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Evaluation of nitric oxide or opioid receptors involvement in antinociceptive properties of silymarin

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Abstract

Objective: It has been shown that *Silybum marianum* or its extracts have hepatoprotective, antioxidant, anticancer, anti-inflammatory and anti-diabetic effects. Nitric oxide (NO) plays an important role in neurotransmission, neuroprotection, neurotoxicity and pathological pain, as a neurotransmitter or neuromodulator in the central nervous system. Therefore, this experiment was performed in order to assess the analgesic effects of single and multiple-dosed ip administration of silymarin and the probable role of nitric oxide or opioid receptors using tail flick assay.

Results: Based on our results, only silymarin 100 mg/kg showed analgesic properties. Since naloxone did not change silymarin's analgesic effects, it is concluded that opioid receptors are not involved. Although in the presence of L-arginine, analgesic effect of silymarin remained intact, but it is not possible to strongly determine the involvement of nitric oxide pathway here. Based on our results, the difference between anti nociceptive properties of single and multiple-dosed treatment of silymarin 100 mg/kg is not significant.

Conclusion: It is concluded that silymarin exert its analgesic effects via other mechanisms. Inhibiting 5-lipooxygenase and neutrophil chemotaxis to inflammation location could be the probable ways of silymarin's action.

Keywords: Silymarin, Tail flick, L-NAME, Opioid receptors

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Introdoction

Milk thistle or St. Mary's thistle are common names for compositae family member, *Silybum marianum* (L.). This plant can be found throughout Mediterranean regions, southern Europe (Karen et al. 2005), North Africa, in the Alborz Mountains, Khuzestan and Azerbaijan provinces of Iran (Ramezani et al., 2008). This plant has been known to have medical properties since 2000 years ago (Gazak et al., 2007) and its medicinal properties have been widely studied. It has been reported that silybum marianum or its extracts have hepatoprotective, antioxidant (Morazzoni and Bombardelli, 1995), anticancer (Zi et al., 1997), anti-inflammatory (De La Puerta, 1996) and anti-diabetic (Maghrani et al., 2004) effects.

Silymarin, a standardized extract from the milk thistle seeds, is a mixture of flavanolignans composed primarily of silibin, silidianin and silichristin and is considered the major active constituent of *silybum marianum* (Karen et al. 2005). It is believed that flavonolignan Silymarin is a valuable naturally occurring substance exhibiting anticancer, anti-inflammatory, antioxidant, immune modulator (Katiyar, 2005), sedative and anti-depressant effects (Anjaneyulu et al., 2003; Sayyah et al., 2009).

One third of the world's population is believed to suffer from some sort of pain and this pain may cause significant disability, morbidity and different healthcare costs (Van Zundert and Cahana, 2005).

There is a great role for nitric oxide (NO) in lots of physiologic and pathologic conditions, such as neurotransmission, synaptic plasticity, neuroprotection, neurotoxicity and pathological pain, as a neurotransmitter or neuromodulator in the central nervous system (Meller and Gebhart, 1993; Prast and Philippu, 2003; Snyder, 1992). It has been reported that neuronal nitric oxide synthase (nNOS) is widely involved in thermal and mechanical hyperalgesia, neuropathic and inflammatory pain [Dolan et al., 2003; Inoue et al., 1998; Kolesnikov et al., 1993; Kolesnikov et al., 1997; Malmberg and Yaksh, 1993; Meller and Gebhart, 1993; Meller et al., 1992; Roche et al., 1996).

According to researches silymarin can markedly inhibit NO and PGE_2 production (Raso et al., 2001), thus in this study we evaluated the impact of silymarin on different pathways that might be involved in the tail flick assay induced pain mechanism. It is clear that over time, the magnitude of the effect produced by a given dose of morphine will diminish, suggesting the development of tolerance (Yaksh and Onofrio, 1987) therefore, probable additive effect of silymarin on morphine analgesic properties was also examined in a multiple-dosed administration model.

Materials and Methods

Male mice (25-30 g) were used in this experiment. Animals were housed in 12h/12h light/dark cycle at 22±2 ^{o}C. Food and water was freely available. The animals were allowed to adapt to the laboratory environment for at least 2 h before testing and were only used once. Since 1941, tail flick has become a famous method to determine the analgesic properties of a chemical by assessing reflex response to a nociceptive factor. In this method, animal tail is exposed to a powerful light beam and the response latency period for flicking the tail off the beam is recorded (Leandri et al., 2011). In this study, baseline latency period was 2-3 seconds and a cut-off time of 10 seconds was used to prevent tissue damage.

Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

In this experiment, these chemicals were used: L-NAME powder (Sigma Co., USA), L-arginine powder (Sigma Co., USA),

Silymarin powder (Sigma Co. USA), tween 80 (Merck Co. Germany), Morphine sulphate ampoule (Daroupakhsh Co., Iran), Naloxone hydrochloride ampoule (Daroupakhsh Co., Iran).

Expriment protocol

Analgesic effect of single dose administration of silymarin alone or its co-administration with either morphine or naloxone

Nine different groups (n=7) were involved in this section. In groups 1, 2 and 3, silymarin at the doses of 25, 50 and 100 mg/kg was injected intraperitoneally (ip), respectively and after 30 minutes, normal saline was injected ip. In groups 4, 5 and 6, the first step was as mentioned above but morphine at the dose of 5 mg/kg was injected in the second step. For group 7, 100 mg/kg was injected ip as the first step and naloxone at the dose of 5 mg/kg as the second step. For the negative control group, normal saline was administered ip in both first and second steps. But in the positive control group, morphine at the dose of 5mg/kg was injected in the second step. Since in the procedure of preparing silymarin solution, tween 80 was used to enhance silymarin's solubility, a control group was set to be evaluated regarding tween 80 analgesic effects. In all groups 45 minutes after the last injection, tail flick test was performed.

Analgesic effect of single dose administration of L-arginine alone or in combination with silymarin and L-NAME alone or in combination with L-arginine

Next Six groups of mice (n=7) received different chemicals as follows. In group 8, L-NAME at the dose of 10 mg/kg was injected ip and after 30 minutes, normal saline was injected ip. In group 9, L-arginine at the dose of 200 mg/kg was injected ip and after 30 minutes, normal saline was injected ip. In group 10, L-arginine at the dose of 200 mg/kg and L-NAME at the dose of 10 mg/kg were simultaneously injected ip and after 30 minutes, normal saline was injected ip. In group 11, L-arginine at the dose of 200 mg/kg and silymarin at the dose of 100 mg/kg were simultaneously injected ip and after 30 minutes, NS was injected ip. Positive and negative controls were the same as discussed in previous paragraph. In all groups 45 minutes after the last injection, tail flick test was done.

Analgesic effect of multiple-dosed administration of silymarin

In group 12, silymarin at the dose of 100 mg/kg was injected ip, twice daily for 3 days and on day 4, normal saline was injected ip. For control groups, normal saline was administered ip twice daily for three days. On day 4, for negative control normal saline and for positive control morphine at the dose of 5mg/kg was injected ip. For all experimental groups after 45 minutes of the last injection on day 4, tail flick test was done.

Statistical analysis

One way ANOVA followed by Tukey-Kramer test, was used for analysis of the data. Differences between means were considered statistically significant if $p<0.05$. Each point is the mean±S.E of seven mice.

Results

Analgesic effect of single dose administration of silymarin alone or its co-administration with either morphine or naloxone

Based on our results, in comparison with negative control, silymarin at the doses of 25 and 50 mg/kg had no significant antinociceptive activity but at 100 mg/kg, significant antinociceptive activity was recorded ($p<0.05$) (Figure 1). Administration of silymarin followed by naloxone shows significant analgesic effects compared with the negative control ($p<0.05$) (Figure 1). According to our results there was no difference between

tween 80 and NS treated groups either (not shown in figures). Co-administration of silymarin at different concentrations with morphine did not change morphine analgesic properties (Figure 2).

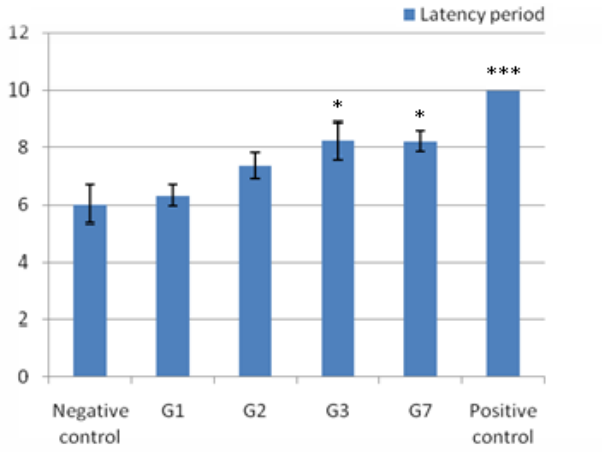

Figure 1. Recorded latency period (s) following single dose administration of silymarin alone (G1: silymarin 25 mg/kg; G2: silymarin 50 mg/kg; G3: silymarin 100 mg/kg) or its co-administration with naloxone (G7: silymarin 100 mg/kg with naloxone 5mg/kg), (negative control: normal saline; positive control: morphine 5 mg/kg). Data showed as mean±S.E. *p<0.05 and ***p<0.001, indicate significant changes compared to the control group (NS).

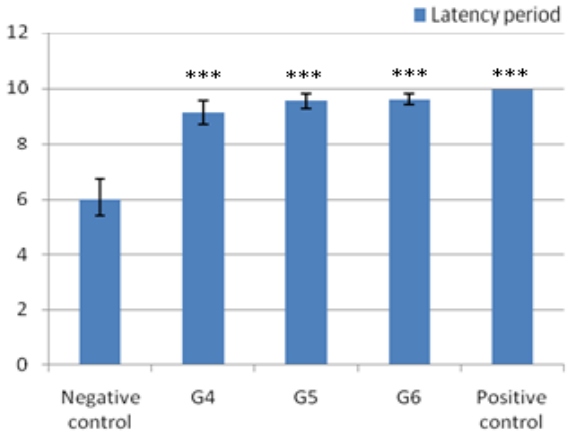

Figure 2. Recorded latency period (s) following single dose co-administration of silymarin and morphine (G4: silymarin 25 mg/kg with morphine 5 mg/kg; G5: silymarin 50 mg/kg with morphine 5 mg/kg; G6: silymarin 100 mg/kg with morphine 5 mg/kg), (negative control: normal saline; positive control: morphine 5 mg/kg). Data showed as mean±S.E. ***p<0.001, indicates significant changes compared to the control group (NS).

Analgesic effect of single dose administration of L-arginine alone or in combination with silymarin and L-NAME alone or in combination with L-arginine

In comparison to normal saline treated group (negative control), L-arginine did not show analgesic properties unless it was co-administered with silymarin ($p<0.05$). Co-administration of L-arginine and L-NAME inhibited L-NAME analgesic effects (Figure 3).

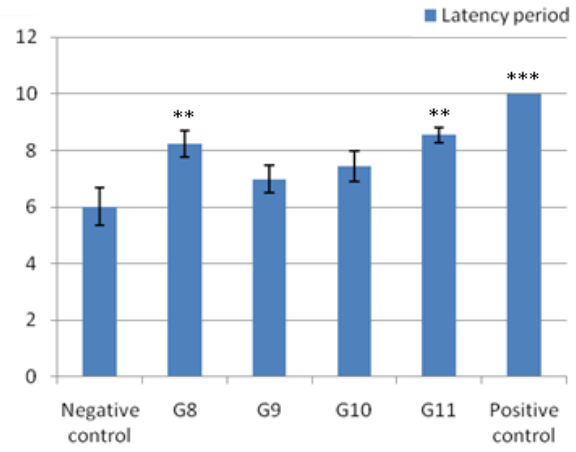

Figure 3. Recorded latency period (s) following single dose administration of L-NAME alone (G8: L-NAME 10 mg/kg) and in combination with L-arginine (G10: L-NAME 10 mg/kg with L-arginine 200mg/kg) or L-arginine alone (G9: L-arginine 200mg/kg) and in combination with silymarin (G11: L-arginine 200mg/kg with silymarin 100 mg/kg), (negative control: normal saline; positive control: morphine 5 mg/kg). Data showed as mean±S.E. **p<0.01 and ***p<0.001, indicate significant changes compared to the control group (NS).

Analgesic effect of multiple-dosed administration of silymarin

Although multiple–dosed administration of silymarin at the dose of 100mg/kg exhibits analgesic properties in comparison with negative control ($p<0.05$), it was not significantly different from single dose administration of silymarin at the dose of 100mg/kg (Figure 4).

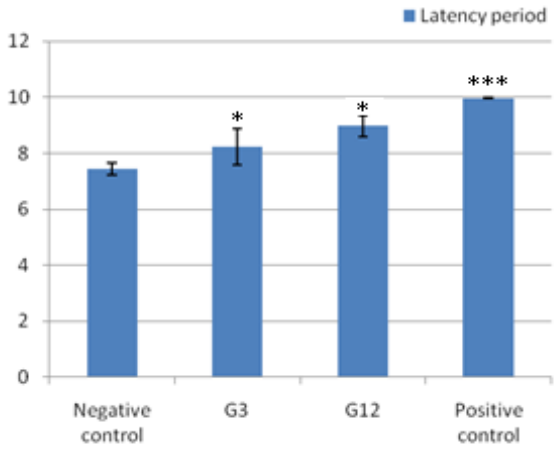

Figure 4. Recorded latency period (s) following multiple-dosed administration of silymarin (G3: silymarin 100 mg/kg single dose; G12: silymarin 100 mg/kg multiple-dosed), (negative control: normal saline; positive control: morphine 5 mg/kg). Data showed as mean±S.E. *p<0.05 and ***p<0.001, indicate significant changes compared to the control group (NS).

Discussion

It has been proven that insufficient analgesia and/or unrelieved pain has negative psychological and physiological effects on patients and results in higher morbidity and mortality when compared to patients whose post-operative pain is sufficiently reduced by analgesics (Werner et al., 2002; Kehlet and Holte, 2001). Looking for naturally occurring substances with analgesic properties, we evaluated the effect of silymarin on latency period in tail flick assay.

Our result revealed that silymarin shows significant analgesic effects only at its highest dose (100 mg/kg) and it didn't follow a dose-response pattern (Figure 1). Also, silymarin in chronic administration caused analgesia but there was no significant difference between its single and multiple-dosed analgesic properties (Figure 4). It might be due to silymarin's short half life (Morishima et al., 2010) which necessitates more frequent administration (injecting silimarin within shorter time intervals than our current research).

In group 7, since silymarin at the dose of 100 mg/kg showed the best analgesic results this dose was selected as the first step and naloxone was injected in the second step. According to our results, naloxone had no effect on silymarin analgesic activity (Figure 1). Hence, silymarin analgesic effect is not via opioid receptors unless it should have been blocked by naloxone.

Silymarin also shows analgesic effects when Co-administered with L-arginine. On the other hand, L-NAME analgesic effect is diminished by L-arginine. Therefore, it is concluded that silymarin causes analgesia not through nictic oxide pathway (Figure 3).

According to previous studies, silymarin antioxidant properties like acting as a free radical scavenger and its potency to increase cellular glutathione content play key roles in its hepatoprotective activity (Montvale , 2000). Also, silymarin reduces superoxide anion radicals and NO production (Hale et al., 2008), activates the antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and inhibits lipid peroxidation (Zhao et al., 2000; Bosisio et al., 1992; Soto et al., 2003). Silymarin causes its anti-inflammatory effects via inhibiting the production of leukotrien, which is a potent inflammatory mediator (Leng-Peschlow , 1996). It has been also reported that, silymarin blocks the release of cytokine by its inhibitory effects on neutrophil infiltration (Hale et al., 2008).

All data from different studies concerning silymarin anti inflammatory effects together with our results, convince us that tail flick is not a proper model to assess silymarin antinociceptive properties and silymarin analgesic activity should be measured by a model in which inflammatory pathways are monitored. Further studies are needed to investigate silymarin antinociceptive effects from different aspects.

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