licking in 0-5 min | No. of paw licking in 15-30 min |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| Chloroform fraction of Ws | 30mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction of Ws | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| Crude extract | 30 mg/kg oral | 13 ± 0* | 1 ± 0* |

**Effect of atropine, ethanol and chloroform fractions of *Withania somnifera* on formalin-induced pain during 1st phase (0-5min) and 2nd phase (15-30 min) in mice**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg) and atropine showed significant (**p < 0.01) decrease in number of paw licking as compared to vehicle control group and chloroform group, and treatment with ehanol fraction of *Withania somnifera* and atropine showed significant (***p < 0.001) decrease in number of paw licking during first phase and during 2nd phase atropine with chloroform fraction showed significant (*p < 0.05) decrease in no. of paw licking and with ethanol fraction showed significant (**p < 0.01) decrease in no. of paw licking.

| Treatment | Dose(mg/kg) | No. of paw licking in 0-5 min | No. of paw licking in 15-30 min |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| Chloroform fraction | 30mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction | 30 mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| Atropine + $CHCl_3$ fraction | 5 mg/kg i.p | 9.8 ± 0.48** | 0.6 ± 0.24* |
| Atropine + Ethanol fraction | 5 mg/kg i.p | 10 ± 0.31*** | 1.2 ± 0.2** |

**Effect of mecamylamine, ethanol and chloroform fractions of Withania somnifera on formalin induced pain during 1st phase (0-5 min) and 2nd phase (15-30 min) in mice**

Treatment with chloroform fraction of Withania somnifera (30mg/kg) and mecamylamine showed significant (*p < 0.05) decrease in number of paw licking as compared to vehicle control group where ethanol fraction showed significant (***p < 0.001) decrease in number of paw licking during 1st phase and 2nd phase.

| Treatment | Dose(mg/kg) | No. of paw licking (0- 5 min) | No. of paw licking (15-30 min) |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| Chloroform fraction of Ws | 30mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction of Ws | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| Mec. + $CHCl_3$ fraction | 1mg/kg i.p | 10.8 ± 0.31* | 0.6 ± 0.24* |
| Mec. + Ethanol fraction | 1mg/kg i.p | 10 ± 0.31*** | 0.8 ± 0.2*** |

**Effect of naloxone, Pentazocine, ethanol and chloroform fractions of Withania somnifera on formalin induced pain during 1st phase (0-5 min) and 2nd phase in mice**

Treatment with chloroform and ethanol fraction

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with naloxone separately showed significant (***p<0.001) increase in number of paw lickings. Naloxone reverses the antinociceptive activity that it showed increase in number of paw licking in mice during 1st and 2nd phase. Treatment with pentazocine showed significant (***p < 0.0001) change as compared with chloroform fraction of Withania somnifera (30mg/kg) and ethanol fraction of Withania somnifera (30 mg/kg) with pentazocine showed decrease in number of paw licking during 1st and 2nd phase of inflammation.

| Treatment | Dose(mg/kg) | No. of paw licking (0-5 min) | No. of paw licking (15-30 min) |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| CHCl3 fraction | 30 mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fractions | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| Naloxone + CHCl3 fraction | 1.5 mg/kg sc | 15 ± 0.31*** | 4.2 ± 0.58* |
| Naloxone + ethanol fraction | 1.5 mg/kg sc | 16.2 ± 1.31*** | 4.4 ± 0.24*** |
| Pentazocine + CHCl3 fract. | 10mg/kg i.p | 7.6 ± 0.24*** | 1.02 ± 0.24*** |
| Pentazocine + ethanol fract. | 10mg/kg i.p | 7.2 ± 0.20*** | 1.0 ± 0.20*** |

**Effect of pCPA, Sertraline, chloroform and ethanol fraction of *Withania somnifera* on formalin induced pain in mice during 1st phase (0-5min) and 2nd (15-30min) phase.**

Treatment with chloroform fraction of *Withania somnifera* (30mg/kg), ethanol fraction of *Withania somnifera* (30mg/kg) with pCPA, showed significant (***p < 0.001) decrease in number of paw lickings as compared to vehicle control group during 1st phase and during 2nd phase. Treatment with sertraline showed significant (***p < 0.001) change as compared with chloroform fraction (30mg/kg) and ethanol fraction of Withania somnifera (30mg/kg) and with vehicle control during 1st and 2nd phase but in 2nd phase treatment of chloroform fraction with sertraline showed significant (*p< 0.05) decrease in number of paw lickings.

| Treatment | Dose(mg/kg) | No. of paw licking in 0-5 min | No. of paw licking in 15-30 min |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| Chloroform fraction of Ws | 30mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction of Ws | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| CHCl3 + pCPA | 150 mg/kg i.p | 4.4 ± 0.24*** | 1.0 ± 0.24* |
| Ethanol + pCPA | 150mg/kg i.p | 8.6 ± 0.24*** | 1.02 ± 0.24* |
| Sertraline + CHCl3 fract. | 10 mg/kg i.p | 7 ± 0.37*** | 1.04± 0.27* |
| Sertraline + Ethanol fract. | 10 mg/kg i.p | 8.2 ± 0.2*** | 1.06 ± 0.27*** |

**Effect of 15 days treatment chloroform and ethanol fraction on formalin induced pain during 1st phase (0-5 min) and 2nd phase (15-30 min) in mice**

Treatment with chloroform and ethanol fraction of Withania somnifera of 15 days treatment showed significant (***p < 0.001) decrease in number of paw licking during 1st and 2nd phase of inflammation

| Treatment | Dose(mg/kg) | No. of paw licking in 0-5 min | No. of paw licking in 15-30 min |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.46 | 6.8 ± 0.37 |
| Chloroform fraction | 30 mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| CHCl3 15 days treatment | 30 mg/kg oral | 4.2 ± 0.2*** | 1.04 ± 0.2*** |
| Ethanol 15 days treatment | 30 mg/kg oral | 4.4 ± 0.24*** | 1.05 ± 0.24*** |

**Effect of Mixed fraction of Withania somnifera on formalin-induced pain in mice during 1st phase (0-5 min) and 2nd phase (15-30 min)**

Treatment with mixed fraction (30mg/kg) showed significant (*p < 0.05) decrease in number of paw licking in mice during 1st and 2nd phase of inflammation.

| Treatment | Dose(mg/kg) | No. of paw | No. of paw |
|---|---|---|---|

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| | | licking in 0-5 min | licking in 15-30 min |
|---|---|---|---|
| Vehicle | 5ml/kg i.p | 21.8 ± 1.39 | 6.8 ± 0.37 |
| Chloroform fraction | 30mg/kg oral | 12.2 ± 0.37*** | 1.6 ± 0.24*** |
| Ethanol fraction | 30mg/kg oral | 12.6 ± 0.24*** | 1.6 ± 0.24*** |
| Mixed fraction | 30mg/kg oral | 1.1 ± 0.5* | 1.1 ± 0.5* |

Values are expressed as mean ± SEM, n = 5; ***p < 0.001 as compared to ethanol and chloroform fraction group (one way ANOVA followed by Dunnett's test).

. % inhibition by Withania somnifera on animal models of pain

| Treatment | Nacl induced Eye Wipes (%) | Formalin induced pain 0-5 min (1st phase) (%) | Formalin induced pain 15-30 min (2nd phase) (%) |
|---|---|---|---|
| Chloroform Fraction | 59 | 44 | 76 |
| Ethanol Fraction | 57 | 42 | 61 |
| Atropine + Chloroform | 15 | 19 | 62 |
| Atropine + Ethanol | 20 | 20 | 53 |
| Mecamylamine + Chloroform | 19 | 11 | 62 |
| Mecamylamine + Ethanol | 27 | 20 | 69 |
| Naloxone + Chloroform | -32 | -22 | -162 |
| Naloxone + Ethanol | -35 | -28 | -69 |
| pCPA + Chloroform | 43 | 63 | 37 |
| pCPA + Ethanol | 22 | 31 | 61 |
| Sertraline+ Chloroform | 63 | 24 | 37 |
| Sertraline + Ethanol | 35 | 34 | 61 |
| Chloroform 15 days treatment | 79 | 80 | 85 |
| Ethanol 15 days treatment | 80 | 79 | 85 |
| Pentazocine + Ethanol | 25 | 42 | 61 |
| Pentazocine + Chloroform | 23 | 37 | 37 |

## 4. DISCUSSION

Pain is the common symptom associated with many diseases and disorders. It is the first sign indicating change in the homeostasis and it indicates abnormalities in the functioning of body. It is suggestive of pathological condition and helps in deciding the treatment of underlying cause. The analgesics commonly used in modern medicines are effective in treating acute pain but effective treatment of chronic pain is still difficult.

Several pharmacological strategies are being explored to treat painful condition, antagonist of certain neurochemical substance involved in pain are being fathomed. The secondary metabolites in the medicinal plant can be good source of analgesic drug. In the present study an attempt is made to evaluate analgesic activity of withanolide isolated from Withania somnifera. Two animal models namely sodium chloride induced eye wiping and formalin induced pain are used for evaluating efficacy of withanolide in trigeminal pain, neurogenic pain, and inflammatory pain.

Standarized fraction of Withania somnifera was purchased and fractinated between chloroform, ethanol and ethyl acetate to obtain non-polar and polar substances of the fraction. The non-polar constituents were tannin, glycoside, carbohydrates and polar constituents were tannin, glycoside, carbohydrates, and phenolic compounds. Both the fractions contained withanolide which were chemically different as indicated by different IR spectra but similar UV spectra. The GC-MS data also indicated presence of withanolides.

In the pharmacological evaluation the chloroform, ethanol, and ethyl acetate fraction of Withania somnifera significantly reduced number of eye wiping. Since the yield of ethyl acetate fraction was very small further studies were carried out using chloroform and ethanol fractions. Present study includes anti-nociceptive activity of Withania somnifera on sodium chloride induced eye wiping and formalin induced pain in mice. Withania somnifera was given orally 30 minutes before injecting formalin in subplantar area and one drop of 5 M solution of NaCl into the eye of mice. Subplantar injection of 0.02 ml formalin produced both neurogenic and inflammatory phases of pain which were relieved by Withania somnifera.

To understand the mechanism of action of several antagonists were used. Since cholinergic system is involved in pain (Bartolini et al., 2011; Sandyk, 1986). In eye wiping test atropine did not reversed antinociceptive activity of any of the fractions, suggesting that cholinergic mechanism was

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not involved in antinociceptive activity of fractions. Pretreatment with mecamylamine (1mg/kg), pCPA (150mg/kg), sertraline (10mg/kg), pentazocine (10mg/kg) increased antinociceptive activity of fractions. The antinociceptive activity of W. somnifera was reversed by naloxone (1.5mg/kg) suggesting involvement of opioidergic mechanism in the anti-nociceptive activity of W. somnifera.

Nicotinic receptors are also involved in pain perception (Mannelli et al., 2011; Cosgrove et al., 2010; Ueda et al., 2007). There are several studies indicating involvement of these receptors in various animal models of pain. Mecamylamine did not significantly modify the effect chloroform and ethanol fraction on number of eye wiping indicating absence of involvement of nicotinic receptors.

Opioid receptors have attracted attention of many researches in developing antinociceptive agents though there is no analgesic which possess activity better than morphine. The addiction liability of narcotic analgesic has been a major disadvantage (Vaille et al., 1956). Withanolide for Withania somnifera fraction does not have conditioned place preference when used alone. There are studies where Withania somnifera fraction prevented acquisition of morphine elicited place preference (Ruiu et al., 2013). The study of Ruiu et al., 2013 showed in the receptor binding assay, the affinity of Withania somnifera fraction for μ opoid and GABAB receptor. The Withania somnifera fraction exhibited greater affinity for GABAB than μ opioid receptor. The opioid antagonist naloxone is most extensively used to study the involvement of opioid receptor. In our study we observed that naloxone significantly reversed the effect of chloroform and ethanol fraction of Withania somnifera in NaCl-induced eye wiping. This strongly indicated effect of chloroform and ethanol fraction on μ receptor.

Serotonergic mechanism is one of the major mechanisms involved in antinociceptive activity (Lynn et al., 1999). pCPA which depletes serotonine content of brain reduces pain perception. pCPA in our study increased antinociceptive activity of chloroform and ethanol fractions. Similar observation has been reported earlier by Santos AR et al., (2005); Stamford, (1995); Marthe, (1974); Frutuoso et al., (2007). Sertraline and fluoxetine are known to exhibit antinociceptive activity (Sawynok et al., 1999; Singh et al., 2001; Mahmood et al., 2010). Sertraline significantly increased antinociceptive activity of chloroform and ethanol fractions, decreased number of eye wiping. This observation strongly suggested that serotonergic drugs synergized antinociceptive activity of Withania somnifera. In very small extent mixed fractions (chloroform, ethanol and ethyl acetate) of Withania somnifera significantly increased the antinociceptive activity as indicated by decrease in number of eye wiping.

Development of tolerance is common problem associated with several drugs acting on CNS (Lane et al., 2005), to evaluate the antinociceptive activity on repeated administration both the extracts were given once daily for 15 days the effect of chronic treatment was much better than the effect of acute treatment thus Withania somnifera has sensitized the antinociceptive activity. Our unpublished study showed increased morphine analgesia by Withania somnifera extract.

In formalin test pharmacological evaluation the chloroform and ethanol, ethyl acetate fraction, crude extract of Withania somnifera significantly reduced number of paw licking. Since the yield of ethyl acetate fraction was very small further studies using ethyl acetate fraction were discontinued. Atropine reduced antinociceptive activity of both the fractions suggesting that they acted via cholinergic mechanism. Mecamylamine, a nicotinic receptor antagonist is known to block coniine-induced antinociception in mice (Arihan et al., 2009). Mecamylamine did not reduced the effect chloroform and ethanol fraction on number of paw licking during the 1st and 2nd phase of pain indicating absence of involvement of nicotinic receptors.

The opioid antagonist naloxone is most extensively used to study the involvement of opioid receptors (Raffa et al., 1992). Naloxone significantly reversed the effect of chloroform and ethanol fraction of Withania somnifera in formalin induced pain test. Naloxone blocks antinociceptive activity of several antinociceptive (Zakaria et al., 2012; Tsouderos et al., 1995). This strongly indicated effect of chloroform and ethanol fraction on μ receptor.

pCPA increased antinociceptive activity chloroform and ethanol fractions as indicated by decrease in number of paw lickings. Similar observations have been reported earlier by Santos AR et al., (2005); Stamford, (1995); Marthe, (1974); Frutuoso et al., (2007). Sertraline and fluoxetine are known to exhibit antinociceptive activity. Sertraline significantly increased antinociceptive activity of chloroform and ethanol fractions suggested by decrease in number of paw lickings, this observation strongly suggested that serotonergic drugs synergized antinociceptive activity of Withania somnifera. To very small extent mixed fractions (chloroform, ethanol and ethyl acetate) of Withania somnifera significantly increased the antinociceptive activity indicated by decrease in number of paw lickings. Development of tolerance is common problem associated with several drugs acting on CNS, to evaluate the antinociceptive activity on repeated administration both extracts were given once daily for 15 days the effect of chronic treatment was much better than the effect of acute treatment thus Withania somnifera has sensitized the antinociceptive activity.

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## 5. CONCLUSION

In conclusion, the antinociceptive activity of ethanol and chloroform fractions which contained withanolides exhibited antinociceptive activity in animal models of trigeminal pain, neurogenic pain, and inflammatory pain. The antinociceptive activity was reversed by naloxone and reduced by atropine suggesting involvement of opioidergic and muscarnic mechanisms. Anti-nociceptive activity is may be due to the flavonoid content of *Withania somnifera.* The study justifies the use of this plant in Ayurveda to treat nociception.

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