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Original Article

Effect of Hydroethanolic Extract of *Citrus Aurantium* Leaves and Magnesium Sulfate in Mice Model of Vincristine-Induced Neuropathy

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Abstract

Neuropathic pain due to vincristine administration is an important dose limiting adverse effect with no definite efficient treatment. *Citrus aurantium* possesses multiple therapeutic potentials and is commonly used in traditional medicine. This study investigate the possible effects of the hydroethanolic extract of *C. aurantium* (CA) leaves and magnesium sulfate ($MgSO_4$) as a known analgesic in vincristine-induced peripheral neuropathy (VIN). Vincristine was administered intraperitoneally (IP) to establish peripheral neuropathy in mice. Effects of CA (50,100 and 150 mg/kg, IP) and $MgSO_4$ (50, 75 and 100 mg mg/kg, IP) were assessed on pain threshold performed by hot plate test. Moreover, the serum levels of total antioxidant capacity (TAC) and malondialdehyde (MDA) were assayed. Administration of CA (100 and 150 mg/kg) showed significant ($p<0.001$) decrease in responses to pain. In addition, $MgSO_4$ in high dose of 100 mg/kg could alleviate the neuropathic symptoms. The result of biochemical tests exerted high TAC level in all CA treated groups ($p<0.01$ in 50 mg/kg and $p<0.001$ for 100 and 150 mg/kg). MDA level was decreased significantly ($p<0.001$) by CA (100 and 150 mg/kg) and $MgSO_4$ (100 mg/kg). However the combination of low dose of CA and $MgSO_4$ exerted no efficient antinociceptive effect. According to the results, it can be concluded that $MgSO_4$ and CA, in an effective dose range, can be effective in controlling the neuropathic pain followed by vincristine, possibly through the modulation of antioxidant balance directed by CA or the NMDA and calcium receptor blocking properties of $MgSO_4$.

Keywords: *Citrus aurantium*, Hot plate, Magnesium sulfate, Mice, Neuropathy, Vincristine.

1. Introduction

Neuropathic pain is caused by damage or pathological alteration of the somatosensory system. Symptoms of neuropathy vary from itching, numbness, burning pain, urinary problems, sexual problems and etc. [1]. Peripheral neuropathic pain is frequently observed in several drug treatments, especially antiviral drugs (Stavudine, Zidovudine, lamovudine), chemotherapeutic drugs like vinca alkaloids, platinum drugs, taxanes and antibiotics such as metronidazole and isoniazid [2].

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Vincristine from vinca alkaloids is one of the most commonly used chemotherapeutic agents in treating variety of malignant diseases including leukemia and lymphoma. However, vincristine may also induce painful peripheral neuropathy. Vincristine induced neuropathy (VIN) is the major dose-limiting side effect which affects the quality of patient's life and even cause the discontinuation of treatment [3]. The incidence of vincristine-induced neurotoxicity, reported from different value of 100% to 10% depending on various items such as patient's criteria, doses and frequency of treatment cycles, accompanying with/without other neuropathic drugs and also the methods of assessing neuropathy [4].

Since chemotherapy-induced peripheral neuropathy (CIPN) is partially or completely unresponsive to conventional analgesic medicines such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, several different agents have been suggested and tested for treatment. Antiepileptic drugs, anti-arrhythmic agents, and tricyclic anti-depressants have being used with limited efficacy and wide spectrum of adverse effects [5]. Due to the high prevalence of VIN, and lack of useful pharmacologic interventions for its prevention or treatment, it would be of great significance to find related effective agents.

Nowadays therapeutic potential of plants as a reservoir for the development of novel medicines are investigated all over the world. Thus far, several previous studies have advocated beneficial effects of herbal medication in CIPN [6, 7]. *Citrus aurantium*, commonly known as sour orange or bitter orange, is a small citrus tree, belonging to Rutaceae family, native to tropical Asia and is also cultivated in all tropical and subtropical countries [8]. The major chemical composition in *C. aurantium* are flavonoids, primarily hesperidin, naringin and alkaloids, mainly synephrine, with different beneficial medical indication in traditional medicine [9]. It was reported that, due to the antioxidant and antiapoptotic activity of naringin, its administration increased nociceptive threshold level, endogenous antioxidant and diminished the oxidative–nitrosative stress, inflammatory mediators as well as apoptosis in diabetic neuropathic pain [10, 11]. Citrus leaves are recognized as natural antioxidants due to possess significant source of bioactive constituents including flavonoids, ascorbic acid, and phenolic constituents [12].

Due to the widespread use of citrus herb in folk medicines for the relief and treatment of pain and inflammation and in other hand upon its known antioxidant property [13, 14] we were prompted to evaluate the effects of *C. aurantium* leaves extract on the vincristine-induced neuropathic pain. Furthermore, as it was demonstrated in different studies that NMDA receptor antagonists can attenuate neuropathic pain [15]; magnesium sulfate with focusing on its NMDA receptor blocking role was used in companion with CA in VIN model.

2. Materials and Methods

2.1. Chemicals and Apparatus

Vincristine sulfate (Sobhan, Iran), Magnesium Sulfate (Pasteur Institute, Iran), normal saline (Ghazi, Iran), Ethanol (Merck,

Germany) were procured for the present study. Hot plate apparatus from Stoelting Co, USA was used. All the reagents used in the present study were of analytical grade. Vincristine, Magnesium Sulfate and CA extract were diluted with normal saline.

2.2. Plant Materials

The leaves of *C. aurantium* were collected in month of October from Sari in Mazandaran province (Iran) and the identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran. A voucher specimen (TUM-ADA 118) for this collection has been retained in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Science.

2.2.1. Extract Preparation

Maceration method was used for extraction. For this purpose air-dried leaves were mildly powdered and 500 g of CA powder extracted with 70 % aqueous ethanol mixture. Obtained extract was completely dried using rotary evaporator at 45 ° C under vacuum and then was weighted and kept in a refrigerator.

2.3. Animals

Experiments were performed on 25-30 g male mice in their 8-9 week, purchased from Pasteur Institute, Tehran, Iran. Animals were housed in 9 groups (n=8) with free access to food and water in a 12 h day and night cycles condition. All procedures were performed in accordance with the institutional guidelines for animal care and use (ethical approval number: IR.TBZMED.VCR.REC.1398.097).

2.3.1. Animal's Grouping

Animals were divided into 9 groups randomly: Group 1: Saline group, received normal saline (NS 10 ml/kg, IP) from day 1 to 10; Group 2: Vincristine group, received Vin (1 mg/kg, IP) single dose on day 4 and in other days received NS (10 ml/kg, IP); Group 3, 4 and 5: received magnesium sulfate ($MgSO_4$; 50, 75 and 100 mg/kg, IP respectively) on day 1 to 3 and 5 to 10 with a single dose of Vin (1 mg/kg, IP) on day 4; Group 6, 7 and 8: received CA extract (50, 100 and 150 mg/kg, IP respectively) on day 1 to 3 and 5 to 10 with a single dose of Vin (1 mg/kg, IP) on day 4; Group 9: received combined regimen of CA extract (50 mg/kg, IP) and magnesium sulfate (50 mg/kg, IP) on day 1 to 3 and 5 to 10 with a single dose of Vin (1 mg/kg, IP) [16, 17].

2.4. Experimental Design

The whole period of experimental study was 15 days. To induce peripheral neuropathy, 1 mg/kg vincristine was administered on day 4 of the study. As in this study, we aimed to evaluate both the prophylactic and therapeutic effects of our targeted agents, the injections have been started from 3 days prior to Vin administration and also continued in next 6 days, till day 10 of experiment. Antinociceptive responses to hot plate were recorded day 1 (prior any injection), day 4 (prior to Vin injection), and day 8, 12 and 15 (prior to related treatments) [18]. Thereafter, under deep anesthesia, blood sampling from heart were performed for estimating Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC).

2.4.1. Hot Plate Test

In this test, mice were individually placed gently on a hot plate (Eddy's hot plate) [19] with the temperature adjusted to 55±1 °C. The latency time to the first sign of exhibiting nociceptive responses of paw licking or jump response were determined. The cut-off time of 30 s was considered in case of no animal responses to avoid tissue damage.

2.4.2. Determination of Malondialdehyde (MDA)

MDA, a thiobarbiturate reactive substance, was measured as a marker for oxidative stress in serum using a method prescribed by Satoh [20]. The lipid peroxide expressed as nanomole per milliliter of serum, were measured spectrophotometrically.

2.4.3. Determination of Total Antioxidant Capacity (TAC)

Serum TAC was measured based on DPPH method (2.2-diphenyl-1-picryl-hydrazyl) [21]. In this method, the scavenging of free radical DPPH (a relatively stable compound in alcoholic solution with a peak absorbance at λ = 517 nm) is determined by a complex of antioxidants in the assayed sample. A decline of absorbance values equivalent to % of DPPH reduction expresses the level of the TAC-DPPH. The test results were expressed as milli moles per liter.

2.5. Statistics

Data are presented as mean ± SEM. One-way ANOVA was used to make comparison between the groups. If the ANOVA analysis indicated significant differences, Tukey test was performed. Differences between groups were considered significant if $p < 0.05$.

3. Results and Discussion

Vincristine as a commonly used anticancer agent possesses neurotoxicity as well as anticancer action due to high binding affinity towards b-tubulin (neuronal cytoskeleton protein) and therefore disorientation of cytoskeletal structure and disruptive action in polymerization of microtubules. Moreover, axonal degeneration can induced by known role of vincristine in modulation of cellular Ca^{2+} level, free radical generation and release of inflammatory mediators [22, 23]. Development of a condition of central sensitization of spinal neurons in vincristine treatment was stated to be an influential factor in appeared spontaneous pain and hyperalgesia [24]. Different studies reported vincristine-induced increase in total calcium level and superoxide anion generation ratio, as well as myeloperoxidase activity [25], lipid peroxidation products, MDA and protein carbonyl contents accumulation [26]. Furthermore, decline in the activities of superoxide dismutase, catalase, and reduced glutathione (GSH) levels reported following vincristine treatment [27]. All these results are declaring the fact that oxidative stress directly involved in the pathogenesis of VIN.

Administration of vincristine resulted in a considerable development of neuropathy ($p<0.001$) as reflected by a decreased latency time in the nociceptive responses of paw licking, lifting, or jumping from the hot plate surface on days 8, 12, and 15 compared to

saline group (Table 1). $MgSO_4$ only in higher dose of 100 mg/kg significantly ($p<0.001$) improved the pain response in comparison to vincristine-treated mice on days 8, 12, and 15. Treatment of CA in doses of 100 and 150 mg/kg significantly ($p<0.001$) restore the physiological thermal pain perception. Combining the low dose of $MgSO_4$ and CA (both 50 mg/kg) resulted in effects somehow near to that of Vin and therefore these two agents could not induce notable synergistic effective role.

As it is demonstrated in figure 1, administration of vincristine had a slight and not significant effect in TAC level compared to saline. This result also repeated in all three concentration of $MgSO_4$. However, as it was expected due to known antioxidant effects of CA, the TAC level were considerably increased in all extract doses ($p<0.01$ in 50 mg/kg and $p<0.001$ in 100 and 150 mg/kg). Interestingly, although not effective in latency time and MDA level test, the combination of $MgSO_4$ and CA induced a significant rise in TAC ratio compared to Vin group (Figure 1).

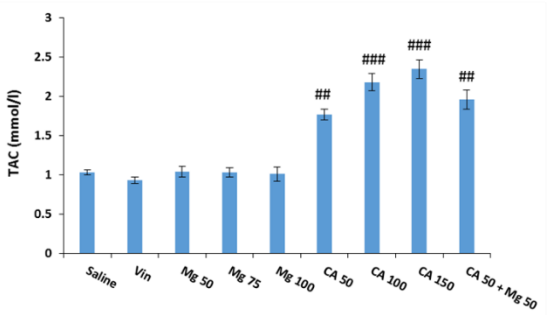

Figure 1. Effects of *Citrus aurantium* (50,100 and 150 mg/kg) and MgSO4 (50, 75 and 100 mg/kg) on Total Antioxidant Capacity levels. Vin: Vincristine, Mg: MgSO4; CA: *Citrus aurantium*. Each value represents the mean ± SEM of 8 mice. $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ compared to Vin group using one way ANOVA followed by Tukey test.

Table 1. Effects of *Citrus aurantium* and $MgSO_4$ on the latency time of mice exposed to the hot plate test

Groups	**Reaction time (s)**				
	Day 0	Day 4	Day 8	Day 12	Day 15
Saline	14.5 ± 0.32	14 ± 0.26	13.5 ± 0.32	13.12 ± 0.54	12.75 ± 0.49
Vin	14.37 ± 0.46	14.12 ± 0.39	10.62 ± 0.18**	9 ± 0.26***	7.75 ± 0.25***
Mg 50	14.62 ± 0.8	14 ± 0.75	10.37 ± 0.37	9.12 ± 0.22	8 ± 0.38
Mg 75	14.62 ± 0.53	14 ± 0.51	10.5 ± 0.42	9.5 ± 0.32	8.12 ± 0.29
Mg 100	14.37 ± 0.82	14 ± 0.65	13.37 ± 0.59$^{\#\#\#}$	13 ± 0.73$^{\#\#\#}$	12.12 ± 0.69$^{\#\#\#}$
CA 50	14 ± 0.46	13.12 ± 0.54	10.37 ± 0.26	8.87 ± 0.39	7.62 ± 0.26
CA 100	14 ± 0.42	13.25 ± 0.52	14.75 ± 0.31$^{\#\#\#}$	15.62 ± 0.18$^{\#\#\#}$	15.5 ± 0.46$^{\#\#\#}$
CA 150	14.12 ± 0.44	13.87 ± 0.47	15.25 ± 0.25$^{\#\#\#}$	15.12 ± 0.22$^{\#\#\#}$	15.12 ± 0.47$^{\#\#\#}$
CA 50 + Mg 50	14.5 ± 0.62	13.75 ± 0.75	9.87 ± 0.39	9.25 ± 0.55	8 ± 0.59

Each value represents the mean ± SEM of 8 mice

** $p<0.01$, and *** $p<0.001$ compared to the Saline group; $^{\#\#\#}$ $p<0.001$ compared to Vin group using one way ANOVA followed by Tukey test.

The vincristine group exhibited remarkable increase in MDA level compared to the saline group. Treatment with $MgSO_4$, as same as latency time effect, only in 100 mg/kg notably decreased the level of MDA compared to Vin ($p<0.001$, Figure 2). Similarly, the CA-treated groups (100 and 150 mg/kg) showed significant ($p<0.001$) decrease in the MDA level compared to the Vin group.

As it was resulted in latency time test, the combination of low doses of $MgSO_4$ and CA induced no significant changes in MDA level (Figure 2).

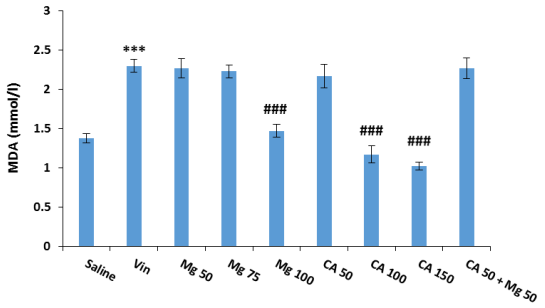

Figure 2. Effects of *Citrus aurantium* (50,100 and 150 mg/kg) and MgSO4 (50, 75 and 100 mg/kg) on Malondialdehyde levels. Vin: Vincristine, Mg: MgSO4; CA: *Citrus aurantium*. Each value represents the mean ± SEM of 8 mice. *** $p<0.001$ compared to the Saline group; ### $p<0.001$ compared to Vin group using one way ANOVA followed by Tukey test.

The results of the present study as well as indicated above, declared that administration of vincristine induced a remarkable degree of neuropathy, manifested as decrease latency time in the nociceptive responses to hot plate test. Furthermore, the trace of oxidative stress in VIN was confirmed by high measured level of MDA. Meanwhile, treatment with extract of CA significantly attenuated VIN and also decreased the MDA level and caused in a notable rise in TAC rate. Likewise, $MgSO_4$ in higher concentration positively affects the VIN and also decreased the MDA level.

In explanation of these obtained results, one of the main involved mechanisms of CA in reducing neuropathy, can be its antioxidant property. Moderate antioxidant activity using 2,2-AzinoBis-3ethylThiazoline-6-Sulphonate (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method was attributed to CA leaf extracts [28]. Linalool, linalyl acetate and alpha terpineol are main constituents in oil from the leaves and also contains high amount of polyphenols and flavonoids [29, 30]. In investigation of anti-inflammatory activity of CA, the flavonoid-type compounds like nobiletin, naringin, and hesperidin were reported. Inhibition of proinflammatory mediators by blocking nuclear factor-kappa B (NF-ĸB) and mitogen-activated protein kinase (MAPK) signaling were also recorded [31]. Furthermore, suppression of mRNA and proteins expression of COX-2 and iNOS and regulation of AMPK and Nrf2-related signaling pathway, all direct us to anti-inflammatory role of CA [32].

Based on our study pattern, the antinociceptive effects of CA can be attributed to its antioxidant property. However, other possible mechanisms such as modulation in calcium level, anti-inflammatory activity and interaction on N-methyl-D-aspartate (NMDA) receptors can be considered and needs to be evaluated.

NMDA receptors play a not clearly defined role in the etiology of neuropathic pains. It is suggested that in some types of neuropathic pain, activation of these receptors or rise in the release of excitatory amino acids may lead to reduced sensitivity to morphine [33]. In confirmation of this suggestion, NMDA receptor antagonists can also alleviate neuropathic pain [34]. In this regard, analgesic effect of Mg^{2+} as a physiologic calcium channel blocker which also interferes with NMDA receptors, has been investigated in different studies [34, 35]. These two mechanisms beside the vasodilatory effect mediated by endothelium-derived nitric oxide pathway, explain analgesic activity of Magnesium sulfate and introduced this agent as an alternative analgesic [36]. Therefore, as were obtained from our results, $MgSO_4$ in higher doses, without any significant impression on TAC and antioxidant relevance can increase the nociception threshold which can be attributed to mentioned mechanisms. As it was reported in previous investigations [37], analgesic effect of $MgSO_4$ exerted in higher doses and this was also repeated in this study. Even combining the low dose of $MgSO_4$ with the low dose of CA did not induce any efficient effect in VIN.

4. Conclusion

According to the results obtained in this study, it can be concluded that magnesium sulfate and extract of *Citrus aurantium* can be effective in controlling the neuropathic pain followed by the vincristine, however there is an effective range for their analgesic effects to be considered. Exerted alleviation in vincristine induced neuropathy, is possibly through the modulation of oxidant/antioxidant balance directed by *Citrus aurantium* or the NMDA and calcium receptor blocking property of $MgSO_4$. Further investigation could determine the exact mechanism and confirm the possible clinical use of these agents in vincristine induced neuropathy.

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