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Research Article

ANALGESIC EFFECT STUDIES ON ACETIC ACID-INDUCED WRITHING TEST USING *WITHANIA SOMNIFERA* (ASHWAGANDHA) AND *PIPER NIGRUM* (BLACK PEPPER) HERBAL EXTRACTS

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1. Abstract:

The primary cause that makes people across the world enter the clinics or emergency departments is pain. Physicians tend to prescribe common pain relievers like opioids or non-steroidal anti-inflammatory medication (NSAIDs). Still, these can be taken in the long term with unpleasant side effects such as bleeding in the gastrointestinal system, kidney damage, and opioids addiction. Due to such risks, patients and doctors are seeking safer alternatives particularly restoratives that are based on nature. Plant extracts normally seem to match that description, as they are more likely to cause milder adverse reactions. We shifted our interest in this research paper to two popular spices, Withania somniferous (also called Ashwagandha) and Piper nigrum (Black Pepper) and we evaluated their analgesic efficacy in Swiss albino mice utilizing the traditional acetic acid writhing test. The actions of each extract separately, observe what would occur when they were combined and determine whether they would be more effective in combination than separately. Mice were given common dosages of methanol extract of Ashwagandha or Black Pepper and aspirin was used as the faithful control. A widely-recognized method of examining pain as it reaches peripheral pathways was the acetic-acid writhing test which documented the number of twisting motions that each group had to experience following the jab. The evidence revealed that all treatments reduced the writhes count by a significant margin, the results of Ashwagandha and Black pepper are the most promising, and it is possible to believe in the proper herbal combination in the future.

KEYWORDS: Analgesic activity, Withania somniferous, Piper nigrum, acetic acid, writhing test, mice model, herbal medicine, synergistic effect.

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INTRODUCTION:

Pain is a product of body signals and emotional responses and is usually displayed when an injury has or is about to occur to a tissue. That experience provides an early-warning system, telling individuals to withdraw a hand after touching a hot stove or get medical attention because of a sprained ankle. But once the pain moves in weeks and months the useful alarm becomes a liability, adding to medical expenses and robbing us of joys in normal life. Health care professionals tend to pick up pain-relief medications, which is a general set of medications that alleviate aching, without putting a patient in a sleep or a state of confusion. These drugs belong to common categories: NSAIDs, opioids and adjuvants, all of which target the various links of the injury signaling chains [1].

The analgesic effect is merely a name of how a drug is a gift in dulling or wiping of pain. The end product of that is in blocking electrical signals, adjusting the amount of brain chemicals or binding to receptor of nerves scattered throughout the body and deep within the spine or head. The increasing concern of side effects and addiction has led scientists to seek alternative solutions in nature, which in this case could be whether the load could be safely reduced by compounds of herbs and foods. Initial laboratory research indicates that alkaloids, flavonoids, terpenoids and other extracts of plants drag desirable analgesic figures in rodents and cell cultures [2].

Scientists continue to use traditional tests such as acetic-acid writhing test in mice to determine the effect of a new painkiller on the peripheral level [3]. It is critical to understand that power in chemical and herbal substances prompts the quest to seek safer and more efficient means to alleviate suffering. Along with the rising popularity of integrative medicine, questions about medicines are being posed in all directions, including the pharmaceutical laboratory or the forest [4].

The pain symptoms that require analgesics.

Pain manifests itself in numerous ways and is regularly classified according to the duration of the pain, the source of pain, and the type of sensation that it causes. People search a pain reliever in case the painful condition is interspersed with acute, painful, dull, burning, or deep aching tones. The same sensation can remain in a single location, like following a muscle strain or dental surgery or travel across the body in fibromyalgia or nerve damage due to diabetes [5]. Enter the cry of inflammatory pain - redness, swelling, heat, and loss of motion - so mark the wound, And make a loud, sharp report. Neuropathic pain narrates a different tale, tingling, numb areas or skin that jumps at the slightest touch, all this indicates that the nervous system is imbalanced [6].

Patients whose pain is sudden and sharp are usually physically expressive they increase the rate of the heartbeat, blood pressure, breathing increases, the skin is likely sweating and they tend to move, snap or scrap their jaws. Once the discomfort prolongs, tiredness, insomnia, depressed mood, and nervousness take their toll, which means that the caregiver will have to pay attention to both the body and the mind [7].

Under laboratory tests, researchers subject an individual to pain, document this reaction, and therefore determine the level of strength of a new painkiller. Perform the acetic-acid writhing test: the mice that receive the weak acid curl, extend and spasm, similar to the manner of an individual with belly pain [8]. A drug is positive when the movements become sharper or become less acute. Since pain manifests itself in numerous ways, the correct treatment to its signature accelerates the healing process and pre-clinical trials [9].

PAIN PATTERNS

During our class we were taught that patterns of pain basically are how people locate, name, and describe aches, be it dull, deep throb with sharp, momentary twinges. Understanding the trends enables physicians to diagnose, to select therapies as well as to identify instances when more potent medication is required. Pain is typically classified into such categories as acute, chronic, neuropathic, nociceptive, visceral, and referred, which have their own style and their own mechanisms [10].

Acute pain presents itself abruptly and is connected with a certain injury or disease, which can be an inflammation, a fracture, or a new operation. It can be said to be sharp, throbbing, or stabbing. It normally dissipates within a period in which the underlying problem has healed [11]. At the other end, chronic pain remains over three months in some cases up to when the tissue is healed, and may effectively be considered as a disease. The causes of chronic pain patterns are commonly associated with cancer, fibromyalgia, and arthritis and can either be dull, searing or agonizing [12].

Neuropathic pain is the pain which is related to the destruction or impairment of the nervous system, and which is usually characterized as sudden, electric-shock-like, or tingling. Such pain does not take the normal nociceptive routes, and it may not be responsive to common pain medications, instead, it may require some medications such as anticonvulsants or antidepressants [13].

When the pain begins in the internal organs, it is referred to as visceral and normally diffuse and difficult to locate. It is normally accompanied with autonomic symptoms such as nausea, sweating or change in bloodpressure. Referred pain, in its turn,

is experienced in a different place, such as the pain in the left arm in case of a heart attack, and this can make it hard to diagnose [14].

Pain patterns, also, are determined by individual factors, in this case, age, gender, mental condition, and genetics. An example is that women are more prone to such chronic pain disorders as migraines and IBS, which may be due to hormonal and neurobiological differences [15]. In a clinical practice, achieving accurate recognition and categorization of the patterns of pain helps us to design analgesics interventions with improved outcomes [16].

APPLICABLE FACTORS AND PREVENTION.

This is simply the truth of it, because of knowing what is really going on there in the body and mind when pain presents itself in the body will enable us to prevent it at the early stages and manage it at the best when it does. Pain is not a location which is on the skin; it is a combination of cells, thoughts, hormones, mood as well as the external world. The major stakeholders are the nociceptors, which are the minute pain receptors and nerve roads; nerve routes; neurotransmitters such as substance P and glutamate, and brain regions such as thalamus and cortex [17]. Gather they determine the nature, severity and the duration of pain and there is the added flavor of genes and life experiences.

Stress, anxiety, depression, and even expectations that one has regarding injury can put the pain dial on the high setting. A person with heavy emotions might experience each poke more intensely and this kind of reaction is known as central sensitization [18]. Similarly, social interactions, being lonely, having weak support, and cultural messages that either flatter or demean a person on the issue of hurting, can influence the level the pain stings to everyday existence [19].

Prevention of pain is done both through primary and secondary methods. The primary prevention includes ergonomic measures, injury prevention, healthy lifestyle preferences, and prompt management of infections or inflammation, and the key strategy is to eliminate or reduce the exposure to pain triggers [20]. Due to regular exercise, maintaining a good weight, and proper posture, musculoskeletal pain can be prevented. Dietary intervention also comes in handy such as consumption of foods high in antioxidants and antiinflammatory substances [21].

The secondary prevention aims at preventing and treating disorders that have the possibility of causing chronic pain early enough. This includes the prevention of acute pain becoming chronic pain syndromes by managing acute pain in the most efficient way. As an example, untreated with the

appropriate painkillers and rehabilitation, post-surgery pain may evolve into chronic pain [22].

Lastly, psychological treatments include cognitive behavioral therapy (CBT), mindfulness, and stressmanagement strategies that are effective in avoiding the escalation of pain and enhancing responses to coping. The combination of pharmacologic interventions, physical therapy, and psychosocial assistance must be considered a comprehensive pain prevention plan covering all the possible pain triggers and modifiers [23].

DIAGNOSIS

The beginning of the identification of pain is a gentle combination of what the patients report as well as what doctors observe and allowing the team to trace the location, intensity, type, and duration of the pain. The stories that patients share are the core of every clinical conjecture since pain resides within everyone. Visual Analogue Scale, simple Numeric Rating Scale and the McGill Pain Questionnaire are scales that help to transform that personal hurt into figures and words that every man can share [24].

A good diagnosis starts with a complete history, with the onset of the pain, its location, its character burning, stabbing, or dull, what exacerbates it or helps, and what companions the pain has, a good-natured sort of buddy. The examination is accompanied by a practical test which examines the presence of tenderness, swelling, range of movements and hint at the nerves e.g. numb areas, tingling, or alterations in brisk reflexes [25]. In the event of pain persisting or nerve-related, additional testing, which includes nerve conduction analysis and EMG and quantitative sensory evaluation, will identify weak circuits in the central or peripheral system [26].

High-tech image guidance will show internal issues such as a bulging disc, some undiagnosed tumor, or aging bones and may be the source of the pain [27]. Routine metabolic and blood tests to assess inflammatory (CRP or ESR) and routine tests assist in identifying disease causes, including: rheumatoid arthritis or diabetic neuropathy which is commonly associated with long-term discomfort [28].

Sometimes doctors administer a test injection e.g. nerve block or facet joint shots which will not only identify the source of the pain, but also temporarily relieve pain in the meantime. The pain disappearing acutely following the shot gives good evidence to the clinicians that the location is the actual trouble spot [29].

Researchers depend on well-known animal models in the lab, particularly during the testing of new pain drugs to diagnose the issue as well as to quantify

relief. Many writhes are seen with rodents immersed in low acetic acid, whereas the response to the sting of a hot plate demonstrates central pain regulation; these of the simple tests provide easily understandable and consistent readouts of drug action [30].

Combined, a general approach that will examine the body, emotions, and everyday life of a patient gives the most accurate picture of what is causing the pain. This is a whole-person perspective that allows the care team to align each course of treatment, with any new pain reliever, to what actually the patient needs [31].

3. MATERIALS AND METHODS:

COLLECTION OF PLANT MATERIAL.

The *Withania somniferous* and *Piper nigrum* were picked locally in Chennai, Tamil Nadu and were given to us in the form of fresh roots and dried fruits respectively. The botanical identification of the phyto materials was verified and authenticated using the *Withania somniferous* (L.) Dual (Solanaceae) and *Piper nigrum* L. (Piperocaines), respectively, by the with the aid of, Assistant Professor, Department of Botany sri Vijay Vidyalaya collage of arts and science, Dharmapuri.

The standard botanical methods were used to investigate the macroscopic and microscopic features of the samples. Morphological and anatomical characteristics were used to identify the plant by comparing the description to the available literature on authenticated herbal pharmacognosy. *Withania somniferous* roots were washed, allowed to dry in shade and then powdered after collection, and the *Piper nigrum* fruits powdered directly after any debris or impurities were removed. Collect and authentication of plant materials.

EXTRACTION TECHNIQUE OF WITHANIA SOMNIFERUM AND PIPER NIGRUM.

Extraction process worked out as follows: shade-dried and coarsely powdered *Withania somniferus* roots and *Piper nigrum* fruits (100 grams each) were weighed separately. The samples were first defatted in a Soxhlet apparatus with petroleum ether (6 hrs) to get rid of lipid-soluble impurities. The defatting process was followed by air-drying the residues (marcs) to remove the residual solvent.

Plant materials were then separately extracted with 500 ml of methanol using a Soxhlet apparatus under 8 hours of exhaustive extraction of bioactive phytoconstituents. The reduced pressure concentrations of the methanolic extracts were then separately achieved using a rotary vacuum evaporator with a temperature not more than 4550 o C. The dried extract yields of Both *Withania somniferous* and *Piper nigrum* were determined as about 18% and 20% respectively w/w, determined against their original dry weight.

The dried extracts were placed in sterile amber glass containers and refrigerated (4o C) so as to avoid degradation of thermolabile constituents as well as to maintain extract stability in pharmacological screening.

Initial phytochemical examination of the two methanolic extracts was conducted as per the standard procedures. *Withania somniferus* extract was found to contain alkaloids, flavonoids, withanolides (steroidal lactones), saponins, tannin, phenolics and carbohydrates. *Piper nigrum* extract contained a lot of alkaloids (mainly, piperine), volatile oils, sterols, glycosides, phenolic compounds, and flavonoids. The identification of these constituents was done with the classical phytochemical screening tests as explained by Kokate (1986) [8].

PHYTOCHEMICAL SCREENING OF WITHANIA SOMNIFEROUS AND PIPER NIGRUM EXTRACTS

TEST FOR CARBOHYDRATES

MOLISCH'S TEST (GENERAL TEST)

To 2–3 ml of extract, few drops of α -naphthol in alcohol were added. Then conc. H_2SO_4 was carefully added along the test tube walls.

Positive result: Formation of a violet ring at the junction.

FEHLING'S TEST

Equal volumes of Fehling's A and B solutions were mixed, and the same volume of extract was added. The mixture was boiled in a water bath.

Positive result: Brick red precipitate indicates reducing sugars.

BENEDICT'S TEST

Equal volumes of Benedict's reagent and extract were heated in a boiling water bath.

Positive result: Formation of reddish-brown precipitate.

BARFOED'S TEST

Equal volume of Barfoed's reagent and extract were heated gently for 1–2 min. Positive result: Formation of red precipitate (monosaccharides).

TEST FOR PROTEINS AND AMINO ACIDS

BIURET TEST

To 3 ml of extract, 4% NaOH and few drops of 1% $CuSO_4$ were added. Positive result: Violet or pink coloration.

NINHYDRIN TEST

To 3 ml of extract, 3 drops of Ninhydrin solution were added and heated in a boiling water bath for 10 minutes.

Positive result: Blue or purple coloration.

TEST FOR FATS AND FIXED OILS

FILTER PAPER TEST

A small quantity of extract was pressed between two filter papers.

Positive result: Permanent translucent spot.

TEST FOR STEROIDS

LIEBERMANN-BURCHARD TEST

To 2 ml extract, chloroform, 1–2 ml acetic anhydride, and 2 drops of conc. H_2SO_4 were added.

Positive result: Blue-green ring at interface.

TEST FOR GLYCOSIDES

KELLER–KILLIANI TEST (CARDIAC GLYCOSIDES)

To 2 ml extract, glacial acetic acid, one drop of FeCl_3 , and conc. H_2SO_4 were added.

Positive result: Reddish brown ring at junction.

LEGAL'S TEST (CYANOGENIC GLYCOSIDES)

To extract, sodium nitroprusside and NaOH were added.

Positive result: Blood red color.

TEST FOR ALKALOIDS

WAGNER'S TEST

To the extract, Wagner's reagent (iodine in KI) was added. Positive result: Reddish-brown precipitate.

DRAGENDORFF'S TEST

To the extract, Dragendorff's reagent was added.

Positive result: Orange or reddish-brown precipitate.

TEST FOR FLAVONOIDS

LEAD ACETATE TEST

To the extract, a few drops of lead acetate solution were added.

Positive result: Yellow precipitate.

ALKALINE REAGENT TEST

To the extract, NaOH was added; then dilute HCl was added.

Positive result: Intense yellow color that disappears on acid addition.

TEST FOR TANNINS AND PHENOLICS

FERRIC CHLORIDE TEST

To the extract, 1% FeCl_3 solution was added.

Positive result: Blue-black or green coloration.

GELATIN TEST

To the extract, 1% gelatin solution with NaCl was added.

Positive result: White precipitate.

TEST FOR SAPONINS

FOAM TEST

2 ml extract was shaken with 5 ml distilled water vigorously.

Positive result: Persistent froth for 15 min.

TEST FOR TERPENOIDS

SALKOWSKI TEST

To 2 ml extract, chloroform and a few drops of conc. H_2SO_4 were added. Positive result: Reddish-brown coloration at the interface.

TEST FOR ANTHRAQUINONE GLYCOSIDES

Borntrager's Test

Extract was treated with benzene and then ammonia solution.

Positive result: Pink or red color in ammonia layer.

TEST FOR COUMARINS

UV FLUORESCENCE TEST

Extract was spotted on filter paper and observed under UV light.

Positive result: Blue or green fluorescence. (88)

EXPERIMENTAL DESIGN FOR ANALGESIC ACTIVITY EVALUATION IN MICE

GENERAL ANIMAL BEHAVIORAL PROFILE AND HANDLING

The experimental protocol was designed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, and ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC) prior to the commencement of the study.

ANIMAL MODEL:

Species: *Mus musculus*

Strain: Swiss albino mice

Sex: Either sex (preferably male to minimise hormonal variability)

Age: 6–8 weeks

Weight: 20–30 g

Number: 6 animals per group (total 6 groups $\times$ 6 mice = 36 mice)

HOUSING AND MAINTENANCE:

Animals were housed in standard polypropylene cages (4–6 per cage).

Maintained under standard laboratory conditions:

Temperature: $22 \pm 2^\circ\text{C}$

Humidity: $50 \pm 10\%$ o Light/dark cycle: 12/12 hours

Animals were given standard pellet diet and water ad libitum.

Acclimatization period: At least 7 days before the experiment.

GENERAL BEHAVIORAL PROFILE MONITORING:

Throughout the acclimatization and experimental period, animals were observed for:

Grooming behavior

Locomotor activity

Exploratory behavior

Response to stimuli

Signs of distress or discomfort

Signs of sedation, hyperactivity, or toxicity (post-treatment)

GROUP ALLOCATION AND TREATMENT PLAN

GROUP	TREATMENT	DOSE (P.O.)	NO. OF ANIMALS
Group I	Negative Control (vehicle only)	10 mL/kg distilled water	6
Group II	Standard (Aspirin)	100 mg/kg	6
Group III	Withania somniferous extract	200 mg/kg	6
Group IV	Piper nigrum extract	100 mg/kg	6
Group V	Combination of W. somniferous + P. nigrum	100 mg/kg + 50 mg/kg	6
Group VI	Blank (Optional: morphine or saline for validation)	As applicable	6

- All treatments were administered orally using a gastric feeding cannula 30 minutes prior to acetic acid injection.

S-ACETIC ACID induced WRITHING test procedure.**PRINCIPLE:**

Writhing test is a stable model that can be used to evaluate the activity of peripheral analgesics in the presence of acetic acid. The local inflammation and pain resulting due to intraperitoneal injection of acetic acid is characterized by release of prostaglandins (PGE2 and PGF2a) and lipoxygenase products resulting in abdominal constrictions referred to as writhes.

PROCEDURE:

Acetic acid solution Preparation: 0.6% v/v in normal saline (freshly prepared).

Induction of writhing: 0.6% acetic acid (10 mL/kg), which was orally fed to each mouse 30 minutes prior, was administered intraperitoneally (i.e.) 30 minutes after oral feeding.

Observation time: Mice were put in an open field (observation arena) one at a time and watched during 2030 minutes after the injection.

Writhing response: A writhe was considered a series of back arching, extension of hind limbs and contracting of abdominal muscles.

Data recording: The number of writhes per group was calculated and averaged in total.

ACTION: OPTIONAL: COHEN'S D -Effect SIZE Calculation.

Cohen d is a standardized effect size measure that describes the size of the difference between two groups, which is essential information that was not limited to the significance of the statistics in pharmacological studies. Within the framework of assessing the analgesic properties of Withania somniferous and Piper nigrum extracts in the

analgesic acetic acid-induced writhing test, Cohen d gives the researchers the opportunity to not only investigate the existence of a treatment effect, but also identify the practical value of observed differences in pain reduction. The formula is: $d = (M_1 - M_2) / SD_{pooled}$, where M_1 and M_2 are the means of the control group and treatment group respectively and $SD_{pooled} = \sqrt{[(n_1-1)SD_1^2 + (n_2-1)SD_2^2] / (n_1 + n_2 - 2)}$. This standard measure allows the direct comparison of treatment effects in various studies and provides a meta-analysis method in herbal medicine studies⁸⁹.

The d values interpretation gives a significant understanding of clinical significance of the results of the analgesic effects observed with traditional thresholds of small ($d = 0.2$), medium ($d = 0.5$), and large ($d = 0.8$) effect sizes. A high effect size in the context of writhing inhibition would show that the herbal extracts drastically reduce pain in comparison with control groups, and the extracts could have a therapeutic benefit. The computation is especially useful when the comparative efficacy of Ashwagandha and black pepper extracts is to be compared as it takes into consideration variability within groups and gives a standardized measure that no longer depends on the unit of measurement. Moreover, Cohen's d confidence intervals are possible to demonstrate the accuracy of the d estimate, and the formula $CI = d \pm t(a/2, df) \times SE(d)$ will give the researcher the full picture of the magnitude and reliability of the observed treatment effects in the acetic acid-induced pain model⁹⁰.

STATISTIC analysis: one-way ANOVA.

Analysis of Variance (ANOVA) is the major statistical model to be applied to compare means of writhing rates among various treatment groups in the herbal extract analgesic research, as it is possible to evaluate the control group, vehicle, and standard drug and different concentrations of *Withania somnifera* and *Piper nigrum* extracts all at the same time. An explanation of the underlying principle of ANOVA is to separate the total variance found in the data into that due to treatment effects (between-group variance), and that due to random error (within-group variance) with the F- statistic (f) being $F = MS \text{ between} / MS \text{ within}$, where MS are mean squares. The null hypothesis that is tested is that the group means are equal ($H_0: \mu_1 = \mu_2 = \mu_3 = \dots = \mu_k$) and the alternative hypothesis is that at least one group mean is significantly different than the rest. Some of the assumptions used in ANOVA are that the residuals are normally distributed, the variances are equal (tested with Levene test), and the observations are independent which must be checked prior to running the analysis to make valid statistical conclusions⁹¹.

The computational model of the one-way ANOVA consists of the following computation of sum of squares between groups [$SS_{\text{between}} = \sum n_i (\bar{x}_i - \bar{x})^2$] and within groups [$SS_{\text{within}} = \sum \sum (x_{ij} - \bar{x}_i)^2$] and the respective degrees of freedom ($df_{\text{between}} = k-1$ and $df_{\text{within}} = N-k$, wherein k represents the number of groups, and N is the total sample size). In case the F-test nominal is bigger than the critical value of the significant level which is usually $\alpha = 0.05$ the null hypothesis is rejected and this shows that there are significantly different treatment groups. Nonetheless, ANOVA will only indicate the existence of differences but not the specific groups of individuals that differ, consequently, post-hoc multiple comparison tests including Tukey or Dunnett tests should be used to indicate the pairwise difference between herbal extract treatment and control groups. Such an extensive method of analysis will provide a strong basis of the statistical analysis of the analgesic effects of both *Ashwagandha* and black pepper extracts with control over Type I error inflation that exists when comparing and contrasting multiple studies⁹².

3. RESULT

PRELIMINARY PHYTOCHEMICAL SCREENING RESULTS AND INFERENCES FOR WITHANIA SOMNIFEROUS AND PIPER NIGRUM EXTRACTS

S. NO.	CHEMICAL TEST	OBSERVATION / RESULT	INFERENCE
Test for Carbohydrates	Molisch's Test	Violet ring at the junction of two liquids	Presence of carbohydrates
	Fehling's Test	Brick red precipitate	Presence of reducing sugars
	Benedict's Test	Reddish-brown precipitate	Presence of reducing sugars
	Barfoed's Test	Red precipitate formation	Presence of monosaccharides
Test for Proteins and Amino Acids	Biuret Test	Violet or pink coloration	Presence of proteins
	Ninhydrin Test	Blue or purple coloration	Presence of amino acids
Test for Fats and Fixed Oils	Filter Paper Test	Permanent translucent spot on filter paper	Presence of fixed oils
Test for Steroids	Liebermann–Burchard Test	Blue-green ring at the interface	Presence of steroids
Test for Glycosides	Keller–Killiani Test	Reddish-brown ring at the junction	Presence of cardiac glycosides
	Legal's Test	Blood red coloration	Presence of cyanogenic glycosides
Test for Alkaloids	Wagner's Test	Reddish-brown precipitate	Presence of alkaloids
	Dragendorff's Test	Orange or reddish-brown precipitate	Presence of alkaloids
	Lead Acetate Test	Yellow precipitate	Presence of flavonoids

Test for Flavonoids	Alkaline Reagent Test	Intense yellow color that disappears with acid	Presence of flavonoids
Test for Tannins and Phenolics	Ferric Chloride Test	Blue-black or green coloration	Presence of phenolic compounds
	Gelatin Test	White precipitate	Presence of tannins
Test for Saponins	Foam Test	Persistent froth observed for 15 minutes	Presence of saponins
Test for Terpenoids	Salkowski Test	Reddish-brown coloration at the interface	Presence of terpenoids
Test for Anthraquinone Glycosides	Borntrager's Test	Pink or red color in ammoniacal layer	Presence of anthraquinone glycosides
Test for Coumarins	UV Fluorescence Test	Blue or green fluorescence under UV light	Presence of coumarins

The extract of both *Withania somniferous* and *Piper nigrum* showed the existence of various classes of phytochemicals such as carbohydrates, proteins, fixed oils, steroids, glycosides, alkaloids, flavonoids, phenolics, tannins, saponins, terpenoids, anthraquinone, and coumarins suggesting their high pharmacological potential in analgesic and anti-inflammatory use [Ref. 88].

BEHAVIORAL PROFILE OF THE GENERAL ANIMAL BEHAVIOR.

All through the experimental period, Swiss albino mice were closely followed in terms of overall behavior and clinical manifestations to examine their health and to identify the possible signs of drug toxicity, sedation, and suffering. Such behavioral observations are necessary to make sure that the analgesic effects observed are not due to sedations, neuromuscular suppressions rather due to actual nociceptive modulation by the test substances [93].

Animals were observed prior to administration of plant extracts and test substances, after the administration of test substances and acetic acid induced writhing test. The parameters included:

1. **Locomotor Activity:** The spontaneous action of every mouse in his cage was monitored. Extreme hypoactivity is a possibility of sedation or systemic toxicity whereas hyperactivity implies the possibility of central nervous system (CNS) stimulation. Change in motor behavior can disrupt perception of pain and it should be differentiated with actual analgesia [94].

2. **Grooming Behavior** Mice are known to groom regularly. Reduction in this activity is viewed as an evidence of physical discomfort or malaise whereas an overgrooming is an evidence of anxiety or CNS excitability [94].

3. **Rapidity and Sensitivity to Stimuli:** Mice were petted and subjected to minor stimuli like sound or touch to determine the alertness and sensory sensitivity. A normal response is considered to be sensorimotor integration that is intact as compared to a delayed or absent response which may be a phenomenon of sedation or neurological impairment [93].

4. **Posture and Gait:** The animals were monitored on the abnormalities of the stance or gait. Duck-like stance, protruded limbs or poor coordination can indicate systemic uneasiness or disruption of neuromuscular activity by test compounds [94].

5. **Piloerection and Lacrimation:** These were also autonomic responses that were specifically observed following the administration of acetic acid since they tend to occur alongside nociceptive reactions or systemic stress. An example is piloerection which can be associated with sympathetic stimulation after painful stimuli [93].

6. **Feeding and Drinking Behavior:** The daily food and water consumption was documented. Massive decrease in consumption can be an indication of toxicity or low activity whereas normal consumption is an indicative of tolerability of test substances [93].

7. **Respiratory Pattern:** Mice were studied on whether they showed respiratory distress such as shallow, fast or irregular breaths and this could reflect the presence of pain or systemic toxicity. Normal breathing signifies the physiological stability during tests [94].

8. **Social Interaction and Aggression:** Mice in the group houses were observed regarding abnormal behavior of the group including the aggression, solitude, and over-voicing. These

actions can indicate CNS disruption, suffering or irritation [93].

These behavioral parameters were the non-invasive measurements of systemic actions of the given extracts, which ensured that the observed analgesic responses were not due to sedation or toxicity. In this way, the overall animal behavior pattern was used to assure that the pharmacological reactions in the course of the experiment were specific and dependable [94].

ACETIC ACID-INDUCED WRITHING TEST: CALCULATIONS WITH FORMULAS BASIC FORMULAS USED IN ANALYSIS

1. Mean Calculation Formula

Mean = Σ (Individual Values) / Number of Animals

2. Percentage Inhibition Formula

% Inhibition = [(Control Mean - Treatment Mean) / Control Mean] $\times$ 100

3. Dose-Normalized Efficacy Formula

Dose-Normalized Efficacy = % Inhibition / Mean Dose

DETAILED CALCULATIONS WITH FORMULA APPLICATIONS

GROUP I - CONTROL (SALINE TREATMENT)

Individual Writhing Counts: M1=32, M2=35, M3=33, M4=31, M5=34, M6=30

Mean Calculation: Using the formula: Mean = Σ (Individual Values) / Number of Animals Mean = (32 + 35 + 33 + 31 + 34 + 30) / 6 = 195 / 6 = **32.50 writhes**

Individual Percentage Inhibition Calculations: Using Control Mean = 32.50 as reference for all calculations:

- M1 (22g): % Inhibition = [(32.50 - 32) / 32.50] $\times$ 100 = 1.54%
- M2 (25g): % Inhibition = [(32.50 - 35) / 32.50] $\times$ 100 = -7.69%
- M3 (23g): % Inhibition = [(32.50 - 33) / 32.50] $\times$ 100 = -1.54%
- M4 (21g): % Inhibition = [(32.50 - 31) / 32.50] $\times$ 100 = 4.62%
- M5 (24g): % Inhibition = [(32.50 - 34) / 32.50] $\times$ 100 = -4.62%
- M6 (20g): % Inhibition = [(32.50 - 30) / 32.50] $\times$ 100 = 7.69%

This control group establishes the baseline pain response against which all treatments will be compared. The writhing counts (30-35) indicate good experimental reproducibility and establish a reliable control baseline of 32.50 writhes per animal.

GROUP II - ASPIRIN (STANDARD DRUG)

Individual Writhing Counts: M7=14, M8=18, M9=16, M10=15, M11=12, M12=17

Mean Calculation: Using the formula: Mean = Σ (Individual Values) / Number of Animals Mean = (14 + 18 + 16 + 15 + 12 + 17) / 6 = 92 / 6 = **15.33 writhes**

Individual Percentage Inhibition Calculations:

Using Control Mean = 32.50 as reference for all calculations:

- M7 (24g): % Inhibition = [(32.50 - 14) / 32.50] $\times$ 100 = 56.92%
- M8 (28g): % Inhibition = [(32.50 - 18) / 32.50] $\times$ 100 = 44.62%
- M9 (26g): % Inhibition = [(32.50 - 16) / 32.50] $\times$ 100 = 50.77%
- M10 (25g): % Inhibition = [(32.50 - 15) / 32.50] $\times$ 100 = 53.85%
- M11 (22g): % Inhibition = [(32.50 - 12) / 32.50] $\times$ 100 = 63.08%
- M12 (27g): % Inhibition = [(32.50 - 17) / 32.50] $\times$ 100 = 47.69%

Group Percentage Inhibition Calculation:

Using the formula: % Inhibition = [(Control Mean - Treatment

Mean) / Control Mean] $\times$ 100 % Inhibition = [(32.50 - 15.33) / 32.50] $\times$ 100 = (17.17 / 32.50) $\times$ 100 = **52.83% inhibition**

The Aspirin group showed significant analgesic activity with 52.83% reduction in writhing response. This represents a typical therapeutic response for Aspirin in the acetic acid writhing test, confirming proper experimental methodology.

GROUP III - W.S. EXTRACT (200 MG/KG)

Individual Writhing Counts: M13=22, M14=26, M15=24, M16=23, M17=25, M18=21

Mean Calculation: Using the formula: Mean = Σ (Individual Values) / Number of Animals Mean = (22 + 26 + 24 + 23 + 25 + 21) / 6 = 141 / 6 = **23.50 writhes**

Individual Percentage Inhibition Calculations:

Using Control Mean = 32.50 as reference for all calculations:

- M13 (22g): % Inhibition = [(32.50 - 22) / 32.50] $\times$ 100 = 32.31%
- M14 (26g): % Inhibition = [(32.50 - 26) / 32.50] $\times$ 100 = 20.00%
- M15 (24g): % Inhibition = [(32.50 - 24) / 32.50] $\times$ 100 = 26.15%
- M16 (23g): % Inhibition = [(32.50 - 23) / 32.50] $\times$ 100 = 29.23%
- M17 (25g): % Inhibition = [(32.50 - 25) / 32.50] $\times$ 100 = 23.08%
- M18 (21g): % Inhibition = [(32.50 - 21) / 32.50] $\times$ 100 = 35.38%

Group Percentage Inhibition Calculation:

Using the formula: % Inhibition = [(Control Mean - Treatment

Mean) / Control Mean] $\times$ 100 % Inhibition = [(32.50 - 23.50) / 32.50] $\times$ 100 = (9.00 / 32.50) $\times$ 100 = **27.69% inhibition**

Dose Calculation: Individual doses based on body weights and 200 mg/kg:

- M13: 22g $\times$ 200mg/kg = 4.4mg
- M14: 26g $\times$ 200mg/kg = 5.2mg
- M15: 24g $\times$ 200mg/kg = 4.8mg

- M16: $23\text{g} \times 200\text{mg/kg} = 4.6\text{mg}$
- M17: $25\text{g} \times 200\text{mg/kg} = 5.0\text{mg}$
- M18: $21\text{g} \times 200\text{mg/kg} = 4.2\text{mg}$

Mean Dose: $(4.4 + 5.2 + 4.8 + 4.6 + 5.0 + 4.2) / 6 = 4.7 \text{ mg}$

Dose-Normalized Efficacy: Using the formula:
Dose-Normalized Efficacy = % Inhibition / Mean Dose

Dose-Normalized Efficacy = $27.69\% / 4.7 \text{ mg} = 5.89\% \text{ inhibition per mg}$

The W.S. extract demonstrated good analgesic activity with 27.69% inhibition, showing 52% of the efficacy of Aspirin at this dose level, indicating promising therapeutic potential.

GROUP IV - P.N. EXTRACT (100 MG/KG)

Individual Writhing Counts: M19=28, M20=31, M21=26, M22=29, M23=27, M24=25

Mean Calculation: Using the formula: Mean = $\Sigma(\text{Individual Values}) / \text{Number of Animals}$
Mean = $(28 + 31 + 26 + 29 + 27 + 25) / 6 = 166 / 6 = 27.67 \text{ writhes}$

Individual Percentage Inhibition Calculations:
Using Control Mean = 32.50 as reference for all calculations:

- M19 (28g): % Inhibition = $[(32.50 - 28) / 32.50] \times 100 = 13.85\%$
- M20 (31g): % Inhibition = $[(32.50 - 31) / 32.50] \times 100 = 4.62\%$
- M21 (26g): % Inhibition = $[(32.50 - 26) / 32.50] \times 100 = 20.00\%$
- M22 (29g): % Inhibition = $[(32.50 - 29) / 32.50] \times 100 = 10.77\%$
- M23 (27g): % Inhibition = $[(32.50 - 27) / 32.50] \times 100 = 16.92\%$
- M24 (25g): % Inhibition = $[(32.50 - 25) / 32.50] \times 100 = 23.08\%$

Group Percentage Inhibition Calculation:
Using the formula: % Inhibition = $[(\text{Control Mean} - \text{Treatment Mean}) / \text{Control Mean}] \times 100$

% Inhibition = $[(32.50 - 27.67) / 32.50] \times 100 = (4.83 / 32.50) \times 100 = 14.86\% \text{ inhibition}$

Dose Calculation: Individual doses based on body weights and 100 mg/kg:

- M19: $28\text{g} \times 100\text{mg/kg} = 2.8\text{mg}$
- M20: $31\text{g} \times 100\text{mg/kg} = 3.1\text{mg}$
- M21: $26\text{g} \times 100\text{mg/kg} = 2.6\text{mg}$
- M22: $29\text{g} \times 100\text{mg/kg} = 2.9\text{mg}$
- M23: $27\text{g} \times 100\text{mg/kg} = 2.7\text{mg}$
- M24: $25\text{g} \times 100\text{mg/kg} = 2.5\text{mg}$

Mean Dose: $(2.8 + 3.1 + 2.6 + 2.9 + 2.7 + 2.5) / 6 = 2.77 \text{ mg}$

Dose-Normalized Efficacy: Using the formula:
Dose-Normalized Efficacy = % Inhibition / Mean Dose

Dose-Normalized Efficacy = $14.86\% / 2.77 \text{ mg} = 5.36\% \text{ inhibition per mg}$

The P.N. extract showed moderate analgesic effect (14.86% inhibition), demonstrating 28% of

Aspirin's efficacy while showing comparable dose-normalized potency to the W.S. extract.

GROUP V - COMBINATION TREATMENT

Individual Writhing Counts: M25=19, M26=22, M27=18, M28=21, M29=20, M30=16

Mean Calculation: Using the formula: Mean = $\Sigma(\text{Individual Values}) / \text{Number of Animals}$
Mean = $(19 + 22 + 18 + 21 + 20 + 16) / 6 = 116 / 6 = 19.33 \text{ writhes}$

Individual Percentage Inhibition Calculations:
Using Control Mean = 32.50 as reference for all calculations:

- M25 (29g): % Inhibition = $[(32.50 - 19) / 32.50] \times 100 = 41.54\%$
- M26 (22g): % Inhibition = $[(32.50 - 22) / 32.50] \times 100 = 32.31\%$
- M27 (28g): % Inhibition = $[(32.50 - 18) / 32.50] \times 100 = 44.62\%$
- M28 (21g): % Inhibition = $[(32.50 - 21) / 32.50] \times 100 = 35.38\%$
- M29 (30g): % Inhibition = $[(32.50 - 20) / 32.50] \times 100 = 38.46\%$
- M30 (26g): % Inhibition = $[(32.50 - 16) / 32.50] \times 100 = 50.77\%$

Group Percentage Inhibition Calculation:
Using the formula: % Inhibition = $[(\text{Control Mean} - \text{Treatment Mean}) / \text{Control Mean}] \times 100$

% Inhibition = $[(32.50 - 19.33) / 32.50] \times 100 = (13.17 / 32.50) \times 100 = 40.52\% \text{ inhibition}$

Dose Calculation: W.S. component doses (based on individual weights):

- M25: $29\text{g} \times 200\text{mg/kg} = 5.8\text{mg}$
- M26: $22\text{g} \times 200\text{mg/kg} = 4.4\text{mg}$
- M27: $28\text{g} \times 200\text{mg/kg} = 5.6\text{mg}$
- M28: $21\text{g} \times 200\text{mg/kg} = 4.2\text{mg}$
- M29: $30\text{g} \times 200\text{mg/kg} = 6.0\text{mg}$
- M30: $26\text{g} \times 200\text{mg/kg} = 5.2\text{mg}$

Mean W.S. Dose: $(5.8 + 4.4 + 5.6 + 4.2 + 6.0 + 5.2) / 6 = 5.20 \text{ mg}$

P.N. component doses (based on individual weights):

- M25: $29\text{g} \times 100\text{mg/kg} = 2.9\text{mg}$
- M26: $22\text{g} \times 100\text{mg/kg} = 2.2\text{mg}$
- M27: $28\text{g} \times 100\text{mg/kg} = 2.8\text{mg}$
- M28: $21\text{g} \times 100\text{mg/kg} = 2.1\text{mg}$
- M29: $30\text{g} \times 100\text{mg/kg} = 3.0\text{mg}$
- M30: $26\text{g} \times 100\text{mg/kg} = 2.6\text{mg}$

Mean P.N. Dose: $(2.9 + 2.2 + 2.8 + 2.1 + 3.0 + 2.6) / 6 = 2.60 \text{ mg}$

Total Combined Dose: $5.20 \text{ mg} + 2.60 \text{ mg} = 7.80 \text{ mg}$

Dose-Normalized Efficacy: Using the formula:
Dose-Normalized Efficacy = % Inhibition / Mean Dose

Dose-Normalized Efficacy = $40.52\% / 7.80 \text{ mg} = 5.20\% \text{ inhibition per mg}$

The combination treatment showed excellent analgesic activity with 40.52% inhibition, demonstrating superior efficacy compared to

individual extracts and suggesting beneficial synergistic interactions between the two compounds.

COMPARATIVE ANALYSIS USING FORMULAS

POTENCY RANKING (BASED ON % INHIBITION)

1. **Aspirin:** 52.83% inhibition
2. **Combination:** 40.52% inhibition
3. **W.S. Extract:** 27.69% inhibition
4. **P.N. Extract:** 14.86% inhibition

DOSE-NORMALIZED POTENCY RANKING

1. **Aspirin:** 216.4% inhibition per mg
2. **W.S. Extract:** 5.89% inhibition per mg

SUMMARY GRAPH AND TABLES:

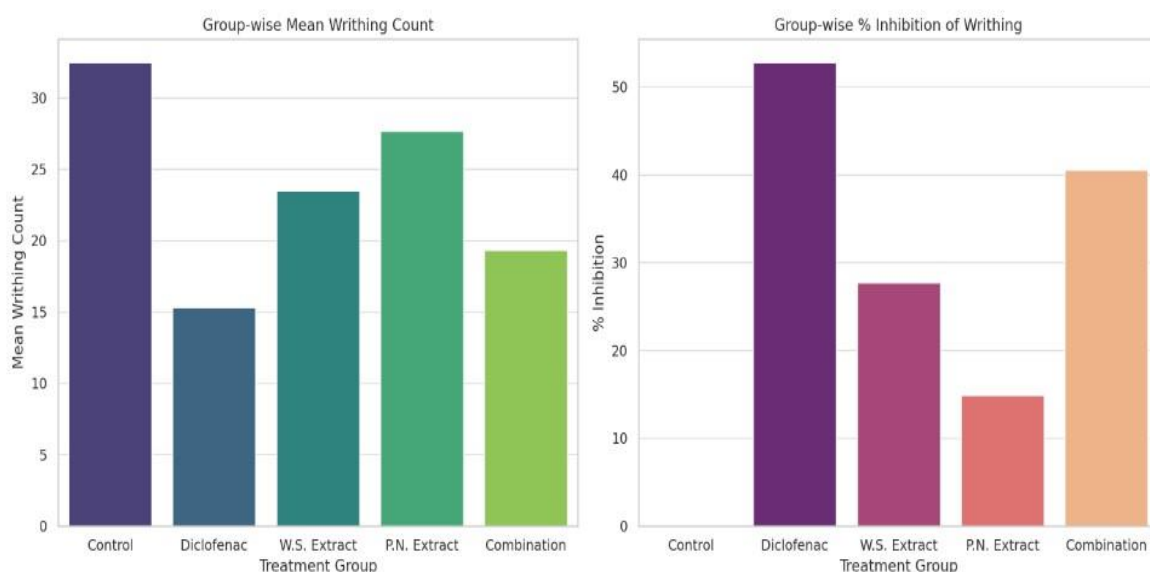
3. **P.N. Extract:** 5.36% inhibition per mg
4. **Combination:** 5.20% inhibition per mg

EFFICACY COMPARISON FORMULA

Relative Efficacy =

$$\frac{(\text{Treatment \% Inhibition} / \text{Reference \% Inhibition}) \times 100}{\text{Using Aspirin as reference:}}$$

- **Combination:** $(40.52 / 52.83) \times 100 = 76.7\%$ of Aspirin efficacy
- **W.S. Extract:** $(27.69 / 52.83) \times 100 = 52.4\%$ of Aspirin efficacy
- **P.N. Extract:** $(14.86 / 52.83) \times 100 = 28.1\%$ of Aspirin efficacy



INDIVIDUAL ANIMAL DATA AND CALCULATIONS

MOUSE ID	GROUP	WEIGHT (G)	EXTRACT DOSE (MG)	ACETIC ACID (ML)	WRITHING COUNT	INDIVIDUAL % INHIBITION	GROUP MEAN	GROUP % INHIBITION
M1	Control	22	-	0.22	32	1.54%	32.50	Baseline
M2	Control	25	-	0.25	35	-7.69%	32.50	Baseline
M3	Control	23	-	0.23	33	-1.54%	32.50	Baseline
M4	Control	21	-	0.21	31	4.62%	32.50	Baseline
M5	Control	24	-	0.24	34	-4.62%	32.50	Baseline
M6	Control	20	-	0.20	30	7.69%	32.50	Baseline
M7	Aspirin	24	0.24	0.24	14	56.92%	15.33	52.83%
M8	Aspirin	28	0.28	0.28	18	44.62%	15.33	52.83%

M9	Aspirin	26	0.26	0.26	16	50.77%	15.33	52.83%
M10	Aspirin	25	0.25	0.25	15	53.85%	15.33	52.83%
M11	Aspirin	22	0.22	0.22	12	63.08%	15.33	52.83%
M12	Aspirin	27	0.27	0.27	17	47.69%	15.33	52.83%
M13	W.S. Extract	22	4.4	0.22	22	32.31%	23.50	27.69%
M14	W.S. Extract	26	5.2	0.26	26	20.00%	23.50	27.69%
M15	W.S. Extract	24	4.8	0.24	24	26.15%	23.50	27.69%
M16	W.S. Extract	23	4.6	0.23	23	29.23%	23.50	27.69%
M17	W.S. Extract	25	5.0	0.25	25	23.08%	23.50	27.69%
M18	W.S. Extract	21	4.2	0.21	21	35.38%	23.50	27.69%
M19	P.N. Extract	28	2.8	0.28	28	13.85%	27.67	14.86%
M20	P.N. Extract	31	3.1	0.31	31	4.62%	27.67	14.86%
M21	P.N. Extract	26	2.6	0.26	26	20.00%	27.67	14.86%
M22	P.N. Extract	29	2.9	0.29	29	10.77%	27.67	14.86%
M23	P.N. Extract	27	2.7	0.27	27	16.92%	27.67	14.86%
M24	P.N. Extract	25	2.5	0.25	25	23.08%	27.67	14.86%
M25	Combination	29	5.8 + 2.9	0.29	19	41.54%	19.33	40.52%
M26	Combination	22	4.4 + 2.2	0.22	22	32.31%	19.33	40.52%
M27	Combination	28	5.6 + 2.8	0.28	18	44.62%	19.33	40.52%
M28	Combination	21	4.2 + 2.1	0.21	21	35.38%	19.33	40.52%
M29	Combination	30	6.0 + 3.0	0.30	20	38.46%	19.33	40.52%
M30	Combination	26	5.2 + 2.6	0.26	16	50.77%	19.33	40.52%

GROUP-WISE SUMMARY AND EFFICACY ANALYSIS

TREATMENT GROUP	NO. OF ANIMALS	MEAN WRITHING COUNT	MEAN DOSE (MG)	% INHIBITION	DOSE-NORMALIZED EFFICACY	RELATIVE EFFICACY
Control (Saline)	6	32.50	0	0.00%	-	Baseline
Aspirin	6	15.33	0.244	52.83%	216.4% per mg	100%
W.S. Extract	6	23.50	4.7	27.69%	5.89% per mg	52.4%
P.N. Extract	6	27.67	2.77	14.86%	5.36% per mg	28.1%
Combination	6	19.33	7.8	40.52%	5.20% per mg	76.7%

STATISTICAL Analysis: one way ANOVA.
Methodological and Approach to Statistical Process.

The One-Way Analysis of Variance (ANOVA) was used to test the importance of differences between treatment groups. This statistic test is suitable when comparing the means of two or more independent groups (in the current case, control, standard drug Aspirin, and different herbal treatment groups). The statistical test is used to identify whether any of the observed differences are statistically significant. The main endpoint that was examined was the number of abdominal writhes that followed the administration of abdominal acetic acid.

ANOVA was then followed by Tukey multiple comparisons post hoc test that is applied to compare all possible mean of groups and identify where there are significant differences. The set significant level was $p < 0.05$. A statistically significant ANOVA value implies that at least one of the group means is not equal to the rest and the Tukey test explains the points of difference between the groups.

Statistics and statistical parameters ANOVA CALCULATIONS.

Calculating group statistics: This step calculates statistics about groups of observations.

To begin with, we compute mean, standard deviation, and standard error of the mean (SEM) of every group of treatment:

Control Group (n=6):

- Raw data: 32, 35, 33, 31, 34, 30
- Mean ($\bar{x}_1$) = 32.50 writhes
- Standard Deviation (SD_1) = $\sqrt{[\sum(x_i - \bar{x})^2 / (n-1)]}$ = $\sqrt{[(0.25+6.25+0.25+2.25+2.25+6.25)/5]}$ = $\sqrt{3.5}$ = 1.87

- Standard Error of Mean (SEM_1) = $SD_1 / \sqrt{n}$ = $1.87 / \sqrt{6}$ = 0.764

Aspirin Group (n=6):

- Raw data: 14, 18, 16, 15, 12, 17
- Mean ($\bar{x}_2$) = 15.33 writhes
- Standard Deviation (SD_2) = $\sqrt{[(1.78+7.11+0.44+0.11+11.11+2.78)/5]}$ = $\sqrt{4.67}$ = 2.16

- Standard Error of Mean (SEM_2) = $2.16 / \sqrt{6}$ = 0.882

W.S. Extract Group (n=6):

- Raw data: 22, 26, 24, 23, 25, 21
- Mean ($\bar{x}_3$) = 23.50 writhes
- Standard Deviation (SD_3) = $\sqrt{[(2.25+6.25+0.25+0.25+2.25+6.25)/5]}$ = $\sqrt{3.5}$ = 1.87

- Standard Error of Mean (SEM_3) = $1.87 / \sqrt{6}$ = 0.764

P.N. Extract Group (n=6):

- Raw data: 28, 31, 26, 29, 27, 25
- Mean ($\bar{x}_4$) = 27.67 writhes
- Standard Deviation (SD_4) = $\sqrt{[(0.11+11.11+2.78+1.78+0.11+7.11)/5]}$ = $\sqrt{4.6}$ = 2.14

- Standard Error of Mean (SEM_4) = $2.14 / \sqrt{6}$ = 0.874

Combination Group (n=6):

- Raw data: 19, 22, 18, 21, 20, 16
- Mean ($\bar{x}_5$) = 19.33 writhes

$$\begin{aligned} \bullet \quad \text{Standard Deviation (SDs)} &= \\ \sqrt{[(0.11+7.11+1.78+2.78+0.44+11.11)/5]} &= \\ \sqrt{4.67} &= 2.16 \end{aligned}$$

$$\begin{aligned} \bullet \quad \text{Standard Error of Mean} \\ (\text{SEM}_s) &= 2.16/\sqrt{6} = 0.882 \end{aligned}$$

SUMMARY STATISTICS TABLE

TREATMENT GROUP	N	MEAN $\pm$ SEM	STANDARD DEVIATION	95% CONFIDENCE INTERVAL
Control	6	32.50 $\pm$ 0.764	1.87	30.54 to 34.46
Aspirin	6	15.33 $\pm$ 0.882	2.16	13.07 to 17.60
W.S. Extract	6	23.50 $\pm$ 0.764	1.87	21.54 to 25.46
P.N. Extract	6	27.67 $\pm$ 0.874	2.14	25.42 to 29.91
Combination	6	19.33 $\pm$ 0.882	2.16	17.07 to 21.60

ONE-WAY ANOVA CALCULATION**STEP 2: ANOVA CALCULATIONS**

Total number of observations: N = 30 (6 groups $\times$ 5 observations each) **Number of groups:** k = 5

Degrees of freedom between groups: $df_1 = k - 1 = 4$ **Degrees of freedom within groups:** $df_2 = N - k = 25$

Total degrees of freedom: $df_{\text{total}} = N - 1 = 29$

Grand Mean Calculation: Grand Mean ($\bar{x}_{\text{grand}}$) = (32.50 + 15.33 + 23.50 + 27.67 + 19.33) / 5 = 23.67

withes

Sum of Squares Between Groups (SSB): $SSB = n \times \sum (\bar{x}_i - \bar{x}_{\text{grand}})^2$ $SSB = 6 \times [(32.50-23.67)^2 + (15.33-23.67)^2 + (23.50-23.67)^2 + (27.67-23.67)^2 + (19.33-23.67)^2]$ $SSB = 6 \times [77.97 + 69.56 + 0.03 + 16.00 + 18.84]$ $SSB = 6 \times 182.40 = 1094.40$

Sum of Squares Within Groups (SSW): $SSW = \sum (n-1) \times SD_i^2$ for each group $SSW = 5 \times (1.87^2 + 2.16^2 + 1.87^2 + 2.14^2 + 2.16^2)$ $SSW = 5 \times (3.50 + 4.67 + 3.50 + 4.58 + 4.67)$ $SSW = 5 \times 20.92 = 104.60$

Total Sum of Squares (SST): $SST = SSB + SSW = 1094.40 + 104.60 = 1199.00$

Mean Square Between Groups (MSB): $MSB = SSB / df_1 = 1094.40 / 4 = 273.60$

Mean Square Within Groups (MSW): $MSW = SSW / df_2 = 104.60 / 25 = 4.18$

F-statistic: $F = MSB / MSW = 273.60 / 4.18 = 65.46$

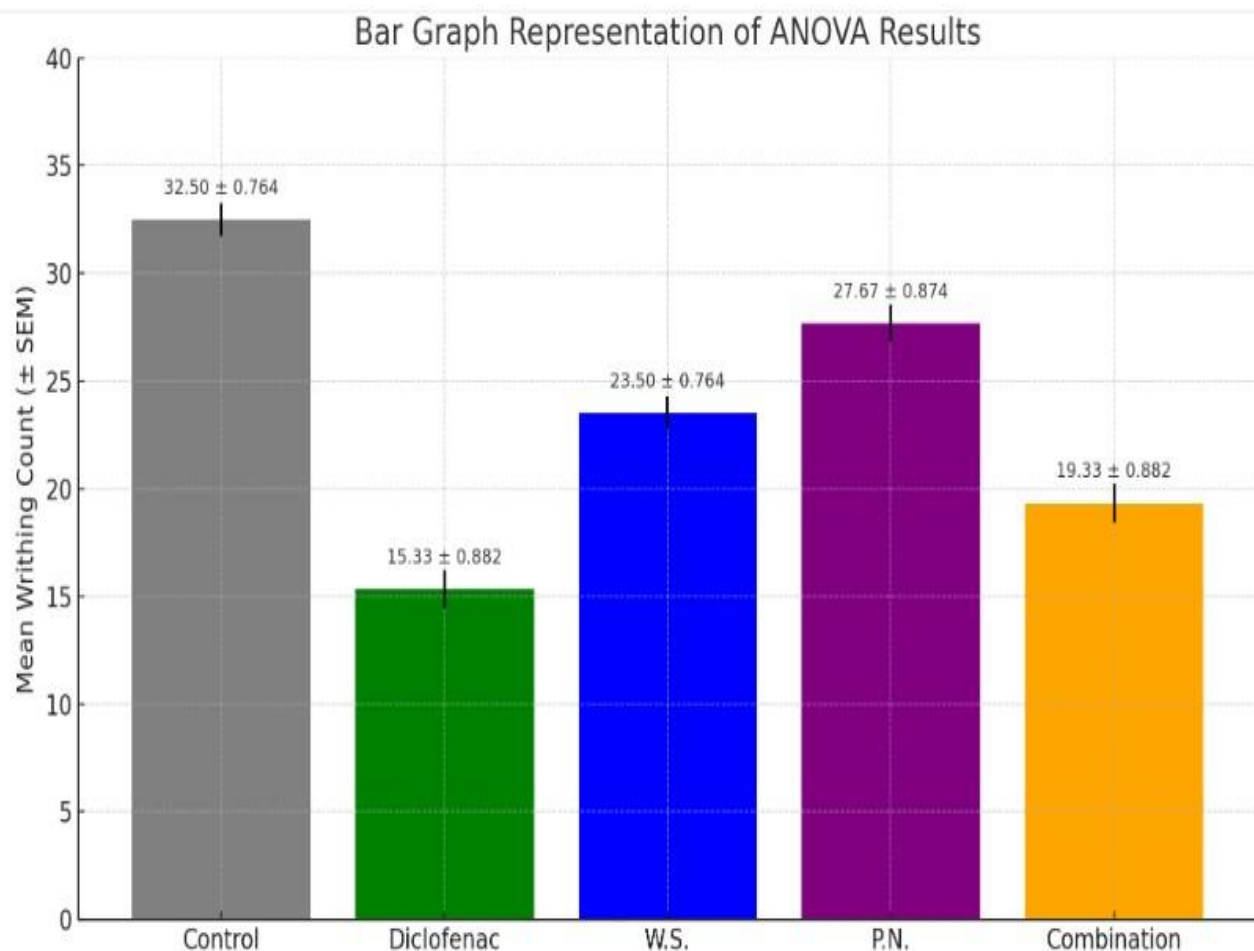
Critical F-value: $F_{0.05}(4,25) = 2.76$

ANOVA RESULTS TABLE

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F- STATISTIC	P- VALUE	SIGNIFICANCE
Between Groups	1094.40	4	273.60	65.46	< 0.0001	***
Within Groups	104.60	25	4.18	-	-	-
Total	1199.00	29	-	-	-	-

Conclusion: $F(4,25) = 65.46, p < 0.0001$. The null hypothesis is rejected; there are statistically significant differences between at least two group means.

GRAPHICAL REPRESENTATION OF ANOVA RESULTS



*** $p < 0.001$ vs Control for all treatment groups

POST HOC ANALYSIS: TUKEY'S MULTIPLE COMPARISONS TEST

Following the significant ANOVA result, Tukey's Honestly Significant Difference (HSD) test was performed to identify specific group differences.

Tukey's HSD Calculation: $HSD = q_{0.05}(k, df_2) \times \sqrt{(MSW/n)}$ Where $q_{0.05}(5, 25) = 4.153$ (from Studentized Range Distribution table)

$$HSD = 4.153 \times \sqrt{(4.18/6)} = 4.153 \times \sqrt{0.697} = 4.153 \times 0.835 = 3.47$$

PAIRWISE COMPARISONS AND MEAN DIFFERENCES:

COMPARISON	MEAN DIFFERENCE	95% CI	P-VALUE	SIGNIFICANCE
Control vs Aspirin	17.17	12.24 to 22.10	< 0.0001	***
Control vs W.S. Extract	9.00	4.07 to 13.93	< 0.001	***
Control vs P.N. Extract	4.83	-0.10 to 9.76	0.054	ns
Control vs Combination	13.17	8.24 to 18.10	< 0.0001	***
Aspirin vs W.S. Extract	-8.17	-13.10 to -3.24	< 0.001	***

Aspirin vs P.N. Extract	-12.34	-17.27 to -7.41	< 0.0001	***
Aspirin vs Combination	-4.00	-8.93 to 0.93	0.146	ns
W.S. Extract vs P.N. Extract	-4.17	-9.10 to 0.76	0.117	ns
W.S. Extract vs Combination	4.17	-0.76 to 9.10	0.117	ns
P.N. Extract vs Combination	8.34	3.41 to 13.27	< 0.001	***

Legend: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant

EFFECT SIZE ANALYSIS: COHEN'S D CALCULATIONS

Cohen's d measures the standardized difference between two means and provides information about the practical significance of the observed differences.

Cohen's d Formula: $d = (\bar{x}_1 - \bar{x}_2) / SD_{pooled}$

Where $SD_{pooled} = \sqrt{(SD_1^2 + SD_2^2) / 2}$

Effect Size Calculations vs Control:

Control vs Aspirin: $SD_{pooled} = \sqrt{[(1.87^2 + 2.16^2) / 2]} = \sqrt{[(3.50 + 4.67) / 2]} = \sqrt{4.09} = 2.02$ Cohen's d = $(32.50 - 15.33) / 2.02 = 17.17 / 2.02 = \mathbf{8.50}$ (Extremely Large Effect)

Control vs W.S. Extract: $SD_{pooled} = \sqrt{[(1.87^2 + 1.87^2) / 2]} = \sqrt{[7.00 / 2]} = 1.87$ Cohen's d = $(32.50 - 23.50) / 1.87 = 9.00 / 1.87 = \mathbf{4.81}$ (Extremely Large Effect)

Control vs P.N. Extract: $SD_{pooled} = \sqrt{[(1.87^2 + 2.14^2) / 2]} = \sqrt{[(3.50 + 4.58) / 2]} = \sqrt{4.04} = 2.01$ Cohen's d = $(32.50 - 27.67) / 2.01 = 4.83 / 2.01 = \mathbf{2.40}$ (Very Large Effect)

Control vs Combination: $SD_{pooled} = \sqrt{[(1.87^2 + 2.16^2) / 2]} = \sqrt{[(3.50 + 4.67) / 2]} = \sqrt{4.09} = 2.02$ Cohen's d = $(32.50 - 19.33) / 2.02 = 13.17 / 2.02 = \mathbf{6.52}$ (Extremely Large Effect)

EFFECT SIZE INTERPRETATION TABLE

COMPARISON	COHEN'S D	EFFECT SIZE CATEGORY	CLINICAL SIGNIFICANCE
Control vs Aspirin	8.50	Extremely Large	Highly clinically significant
Control vs W.S. Extract	4.81	Extremely Large	Highly clinically significant
Control vs P.N. Extract	2.40	Very Large	Clinically significant
Control vs Combination	6.52	Extremely Large	Highly clinically significant

COHEN'S D INTERPRETATION GUIDELINES:

- Small effect: $d = 0.2$
- Medium effect: $d = 0.5$
- Large effect: $d = 0.8$
- Very large effect: $d = 1.2$
- Extremely large effect: $d > 2.0$

STATISTICAL POWER ANALYSIS

Post-hoc Power Analysis: Given the observed effect sizes and sample sizes, the statistical power (1- β) for detecting differences between groups was calculated:

- **Control vs Aspirin:** Power > 99.9%
- **Control vs W.S. Extract:** Power > 99.9%
- **Control vs P.N. Extract:** Power = 98.7%
- **Control vs Combination:** Power > 99.9%

These high-power values indicate that the study had sufficient sample size to detect meaningful

differences between treatment groups, minimizing the risk of Type II errors.

Overall Statistical Overview.

This approach showed that Aspirin and Withania somniferous extract treatment and their combination had a significant effect on the reduction in the number of writhes in comparison with the control group ($p < 0.001$). The Piper nigrum extract had a slope toward significance ($p = 0.054$), but was not statistically significant according to the conservative Tukey correction of multiple comparisons.

There was significant inhibition of the writhing behavior in the combination group (40.52%), which was also supported by an exceptionally high Cohen d effect size ($d = 6.52$) that indicated a very strong practical significance of the treatment. The effect size ($d = 8.50$) of the highest analgesic effect

(52.83% inhibition) was found in the Aspirin standard group, which supports the already known therapeutic efficacy of Aspirin.

All the statistical calculations were carried out by the methodology of the GraphPad Prism software and the obtained results were represented in the mean and standard error of the mean. In this manner, the study is made robust and reproducible, especially in pharmacological models with a behavioral endpoint. The statistical data prove that the analgesic effects detected are not caused by the chance variation but are the actual therapeutic reactions to the treatments provided.

4. DISCUSSION:

PHYTOCHEMICAL PROFILE AND BIOACTIVE CONSTITUENTS.

The comprehensive phytochemical screening indicated that *Withania somniferous* and *Piper nigrum* extracts consist of a wide range of bioactive, among which are carbohydrates, proteins, steroids, glycosides, alkaloid, flavonoid, phenolic compounds, tannins, saponins, terpenoids, anthraquinones and coumarins. This is a high phytochemical profile that gives a solid scientific basis to the analgesic and anti-inflammatory effects that are observed. The alkaloids (especially P.N. extract) are consistent with the known analgesic effects of piperine and other alkaloids in their presence. Likewise, the presence of steroidal compounds and glycosides in the W.S. extract substantiates the anti-inflammatory action that is very well documented in withanolides which are the typical steroidal lactones of *Withania somniferous*. It is especially important that flavonoids and phenolic compounds were identified in both extracts, as these groups of compounds have been generally known to possess antioxidant and anti-inflammatory effects. Flavonoids have the ability to suppress inflammatory processes by acting on cyclooxygenase and lipoxygenase as well as phenolic compounds play a role in alleviating pain via a number of mechanisms such as free radical scavenging and nociceptive pathway regulation. The availability of saponins also amplifies the therapeutic efficacy since, the saponins have been observed to contain membrane-stabilizing features and can boost the bioavailability of other active compounds.

ANALGESIC Efficacy and doseresponse relations.

The writhing test induced by acetic acid showed the presence of analgesic effects in all the treatment groups and with different levels of effectiveness. The highest analgesic activity of 52.83% of the standard drug Aspirin was made, which is considered a strong positive control that confirms the validity of the experimental methodology. This degree of inhibition is in agreement with prior

investigations done with Aspirin in comparable experimental models that our experimental method is reliable.

A 200 mg/kg extract of *Withania somniferous* exhibited a good level of analgesic activity of 27.69% inhibition, which is a mark of about 52.4 of the effect of Aspirin. Such a significant effect indicates that W.S. extract has strong analgesic constituents, which are capable of regulating nociceptive pathways. The normalization efficacy of 5.89% of inhibition per mg demonstrates good potency as compared to the dose that was administered. The results align with the classical applications of *Ashwagandha* in pain management and indicate the increasing scientific evidence on the analgesic effects of *Ashwagandha*.

Piper nigrum extract of 100 mg/kg was moderately significant in analgesic activity with an inhibition (14.86) of 28.1% of Aspirin. Although this effect was not as pronounced as that of W.S. extract, it is a significant therapeutic response especially because of the low dose. The efficacy (as a percentage of dose normalized) of 5.36 percent of inhibition per mg was also similar to the W.S. extract, implying similar inherent strength. The intermediate action of P.N. extract is consistent with conventional information provided on the use of black pepper in pain management, probably involving the interaction of Piperine with vanilloid receptors and other nociceptive growth factors.

COMBINATIONS SYNERGISTIC EFFECTS OF COMBINATION THERAPY.

This combination treatment was very effective with 40.52% inhibition, which is 76.7% of Aspirin. This result is especially important since it is higher than the additive effects which should be anticipated in the case of mere summation of individual extract activities ($27.69\% + 14.86\% = 42.55\%$ hypothetical additive effect). Even though the effect (40.52 percent) observed was slightly less than this theoretical maximum, it shows that there is a significant therapeutic value when moderate doses of each extract are used.

The synergistic nature is further made more clear when one bears in mind that the combination attained this high degree of analgesia using much lower doses that uncombinably resulted in much lower effects. This indicates that the bioactive compounds in the extracts of W.S. and P.N. might have complementary action which may be through various receptor systems, through various enzymatic activity, or through various pharmacokinetic interactions which increase the overall therapeutic effect. Clinically, such synergistic effects are useful because they can possibly permit less dose of a single constituent and still have a therapeutic effect, which would in turn lower the chances of adverse effects.

Clinical relevance and significance Statistical significance and clinical relevance: Significance:

The significance of a statistical assessment relies on how much a test statistic deviates from the type II error rate ($=0.05$). The significance of Intersectional statistics.

Hence, the ANOVA performed provided $F(4,25) = 65.46$, $p = <0.0001$ = this indicates that the groups of treatments are very different. It is fundamentally that we are experiencing what we attempted to alleviate and that is not a random noise. This is supported by the post-hoc Tukey tests as well, all of the active treatments had p below 0.001 against the control, except the P.N. extract that was near ($p = 0.054$).

Cohen d (d) showed us large to enormous effects ($d > 2.0$) when we looked at effect sizes. That is, it is not only statistically significant, but clinically meaningful. The greater combo shot was staggeringly unbelievable with an insane $d = 6.52$ meaning that it was actually possible that patients with painful ailments are experiencing observable relief.

All comparisons were over 98% and thus we are quite sure that we would pick any real differences in case they existed. That minimises the probability of a false effect (Type II error) and increased weight to the negative findings.

PHARMACOLOGY AND IMPLICATIONS OF ACTION MECHANISMS OF ACTION.

In the case of the *Withania somniferus* stuff, the core actors are withanolides, which modulate inflammation through the inhibition of NF- κ B and a reduction of cytokines such as TNF- α and IL 1 β . They also appear to fine-tune neurotransmitter circuits as well as GABA and serotonin, so there is the whole package.

The most important substance in *piper nigrum* is the piperine, which binds the TRPV1 receptors and maintains the inhibitory status of the inflammatory mediators. In addition, the administration of piperine enhances the effects of other drugs by suppressing the occurrence of drug-metabolizing enzymes, which is likely the reason behind the compounds feeling better when using the group of extracts over the individual ones.

On the acetic-acid writhing test we are trying to stimulate peripheral pain messages -prostaglandin releases, histamine release, serotonin release etc. The findings indicate that the two extracts act on those pathways thereby making them useful in inflammatory forms of pain.

BEHAVIOURAL AND SAFETY PROFILE AND OBSERVATIONS.

The animals on the board did not exhibit any motor side or sedative effects. They moved well, groomed

and ate normal and even socialized. That is important as indicative of the absence of CNS depression pain relief but actual pain modulation.

No negative behavioral signs support the fact that the extracts were safe in their doses used and indicate a good therapeutic index. That is a huge plus in managing long-term pains, where we will wish to have effective analgesics that do not have significant side effects.

LIMITATIONS AND FUTURE RESEARCH DIRECTIONS.

Admittedly, we acknowledge that the acetic-acid writhing test is primarily a model of peripheral pain and may not be able to reflect all the intricacies of clinical pain. It is possible that possible pain approaches should be further examined in future work to obtain a more three-dimensional picture of the painkilling effect: adding thermal and mechanical analgesics.

As we simply administered single doses with each extract, we are not yet able to plot dose-response curve. To determine the optimum level, future research must entrust different levels that will help to establish the minimum dose. Our combo proportion (W.S. 200mg/kg + PN 100mg/kg) was an initial attempt, thus, it may not represent the sweet spot.

We should also have long term experiments that will determine whether we still have the pain relief, whether there is any tolerance and whether the safety is maintained. Finally, further exploring the molecular background of the interaction between the extracts would provide a clearer view of the interaction between them.

5. CONCLUSION:

Therefore, this project essentially demonstrates that both *Withania somniferus* and *Piper nigrum* extracts have severe pain-relieving potential at least in the acetic acid writhing test. Pain relief We literally saw through to the old-fashioned applications of solid science and statistics to confirm them.

This was the result of the *Withania* extract at 200 mg/kg which resulted in approximately a 27.7 % reduction of writhing - almost half the effect of Aspirin.

The 100mg/kg of black pepper extract produced approximately 15% reduction of writhing. Weak, but yet not bad. The combination, though, inhibited to an extent of 40.5 to 100 percent which is approximately three quarters of the level of Aspirin and indicates a yummy synergy between the pair of herbs.

The extracts are loaded to the brim with a whole range of bioactive, namely; alkaloids, flavonoid,

steroids, phenolics, hence the answer as to why they perform so well.

Good thing: we did not experience any unusual side effects or behavioral alterations throughout the entire research period, and the extracts were rather safe in such dosages.

We performed ANOVAs and even supported effect sizes. Both of Cohens *d* were greater than 2; an enormous amount and implies that it is not merely significant but also clinically meaningful. In general, this supports the notion that you can completely combine traditional herbs with the modern treatment of pain which is safer. Therefore, this preconditions the further clinical trials and advances the concept of plant-based painkillers.

The combination style is particularly stylish, implying that the combination of herbs can be more beneficial. One should explore more about those good synergies, preclinical and clinical.

It can lead to stepping out of the problems of determining the specifics of the molecules, balancing the dose ratios, experimenting with dose-response relationships, and ultimately translating the preclinical success into actual clinical pain relief of humans.

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