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Isobolographic analysis of analgesic interactions of silymarin with ketamine in mice

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ABSTRACT: The present study was undertaken to explore the analgesic effect of silymarin and ketamine alone or in combination in mice. Analgesia was measured by using a hot plate and the writhing test. The up-and-down method was used to determine the median effective analgesic dosages (ED_{50s}) of silymarin and ketamine administered intraperitoneally (ip) either alone or together. The ED_{50s} of both drugs were analyzed isobolographically to determine the type of pharmacological interaction between them. The analgesic ED_{50s} for silymarin and ketamine in mice were 57.22 and 1.96 mg/kg, *ip*, respectively. Concomitant administration of the silymarin and ketamine at fixed ration (0.5:0.5) of their individual ED_{50s} was 38.4 mg/kg and 1.28 mg/kg, ip, respectively. Silymarin and ketamine at fixed ration (1:1) of their individual ED_{50s} were 47.54 mg/kg and 1.58 mg/kg, ip, respectively . Depending on the isobolographic analysis and calculating Y value, the type of pharmacological interaction between silymarin and ketamine at a ratio of 0.5:0.5 and 1:1 of their analgesic ED_{50} values of each drug , was antagonistic .In the writhing test the concomitant administration of silymarin and ketamine at 120mg/kg and 4mg/kg , ip, respectively reduce significantly the numbers of writhing in compare with silymarin120 mg/kg,ip and ketamine 4mg/kg, ip separately. The results suggest that the co-administration of silymarin and ketamine was ineffective to reduce the central pain while the concomitant administration of silymarin and ketamine was effective to reduce the visceral pain.

Keywords: silymarin, ketamine, analgesia, isobolographic, hotplate, mice.

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INTRODUCTION

Ketamine is an anesthetic and pain killeragentused in human and animals for anesthesia which characterized by hypnosis and good analgesia with muscle rigidity(Carter & Story, 2013; Persson, 2013; Tawfic, 2013). Ketamine inhibits the polysynaptic actions of the excitatory neurotransmitter acetylcholine (Ach) and glutamate in the spinal cord and block the N-methyl-D-aspartate receptor complex (Hsu, 2013). Ketamine used in human(Aiello & Mays, 1998)and veterinary clinic (Rang, Dale, Ritter, & Moore, 2003) as a general anesthetic agent usually in combination with sedatives, tranquilizers and analgesics. Ketamine combinations with sedatives, tranquilizers and analgesics are also used as balanced anesthetics and restraining agents in wild animals(Carter & Story, 2013; Coetzee, 2013; Short, 1992). Further, ketamine have anticonvulsant activity in mice (Tricklebank, Singh, Oles, Preston, & Iversen, 1989) and chicks (Reder, Trapp, & Troutman, 1980)

Silymarin (milk thistle), a medical herb belonging to the Asteraceae family(Ottai& Abdel-Moniem, 2006), is home Southern Europe, Southern Russia, Asia Minor and Northern Africa (Abenavoli, Capasso, Milic, & Capasso, 2010).Silymarin is mixture of silibinin A and B (silybin A and B), silydianin and silychristin. Other flavonolignans include isosilybin A and B, isosilychristin and taxifolin(Ottai & Abdel-Moniem, 2006; Stolf, Cardoso, & Acco, 2017).

Silymarin was used for the management of numerous liver disorders including cirrhosis, hepatitis (Herz, Heywood, Harborne, & Turner, 1977; Luper, 1998) Except hepatoprotective effects, it has cardioprotective, neuroprotective, cytoprotective, anti-inflammatory , analgesic and anti-carcinogenic effects (Manna, Mukhopadhyay, Van, & Aggarwal, 1999; Naser & Amin, 2019; Škottová et al., 2003)A useful practice is a combination of two drugs with the same therapeutic effect where in each agent is administered to obtain additive, synergistic or antagonism interaction in a fixed ratio. If the combination resulted in addition or synergism, the doses employed by each drug are reduced, then the side effects are decline; this type of study is calledisobolographic analysis (Raffa, 2001).

The purpose of the presentstudy was to explore the analgesic effect of silymarin and ketamine combination in mice. The models of pain induction used were the hot plateand chemical-induced writhing, which are predictive of acute pain responses with involvement of central and peripheral systems, respectively(Acharya et al., 2011; Barrot, 2012; de Campos Buzzi et al., 2010; Jain, Kulkarni, & Singh, 2001).

MATERIALS AND METHODS

46 Male and female Swiss albino mice weighing 28–33 g were housed at a temperature of 20 ± 2°C and 10/14 h light/dark cycle, with water and food ad libitum. All experiments complied with institutional regulations addressing animal use, and the mice received suitable attention and humane care. The Scientific Committee of the physiology, biochemistry and pharmacology of the College of Veterinary Medicine at the University of Mosul has reviewed and approved the protocol of this study. The required doses of The commercial powder of silymarin (175mg, 21ST Century HealthCare, Inc.) was dissolved in propylene glycol(Sigma chemical CO. 99%) . Ketamine (10 % injectable solution, DOPHARMA Netherland.) was further diluted in saline solution (Pharmaceutical Solution Industry, Saudi Arabia) to obtain the desired concentrations of the drug. The volume of administration of each drug was at 5 ml/kg body weight given intraperitoneally (ip). Two experimenters simultaneously observed the responses of the mice during the experiments which were conducted between 9–12 a.m.

Experiments

The up-and-down method (Dixon, 1980)was used to determine the median effective analgesic dosages (ED_{50s}) of silymarin and ketamine (administered either alone, or concomitantly – silymarin followed immediately with ketamine) in mice. The initial dose of silymarin was at 100 mg/kg, *ip*whereas that of ketamine was at 2 mg/kg, *ip*. In the combination experiment, the initial dosages of silymarin and ketamine were 30 and 1 mg/kg, *ip*, respectively (50:50 of their individual ED_{50} values) and the initial dosages of silymarin and ketamine were 60 and 2 mg/kg, *ip*, respectively (1:1 of their individual ED_{50} values) . We based our choices for silymarin and ketamine dosages on preliminary experiments in mice, as well as on previous studies (G. Jadhav & Upasani, 2009; Mohammad, Al-Baggou, & Naser, 2012; Sabiu, Sunmonu, Ajani, & Ajiboye, 2015; Vahdati Hassani et al., 2015) . Analgesia was measured by the thermal method using a hot plate (Panlab, S.I.U., Cornella, Spain) held at a temperature of 56°C. Mice were individually placed on the hot plate 15 min after the drug administration and the latency time to the first hind paw removal/

licking and/or jumping response was measured (Barrot, 2012; Le Bars, Gozariu, & Cadden, 2001)The cutoff point latency for the induction of analgesia was equal to or more than 6 s (i.e., positive analgesic response), and the maximum time allowed for the animal to stay on the hot plate was 20 s to prevent tissue damage .. The ED_{50s} of both drugs were subjected to isobolographic analysis to determine the type of interaction involved in the administration of silymarin and ketamine concomitantly (Gonzalez, Zegpi, Noriega, Prieto, & Miranda, 2011; Mohammad, Al-Zubaidy, & Alias, 2007; Tallarida, 1992)A straight line was drawn for the isobolographic analysis between isoeffective analgesic doses (ED_{50}) of silymarin and ketamine given to the mice either alone or in combination. The ED_{50} points of silymarin and ketamine given alone are represented on the x- and y-axes, respectively. The straight diagonal line indicates the line of additivity (zero interaction) at the ED50 values, and the location of the combination points on the left (below) and right (above) sides of the additive line indicates synergistic and antagonistic interactions, respectively (Gonzalez et al., 2011; Mohammad et al., 2007; Tallarida, 1992) The interaction index was calculated by the equation da/Da + db/Db(Tallarida, 1992)Da and Db are the individual ED_{50s} of silymarin and ketamine for the induction of analgesia, respectively, whereas da and db are their combined ED_{50s} for causing analgesia. An interaction index of 1 means additively (no interaction), <1 synergism and > 1 antagonism (Gonzalez et al., 2011; Mohammad et al., 2007; Tallarida, 1992).

To further examine the potential analgesic effect of silymarin and ketamine coadministration (by doublingthe ED_{50s} of both drugs given to mice), additional experiments were conducted to measure the analgesic effect of the combination 15 min after the administration by the thermal and chemical methods (Acharya et al., 2011; Le Bars et al., 2001).Acetic acid-induced writhing test in the mice,Thistest was conducted employing (Koster, 1959) method. Mice were divided into 4 groups of 6 mice each. The first group served as control and was given 10ml/kg i.p normal saline to act as control group. Groups II, received ketamine at 4mg/kg i.p , group III received silymarin at 120 mg/kg i.p and the group IV received ketamine 4mg/kg and silymarin 120 mg/kg i.p concomitantly . 15 minutes later, each mouse was injected with (0.06% acetic acid of 1ml per 100g i.p). The number of abdominal constriction for each mouse was counted 30 minutes after injection of acetic acid . Percentage inhibition of writhing was calculated using the formula. Inhibition % = Mean no of writhes (control) – mean no of writhes (test) / Mean no of writhes (control) × 100.

Statistical Analysis

Data are expressed as mean + SEM. Statistical analysis was done by using one way analysis of variance (ANOVA).P<0.05were considered significant.

RESULTS

Hot plate test

The ED_{50s} for silymarin - and ketamine induced analgesia in mice, as determined by the up and- down method, were 57.22 and 1.96 mg/kg, *ip*, respectively (Table 1). The silymarin-treated mice appeared to be slightly sedated. However, Combined administration of silymarin and ketamine at fixed ratio (50:50) of their individual ED_{50} values were 38.4 and 1.28 mg/kg, *ip*, respectively(Table 2) whereas , Combined administration of silymarin and ketamine at fixed ratio (1:1) of their individual ED_{50} values were 47.54 and 1.63 mg/kg, *ip*, respectively (Table 3). Isobolographic analysis of these ED50s for both drugs (either alone or in combination) at fixed ration either 0.5:0.5 or 1:1 revealed that combined administration of the drugs has antagonistic effect on the induction of analgesia in mice (Fig. 1). This antagonistic effect was indicated by the location of the points representing the combined analgesic ED50s of silymarin and ketamine above the diagonal line that connect their isoeffective analgesic doses (ED50s) given alone (Fig. 1). Further, the calculated interaction index for analgesia at fixed ration either 0.5:0.5 or 1:1 was 1.32 and 1.63 respectively indicating an antagonistic interactions between both drugs (an index of > 1indicates antagonism). The animals did not reach the point of tissue damage, so at the end of the experiments, we didn't have to give an analgesic dose.

Acetic acid-induced writhing response in mice

We found that ketamine and silymarin at 4 mg/kg and 120mg/kg significantly reduced the writhing number as compared to mice treated with normal saline (control),silymarin 120mg/kg and ketamine 4mg/kg (Table 4) . The percentage of inhibition of writhing was 62.1, 63.6 and 84.8 in ketamine at 4mg/kg ,silymarin 120mg/kg and concomitant silymarin 120mg/kg +ketamine 4mg/kg treated mice, respectively (Table 4) (Fig 1).

Table 1. Median effective doses (ED50) of silymarin and ketamine administered ***ip*** alone for induction of analgesia in the hot plate test in mice

Variable	Silymarin	Ketamine
ED50	57.22	1.96
Range of the doses used	100-40= 60mg/kg	2-1.5=0.5mg/kg
Initial dose	100mg/kg	2mg/kg
Last dose	40mg/kg	2mg/kg
Number of mice used	6(xxoxxo)	5(xoxoo)
Increase or decrease in the dose	20mg/kg	0.5mg/kg

aX – analgesia; O – no analgesia. The ED50s were determined by the up-and-down method (Dixon, 1980)

Table 2. Median effective doses (ED50) of silymarin and ketamine administered *ip* concomitantly for induction of analgesia in the hot plate test in mice at fixed ratio (0.5:0.5)

Variable	Silymarin + Ketamine	
ED50	38.4mg/kg	1.28mg/kg
Range of the doses used	45-30= 15mg/kg	1.5-1=0.5mg/kg
Initial dose	30mg/kg	1mg/kg
Last dose	45mg/kg	1.5mg/kg
Number of mice used		5(oxoox)
Increase or decrease in the dose	7.5mg/kg	0.25mg/kg
Y		1.32

Table 3. Median effective doses (ED50) of silymarin and ketamine administered *ip* concomitantly for induction of analgesia in the hot plate test in mice at fixed ratio (1:1)

Variable	Silymarin + Ketamine	
ED50	47.54mg/kg	1.58mg/kg
Range of the doses used	60-30= 30mg/kg	2-1=1 mg/kg
Initial dose	60mg/kg	2mg/kg
Last dose	45mg/kg	1.5mg/kg
Number of mice used		6(xxooxo)
Increase or decrease in the dose	15mg/kg	0.5mg/kg
Y		1.63

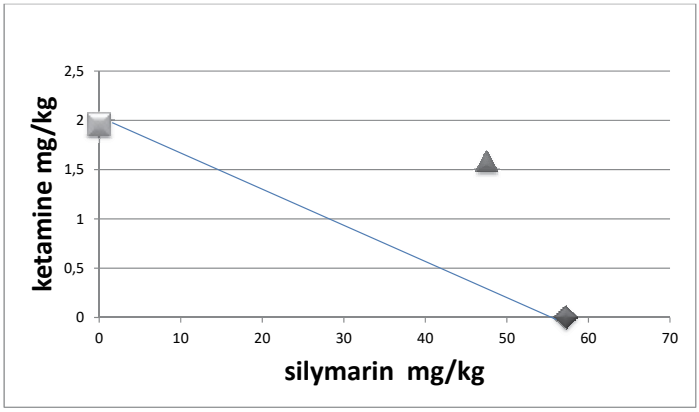

Figure 1. Isobolographic analysis of the analgesic interaction of silymarin and ketamine in mice.silymarin and ketamine was injected intraperitoneally. Points on x- and y-axes represent median analgesic doses (ED50s, mg/kg) of the drugs given alone, whereas the triangular point represents 1:1 of ED50 combinations of both drugs. The diagonal line between the individual ED50s of silymarin and ketamine is antagonistic and the triangular point indicates synergistic interaction.
n=5–6 mice/each ED50 experiment.

Table 4. The analgesic activity of Silymarin and ketamine in visceral pain model-writhing method following concurrent administration in mice

Groups	Onset of writhing	Numbers of writhing	Percentage of inhibition
Normal saline	31.5±3.7	13.2±1.7	0
Ketamine 4mg/kg	30.0±3.7	5.0±0.5*	62.1%
Silymarin 120mg/kg	37.5±3.7	4.8±0.5*	63.6%
Ketamine 4mg/kg+ Silymarin 120mg/kg	25.8±4.7	2.0±0.5*ab	84.8%

n = 6 for each group.The observations are mean ± SEM.
* p<0.05, as compared to control
a p<0.05, as compared to ketamine 4mg/kg .
b p<0.05, as compared to silymarin at 120mg/kg.

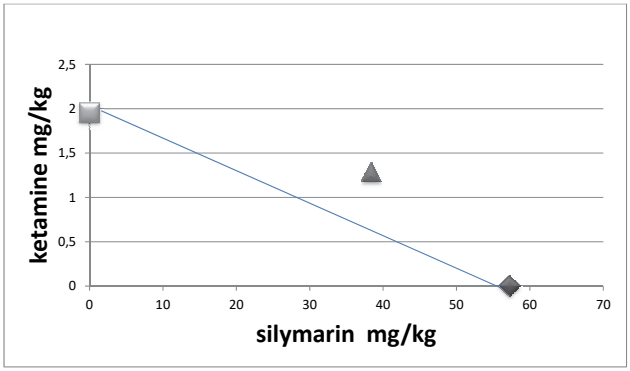

Figure 2. Isobolographic analysis of the analgesic interaction of silymarin and ketamine in mice . silymarin and ketamine was injected intraperitoneally. Points on x- and y-axes represent median analgesic doses (ED50s, mg/kg) of the drugs given alone, whereas the triangular point represents 0.5:0.5 of ED50 combinations of both drugs. The diagonal line between the individual ED50s of silymarin and ketamine is antagonistic and the triangular point indicates synergistic interaction.
n=5–6 mice/each ED50 experiment.

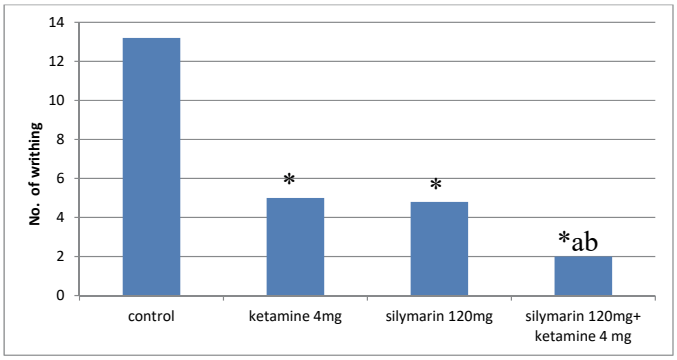

Figure 3. The analgesic activity of Silymarin and ketamine in visceral pain model-writhing method following concurrent administration in mice treated with normal saline , ketamine 4mg , silymarin 120mg and ketamine 4mg+ silymarin 120mg were injected i.v. 30 min before 0.6% acetic acid i.p. After 15 min the no. of abdominal constrictions was counted during 30 min. Results expressed as mean S.E.M. n=6for each group.

DISCUSSION

A large number of herbal medicinal products are reputed to have excellent medicinal value and are used to treat multiple ailments. In herbal medicine, different indigenous drugs are used with considerable success in single and/or combined forms to treat different types of inflammatory and arthritic illnesses(Hussain, Jassim, Numan, Al-Khalifa, & Abdullah, 2009). Silymarin produced considerable analgesic effects (on the writhing test) in mice either alone or in combination with ketamine while the combination of silymarin and ketamine was antagonistic in the hot plate test. Silymarin's individual action is reliable with the reported analgesic effect of the drug on mice, rats and chicks(Amin & Arbid, 2015; Hassani et al., 2015; G. B. Jadhav, Upasani, & others, 2009; Sahib, 2011)

In the acetic acid induced writhing tests, the administration of silymarin and ketamine combinations produced antinociceptive effects that were greater than would be achieved for individual drug administration alone.

The method of hot plate and tail immersion originally described by (Woolfe & Macdonald, 1944) was found to be suitable for the evaluation of analgesics that act centrally but not peripherally. Acetic acid induced writhing is a sensitive approach for the testing of compounds ' peripheral analgesic effect. Peritoneal nociceptor stimulation is indirect and occurs by releasing endogenous substances that stimulate nerve endings (Bhutia, Vijayaraghavan, & Pathak, 2010; Gawade, 2012). silymarin has cardioprotective, neuroprotective, cytoprotective, anti-inflammatoryandanti-carcinogenic effects, analgesic effect and hepatoprotective effects(Corchete, 2008; Fanoudi, Alavi, Karimi, & Hosseinzadeh, 2018; Kren & Walterová, 2005; Semalty, Semalty, Rawat, & Franceschi, 2010) . Silymarin is a free radical scavenger that uses cyclooxygenase and lipoxygenase pathways to various steps in arachidonic acid cascade (O. P. Gupta et al., 2000). Silymarin showed inhibitory effects on macrophage production of IL-1β and PGE2 and blocked IL-1β and cyclooxygenase-2 expression of mRNA in LPS-stimulated RAW 264.7 cells (Mateen, Raina, & Agarwal, 2013). There is a marked increase in the concentration of PGE2 and PGF2a in the peritoneal fluid following injection of acetic acid(Ricciotti, E., & FitzGerald, 2011), and the analgesic effect of silymarin similar to aspirin may be due to the Inhibition of prostaglandin synthesis (Fiebrich & Koch, 1979; Soon, Young, Song-Kyu, Kyu-Hwan, & Mook, 2004).

Ketamine acts to induce analgesia and anesthesia in humans and animals by antagonizing N-methyl-D-aspartate receptors(Aiello & Mays, 1998; Cornick-Seahorn JL:, 2001; Rang et al., 2003) By interacting with the supraspinal μ-opioid receptors, ketamine also induces analgesia(Nieuwenhuijs et al., 2004)In this context, by modulating phosphorylation in cells that express μ-opioid receptors endogenously, ketamine enhances opioid-induced analgesic signaling(Gupta, A., Devi, L. A., & Gomes, 2011).

This is the first study to provide an isobolographic analysis of the analgesic interaction between silymarin and ketamine. These data suggest that silymarin and ketamine in combination may have greater than additive effects in the treatment of visceral hyperalgesic conditions.

In the present study, the analgesic effect of combined administration of silymarin and ketamine was found to be antagonistic in the hotplate test as substantiated by the isobolographic analysis of the individual and combined ED50s of both drugs for the induction of analgesia at a fixed ratio of 0.5:0.5 and 1:1 of their individual ED50s, resulting in an interaction index of >1 (1.32 and 1.63) respectively .The antagonistic effect at level of central nociception may be due to the different mechanism between two drug and may be due to the distribution of silymarin in the CNS.Most flavonoids are metabolized in the gastro-intestinal tract and liver, are absorbed into the bloodstream and can reach the CNS by crossing the blood brain barrier (BBB)(Rodriguez-Mateos et al., 2014). To date, there is little data on the bioavailability of flavonoids and their capacity to reach the CNS.Detection of certain polyphenols and their metabolites indifferent brain regions after oral administration of polyphenolic extracts in murine models indicates that at least some flavonoids are able to cross the BBB(El Mohsen et al., 2002; Ferruzzi et al., 2009).

The pain eliciting models used in this study vary from one another and reflect the central (hot plate test) and peripheral (writhing test) mechanisms involved in pain generation and recognition(Acharya et al., 2011; Dambisya YM, 1994; de Campos Buzzi F, Fracasso M, Filho VC & del Olmo E, 2010). Different research groups have used acetic acid induced writhing test primarily to evaluate antinociceptive of natural compounds worldwide(Danion, Diemunsch, & Brandt, 2000; V. K. Gupta, 2006). Several endogenous noxious mediators such as bradykinin, serotonin, histamine, substance P(Danion et al., 2000; Irifune et

al., 1998) were released by acetic acid. The resulting pain is symbolized by abdominal muscle contraction accompanied by forelimb extension and elongation of the body. Our study did not investigate the mechanisms involved in the synergy between silymarin andketamine. The synergy observed with the writhing test may be associated with a pharmacokinetic or pharmacodynamic interaction. Silymarin may induce a decrease in the glomerular excretion of ketamine and its active metabolite (i.e., norketamine) through prostaglandin synthesis inhibition..

A pharmacodynamic interaction is more likely, as indicated by the peak amplitude synergistic effect. Silymarin acts as analgesic primarily through peripheral inhibition of the cyclooxygenase enzyme (Hussain et al. 2009). . Ketamine has mainly a central site of action through the block of NMDA receptors . A sensible hypothesis to explain our outcomes may be that silymarin decreased nociceptive inputs reaching the central nervous system, thereby improving the effectiveness of ketamine's central action. A reduced frequency of nociceptive inputs is probable to require less blockage of the NMDA receptor to induce an analgesic effect.however, some researcher indicate that repeated administration of silymarin prevents the formalin – induced pain behavior but it is ineffective in the treatment of sciatic neuropathic pain in mice (Hassani et al., 2015) . Another proposed mechanism involves pharmacodynamic interactions between the two drug-affected receptors (Yaksh, 1994)However, in the present study, the combination of silymarin and ketamine not only induced synergistic antinociceptive effects, but also reduced side effects by reducing each drug's dose.

In conclusionsilymarin and ketamine have antagonistic antinociceptive effects at the central pain, but both drugs have synergeticantinociceptive effects at the peripheral pain .These results could be of clinical importance in the species of rodents or could be extended to mammals after further research.

CONFLICT OF INTEREST

None declared.

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