EVALUATION OF ANTI-NOCICEPTIVE ACTIVITY OF CROCUS SATIVUS

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***ABSTRACT:** Extensive use of analgesics like non-steroidal antiinflammatory drugs, opioid is associated with undesirable side effects often limiting their use. Hence the search for indigenous safer analgesic agent is ongoing. It was considered worthwhile to evaluate analgesic activity of Petroleum benzine, Chloroform, Methanol and Sterile water fraction of ethanolic extract of Crocus sativus stigmas.*

This study was conducted to evaluate analgesic activity of Methanol (CSEEMF) and Sterile water (CSEEWF) fraction of ethanolic extract of Crocus sativus in rats and mice.

Antinoceceptive activity of stigmas of CSEEMF and CSEEWF were investigated at doses 50 mg/kg, 100 mg/kg and 200 mg/kg through tailflick method, tail clip method and formalin test in swiss albino mice and writhing test by using acetic-acid in wistar albino Rats. One-way analysis of variance (ANOVA) followed by Dunnett's test was applied in the study. Both $p<0.05$, $p<0.001$ were considered to be significant. In tail-flick method, CSEEMF produced significant ($p<0.05$, $p<0.001$) increase in mean reaction time as compared to control in mice. In tail-clip method, same extract fraction caused significant ($p<0.001$) inhibition of pain in mice at all the doses. CSEEMF produced statistically significant ($p<0.001$) reduction in number of writhes induced by acetic-acid in rats. Moreover, there was significant inhibition of pain in both phases of the formalin induced response in mice. Stigmas of Crocus sativus ethanolic extract methanolic fraction exhibited significant central and peripheral analgesic activity.

I. INTRODUCTION

The International association for the study of pain (1979) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. However, for animals, it is harder to know whether an emotional experience has occurred. Therefore, this concept is often excluded. Pain in animals is an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species specific behaviour, including social behaviour [1].

Pain is an unpleasant sensation no doubt, but on the whole it is usually beneficial to man (or animal). It is mainly a protective mechanism for the body, occurs whenever any tissues are being damaged, and it causes the individual to react to remove the pain stimulus. With many pathological conditions, tissue injury is the immediate cause of the pain, and this result in the local release of a variety of chemical agents, which are assumed to act on the nerve terminals, either activating them directly or enhancing their sensitivity to other forms of stimulation [2]. Pain is a pervasive public health problem, and analgesic drugs play a central role in its treatment.

Historically, the most widely used analgesics have included opioid agonists such as morphine, antiinflammatory steroids such as cortisone, and nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin. Although these drugs are useful across a wide range of conditions, they are not uniformly effective and undesirable side effects often limit their use. Chronic painful conditions (like gout, rheumatoid arthritis etc.) are a few of the oldest known diseases of mankind that affect a majority of the population across the globe. No substantial progress has been observed in the achievement of the permanent restoration and convalescence of these conditions. Consequently, one long-standing focus of drug discovery has been the search for novel analgesics. As a consequence, one of the chief focuses of drug discovery has been the pursuit of new analgesics.

Currently available drugs are not uniformly effective, and adverse effects often pose certain limitations to a continual consumption. So, keeping in the mind this fact, herbal medicines derived from the plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, though relatively little knowledge about their mode of action is available. A bibliographic survey showed that there is large number of reports on the analgesic activity of different Indian traditional plant [2, 3].

So, keeping all the risk and benefit aspects of the presently ongoing molecules in the mind, Crocus sativus, a herbal moiety, was brought into consideration. Saffron is the dried stigma (tiny thread like strands) of the Crocus sativus also called the "fall flower". It is World's most expensive spice by weight used in industry, with many different uses as drug, textile dye and culinary adjunct. It is mainly valued as a food additive for tasting, flavouring and colouring, as well as for its therapeutic properties. Saffron is cultivated in countries such as Iran, Spain, Italy,

Switzerland and India[4]. The saffron and its coloured components can be used to treat various human diseases like anxiety disorders and schizophrenia [5], antiulcer [6], antitumor [7], anticonvulsant [8] and antidepressant [9] activities. Recently it has been seen that Crocus sativus extract also helps to tighten the blood brain barrier [10] and erectile dysfunction [11]. From the previous study [12] ethanolic extract of Crocus sativus ''safranal'' main constituent of it showed significant anti-nociceptive activity. But the studies on Crocus sativus methanol fraction of ethanolic extract and its evaluation in different anti nociceptive models are still lacking. It is necessary to assess analgesic activity of extract fraction of traditional Indian flower Crocus sativus. Hence, the present study was planned to evaluate and compare analgesic activity of Crocus sativus ethanolic extract methanolic fraction (CSEEMF) and sterile water fraction (CSEEWF) in different animal models of pain.

II. MATERIAL AND METHODS

Preparation of the Extract

Freshly procured Crocus sativus from local market stigmas of the flower were dried under shade and cut into small segments with scissor. Sixty four grams of the pulverized stigmas were extracted with 95% ethanol by cold maceration technique consecutively for three days. The ethanolic Crocus sativus extract was subjected for fractionation with different solvents according to their polarity with the help of column chromatography technique for the determination of various constituents[13]. The methanolic fraction and sterile water fraction of ethanolic Crocus sativus extract (CSEEMF) was used for the present study.

Experimental Animals

After getting approval from the animal ethics committee, the experiment was performed in accordance with the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines with IEC _____. Swiss albino mice (18 - 25 g) and wistar albino rats (150 - 200 g) of either sex were procured from animal house. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ºC ± 2C). The animals were given a standard laboratory diet and water ad libitum. Food was withdrawn 12 h before and during the experimental h.

Evaluation of Analgesic Activity Using Pain Models:

Thermal Stimulus:Tail flick method: [14] analgesic activitieswere assessed by tail-flick response method using analgesiometer (Lab tech 198). Swiss albino mice (18-25gm) were placed on the analgesiometer with tail freely protruding out of the holder. Observations were taken by placing the tip of the tail (last 1-2 cm) on the radiant heat source that is heated nicrome wire with platform. The strength of the current passing through the naked nicrome wire was kept constant at 3 amps.

A sharp withdrawal of the tail from the heat source is called as "tail-flick response" and was taken as the endpoint of the experiment. The mice tried to pull the tail away and rotate the head. The time from placing the tail of the mice on the radiant heat source to sharp withdrawal of the tail was recorded as "reaction time" (in sec). Weighed Swiss albino mice were screened for sensitivity test by placing the tip of the tail on the radiant heat source. Any animal that failed to withdraw its tail within 10 sec were rejected from the study. There after mice were randomly divided into 8 groups with 6 animals in each group. In control group distilled water was given by oral route while in standard group aspirin (from USV pharmaceuticals) 75 mg/kg per oral by suspending it with 2% gum acacia was given. Other six groups were served as test groups where CSEEMF and CSEEWF (diluted with distilled water) was given at the dose of 50, 100, 200 mg/kg.

During experiment each animal was tested four times keeping the in between gap of 5 min between the two responses. The mean of all four readings was labeled as "reaction time" (sec) of that animal at given point of time. Again mean of reaction time was calculated to obtain mean reaction time (sec) of that particular group for given point of time. Tail-flick test was performed prior to drug administration and at the end of 30, 60, 90 and 120 min after drug administration.

Percentage maximum Analgesia =

$$\frac{\text{Mean Reaction time (treatment group)} - \text{Mean reaction (pretreatment group)}}{\text{Cut off time} - \text{Mean reaction time (pretreatment group)}} \times 100$$

Mechanical Stimulus:Heffner's Tail Clip Method: [15] Swiss albino mice weighting between 18-25 gm were used in tail clip method. An artery clip was applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to this noxious stimulus by biting the clip. The time from onsets of stimulation to response (Biting of clip) was measured by stopwatch referred as "reaction time."

A sensitivity test was carried out, and animals that did not attempt to dislodge the clip within 10 seconds were discarded. There after mice were randomly divided into 5 groups with 6 animals in each group. In control group Normal saline was given by intra- peritoneal route while in standard group Pentazocine (from USV

pharmaceuticals) 5mg/kg (subcutaneously) was given. Other three groups were served as test groups where CSEEMF (diluted with Normal saline) was given at the dose of 50, 100, 200 mg/kg (intra- peritoneally).

During experiment each animal was tested four times keeping the in between gap of 5 min between the two responses. The mean of all four readings was labeled as "reaction time" of that animal at given point of time. Again mean of 'reaction time' was calculated to obtain "mean reaction time" of that particular group for given point of time. Tailclip test was performed prior to drug administration and at the end of 30, 60, 90 and 120 min after drug administration

$$\text{Percent reaction time} = \frac{C - T}{C} \times 100$$

Where, C - Mean Reaction time of control group T - Mean Reaction time of test group

Chemical Stimulus:Acetic Acid Induced Writhing Test:[16, 17] Antinociceptive activity of opioid agonist, opioid partial agonist, non-steroidal anti-inflammatory drugs (NSAIDs) can be determined by the writhing test 18 . Wistar albino rats weighing between 150 gm to 200 gm of either sex were divided into five groups with six animals in each group. All the animals were fasted overnight with free access to water. Acetic acid 0.2 ml of 0.6% v/v (0.6 ml acetic acid was diluted with 100 ml distilled water) was administered intraperitoneally after 30 min of drug administration in each animal.

In control group normal saline was given intraperitoneally. In standard group aspirin (from USV pharmaceuticals) was given at the dose of 150 mg/kg per oral by suspending it with 2% gum acacia. Other six groups were served as test groups where CSEEMF and CSEEWF (diluted with distilled water) was given at the dose of 50, 100, 200 mg/kg intraperitoneally. Rats were kept separately into the glass chamber and the number of writhes over a period of 10 minutes was recorded taken as the end point of the experiment. Writhe is indicated for scoring purpose as animal reacts with characteristic stretching behaviour i.e. a series of contractions occur that travel along the abdominal wall sometimes accompanied by the turning movements of the body and extension of hind limb.

The results were tabulated and percentage inhibition of writhes was calculated by the following formula:

Percentage inhibition =

$$\frac{\text{Number of writhes in control group} - \text{Number of writhes in test group}}{\text{Number of writhes in control group}} \times 100$$

Formalin Test: [19] The formalin test in mice is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The formalin test was performed in swiss albino mice weighting between 18-25gm. Before administration of formalin mice were randomly divided into 8 groups with 6 mice in each. Half h before testing, mice was pretreated with drugs. In control group normal saline was given intraperitoneally. Standard group received pentazocine at the dose of 5mg/kg subcutaneously. Other five groups served as test groups where CSEEMF and CSEEWF (diluted with Normal saline) was given at the dose of 50, 100, 200mg/kg intraperitoneally. Mice were individually placed in transparent observation chambers for adaptation. In the dorsum of right hind paw of the animal, 0.05 ml of 10% formalin (10ml formaldehyde was diluted with 100 ml distilled water to make 10% formalin) was injected subcutaneously with a micro syringe of 26 gauge.

Each animal was kept in the separate cage for observation of paw licking which was started after injection of formalin and lasts for next five minutes (0 to 5) and it is said to be as early phase. Late phase was started from 10 min after formalin injection lasting from 10 to 40 min after the injection of formalin. Mice show behaviour like licking, flitching lifting and biting of paw. Time spends by animal in licking (sec) in both early and late phase was considered as end point of this experiment. The mean licking time (sec) was calculated in control, Pentazocine and CSEEMF (50, 100, 200 mg/kg) groups. The time which was spent in licking was recorded.

Percent inhibition = C-T/C x100, where

C represents the biting/paw licking response time in (seconds) in the control group (Normal saline)

T depicts the biting/paw licking response time (seconds) in the Pentazocine (5mg/kg) s.c or CSEEMF (50 or 100 or 200mg/kg) treated group.

Statistical Analysis:

The data was expressed as the mean ± SD. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett's test. The probability of $p<0.05$, $p<0.01$and $p<0.001$ or less was considered statistically significant. For the statistical analysis IBM (SPSS version 20) was used.

III. RESULTS

Tail flick method

Table 5.3 illustrates the maximum analgesic effect of aspirin and CSEEMF at different doses. The peak analgesic effect (maximum analgesia) of CSEEMF was observed at the doses of 100 and 200mg/kg following administration

followed by gradual decrease throughout the observation period. At the peak of activity (30 min), CSEEMF showed maximum analgesia of 56.67% and 48.49% at 400mg/kg, 200mg/kg doses respectively. CSEEMF (50mg/kg) also revealed mild significant analgesic effect throughout the observation period. (Table 1a and Table 1b)

Table 1a: Mean reaction time in tail flick method in Swiss albino mice

Group/Treatment	Pre Treatment	Mean reaction Time (Seconds)			
		30 min	60 min	90 min	120 min
Control (10 ml/ kg)	5.15±0.11	5.38±0.13	5.31±0.17	5.32±0.15	5.28±0.12
Aspirin (75 mg/kg)	4.60±0.34	8.40±0.48***	8.12±0.16***	7.78±1.43***	8.144±0.05***
CSEE(100 ml/ kg)	4.77±0.20	5.93±0.38*	5.61±0.37*	5.40±0.33*	5.494±0.24*
CSEE(200 ml/ kg)	4.66±0.29	7.73±0.28***	7.51±0.29***	7.15±0.33***	7.296±0.14***
CSEE(400 ml/ kg)	4.55±0.26	8.20±0.22***	7.93±0.36***	7.80±0.33***	7.884±0.03***
CSWE(100 ml/ kg)	4.93±0.26	5.36±0.40	5.51±0.38	5.21±0.29	5.45±0.29
CSWF(200 ml/ kg)	5.21±0.28	6.37±1.07	6.23±0.86	6.22±0.54	6.56±0.83***
CSWF(400 ml/ kg)	4.93±0.65	6.70±1.34*	6.88±1.15*	6.75±0.58*	6.66±0.42***

Values are expressed as mean±SD (n=6) by ANOVA followed by Dunnett's test by comparing the other groups with control group, taking Control = Control Group and $p>0.05$ not significant, * $p<0.05$, ***$p<0.001$

Table 1b: Percentage maximum analgesia in tail flick method in Swiss albino mice

Group/Treatment	Maximum percentage analgesia (%)			
	30 min	60 min	90 min	120 min
Control (10 ml/ kg)	4.932	2.72	2.91	2.22
Aspirin (75 mg/kg)	59.46	55.08	49.76	55.32
CSEE(100 ml/ kg)	18.64	13.50	10.12	11.41
CSEE(200 ml/ kg)	48.49	45.02	39.33	41.39
CSEE(400 ml/ kg)	56.67	52.48	50.46	51.55
CSWE(100 ml/ kg)	6.9	9.4	8.4	4.46
CSWF(200 ml/ kg)	20.06	17.64	23.35	17.47
CSWF(400 ml/ kg)	29.20	32.17	28.54	30.03

Tail clip method

The results for the effects of different agents by using tail clip method in mice were presented in [Table 5.5]. Analgesic activity in CSEEMF & CSEEWF (100,200 mg/kg) groups were observed at 30,60, 90 and 120 minutes interval, and it was found to be statistically significant as compared to control group ($p<0.05$, and $p<0.001$) at 60 and 90 minutes post- administration.. The maximum mean reaction time was 7.481±0.005 sec seen with 100mg/kg of extract at 90 mins post administration within the test groups. The peak of mean reaction time was observed at 90 minutes interval followed by gradual decrease at 120 minutes.

Table 5.6 shows the percentage reaction time of Pentazocine and CSEEMF at different doses. The analgesic effect of CSEEMF was observed at the doses of 100 and 200 mg/kg following administration followed by gradual decrease throughout the observation period. At the peak of activity (90 min), CSEEMF showed maximum percentage reaction time of 93.06 % and 93% at 200mg/kg, 100mg/kg doses respectively. CSEEMF at 50mg/kg also revealed significant analgesic effect throughout the observation period as compared to control. (Table 2a and Table 2b)

Table 2a: Mean reaction time in tail clip method in Swiss albino mice

Group/Treatment	Pre Treatment	Mean reaction Time (Seconds)			
		30 min	60 min	90 min	120 min
Control (10 ml/ kg)	3.90± 0.30	3.78±0.41	3.95±0.05	3.96±0.31	4.41±0.47
Pentazocine 5mg/kg S.C	3.85±0.128	7.25±0.31***	7.06±0.83***	7.52±0.03***	6.58±0.33***
CSEE(100 ml/ kg)	4.33±0.084	6.91±0.58***	6.56±0.24***	6.647±0.04***	5.91±0.39***
CSEE(200 ml/ kg)	3.86±0.09	7.41±0.56***	7.46±0.30***	7.48±0.30***	7.21±0.14***
CSEE(400 ml/ kg)	3.87±0.19	7.35±0.62***	7.16±0.54**	7.47±0.072***	6.75±0.35***
CSWE(100 ml/ kg)	4.33±0.410	4.46±0.87	5.06±1.11*	5.3±0.84***	5.03±0.92
CSWF(200 ml/ kg)	3.85±0.10	4.71±0.85	5.11±0.29*	5.48±0.27***	5.34±0.15

CSWF(400 ml/ kg)	4.05±0.14	5.7±0.73***	6.16±0.54***	6.16±0.23***	6.13±0.36

One Way ANOVA followed by Dunnett's test While Control = Control Group Values are expressed as mean±SD (n=6) AS p>0.05 not significant ***p<0.001

Table 2b: Percentage reaction time in tail clip method in Swiss albino mice

Group/Treatment	Maximum percentage analgesia (%)			
	30 min	**60 min**	**90 min**	**120 min**
Control (10 ml/ kg)				
Pentazocine 5mg/kg S.C	88.31	83.36	95	71
CSEE(100 ml/ kg)	59.59	51.52	53	36.52
CSEE(200 ml/ kg)	92	93.27	93	86
CSEE(400 ml/ kg)	89.77	85.05	93.06	74.28
CSWE(100 ml/ kg)	3.00	16.85	16.16	22.40
CSWF(200 ml/ kg)	22.33	32.72	38.70	42.33
CSWF(400 ml/ kg)	40.74	52.09	51.35	52.09

Acetic acid-induced writhing reflex in rats

The results of the effects of different agents by acetic acid-induced writhing reflex in rats are presented in [Table 3]. The results of the visceral nociception induced by injection of acetic acid-induced writhing model revealed that all the three test doses and standard drug aspirin 150mg/kg showed statistically significant results with reduced number of abdominal constriction and stretching of hind limb when compared to control group ($p \leq 0.001$)[Table 5]. The percentage of inhibition of writhing was maximum at dose of 200mg/kg (74.97%) followed by dose of 100mg/kg (72.42%) and 50mg /kg in dose dependent manner when compared with control group.

Table 3: Effect of acetic acid induced writhing in Wistar albino rats

Groups	Number of writhes	Percentage Inhibition (%)
Control Normal saline 10ml/kg	25.89±1.09	---
Standard (Aspirin) 150mg/kg	5.74±0.88***	77.80
CSEE(100 ml/ kg)	14.81±1.19***	42.79
CSEE(200 ml/ kg)	7.138±0.96***	72.42
CSEE(400 ml/ kg)	6.480±0.53***	74.97
CSWE(100 ml/ kg)	20±1.67***	22.75
CSWF(200 ml/ kg)	19.66±2.06***	24.06
CSWF(400 ml/ kg)	12.16±2.22***	53.03

Values are expressed as mean±SD (n=6) by One Way ANOVA followed by Dunnett's test for comparing other groups with the control group. Significances ***p<0.001

Formalin-induced nociception model

The anti-nociceptive effect of CSEEMF using formalin-induced nociception model is presented in Table 4.The injection of formalin into the dorsal surface of a hind paw produced an adequate behavioral response with licking of the paw. Two distinct phases of high paw licking activity were observed i.e. early phase (lasting for the first 5 min after injection of formalin) where responses were observed at 2-3 minutes and late phase started from 10 minutes of formalin injection lasting from 20 to 40 min after the injection of formalin). All the doses of CSEEMF (50,100,200mg/kg) showed obvious dose-dependent and highly statistically significant ($p < 0.001$) antinociceptive activities both during the early and late phase as compared to control group. However Pentazocine (5mg/kg) group showed highest percentage inhibition of paw licking in both early and late phases as compared to test groups. Different doses of CSEEMF revealed dose dependent reduction in paw licking time during both early and late phases.

Table 4: The effect of different agents in formalin induced paw licking model in Swiss albino mice

Group /Treatment	Early Phase (0-5 min)		late Phase (10-40 min)	
	Licking time in (Sec)	Percentage inhibition (%)	Licking time in (Sec)	Percentage inhibition (%)
Control (10 ml/ kg)	58.33±4.96	0	77.16±3.92	0
Pentazocine 5mg/kg S.C	13.33±2.58***	78.57	17.66±3.63***	77.10
CSEE(100 ml/ kg)	34.33±2.58***	41.14	34±2.36***	55.93
CSEE(200 ml/ kg)	23.83±2.31***	59.14	25.83±2.48***	66.52
CSEE(400 ml/ kg)	17±2.60***	70.85	21.66±2.16***	71.92
CSWE(100 ml/ kg)	47±2.378***	19.42	54±7.89***	30.01

CSWF(200 ml/ kg)	42.5±6.28***	27.13	31.66±9.52***	58.96
CSWF(400 ml/ kg)	33.33±7.17***	42.85	22±5.01***	71.48

One Way ANOVA followed by Dennett's test for both phases. Significant differences between control and drug treated groups are expressed as mean±SD (n=6) ***p<0.001

DISCUSSION

Tail Flick Method

The thermal model of the tail flick tests is considered to be a spinal reflex, which involves higher neural structures identifying mainly central. The μ receptor has generally been regarded as the receptor type associated with pain relief and has been shown to be potent in regulating thermal pain.

No analgesic effects mediated by the μ receptors include respiratory depression Inhibition of intestinal motility and most importantly for therapeutic considerations its induction of physical dependence. Activation of μ_2 opioid subtype leads to spinal analgesia and commonly through constipation adverse effect.

In the present study the Crocus sativus ethanolic extract methanol and water fractions increased reaction latency to thermal pain induced by the tail flick method in mice, which is a specific central antinociceptive test. Inhibition of histamine or kinin pathway may reduce pain. Analgesic effect of CSEEMF (200 and 400 mg/kg) and CSEEMF (200 mg/kg) was observed at 30,60, 90 and 120 minutes interval, when it was found to be statistically significant as compared to control group ($p<0.05$ and $p< 0.001$). Antinoceceptive effect of CSEEMF (100 and 200 mg/kg) observed at 30, 60, 90 minutes interval, when it was found to be statistically non significant as compared to control group.

The Ethanolic extract of *Crocus sativus* produced dose-dependent increase in mean reaction time in CSEEMF as well as CSEEWF group in Swiss albino mice at 100 and 200 mg/kg doses. The antinociceptive activity observed with CSEEMF (200mg/kg) was maximum at 30 minutes and it was statistically significant when compared to control group ($p<0.05$, $p<0.001$)

The mean reaction time for the CSEEMF at 50mg/kg also exhibited mild significant analgesic effect throughout the observation period as compared to control. Whereas 50mg/kg dose in CSEEWF does not represent significant effect.

The Maximum analgesic effect of CSEEMF (200mg/kg) was observed after 30 minutes of intra-peritoneal injection and gradually decreased towards 60, 90 and 120 minutes. This mean reaction time exhibited similar trends for the dose of 100mg /kg of CSEEMF producing Maximum possible effect at the same time point.

Table 1 illustrates the maximum analgesic effect of aspirin and CSEEMF at different doses. The peak analgesic effect (maximum analgesia) of CSEEMF and CSEEWF was observed at the doses of 100 and 200mg/kg followed by gradual decrease throughout the observation period. At the time interval of (30 min) .However CSEEWF 200mg/kg dose revealed the peak analgesic activity 32.17% at 60(min) followed by gradual decrease. Whereas CSEEWF 100mg/kg showed peak effect at (30) min. CSEEMF showed maximum analgesia of 56.67% and 48.49% at 200mg/kg and 100mg/kg doses respectively. CSEEMF (50mg/kg) also revealed mild significant analgesic effect throughout the observation period. Whereas CSEEWF (100mg/kg) and CSEEWF (100mg/kg) does not reveal significant results when compared with control.

The tail flick method of analgesia is very effective in estimating the efficacy and potency of centrally acting analgesic drugs. In this study, CSEEMF at higher doses (100 and 200mg/kg) increased the pain threshold in highly significant manner which was comparable to aspirin. The effectiveness of analgesic agents in the tail flick pain model is highly correlated with relief of human pain The μ receptor stimulation is generally associated with pain relief and has been shown to be potent in regulating thermal pain[20].

Tail clip method

Analgesic activity in CSEEMF (100,200 mg/kg) groups were observed at 30,60, 90 and 120 minutes interval, found to be statistically significant as compared to control group ($p<0.05$ and $p<0.001$) at 60 and 90 minutes post-administration.

The mean reaction time was increased in the test groups treated with 100, 200 mg/kg of extract and highly statistically significant at 120 minutes post administration are comparable with Pentazocine group. The maximum mean reaction time was 7.48±0.074 sec seen with 100mg/kg of extract at 90 mins post administration within the test groups. However the maximum mean reaction time was 6.16±0.54 sec seen with 200mg/kg of extract at 60 mins post administration within the CSEEWF groups. The peak of mean reaction time was observed at 90 minutes interval followed by gradual decrease at 120 minutes. however for CSEEWF the peak of mean reaction time was observed at 60 min which was 52.09 followed by decrease 51.35. Table 4 shows the percentage reaction time of Pentazocine and CSEEMF at different doses. The analgesic effect of CSEEMF was observed at the doses of 100

and 200 mg/kg following administration followed by gradual decrease throughout the observation period. At the peak of activity (90 min), CSEEMF showed maximum percentage reaction time of 93.06 % and 93% at 200mg/kg, 100mg/kg doses respectively. CSEEMF at 50mg/kg also revealed significant analgesic effect throughout the observation period as compared to control. Whereas CSEEMF also showed maximum percentage reaction time of 52.09% and 42.33%

In tail clip method, the maximum analgesic effect was observed at all the doses of CSEEMF following administration followed by gradual decrease at 120 minutes observation period. It was highly significant ($p<0.001$) with all the dose of CSEEMF weight when compared with control group indicating anti-nociceptive activity of CSEEMF.

Similarly in case of CSEEWF same pattern of analgesic activity was observed with maximum effect observed at 90 min duration by gradual decrease in the response with all the three doses. The peak of activity of CSEEMF was noted 90 minutes at 100 and 200 mg/kg.

In order to reveal centrally mediated antinociceptive responses Tail-clip model is useful technique, which targets mainly on modifications above the spinal cord level. flicking of the tail by a brief vigorous movement is observed when thermal radiation is applied to the tail of an animal . The considerable increase in pain threshold created by CSEEMF in these models Reveal the involvement of central pain pathways. Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems. It is assumed that these extract fractions might be showing their effect by acting on central mechanisms which involves these receptor systems or may be by peripheral mechanisms which is associated by the inhibition of prostaglandins, leucotrienes, and other endogenous substances that are key stimulants in inflammation and pain[21].

Writhing test

The peripheral analgesic activity may be mediated through inhibition of cyclooxygenases and/or lipoxygenase, while central analgesic action can be propagated through inhibition of central pain receptors[22].Therefore peripheral (acetic acid induced writhing) was selected to observe the analgesic effect of CSEEMF and CSEEWF.The abdominal constriction response produced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics[23] generally, acetic acid results into pain by releasing endogenous substances like serotonin, histamine, cytokines, prostaglandins (PGs), bradykinins along with Nitric oxide and substance P, which excites nerve endings.

Peritoneal receptors are said to be occupied in the abdominal constrictions response. The method is concerned with prostanoids in general, that is bigger levels of PGE2 and PGF2α in peritoneal fluids have taken place[24,25]. The abdominal constrictions response induced by acetic acid in rats' results from an acute inflammatory reaction with production of prostaglandins E2 and F2 in the peritoneal fluid. For pain process prostaglandin along with bradykinins were suggested to take part in an important role. NSAID like aspirin leads to a relief from pain by suppressing the formation of pain inducing substances in the peripheral tissues[26]. In this study, the significant decrease in the scoring of writhes by CSEEMF and CSEEWF (100, 200, 400mg/kg) suggested that the antinociceptive effect of the extract fractions may be peripherally mediated via the inhibition of synthesis and release of PGs and other endogenous substances. The maximum inhibition of writhing by CSEEMF was observed at 200mg/kg which was 74.97% followed by 100mg/kg which was 72.42% with $p< 0.001$ as compared to control group where as with CSEEMF percentage inhibition observed was 53.03% by 200mg/kg dose followed by 24.06% and 22.75% by 100mg/kg and 200mg/kg dose of water fraction and it was comparable with control group. However the maximum percentage inhibition was observed by aspirin group followed by CSEEMF 100mg/kg and CSEEWF 200mg/kg $P< 0.001$ when compared with control group. It can be thus inferred that the CSEEMF and CSEEWF has peripheral mechanism of anti-nociceptive activity analogous to aspirin by inhibition of prostaglandin synthesis or action of prostaglandin.

The antinociceptive effects of DHC may result from an inhibition of the production of inflammatory mediators (e.g., TNF-α, IL-1β) released into the peritoneal cavity. Several categories of drugs, including muscle relaxants and adrenergic receptor agonists, can also inhibit writhing. Due to the lack of specificity of this test, positive results in the writhing test require confirmation in other models.

Formaline Test

The effect of CSEEMF as anti-nociceptive by means of formalin triggered nociception model is represented in Table 22/graph- (9A, B). All the doses of CSEEMF (100,200,400mg/kg) and CSEEWF (100,200,400mg/kg) represented obvious dose-dependent and highly statistically significant ($p < 0.001$) antinociceptive activities both during the early and late phase as compared to control group. However Pentazocine (5mg/kg) group showed highest percentage inhibition of paw licking in both early and late phases as compared to test groups. Different doses of CSEEMF revealed dose dependent reduction in paw licking time during both early and late phases.

Among all the test doses CSEEMF at the dose of 200mg/kg represented maximum percentage inhibition followed by CSEEWF 71.48% at the dose of 200 mg/kg. The CSEEMF markedly reduced the formalin induced paw licking response in both early and late phase. A Formalin test which is divided into two phases measures pain of both neurogenic (early phase) and of inflammatory origin (late phase). The first phase (0 – 5min).This occurs due to chemical stimulation of nociceptor causing c fiber activation .Late phase which starts after 10 to 15 minutes of formalin administration and lasts for 20 to 40 minutes .This phase appears due to combination of an inflammatory reaction in the peripheral tissues and functional changes during the early phase. Non steroidal antiinflimmatory drugs are effective in second phase. Whereas first phase remains unaffected.[27-29].

Hence from the above models, it was revealed that CSEEMF showed both central as well as peripheral analgesic effect. Formalin test is sensitive to opioid agents, Non-steroidal anti-inflammatory drugs and rest other mild analgesics. This test shows two separate phases, possibly revealing different types of pain. The earlier phase reflects a direct effect of Our results showed that Ethanolic extract of Crocus sativus fractions have anti-nociceptive activity in chemical induced methods like (formalin and acetic acid tests) that may be mediaated more peripherally[30].Based on these results it can be summarized that this method enable one to distinguish the site of action of analgesics whether it is central, peripheral or both central and peripheral.

IV. CONCLUSION

The present study supported the use of CSEEMF and CSEEWF in painful conditions which are mediated via peripheral and central inhibitory mechanisms. It is quite apparent that the CSEE MF possesses significant analgesic effect against different stimuli. This was evidenced by a significant increase in the reaction time by the stimuli in different experimental models and by the significant analgesic activities. Moreover the CSEEWF has revealed the presence of Crocin like functional group from spectroscopy. CSEEMF analysis revealed the presence of Picrocin Like structure from spectroscopy.

The Anti-nociceptive effect of CSEEMF are more when compared with CSEEWF However, pharmacodynamic studies are needed to understand the mode of action. Further studies needs to be done in different species of animals and human beings to confirm the anti – nociceptive effect of CSEEMF which may result in the development of potent analgesic agents.

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