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Analgesic Effect of *Caryophyllus aromaticus* by Formalin Test in Albino Rats

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Abstract: Objective: To study the analgesic effect of *Caryophyllus aromaticus* (flower buds) by formalin test in albino rats. Albino rats of either sex were taken and divided into 7 groups with 6 animals in each for central and peripheral analgesic activity by formalin test. 5% formalin is injected into the right dorsal surface of a hind paw of rat after half an hour of drug administration and the time the animal spent licking the paw was recorded as early phase (0-5 min after injection) and late phase (15-30 min after injection). *Caryophyllus aromaticus* was used in a dose of 150,300 and 600mg/kg i.p. The drug used for central analgesic activity was Pethidine (5mg/kg, i.p) and Naloxone (1mg/kg) with 300mg/kg of *Caryophyllus aromaticus* i.p to study the central analgesic activity. The drug used for peripheral analgesic activity was Ketorolac (10mg/kg, i.p). A control group was maintained in all models. Results revealed that in *Caryophyllus aromaticus*300mg/kg significantly decreased the reaction time of early phase in formalin test similar to Pethidine whereas the combination of Naloxone and *Caryophyllus aromaticus* 300mg/kg increased the reaction time of early phase, indicating that Naloxone inhibits the analgesic effect of *Caryophyllus aromaticus*. *Caryophyllus aromaticus* 300mg/kg significantly decreased the reaction time of late phase also. In Conclusion: *Caryophyllus aromaticus* (flower buds) possesses significant central and peripheral analgesic activity and acts through opioid receptors.

Key words: Caryophyllus aromaticus · Analgesia · Naloxone · Pethidine · Ketorolac

INTRODUCTION

Pain is an unpleasant sensation and a very common phenomenon. There is no doubt that pain acts as a warning signal against disturbances either in the body or in the external environment of an individual. The principal objective of the treatment of pain is to remove or abolish the cause of pain. But it is not always possible to do so; hence, analgesics are used for the symptomatic treatment of pain. Opioids are the most potent and commonly used group of analgesic drugs e.g. Morphine and Pethidine. But their analgesic action is associated with a greater degree of adverse drug reactions, most of which are dose dependent.

Increased pain in response to noxious stimulation following peripheral tissue injury depends on an increase in the sensitivity of primary afferent nociceptors at the site of injury (peripheral sensitization) [1, 2] and on an increase in the excitability of neurons in the CNS (central sensitization) [3, 4]. Central sensitization is triggered by inputs from nociceptive afferents and is associated with a reduced threshold of dorsal horn neurons to noxious stimulation [5-7], an expansion of the receptive fields of dorsal horn neurons [8, 9] a summation of slow postsynaptic potentials resulting in a cumulative depolarization and a prolonged after discharge or "windup" of dorsal horn neurons [10] and an increased excitability of the flexion reflex in response to peripheral stimulation [3, 11].

The use of plant products is increasing in many segments of the population. According to an estimate, 80% of the world's population relies upon plants for their medication. Most of the synthetic drugs used at present for analgesic and anti-nociceptive effect have many side and toxic effects. Plants still represent a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs [12]. Many medicines of plant origin with analgesic and anti-nociceptive activity had been used since long time without any adverse effect. North East India is considered as one of the "hotspots" for biodiversity in India, since out of the 1500 species of medicinal plants available in

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India, almost 350 species belong to Assam and many of these traditionally used plants have not yet been studied scientifically which can be developed as a potential drug after scientific validation.

One of the spices, widely used as flavoring agent is *Caryophyllus aromaticus* (flower buds) commonly known as *clove bud*. Apart from its use as spice, it is shown to possess a wide range of pharmacological effects such as antimicrobial, antipyretic, antioxidant, antithrombotic, anaesthetic and anticancerous activities [12-16]. Further, it is widely believed that *clove* is a potent analgesic agent in Chinese and herbalist medicine. The present study was undertaken to study on analgesic effect of *Caryophyllus aromaticus* (flower buds) by formalin test in albino rats.

MATERIALS AND METHODS

Experimental Animals: Adult male or female (non pregnant) albino rats of Wistar strain weighing 200 g were used for the study. Animals were purchased from King Institute of Preventive Medicine, Guindy and Chennai and maintained in the Central Animal House, Sree Balaji Medical College and Hospital, Chennai, India. The animals were individually housed under controlled temperature and hygienic conditions in polypropylene cages under 12-hour light and dark cycles. They all receive a standard pellet diet and water. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Preparation of Extract: Clove(flower buds) was purchased from the local market at Sri Lanka. The (flower buds) was dried and finely powdered in a mechanical mixer. The dried powder (2 kg) is soaked in ethanol (50%) and kept aside for 7 days. After 7 days, the solvent from the total extract is distilled off and the concentrate is evaporated on a water bath to a syrup consistency and then evaporated to dryness (150mg). This extract will be administered intraperitoneally to rats. All the other chemicals like formalin, Pethidine, Ketorolac and Naloxone were of analytical grade and were procured from local commercial companies.

Selection of Doses: For the assessment of analgesic activity, three dose levels were chosen in a such a way that, middle dose(1/10 LD_{50})… is one-tenth of the maximum dose during acute toxicity study and a low dose, which is 50% of the one-tenth dose and the high dose, which is twice that of the one-tenth dose (150mg/kg, 300mg/kg, 600mg/kg) [17].

Screening Method for Analgesic Activity

Formalin Test: Thirty min after intra peritoneal administration of drugs (Normal saline or *Caryophyllus aromaticus* or Ketorolac or Pethidine); the right hind paw is injected with 0.05 ml of 5% formalin in saline subcutaneously using a 27 gauge ½-inch needle. The animal is placed in a transparent polypropylene cage next to a mirror so that the animal could be observed from all angles. The animals are continuously observed from the time of formalin injection to 50 min. The behavior of the animals is recorded by stop watch manually and entering data into a computer program that tabulated the time animal spent licking and biting the injected foot. The time spent licking and biting is monitored continuously and recorded as seconds per 5 minutes from minute 1 through minute 50 [18-22].

It is consistently been found that two distinct phases of licking and biting occur phase 1 a short but immediate response lasting the first 5 min after the hind paw is injected; phase 2 a prolonged response starting at approximately minute 15 and ending at about minute 30. Between phases 1 and 2, there is an intermittent period from minute 6 to minute 10 where little nociceptive behavior is observed. It is theorized that the two different phases represent two qualitatively distinct types of pain. Phase 1 is a direct stimulation of the nerve by the formalin and phase 2 is an inflammatory reaction-induced pain.

Study Design:

Number of groups: 7
Number of animals in each group: 6
Total number of animals: 42

Part 1: Finding out the Effective Dose among Three Doses of *Caryophyllus aromaticus* with Control Group: All medicinal agents used in this experiment, including saline, will be administered by intraperitoneal injection(ip). The control group, consisting of six animals (N= 6), is given 0.5 ml of 50% ethanol. The experimental group of *Caryophyllus aromaticus* is divided into three subgroups: 150,300 and 600 mg/kg. Each subgroup of *Caryophyllus aromaticus* consisted of six animals (N = 6).

Part 2: Comparison of Effective Dose of *Caryophyllus aromaticus* with Pethidine, Ketorolac, Naloxone and Control Group: All medicinal agents used in this

experiment, including saline, will be administered by intraperitoneal (ip) injection. The control group, consisting of six animals (N = 6), is given 0.5 ml of 50% ethanol. The experimental group of *Caryophyllus aromaticus*, Pethidine, Ketorolac and *Caryophyllus aromaticus*+ Naloxone consisted of six animals (N = 6).

Part 1: Finding out the Effective Dose among Three Doses of *Caryophyllus Aromaticus* with Control Group:
Groups:
Group A: Control (50% ethanol, 0.5ml, i.p)

Group B: *Caryophyllus aromaticus* (flower buds) (150mg/kg)

Group C: *Caryophyllus aromaticus* (flower buds) (300mg/kg)

Group D: *Caryophyllus aromaticus* (flower buds) (600mg/kg)

In all these groups the drugs will be given intraperitoneally 30 mins before the administration of formalin and the time spent by the rats to lick the paw from 0-5mins and 15-30mins after administration of formalin will be recorded.

Part 2: Comparison of Effective Dose of *Caryophyllus Aromaticus* with Pethidine, Ketorolac, Naloxone and Control Group:
Groups:

Group A: Control (50% ethanol, 0.5ml, i.p)

Group 1 (B/C/D): *Caryophyllus aromaticus*(flower buds) (effective dose, i.p)

Group 2: Pethidine (5 mg/kg, i.p)

Group 3: Ketorolac (10 mg/kg, i.p)

Group 4: *Caryophyllusaromaticus* (effective dose, i.p) + Naloxone (1mg/kg, i.p)

In all these groups the drugs will be given intraperitoneally 30 mins before the administration of formalin and the time spent by the rats to lick the paw from 0-5mins and 15-30mins after administration of formalin will be recorded.

RESULTS

Analgesic Effect: This study was carried out with an attempt to evaluate the analgesic activity of *Caryophyllus aromaticus*in comparison to standard drug, Pethidine, Ketorolac and Naloxone. Results obtained from the study were summarized below.

The values of Formalin test were expressed as animal spent licking the paw in seconds. Results of estimations have been reported as mean±SEM and significance of six animals in each group. Graphical representation of mean ± SEM of animal spent licking the paw in seconds is shown in figures 1,2 for various groups.

For determining significance of intergroup difference each parameters were analyzed separately and one way analysis of variance (ANOVA) was carried out. Descriptive statistics was used to find out the mean of each group and Post hoc multiple comparison (LSD) was done to find out the significance and difference between the groups. The "p" value less than 0.05 were considered to be significant.

Part 1: Finding out the Effective Dose among Three Doses of *Caryophyllus Aromaticus* with Control Group:
Analyzing Figure 1, We Got the Results as Follows: Comparing the mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus*150mg/kg, 300mg/kg and 600mg/kg with the control group, it showed a significance of $p<0.000$ to the control group with mean difference of 41.33sec, 50.66sec and 51.83sec respectively in phase 1 and 82.83sec, 106.83sec and 105.50 sec respectively in phase 2.

Among the mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus*150mg/kg, 300mg/kg and 600mg/kg. *Caryophyllus aromaticus* 300mg/kg and 600mg/kg showed a significance of $p<0.05$ to *Caryophyllus aromaticus* 150mg/kg with mean difference of 9.33 sec and 10.50sec respectively for Phase 1 and $p<0.05$ with mean difference of 24.00sec and 22.66sec for Phase 2.

The mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus* 300mg/kg and 600mg/kg showed statistical not significant value of $p= 0.787$ with mean difference of 1.16sec in Phase 1 and $p= 0.901$ with mean difference of 1.33sec in Phase 2.

For all these four group the one way ANOVA value F and significance for Phase 1 and Phase 2 were 65.67, 45.05 and $p<0.000$, $p<0.000$ respectively with degree of freedom 3.

Part 2: Comparison of Effective Dose of *Caryophyllus Aromaticus* with Pethidine, Ketorolac, Naloxone and Control Group:

Analyzing Figure 2, We Got the Results as Follows: Comparing the mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus* 300 mg/kg, Pethidine 5mg/kg, *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg with the control group, it showed a significance of p<0.000 for *Caryophyllus aromaticus* 300 mg/kg and Pethidine 5mg/kg to the control group in both the phases and with mean difference of 50.66sec and 55.33sec respectively in phase 1 and 106.83sec and 109.16sec respectively in phase 2.

*Caryophyllus aromaticus*300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg group was not significant when compared with control group in phase 1 with P value of 0.066 and 0.091 respectively and with mean difference of 7.83sec and 7.13sec respectively but these groups where significant when compared with control group in phase 2 with p<0.000 and mean difference of 105.16 sec and 72.33sec respectively.

Among the mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus* 300mg/kg, Pethidine 5mg/kg, *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg. *Caryophyllus aromaticus*300mg/kg showed a significance of p<0.000 to *Caryophyllus aromaticus*300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg with mean difference of 42.83sec and 43.50sec respectively for Phase 1 but Ketorolac 10mg/kg only was significant with P<0.05 to *Caryophyllus aromaticus* 300mg/kg in phase 2 with mean difference of 34.50sec. *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and Pethidine 5mg/kg was not significant with p value 0.872 and 0.821 respectively to *Caryophyllus aromaticus* 300mg/kg with mean difference of 1.66sec and 2.33sec respectively for Phase 2. Pethidine 5mg/kg was not significant with p value 0.263 to *Caryophyllus aromaticus* 300mg/kg with mean difference of 4.66sec for phase 1.

The mean seconds animal spent licking the paw in both the phases of Pethidine 5mg/kg showed a significant value of p<0.000 to *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg with mean difference of 47.50sec and 48.16sec respectively in Phase 1 but Ketorolac 10mg/kg only was significant with P<0.05 to Pethidine 5mg/kg group in phase 2 with mean difference of 36.83sec. *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg was not significant with p value of 0.699 to Pethidine 5mg/kg with mean difference of 4.00sec in phase 2.

Comparing the mean seconds animal spent licking the paw in both the phases of *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and Ketorolac 10mg/kg showed no significance with p value of 0.871 and mean difference of 0.66sec in Phase 1 and with significant p value of <0.05 and mean difference of 32.83sec in Phase 2.

For all these five group the one way ANOVA value F and significance for Phase 1 and Phase 2 were 84.68, 41.40 and p<0.000, p<0.000 respectively with degree of freedom 3.

DISCUSSION

The purpose of this study was to objectively assess the effectiveness of *Caryophyllus aromaticus* (flower buds) in treating two different types of nociception using the rat formalin test. Most reported studies used licking and biting as the measure of assessing nociception. Results of our studies with *Caryophyllus aromaticus*(flower buds) are consistent with other studies using opioid type analgesic and NSAIDs [23].

The data obtained for the control (Figure 1) showed a biphasic nociceptive response with an immediate and short burst of activity lasting approximately the first 5 min (phase 1), followed by a prolonged period of activity (phase 2) starting at minute 11, peaking between 15 to 30 min and subsiding by 50 min after the injection. Little nociceptive behavior was observed during a 5-min intermittent period from minute 6 to minute 10.

It has been shown that the nociception seen in phase 1 is a result of direct nerve stimulation by the formalin [24] Opioid type analgesic modulates the perception of painful stimuli through interaction with opioid receptors in the central nervous system. Thus, it is effective in eliminating pain associated with direct nerve stimulation.

It has been demonstrated that the nociception produced in phase 2 of the formalin test is a result of chemical insult resulting in tissue damage [25]. Tissue destruction produces mediators of inflammation such as histamine, [26, 27] brady kinines, 28 prostaglandins, [29, 30] and serotonin [31] NSAIDs, blocks the production of prostaglandins[32], therefore, sensitization of the peripheral nervous tissue is reduced, resulting in less nerve stimulation and ultimately less pain.

In addition to the peripheral activity, it has been proposed that significant modification of central system neurons occurs. There is a reduced threshold of dorsal horn neurons to stimulation triggered by inputs from afferent neurons [33, 34] an expansion of the receptive fields of dorsal horn neurons, [35, 36] and a summation of

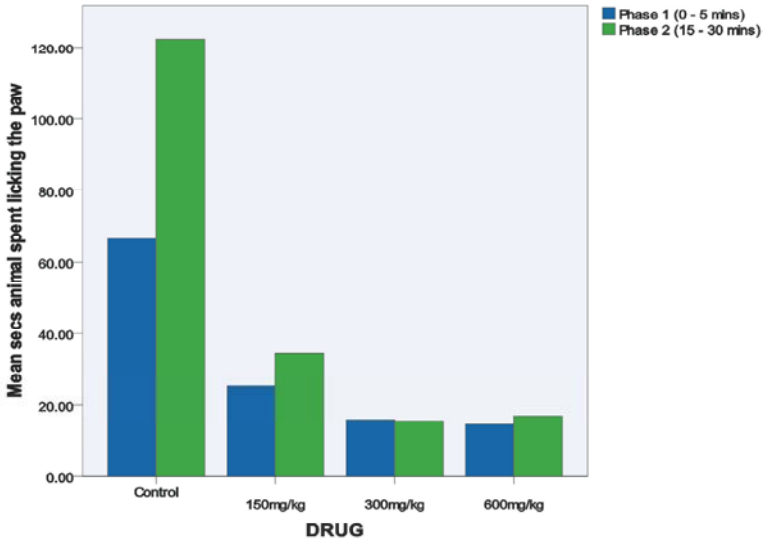

Fig 1: Effects of early response (Phase 1= 0-5 min after injection) and late response (Phase 2= 15-30 min after injection) in the formalin test after intraperitoneally administered of *Caryophyllus aromaticus*150mg/kg, 300mg/kg, 600mg/kg and control group. Each point represents the amount of time (s) the animals spent licking the injected paw (mean ± S.E.M.).

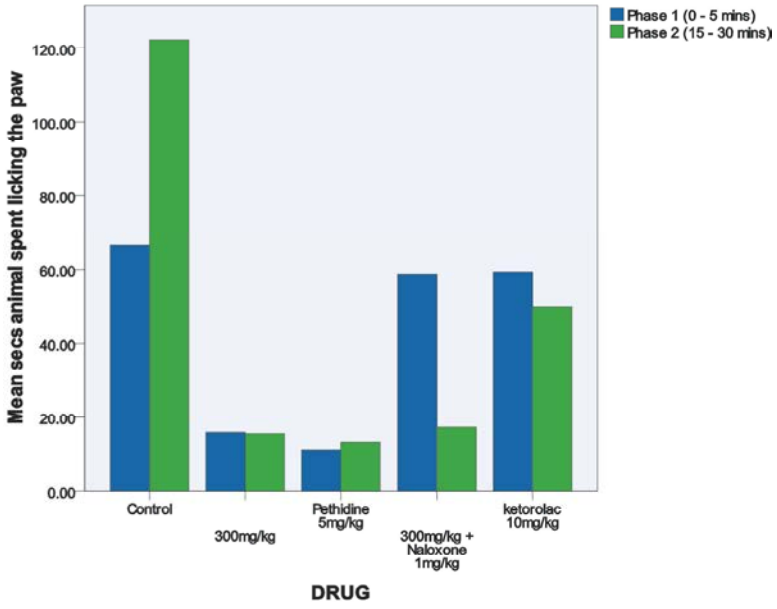

Fig 2: Effects of early response (Phase 1= 0-5 min after injection) and late response (Phase 2= 15-30 min after injection) in the formalin test after intraperitoneally administered of *Caryophyllus aromaticus*300mg/kg, Pethidine 5mg/kg, *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg, Ketorolac 10mg/kg and control group. Each point represents the amount of time (s) the animals spent licking the injected paw (mean ± S.E.M.).

slow postsynaptic potentials, resulting in a cumulative depolarization and a prolonged after-discharge of dorsal horn neurons. The latter is referred to as "windup. "[37] Studies have been ongoing to elucidate the compounds involved in this central nervous system phenomenon and include N Methyl-D-Aspartate antagonists and amino acids [38]. Opioid analgesics may be involved with modulating this process, in addition to their other known central nervous system mechanisms of action.

To find out the effective dose of *Caryophyllusaromaticus* (flower buds) we calculated three doses from the toxicity study. Acute oral toxicity dosages were 0.5, 1.0 and 3 g/kg while the chronic oral toxicity dosage was 100 mg/kg/day. For the assessment of analgesic activity, three dose level were chosen in a such a way that, middle dose is one-tenth of the maximum dose during acute toxicity study (*Caryophyllus aromaticus* 300mg/kg) and a low dose, which is 50% of the one-tenth dose (*Caryophyllus aromaticus* 150mg/kg) and the high dose, which is twice that of the one-tenth dose (*Caryophyllus aromaticus*600mg/kg).

Formalin test was done for the three doses of *Caryophyllusaromaticus* and compared with control group. All the doses of *Caryophyllus aromaticus* administration caused a significant ($p< 0.000$) reduction in licking and biting response during phase 1 and phase 2 compared with control group. Among the doses of *Caryophyllus aromaticus*, 300mg/kg and 600mg/kg was found to be significant in reduction of nociceptive response during phase 1 and phase 2 when compared with 150mg/kg. There was no significant ($p = 0.787, 0.901$) reduction of nociceptive response in phase 1 and phase 2 between 300mg/kg and 600mg/kg with mean difference of 1.16sec in Phase 1 and 1.33sec in Phase 2. Since there is no much difference between the reaction time, *Caryophyllus aromaticus* 300mg/kg is considered as the effective dose instead of going to higher dose 600mg/kg. (Fig 1).

Next we compared the effective dose of *Caryophyllus aromaticus* 300mg/kg, Pethidine 5mg/kg, Ketorolac 10mg/kg, *Caryophyllus aromaticus* 300mg/kg + Naloxone 1mg/kg and the control group. This comparison is to see whether *Caryophyllusaromaticus*is similar to Pethidine (an opioid analgesic) or Ketorolac (NSAID) and to establish the mechanism of action by using Naloxone (an opioid receptor antagonist).

Pethidine and *Caryophyllus aromaticus* 300mg/kg caused a significant ($p < 0.000$) reduction in licking and biting response during phase 1 and phase 2 compared to control group with mean difference of 55.33sec and 50.66sec respectively for phase 1 and 109.16sec and 106.83sec respectively for phase 2. Comparison between Pethidine and *Caryophyllus aromaticus* 300mg/kg was not significant ($p = 0.263, 0.821$) in both phases with mean difference of only 4.66sec and 2.33sec in phase 1 and phase 2 respectively. So Pethidine and *Caryophyllusaromaticus* acts identical and has both central and peripheral analgesic action (Fig 2).

Ketorolac administration caused significant ($p< 0.000$) reduction in nociceptive response during phase 2 only, when compared to control group. Phase 1 was not significant ($p= 0.091$) with mean difference of only 7.16sec when compared to control group. Pethidine and *Caryophyllus aromaticus*300mg/kg caused a statistically significant ($p< 0.004$) reduction in nociceptive response during phase 1 and phase 2 compared to Ketorolac with mean difference of 48.16sec and 43.50sec respectively for phase 1 and 36.8sec and 34.5sec respectively for phase 2. These data support the hypothesis that Ketorolac is limited in the type of nociception it can be used to treat and that, even in the inflammatory type of pain where it has its effects, the maximum efficacy is limited compared to Pethidine, an opioid analgesic. Thus, Pethidine and *Caryophyllus aromaticus*300mg/kg is effective as both central and peripheral analgesic action, while Ketorolac is effective as peripheral analgesic action only (Fig 2).

To find out the mechanism of action we added Naloxone (an opioid receptor antagonist) with *Caryophyllus aromaticus* 300mg/kg. Naloxone antagonizes opioid effects by competing for the mu, kappa and sigma opiate receptor sites in the CNS, with the greatest affinity for the mu receptor.The injection of formalin causes an immediate and intense increase in the spontaneous activity of C fiber afferent [39] and evokes a distinct quantifiable behavior indicative of pain [40]. It has been suggested that the early phase is caused by a direct effect of formalin on nociceptors, whereas the late phase is a tonic response in which inflammatory processes are involved and neurons in the dorsal horns of the spinal cord are activated (Fig 2).

Caryophyllus aromaticus 300mg/kg + Naloxone did not reduce the nociceptive behavior in phase 1 when compared to control group. It was not significant ($P = 0.066$) and the mean difference was only 7.83sec. When compared with *Caryophyllus aromaticus* 300mg/kg and Pethidine group it was significant ($p< 0.000$) with mean difference of 42.83sec and 47.5sec respectively. The central action is probably mediated via opioid receptors as seen with the formalin test. When Naloxone was given along with the test drug, there was significant increase in nociceptive behavior in phase 1 as compared to test drug alone and Pethidine. This indicates the involvement of endogenous opioid peptides in mediation of antinociceptive response of *Caryophyllus aromaticus* (Fig 2).

From this study, it was found that in nociception state the (flower buds) of *Caryophyllus aromaticus* shows significant decrease in nociception in both the

phases. Moreover, it also shows reduction of nociception in both phases by the standard drug Pethidine.

After receiving all the literature available in support of *Caryophyllus aromaticus* (flower buds) as analgesic, it was found that *Caryophyllus aromaticus* (flower buds) has beneficial effect in reducing nociception. *Caryophyllus aromaticus* (flower buds) thus holds the hope of new generation of drugs.

However, there is need for further studies on experimental animals and human beings that may provide more definitive and sure data regarding its usefulness, exact mode of action for its better economic and therapeutic utilization.

Hence *Caryophyllusaromaticus* (flower buds) may have a promising role in the management of Pain especially in countries like India where conventional treatment is not easily accessible to the general population.

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