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Original Research Article

In vivo analgesic activities and safety assessment of Vitis vinifera L and Punica granatum L fruits extracts

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Abstract

Purpose: *To investigate the analgesic properties of hydro-alcohol fruit extracts of Vitis vinifera (grape) and Punica granatum (pomegranate) in albino male mice.*
Methods: *The analgesic activity of the fruit extracts was examined in vivo using thermal stimulus assays (tail immersion and hot plate) and chemically-induced writhing test. The extracts were administered orally at doses of 1.0, 2.0 and 3.0 g/kg. Acetylsalicylic acid (0.1 g/kg, per os) was used as analgesic drug.*
Results: *In acetic acid writhe test, pre-treatment with both extracts significantly decreased ($p < 0.0001$) in a dose-dependent manner the number of writhes when compared to control. Vitis vinifera extract treatment caused pain inhibition index values of 36.52 % (1.0 g/kg), 66.67 % (2.0 g/kg) and 89.71 % (3.0 g/kg). Corresponding values for Punica granatum extract treatment were 45.39 % (1.0 g/kg), 70.93 % (2.0 g/kg) and 86.88 % (3.0 g/kg). Acetylsalicylic acid treatment produced 76.06 % of pain inhibition. There were no significant differences ($p < 0.05$), at equivalent doses of 2.0 and 3.0 g/kg between Vitis vinifera and Punica granatum extract treatments regarding reduction in number of writhes. In hot-plate test, both extracts increased reaction latency time. In tail-immersion assay, Punica granatum significantly increased response to heat stimulus at doses of 2.0 g/kg ($p < 0.05$) and 3.0g/kg ($p < 0.001$) compared to control. However, Vitis vinifera did not produce such effects at any of the doses used.*
Conclusion: *These results show that the hydro-alcohol fruit extract of Punica granatum has a superior analgesic effect to that of Vitis vinifera extract.*

Keywords: *Vitis vinifera, Pomegranate, Punica granatum, Thermal stimulus assay, Analgesic activity, Pain*

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INTRODUCTION

Drugs presently used for management of pain and inflammatory conditions are either NSAIDs or opiates [1]. Most of these medicines have risk of adverse side effects (like gastric lesions caused by NSAIDs and tolerance and dependence induced by opiates); and their analgesic effects are not effective in all cases [2]. According to World Health Organization, traditional herbal remedies are still extensively used, especially in rural regions with restricted access to modern medicines [3]. Investigation of pain relief potential of plant-based remedies used in traditional medicine is one viable way of discovering new analgesic agents which could be beneficial in pain management [4].

An ethno-pharmacological survey of plant-based remedies commonly used to relieve pain, conducted as part of our research program on traditional medicinal plants of the Maghreb region

has resulted in establishment of a priority list of plants, among are two well-known species, *Vitis vinifera* (grape) and *Punica granatum*, (pomegranate). These plants are extensively cultivated in Tunisia and Morocco for their socio-economic values. Both species produce juicy and sweet fruits. Their therapeutic uses in traditional medicine are well documented in the literature of North Africa traditional medicine through several comprehensive reviews [5-8].

Vitis vinifera L. (Vitaceae), known locally as "Dalya", is a climbing shrub native to the Mediterranean region, Central Europe and South-Western Asia [9]. The leaves, fruits and seeds of this plant possess astringent, homeostatic and anti-inflammatory properties [9,10]. Parts of the plant are traditionally utilized to stop bleeding, inflammation, and also remedy for painful conditions due to hemorrhoids and headaches [7]. Studies have shown that the leaves and fruits of *Vitis vinifera* are rich in flavonoids (kampferol-3-0-glucosides, quercetin-3-O-glucosides); tannins (procyanidolic oligomers); stilbenes (resveratrol and viniferins); phenolic acids (tartaric acid, malic acid, succinic acid, citric acid and oxalic acid); and phenylacrylic acid derivatives (p-coumaroyl acid, caffeoyl acid and feruloylsuccinic acid) [11,12]. From a pharmacological point of view, *in vitro* and *in vivo* studies have reported a wide range of biological effects, including anti-inflammatory, anti-oedematous, hepatoprotective, antimicrobial, antioxidative, vasorelaxant and spasmolytic effects [13].

Punica granatum L. (Lythraceae), locally called "Rouman", is a fruit-bearing shrub native to the Middle East but now widely cultivated in warm regions of the world, particularly throughout the Mediterranean region [14-16]. Pomegranate is a used for the nutritional and medicinal benefits of its fruits [16,17]. The entire fruit is used in folklore medicine as remedy for various diseases such as dyspepsia, ulcer, hepatic damage, jaundice and diarrhea, as well as relief of pain from sore throat and menstruation [7,18]. The chemistry and pharmacology of *Punica granatum* has been recently reviewed [19]. The fruit contains polyphenols such as ellagic acid, punicic acid, ellagitannin, punicalagin, anthocyanidins, oestrogenic flavonols and flavones [19,20]. Pharmacological studies have established its antimicrobial, antioxidant, anti-inflammatory, anti-helminthic and molluscicidal properties [18,19]. Other benefits of pomegranate include chemopreventive potential of pomegranate ellagitannin against prostate cancer [21].

The current study was carried out to investigate the possible protective roles of hydro-alcohol fruits extracts of *Vitis vinifera* and *Punica granatum* against pain induced in mice by chemical and thermal methods.

EXPERIMENTAL

Plant materials

Plant materials were purchased from local market from the 2012 harvest season. Samples of black grape (*Vitis vinifera L*.) and pomegranate (*Punica granatum L*), were authenticated by a taxonomist.

Mature fresh fruits of the two species were collected in bulk, and washed under running tap water to remove dirt and adhering materials. The edible portion of the fruits was freeze-dried, powdered using a dry grinder and stored at low temperature (-25 ºC) until extracted.

Drugs and chemicals used

Acetic acid, acetylsalicylic acid and other chemicals used for extraction purpose and phytochemical screening were purchased from Sigma Aldrich.

Extraction process

The extraction process was carried out according to the methods of Babero *et al* and Ma *et al* [22,23]. Samples (25 g) were extracted by maceration for 24 h with 500 ml of methanol: water (70:30 v:v) in an automatic shaker. The extraction was assisted by ultrasound for 30 minutes at room temperature, and the debris was re-extracted twice under identical conditions.

The extracts from each plant were combined and filtered, and the combined filtrate was concentrated in a rotary evaporator under vacuum at low temperature (< 40 °C) to yield the crude extract which was subjected immediately to lyophilization. Freeze dried samples were kept at low temperature (-25 °C) until required for further experiments. Extracts of both species were reconstituted in distilled water for the evaluation of analgesic activity.

Phytochemical screening

Phytochemical analysis of crude extracts of both species was done using standard reactions of biological active groups i.e. flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, and coumarins [24,25].

Experimental animals

Adult albino mal mice, weighing between 20-32 g and aged (4-5) weeks were used for the studies. The animals were kept in standard polypropylene cages at room temperature (24 ± 2°C) in a 12 h light/dark cycle. They were allowed access to standard pellet diet and water *ad libitum,* and were acclimatized to the laboratory conditions for seven days before the experiment. The study was carried out following the guidelines prescribed in Guide for the Care and Use of Laboratory Animals [26].

Acute toxicity study

After an overnight fast, healthy animals were weighed and randomly distributed into 5 groups of six animals each (one control group and four treated groups). The animals were fed by oral gavages using a specially designed mice needle. The control and extract-treated groups received *per os* distilled water and serial doses (0.5, 2.5, 5.0 and 10.0g/kg) of extracts reconstituted in distilled water, respectively. Animals observation was carried within the first 30 minutes, then periodically during the first 24 hours and once daily for two weeks. Death or changes in general behavior and other physical activities were noted [27,28].Mice of all groups were weighted on days 7^{th} and 14^{th}. At the end of the experiment, the animals were scarificed and their internal organs including heart, liver, kidneys, lungs, spleen were examined [29,30].

Determination of analgesic activity

Analgesic activity was measured by three different methods: chemically-induced writhing, hot-plate and tail-immersion assays, as previously reported [31]. The peripheral analgesic effect of the extracts was evaluated by chemically- induced writhing test [32-34], while the involvement of central mechanisms was studied by using the hot-plate and tail-immersion tests. The latter assays are known to activate supra-spinal and spinal nociceptive pathways, respectively [35]. Tests were conducted after 24 h fast; healthy animals were then weighed and randomly distributed into groups of six animals each (n = 6).

Acetic acid-induced writhing test

The method described initially by Collier *et al* [36], was used. Writhing was elicited by an intraperitoneal (*i.p*) injection of 1 % acetic acid solution. Animals (6 per groups) were pretreated with fruits extracts of *Vitis vinifera* or *Punica granatum* (1.0, 2.0 and 3.0 g/kg, *per os*, single dose) reconstituted in distilled water, or acetylsalicylic acid (standard drug, 0.1 g/kg, *per os*). Then they were allowed to adapt for 60 min before intraperitoneal (*i.p.*) injection of acetic acid solution. The number of writhes in 20 min was counted for each mouse. Pain inhibition index [PII] was expressed as in Eq 1.

$$PII = [(Nc-Nt)/Nc] \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where *N*c represents the number of writhes observed for control group, and *N*t the number of writhes in test groups (fruits extracts, acetylsalicylic acid).

Evaluation of central analgesic activity

Central analgesic activity was monitored using two standard mechanical methods, the hot plate and tail immersion tests.

Hot plate test

Hot plate test was performed according to the method described earlier [33]. Five groups of mice (6 mice per group) were used. They received, one hour before test, fruit extracts of *Vitis vinifera* or *Punica granatum* at different concentrations (1.0, 2.0 and 3.0 g/kg, single dose, *per os*), distilled water (control), or acetylsalicylic acid as anti-inflammatory drug (0.1 g/kg, *per os*). The animals were placed on a heated surface of a hot plate maintained at 55.0 ± 0.5 °C. Pain threshold was considered reached when the animals licked their hind paws or jumped out [37].

Tail immersion test

Tail immersion test was performed according to the method described before [34]. Five groups of mice (6 mice per group) were used. They received, one hour before testing, fruits extracts of *Vitis vinifera* or *Punica granatum* at different doses (1.0, 2.0 and 3.0 g/kg, single dose, *per os*), distilled water (control), or acetylsalicylic acid as anti-inflammatory drug (0.1 g/kg, *per os*). The lower portion of the animal tail was immersed gently in a hot water bath maintained at 55.0 ± 0.5 °C. The reaction time taken by the animal to withdraw its tail was recorded (using a chronometer), after administration of treatments [38].

Statistical analysis

The results of pharmacological testing were expressed as mean ±SD and analyzed by Tukey test *(HSD)* to determine the level of significance. A value of $P<0.05$ was considered to be

significantly. Statistical analyses were performed using *XL Stat* version.

RESULTS AND DISCUSSION

Acute toxicity

Fruits extracts of *Vitis vinifera* and *Punica granatum* showed no mortality within 24 h at all the doses tested. Moreover, there were no visible signs of acute toxicity up to 10.0 g/kg along 14 days observation. Substances with oral LD_{50} higher than 5.0 g/kg are considered practically nontoxic [39]. Macroscopic examination of heart, liver, kidneys, lungs and spleen also revealed no abnormalities.

Acetic acid induced writhing

Results of acetic acid-induced abdominal writhing test are shown in Table 1 and Figure 1.

Both fruits extracts treatments (1.0, 2.0 and 3.0 g/kg, *per os*) reduced significantly ($p < 0.0001$) and in a dose-dependent manner, the number of writhes induced by acetic acid when compared to control. *Vitis vinifera* extract treatment produced PII values of 36.52 % (1.0 g/kg), 66.67 % (2.0 g/kg) and 89.71 % (3.0 g/kg). The corresponding PII values for *Punica granatum* extract treatment were 45.39 % (1.0 g/kg), 70.93 % (2.0 g/kg) and 86.88 % (3.0 g/kg). Acetylsalicylic acid treatment yielded 76.06 % (0.1 g/kg) pain inhibition. There were no significant differences, at equal doses of 2.0 g/kg and 3.0 g/kg,

Table 1: Anti-nociceptive effect of fruits extracts and acetylsalicylic acid on acetic acid-induced pain in mice

Compound and herbal extract	Dose (g/kg)	Number of writhings* (PII%)
Control (distilled water)	-	94.00 ± 3.03 (-)
Acetylsalicylic acid	0.1	22.50 ± 1.00 $^{\#}$ (76.06%)
Vitis vinifera	1.0	59.67 ± 4.76$^{\#\blacksquare}$ (36.52%)
	2.0	31.33 ± 5.95$^{\#\blacksquare\blacksquare}$ (66.67%)
	3.0	09.67 ± 1.97$^{\#\blacksquare}$ (89.71%)
Punic agranatum	1.0	51.33 ± 0.82$^{\#\blacksquare}$ (45.39%)
	2.0	27.33 ± 5.43$^{\#}$ (70.93%)
	3.0	12.33 ± 4.41$^{\#\blacksquare}$ (86.88%)

**Values are expressed as mean ± SD (Tukey HSD-test, n =6); $^{\#}P<0.0001$ vs. control group; $^{\blacksquare}p < 0.0001$, $^{\blacksquare\blacksquare}p < 0.01$ vs. acetylsalicylic acid (standard drug) treated group; PII = pain inhibition index (%)*

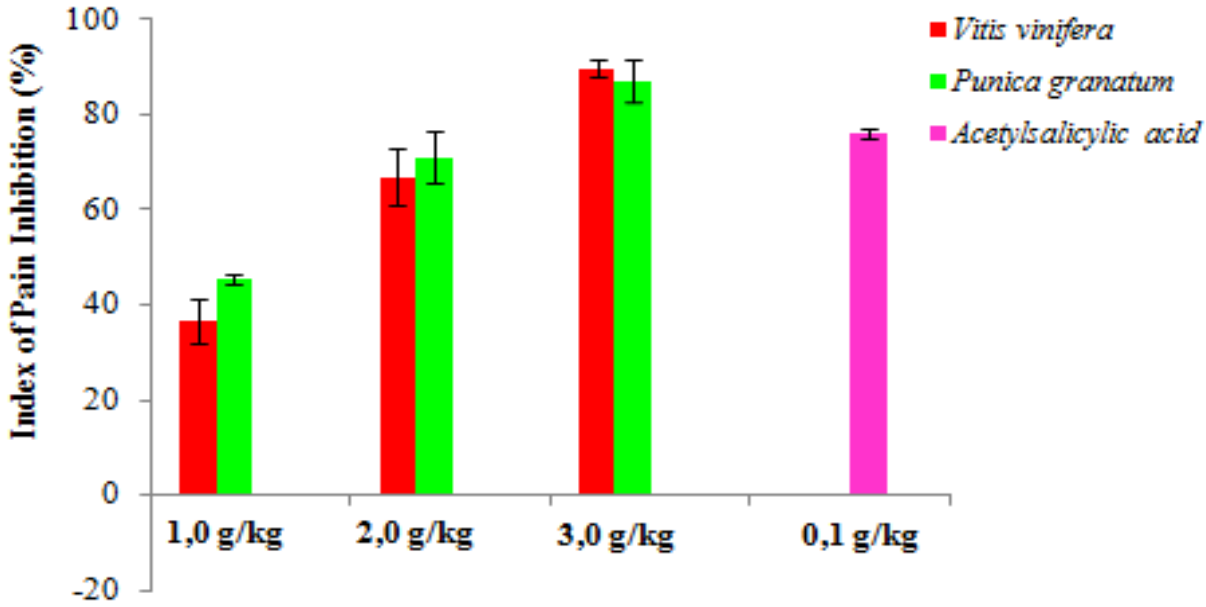

Figure 1: Peripheral analgesic activity of fruits extracts expressed as inhibition of acetic acid-induced writhing in mice

between the two extract treatments regarding reduction of the number of writhes. It is noteworthy extracts administered at a dose of 3.0 g/kg were significantly (*P*<0.0001) better than acetylsalicylic acid (0.1 g/kg).in the inhibition of writhing.

Central analgesic activity (Tail immersion and hot plate tests)

Results of tail immersion and hot plate tests are displayed in Table 2, and in Figure 2 and Figure 3. The tail immersion assay revealed no significant differences in reaction time between the control group and *Vitis vinifera*-pretreated groups over the range of tested doses (1.0, 2.0 and 3.0 g/kg), as well as between the fruit extracts. Similarly, no significant difference was seen between the acetylsalicylic acid group and control group. However, mice groups that received *Punica granatum* extract showed a significant increase in reaction time relative to the control group. The reaction times for *Punica granatum* extract groups at doses of (2.0, 3.0 g/kg) were superior to that of the acetylsalicylic acid group.

In the hot plate assay, the *Vitis vinifera* and *Punica granatum* groups showed significant increases in reaction time when compared with the control group. Compared to *Vitis vinifera*, *Punica granatum* extract was significantly more potent at the same doses. At all treated doses the protection against pain was dose-dependent, and higher than corresponding values for the acetylsalicylic acid group.

Table 2: Central analgesic activity of herbal extracts, measured by hot plate and tail immersion tests

Compound and plant extract	Dose (g/kg)	Reaction time *	
		Hot plate test	***Tail immersion test***
Control	-	02.98 ± 0.47	02.12 ± 0.51
Acetylsalicylic acid	0.1	01.92 ± 0.61$^{\Delta\Delta}$	02.80 ± 1.17
Vitisvinifera	1.0	03.16 ± 0.82	02.38 ± 0.61
	2.0	04.17 ± 1.09	02.43± 0.60
	3.0	12.77 ± 2.79$^{\Delta\Delta\Delta\Delta\#\#\#\#}$	03.06 ± 1.32
Punicagranatum	1.0	04.13 ± 0.6$^{\#\#}$	02.39 ± 0.49
	2.0	10.32 ± 1.27$^{\Delta\Delta\Delta\Delta\#\#\#\#\S\S}$	03.31 ± 0.96$^{\Delta}$
	3.0	16.18 ± 1.02$^{\Delta\Delta\Delta\Delta\#\#\#\#\S}$	03.90± 0.51$^{\Delta\Delta\Delta}$

* RT in seconds expressed as mean ± SD, *Tukey (HSD)-test*n=6; $^{\Delta}$ P < 0.05,$^{\Delta\Delta}$ P <0.01,$^{\Delta\Delta\Delta}$P <0.001, $^{\Delta\Delta\Delta\Delta}$P <0.0001: compared to control group; $^{\#}$ P < 0.05,$^{\#\#}$ P < 0.01, $^{\#\#\#}$ P < 0.001, $^{\#\#\#\#}$P<0.0001: compared to acetylsalicylic acidtreated group.§P< 0.01,§§P < 0.0001: compared to *Vitis vinifera* treated group

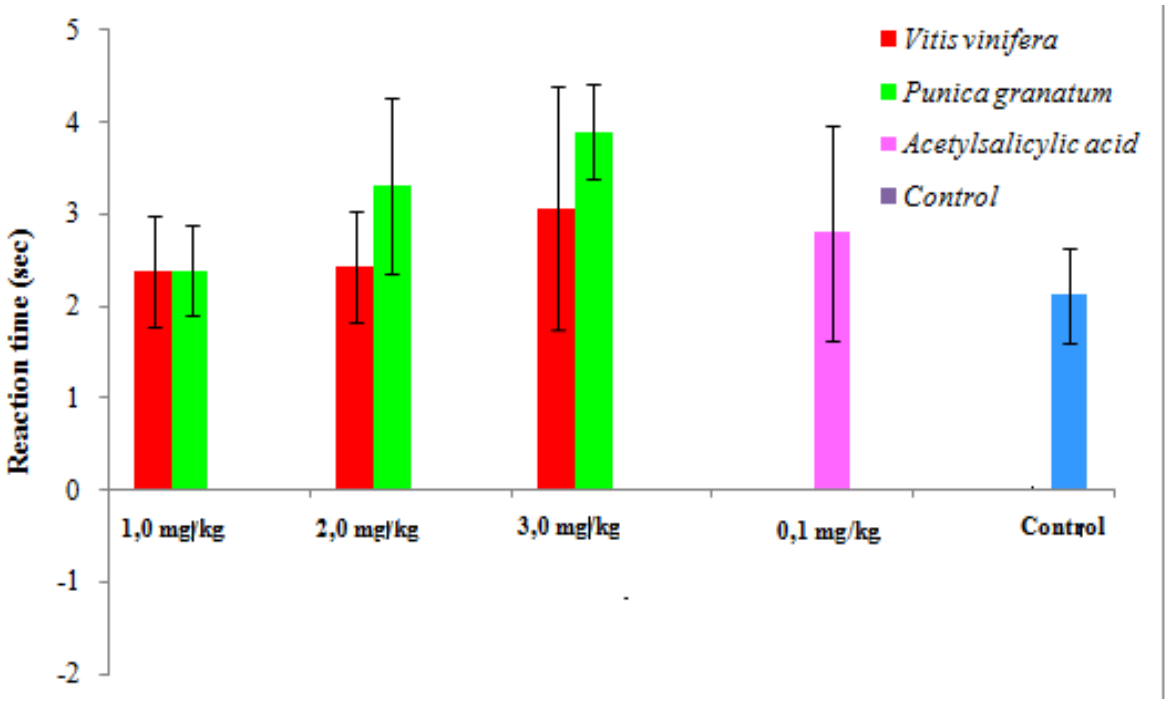

Figure 2: Central analgesic activity of fruits extracts, as measured by tail immersion test

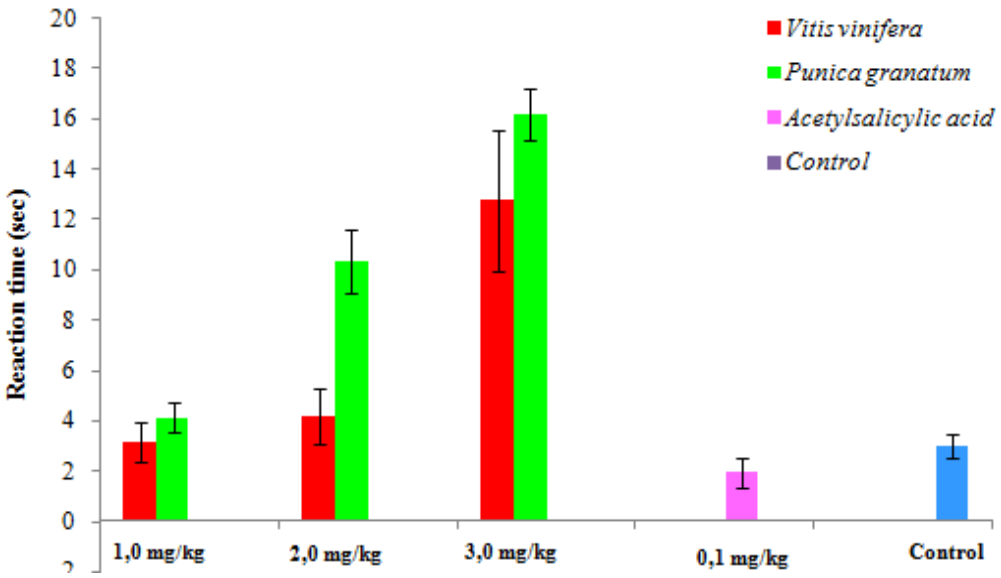

Figure 3: Central analgesic activity of fruits extracts, measured by hot plate test

The peripheral analgesic effect of the extracts was tested by using acetic acid-induced writhing test. This assay is widely accepted as a model for visceral pain [40,41]. The involvement of central mechanisms was studied by using the hot-plate and tail-immersion tests, known to activate supra-spinal nociceptive and spinal nociceptive pathways, respectively [40,41]. Although no significant difference was noted between the hydro-alcoholic fruit extracts of *Punica granatum* and *Vitis vinifera* at doses of 2.0 g/kg and 3.0 g/kg in acetic acid writhing test, the extracts produced significant ($p < 0.0001$) and dose-related peripheral analgesic effect compared to control. These results are in agreement with previous studies on antinociceptive effect using the entire fruit, peels or seeds extracts of *Punica granatum* [45,46], and leaves of *Vitis vinifera* [46]. Abdominal constriction response is induced through activation of local peritoneal receptors by mediators of pain [42]. Thus, the peripheral analgesic effect of hydro-alcoholic fruit extracts of *Punica granatum* and *Vitis vinifera* could be mediated by inhibition of the release of these endogenous nociceptive mediators [43].

In hot-plate test, the results show clearly that the fruit extracts of both species exert potent analgesic effects, thus confirming their central activity through supra-spinal nociceptive activation. It is worthy of note that although *Vitis vinifera* fruit extract produced statistically significant effects at a dose of 3.0 g/kg, it was less potent than *Punica granatum*.

In tail-immersion assay, hydro-alcoholic fruit extract of *Punica granatum* exhibited significant antinociceptive effects only at doses of 2.0 and 3.0 g/kg, whereas *Vitis vinifera* did not produce such effects over the tested doses.

This study clearly shows that hydro-alcoholic fruit extract of *Punica granatum* possesses analgesic potential which suggests the existence of both peripherally and centrally-mediated mechanisms as demonstrated by acetic acid writhing, tail immersion and hot plate assays. This finding is in agreement with previous reports on extracts from different parts of *Punica granatum* [47,55].

Results for fruit extract of *Vitis vinifera* suggest an analgesic effect with peripheral and central based mechanisms consistent withy supra-spinal nociceptive pathway.T hese results are in agreement with past studies using leaf-based methanol extract of *Vitis vinifera* [46,49,56].

Results of phytochemical screening of methanol fruit extracts of *Vitis vinifera* and *Punica granatum* are similar to be those from previous works [11,12,19,20]. The chemistry of pomegranate and grape is characterized by the presence of high amounts of phenolic compounds, including tannins and flavonoids. These plant constituents are known to possess analgesic and anti-inflammatory effects in experimental animals [47,48,51,54,56,57], and may be associated with the observed analgesic activities.

CONCLUSION

The findings of this study demonstrate that the hydro-alcohol fruit extracts of *Vitis vinifera* and *Punic agranatum* exhibit significant analgesic

activities in mice. These findings confirm results of previously reported studies on analgesic activity of *Vitis vinifera* and *Punica granatum*, and indicate that these two plants may offer alternative herbal treatments for the management of pain and inflammatory conditions.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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