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Full Length Research Paper

Involvement of pro-inflammatory cytokines and nociceptive pathways on the pharmacological activity of hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione

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Hydantoins are often reported as potent anticonvulsant drugs; however, recent studies have highlighted their antinociceptive potential. Based on these reports, this study investigated the antinociceptive and anti-inflammatory activities of the hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione (IM-7) using animal models. Treated mice submitted to the acetic acid-induced writhing test showed increase ($p<0.01$ or $p<0.001$) in the latency to the first writhing and reduction in the number of abdominal writhing ($p<0.001$). Furthermore, all doses reduced the nociceptive response in the first ($p<0.05$ or $p<0.001$) and second ($p<0.001$) phases of the formalin test. This effect was inhibited by pretreatment with the antagonists naloxone, sulpiride and caffeine. Additionally, IM-7 (75, 150 and 300 mg/kg, i.p.) reduced ($p<0.05$ or $p<0.001$) the glutamate-induced nociceptive response. Carrageenan-induced paw edema was strongly reduced following treatment with all doses of IM-7 ($p<0.05$, $p<0.01$ or $p<0.001$), and so were leukocyte migration and levels of interleukin-1β (300 mg/kg: $p<0.001$) and tumor necrosis factor-α (300 mg/kg: $p<0.01$) in carrageenan-induced peritonitis. Therefore, IM-7 decreased the nociceptive response via a mechanism involving the opioid, dopaminergic, and adenosinergic receptors. Its anti-inflammatory action also contributed by decreasing the release of pro-inflammatory cytokines.

Key words: Pain, inflammation, IM-7, nociception.

INTRODUCTION

Pain is the symptom that most often leads patients to seek medical care (Rief et al., 2012). It currently affects about 100 million Americans, generating an estimated economic impact of 560 to 635 billion dollars per year (Dubois and Follett, 2014). Although there is an extensive therapeutic arsenal available for the treatment of pain, it

is accompanied by a range of undesirable side effects. Some examples include the gastric irritation that can ensue as a result of non-steroidal anti-inflammatory drug (NSAID) usage or opioid tolerance. This issue is, therefore, one of the factors that motivates research into the development of new drugs for pain relief.

It has been reported previously that anticonvulsants may be useful in some painful conditions such as trigeminal neuralgia and diabetic neuropathy (Bhattacharya et al., 2009). Phenytoin, for example, blocks presynaptic voltage-gated sodium channels, thereby inhibiting the pathological release of excitatory neurotransmitters, such as glutamate, which are directly involved in nociceptive pathways (Sakaue et al., 2004; Webb and Kamali, 1998). This drug belongs to the class of drugs known as the hydantoins, which are compounds with a chemical structure containing a derivative of the imidazolidine ring. These substances have been extensively studied, both in regards to their chemical structure and their pharmacological properties. Some of these properties include acting as a muscle relaxant, antiarrhythmic, antibacterial, antitumor and, particularly, anticonvulsant (Oliveira et al., 2008).

While studies investigating the anticonvulsant activity of hydantoins are common in the scientific literature, especially because of the established efficacy of phenytoin against partial and generalized tonic-clonic seizures, increasing efforts have been undertaken to characterize the antinociceptive profile of new hydantoins. Behavioral studies have demonstrated that hydantoins are able to reduce nociceptive behavior and inflammation in animal models (Guerra et al., 2011; Sudo et al., 2010; Sztanke et al., 2005; Walls et al., 2012). These results were similar to those observed by our research group in mice subjected to the acetic acid-induced writhing test, formalin and hot plate tests, and carrageenan-induced peritonitis after treatment with the hydantoin derivatives IM-3 and HPA-05 (Maia, 2013; Queiroz et al., 2015; Salgado, 2011).

Studies concerning the antinociceptive effects of hydantoins are still scarce; however, there is a strong indicator of the potential of these substances. Thus, in this study the antinociceptive and anti-inflammatory properties of the hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione (IM-7) were investigated in mice.

MATERIALS AND METHODS

Hydantoin derivative IM-7

The synthetic compound IM-7 was provided by Prof. Dr. Petrônio Filgueiras de Athayde Filho (Organic Synthesis Laboratory, Federal University of Paraíba, Brazil). Synthesis was performed as previously described (de Sousa et al., 2009).

Animals

Male Swiss mice (25 to 35 g, approximately 3 months old) were obtained from the vivarium of Prof. Dr. Thomas George (UFPB), where they were monitored and kept at a constant temperature (21 ± 1°C), exposed to light/dark cycles of 12 h each (light phase from 0600 to 1800), and given water and food ad libitum. Animals were used only once and euthanized at the end of each experiment. All procedures were approved by the Animal Experimentation Ethics Committee (CEPA/UFPB/BRAZIL, protocol number 0601/11).

Drugs and reagents

The following materials were used: Acetic acid (Reagen, Brazil); cytokines assay kits (e-Bioscience, USA); atropine, bicuculline, caffeine, carrageenan, dexamethasone, glibenclamide, glutamate, sulpiride and yohimbine (Sigma, USA); formaldehyde P.A. (Vetec, Brasil); morphine hydrochloride (Merck, USA) and naloxone hydrochloride (Research Biochemical, USA). The substances were solubilized in distilled water (LTF/UFPB, Brazil) and administered intraperitoneally (i.p.) at 0.1 mL per 10 g of body weight (except formaline, glutamate and carrageenan: 20 µL, via intraplantar; and naloxone and dexamethasone used subcutaneously). IM-7, glibenclamide and sulpiride were dissolved in Tween 80 (Vetec, Brazil) and distilled water.

Evaluation of the antinociceptive activity of IM-7

Acetic acid-induced writhing test

The injection of 0.85% acetic acid into the peritoneal cavity of mice triggers a nociceptive response characterized by abdominal contortions followed by stretching of the hind limbs (Koster et al., 1959). Mice were distributed into groups (n=8), and treated with a vehicle (3.4% v/v Tween 80), morphine (10 mg/kg), or IM-7 at doses of 75, 150, and 300 mg/kg. After 30 min (morphine treatment group) or 60 min (vehicle and IM-7 treatment groups), the animals were administered an intraperitoneal (i.p.) injection of 0.85% acetic acid (0.1 mg/mL) and were observed for 15 min. The latency to the first writhing and the number of abdominal contortions that occurred in the interval of 5 to 15 min after the administration of acetic acid were recorded. A decrease in the number of contortions was considered indicative of an antinociceptive effect.

Formalin test

This methodology, described by Dubuisson and Dennis (1977) and adapted by Hunskaar et al. (1985) for mice, involves inducing nociception by the subcutaneous (s.c.) injection of formalin 2.5% (20 µl) into the hind paws of mice. Animals respond to this stimulus by licking the injected paw, and the time spent in this activity is recorded in 2 phases. The first phase (0 to 5 min) occurs due to the direct stimulation of nociceptors, while the second phase (15 to 30 min) involves the participation of inflammatory mediators. Mice were distributed into groups (n=8), and injected with a vehicle (3.4% v/v

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solution of Tween 80), morphine (10 mg/kg) or IM-7 (75, 150, and 300 mg/kg). An intraplantar (i.pl.) injection of formalin 2.5% (20 µL) was administered into the right hind paw of the mice 30 min (morphine group) or 60 min (vehicle and IM-7 groups) after treatment. The times spent in the 2 phases were recorded.

Glutamate-induced nociception

Glutamate is the major excitatory neurotransmitter involved in nociceptive signal transmission (Beirith et al., 2002). In order to evaluate the influence of IM-7 on the glutamatergic system, mice were treated with an i.p. injection of a vehicle (3.4% v/v solution of Tween 80) or IM-7 (75, 150, or 300 mg/kg). After 60 min, 20 µl of

glutamate solution (20 µmol/paw) was injected into the right hind paw of the mice. Animals were immediately placed into individual observation boxes and the nociceptive response (paw licking time) was recorded for 15 min.

Mechanism of action

To demonstrate the possible mechanism of action involved in the IM-7-induced antinociceptive effect we used pharmacological tools and formalin. Briefly, 15 min prior to treatment with IM-7 (75 mg/kg, i.p.), pattern drugs were administered to the mice. These included naloxone (5 mg/kg, s.c.), an opioid receptor antagonist; glibenclamide (10 mg/kg, i.p.), an ATP-sensitive potassium channel (K_{ATP}) blocker; sulpiride (20 mg/kg, i.p.), a D2 dopamine receptor antagonist; yohimbine (0.15 mg/kg, i.p.), an α_2-adrenergic receptor antagonist; atropine (5 mg/kg, i.p.) a muscarinic receptor antagonist; bicuculline (1 mg/kg, i.p.)a gamma amino butyric acid-A receptor ($GABA_A$) antagonist; and caffeine (10 mg/kg, i.p.), an adenosine receptor antagonist.

Evaluation of the anti-inflammatory activity of IM-7

Carrageenan-induced paw edema

The injection of carrageenan elicits an inflammatory reaction characterized by edema, erythema, and hyperalgesia, which can be reversed by steroid and non-steroid anti-inflammatory agents (Winter et al., 1962). Mice were distributed into groups (n=8) and treated as follows: negative control group, 3.4% v/v Tween 80, i.p.; positive control group 3.4% v/v Tween 80, i.p. and IM-7 (75 mg/kg, i.p.); IM-7 (150 mg/kg, i.p.); IM-7 (300 mg/kg, i.p.); and dexamethasone (2 mg/kg, s.c.). Mice received 20 µL of a 1% solution of carrageenan in the subplantar region of the right hind paw and 20 µL of saline into the left hind paw 60 min after treatment. The negative control group received saline in both paws (data not shown). Hind paw thickness was recorded for each mouse with a digital micrometer just before carrageenan injection (baseline measurement, data not shown), and then after 1, 2, 3, 4, 5, 24, 48, and 72 h. Edema was calculated as the difference between right and left hind paw thickness.

Carrageenan-induced peritonitis

Carrageenan-induced peritonitis was used to determine if IM-7 could inhibit cell migration that results from the animal's exposure to an acute inflammatory stimulus. Mice (n=6) were distributed into groups and treated with a vehicle (3.4% v/v Tween 80); IM-7 at 75, 150, or 300 mg/kg i.p.; or dexamethasone (2 mg/g, s.c.). After 60 min, the inflammatory process was induced by an i.p. injection of 1% carrageenan. Four hours later, animals were euthanized and the peritoneal exudate was removed after washing the peritoneal cavity with 3 mL of phosphate buffered saline (PBS). The peritoneal exudate was centrifuged at 1500 rpm for 5 min at 4°C, and the supernatant was stored at -20°C for further measurement of the pro-inflammatory cytokines. Precipitated cells were diluted with Turk's solution (1:10) to determine the total cell count in a Neubauer chamber under an optical microscope.

Differential cell counts in the peritoneal exudate

For each animal, the peritoneal exudate was diluted 1:10 in PBS and 100 µL of this cell suspension was cytocentrifuged (Citopin, Bio Research) at 1500 rpm for 15 min to prepare slides. The slides were then stained with May-Grunwald Giemsa to allow differential cell counts of polymorphonuclear and mononuclear leukocytes using an optical microscope.

Pro-inflammatory cytokine assay

Tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels in the peritoneal fluid were assessed using specific cytokine ELISA assay kits and a microplate reader set at 450 nm (VERSAmax Tunable, Molecular Devices). The protocol was performed according to the manufacturer's instructions. The levels of cytokines were calculated from the standard curves and expressed as the total amount per milliliter (pg/ml).

Statistical analysis

Differences between the means of the groups were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's test or Tukey's multiple comparisions test depending on the case. Results were expressed as the mean ± standard error of mean (SEM) and considered significant when $p < 0.05$.

RESULTS

Effects of IM-7on the acetic acid-induced writhing test

The latency to the first writhing was significantly increased in mice treated with doses of 75 mg/kg (357.4 ± 34.6 s; $p < 0.05$), 150 mg/kg (518.5 ± 87.9 s; $p < 0.01$) and 300 mg/kg (767.9 ± 71.2 s; $p < 0.001$) of IM-7 when compared to the control group (221.3 ±15.0 s) (Figure 1A). The number of contortions decreased in animals treated with IM-7. While the vehicle-treated group showed an average of 44.8 ± 4.0 contortions, the groups treated with doses of 75, 150, and 300 mg/kg IM-7 exhibited 16.8 ± 3.5, 7.1 ± 2.9, and 2.6 ± 2.0 contortions during the 10 min of observation, respectively (Figure 1B). Animals treated with morphine (10 mg/kg, i.p.) exhibited no abdominal contortions.

Effects of IM-7 on formalin-induced nociception

According to data shown in Figure 2A, in the first phase of this test all doses of IM-7 (75, 150, and 100 mg/kg, i.p.)

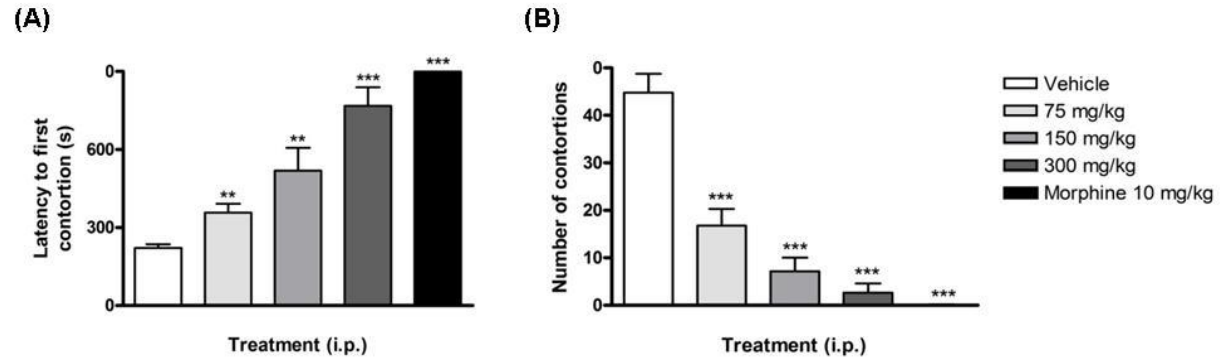

Figure 1. Figure (A): the effect of IM-7 on the latency to the first contortion in acetic acid-induced writhing test. Figure (B): the effect of IM-7 on the number of contortions in acetic acid-induced writhing test. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. **p<0.01, ***p<0.001 versus control group.

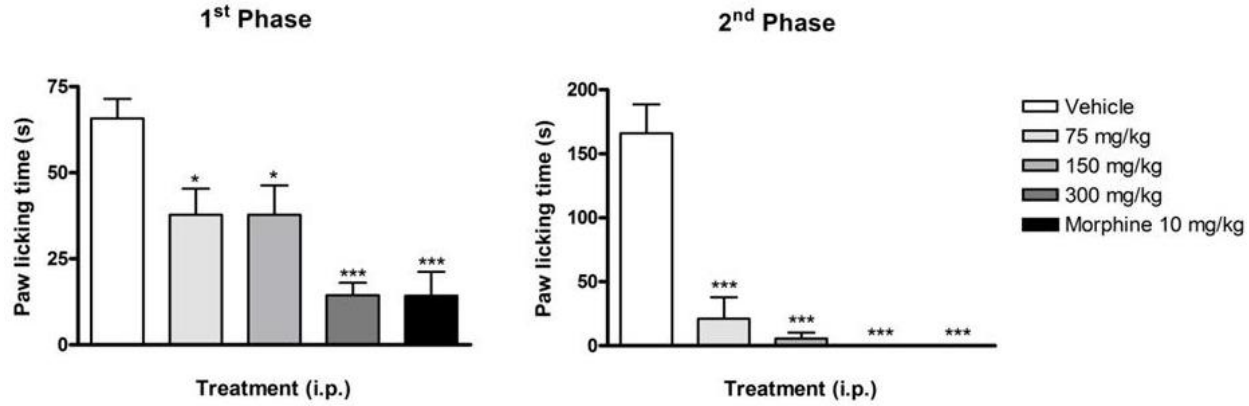

Figure 2. Effect of IM-7 on the licking response induced by intraplantar injection of formalin in the first (neurogenic) and in the second (inflammatory) phases of formalin test. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. *p<0.05, ***p<0.001 versus control group.

were able to reduce the paw licking time (37.8 ± 7.6 s, 37.8 ± 8.6 s, and 14.4 ± 3.6 s, respectively) when compared to the control group (65.8 ± 5.7 s). A similar reduction was observed with morphine (14.3 ± 6.9 s). In the second phase, the paw licking time also decreased significantly (p <0.001) at all tested doses of IM-7 when compared to the control group (165.9 ± 22.7 s). The obtained values for mice treated with doses of 75 and 150 mg/kg were 21.1 ± 16.8 and 5.5 ± 4.7 s, respectively. Animals that received morphine or IM-7 at the dose of 300 mg/kg did not lick their paws in this phase (Figure 2B).

Effect of IM-7 on glutamate-induced nociception

At all tested doses, IM-7 (75 mg/kg, 16.1 ± 5.1 s; 150 mg/kg, 16.3 ± 8.1 s; 300 mg/kg, 0.75 ± 0.4 s) significantly inhibited nociceptive behavior induced following intraplantar injection of glutamate in mice, when compared to the vehicle-treated group (42.1 ± 9.7 s, p <0.05, Figure 3).

Investigation of the mechanism of action of IM-7

The pretreatment of mice with naloxone (5 mg/kg, s.c., opioid receptor antagonist), sulpiride (20 mg/kg, i.p., D2 dopamine receptor antagonist) or caffeine (10 mg/kg, i.p., adenosine receptor antagonist) 15 min prior to the administration of IM-7 (75 mg/kg, i.p.) significantly inhibited the antinociceptive response induced by IM-7 in the first phase of the formalin test (Figure 4). However, the pretreatment with glibenclamide (10 mg/kg, i.p., K_{ATP} channel blocker), atropine (5 mg/kg, i.p., muscarinic receptors antagonist), yohimbine (0.15 mg/kg, i.p., α_2-adrenergic receptor antagonist) or bicuculline (1 mg/kg, i.p., GABAA receptor antagonist) did not alter the effect

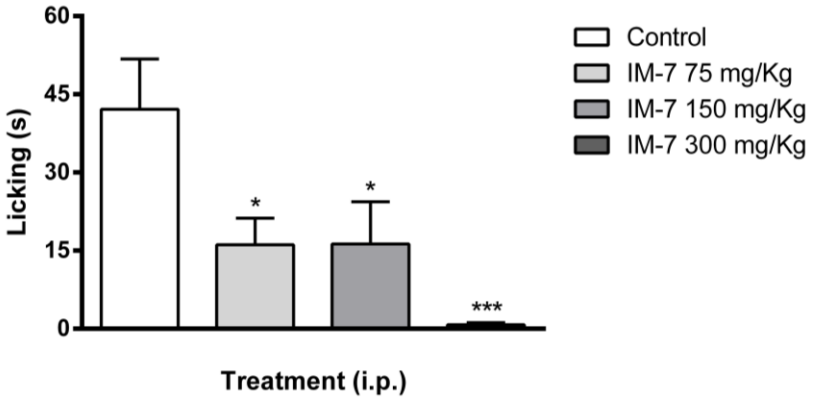

Figure 3. Effect of IM-7 on the licking response induced by intraplantar injection of glutamate in mice. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. *p<0.05, ***p<0.001 versus control group.

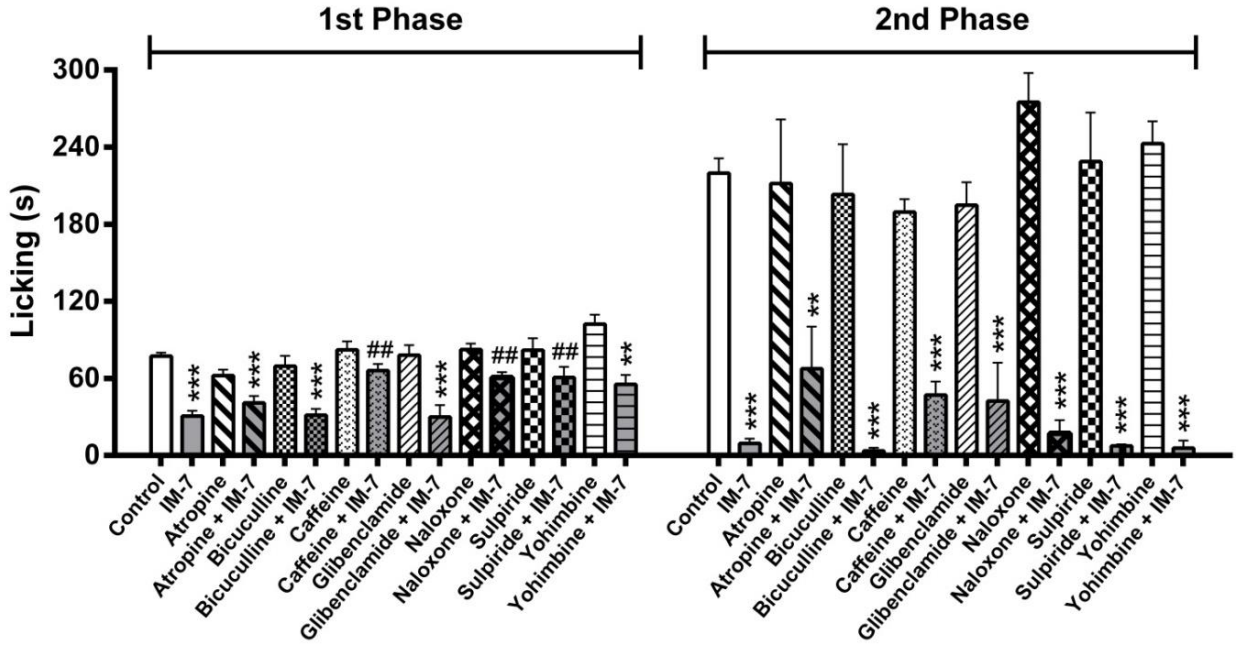

Figure 4. Effect of pretreatment of mice with atropine (5 mg/kg, i.p.), bicuculline (1 mg/kg, i.p.), caffeine (10 mg/kg, i.p.), glibenclamide (10 mg/kg, i.p.), naloxone (5 mg/kg, s.c.), sulpiride (20 mg/kg, i.p.) and yohimbine (0.15 mg/kg, i.p.) on the antinociceptive behavior induced by IM-7 in the formalin test. Animals were pretreated with antagonists 15 min prior treatment with IM-7. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. **p<0.01, ***p<0.001 versus vehicle-treated group; ##p<0.01 when comparing antagonist+IM-7 group with IM-7 group.

of IM-7 in this test (Figure 4).

Effect of IM-7 on carrageenan-induced paw edema

As shown in Figure 5, the administration of IM-7 (i.p.) significantly inhibited carrageenan-induced paw edema from the first hour after carrageenan injection until the fifth hour of observation at all tested doses ($p < 0.05$). This effect persisted until 24 h at a dose of 150 mg/kg, and until 48 h in mice treated with 300 mg/kg.

Effect of IM-7 on carrageenan-induced cellular influx into the peritoneal cavity

As shown in Figure 6, leukocyte migration induced by intraperitoneal carrageenan administration (8.0 ± 0.6 ×

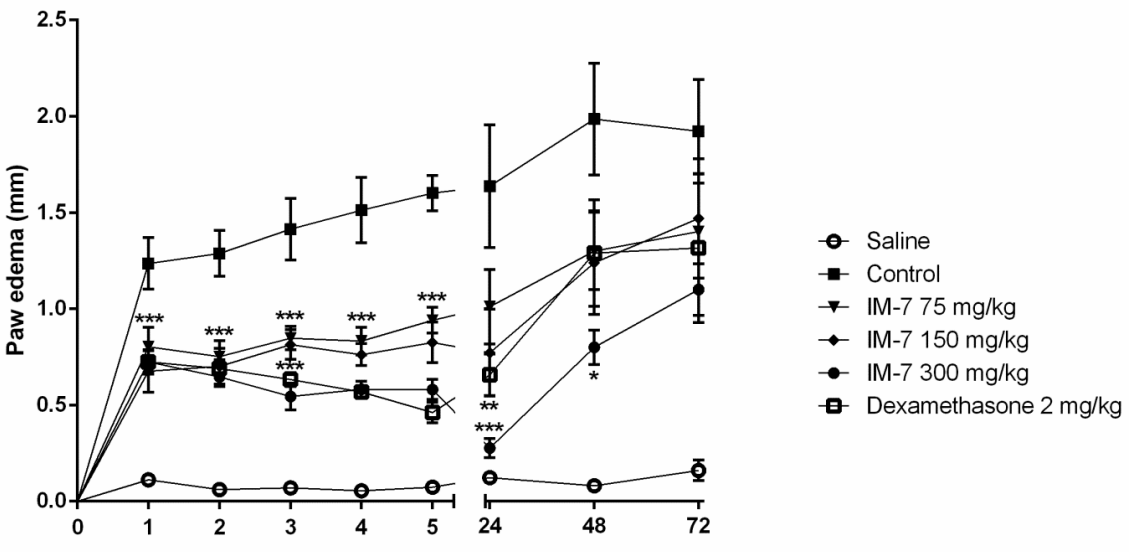

Figure 5. Effect of IM-7 on carrageenan-induced paw edema in mice. Results are shown as mean ± S.E.M., and the % of inhibition when compared to control group is shown in parenthesis ANOVA one-way followed by Tukey's multiple comparisions test. *p<0.05; **p<0.01; ***p<0.001 versus vehicle-treated group.

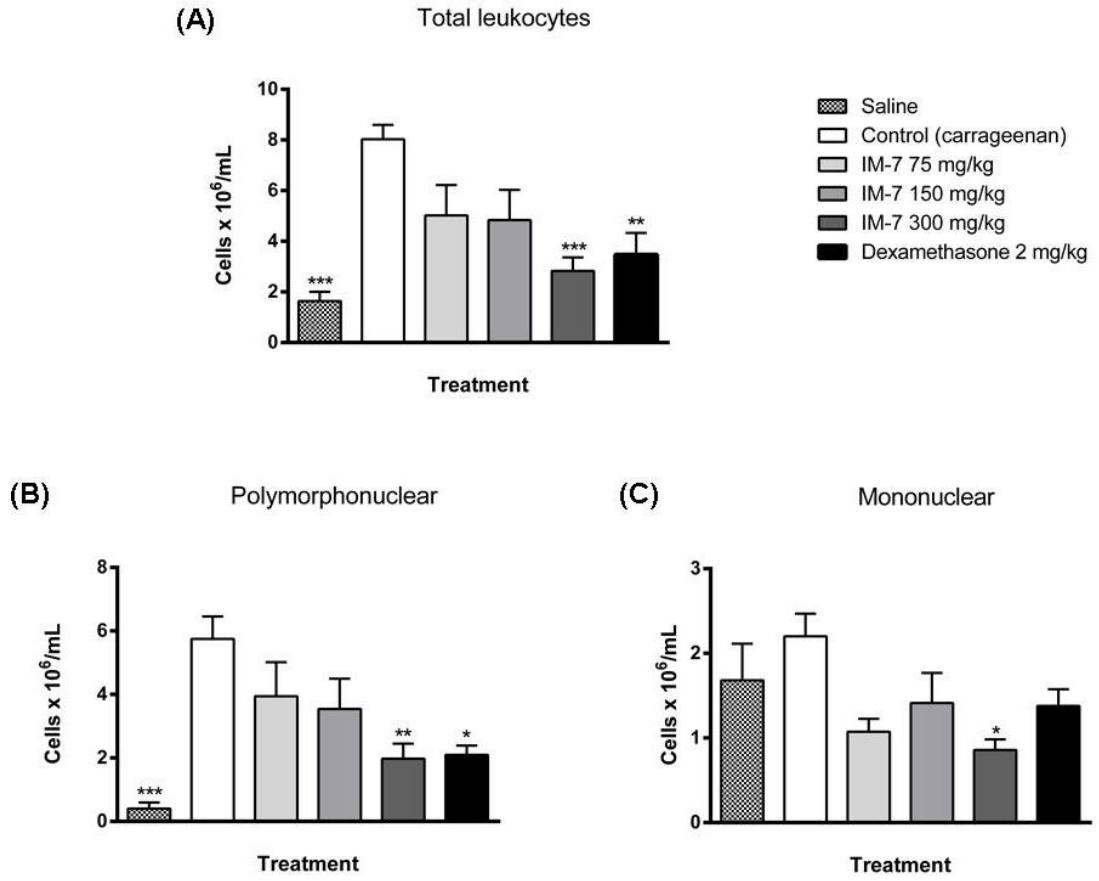

Figure 6. Effect of intraperitoneal treatment of mice with IM-7 on carrageenan-induced inflammatory cells influx into peritoneal cavity. (A) Total leukocyte, (B) Polymorphonuclear cells, (C) Mononuclear cells. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. *p<0.05, **p<0.01, ***p<0.001 versus control group.

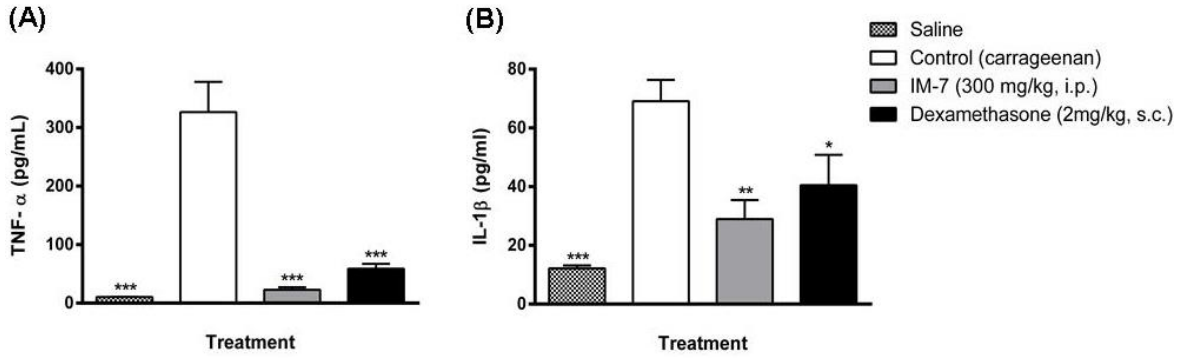

Figure 7. Effect of IM-7 on the levels of the pro-inflammatory cytokines TNF-α (A) and IL-1β (B) in the carrageenan-induced peritonitis model in mice. Each column represents mean ± S.E.M. ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ control group.

10^6 cells/mL) was significantly reduced following treatment with 300 mg/kg of IM-7 ($2.8 \pm 0.5 \times 10^6$ cells/mL; $p < 0.001$). This effect seems to be due to the reduction of the influx of both polymorphonuclear (control, $5.8 \pm 0.7 \times 10^6$ cells/mL; IM-7 300 mg/kg, $2.0 \pm 0.5 \times 10^6$ cells/mL; $p < 0.01$) and mononuclear (control, $2.2 \pm 0.3 \times 10^6$ cells/mL; IM-7 300 mg/kg, $0.9 \pm 0.1 \times 10^6$ cells/mL; $p < 0.05$) cells into the peritoneal cavity. Treatment with doses of 75 and 150 mg/kg of IM-7 did not show significant decreases in cellular migration in this model.

Effect of IM-7 on pro-inflammatory cytokine levels in the peritoneal fluid

Figure 7 shows that intraperitoneal injections of carrageenan significantly increased the amount of the pro-inflammatory cytokines TNF-α (326.4 ± 51.7 pg/mL) and IL-1β (69.1 ± 7.2 pg/mL) in peritoneal exudate when compared to the saline group (10.6 ± 0.3 pg/mL and 12.1 ± 1.0 pg/mL, respectively). Treatment with 300 mg/kg of IM-7 significantly reduced the TNF-α level (93% decrease; $p < 0.001$) when compared to the control group. At the same dose, IM-7 also reduced the IL-1β level (58% decrease; $p < 0.01$) in the peritoneal exudate. Dexamethasone (2 mg/kg), a well-known anti-inflammatory drug, reduced both TNF-α (82% decrease; $p < 0.001$) and IL-1β (41% decrease; $p < 0.05$) levels.

DISCUSSION

In this study, the antinociceptive and anti-inflammatory effects of the hydantoin derivative, IM-7 in mice were investigated. It was observed that i.p. treatment of mice with IM-7 to decrease the number of abdominal contortions induced by the intraperitoneal injection of 0.85% acetic acid. The injection of i.p. acetic acid enhances the release of several pro-inflammatory mediators such as prostaglandin (PG)E_2, $PGF_{2\alpha}$, TNF-α, IL-1β, and IL-8 (Deraedt et al., 1980; Ribeiro et al., 2000). Additionally, it also enhances the release of the excitatory neurotransmitters, aspartate and glutamate, in the cerebrospinal fluid by sensitizing and stimulating primary afferent neurons (Gu et al., 2013).

Despite its high sensitivity to centrally and peripherally acting drugs, the acetic acid-induced writhing test has poor specificity. This is due to substances lacking analgesic effects, like sedatives, antidepressants, antihistamines, muscle relaxants, and neuroleptic agents, being able to inhibit abdominal writhing and, therefore, producing false-positive results (Le Bars et al., 2001). On the other hand, the formalin test, a widely used model of persistent pain, is more selective and permits the differentiation between 2 types of antinociceptive mechanisms: neurogenic and anti-inflammatory (Hunskaar et al., 1985, 1987).

The antinociceptive effect of IM-7 was also detected by the formalin test, since all tested doses significantly reduced the paw licking response in both phases, in similarity to morphine. Centrally acting drugs such as opioids inhibit both phases of the formalin test, while peripherally acting drugs, such as NSAIDs and corticosteroids, only inhibit the second phase (Hunskaar et al., 1987). Thus, these results demonstrated that IM-7 exerts effects both at the central and peripheral level, by interfering with direct nociceptive and inflammatory mediators or receptors. Glutamate is the major excitatory neurotransmitter involved in nociceptive signal transmission. Beirith et al. (2002) demonstrated that the intraplantar injection of glutamate into the mouse paw produces a nociceptive response (licking) and paw edema. These effects seem to be mediated by both ionotropic and metabotropic glutamate receptors and involve peripheral, spinal, and supraspinal sites. When given intraperitoneally, IM-7 significantly inhibits the nociceptive response induced by the peripheral injection of glutamate. To further characterize the underlying mechanisms of the antinociception induced by IM-7, a series of antagonists/blockers of known pathways

involved in pain regulation were employed. The antinociceptive response induced by IM-7 in the formalin test was significantly inhibited by the pretreatment of mice with naloxone, caffeine, or sulpiride. This supports the proposal that opioid, adenosine, and D2 dopamine receptors play a role in the mechanism of action of IM-7, since their activation reduce the transmission of nociceptive impulse. Pretreatment with atropine, yohimbine, bicuculline, or glibenclamide did not modify the antinociceptive effect induced by IM-7, therefore, eliminating the participation of muscarinic, α_2-adrenergic, and $GABA_A$ receptors, as well as K_{ATP} channels. However, Sudo et al., (2010) demonstrated the involvement of α_{2A}-adrenergic receptors in the antinociceptive response evoked by the hydantoin derivative PT-31 in mice. In their study, pretreatment with yohimbine or BRL 44408 (selective α_{2A}-adrenergic receptor antagonists) significantly reduced the effect of PT-31. These results explain only the central antinociceptive mechanism of IM-7, since all antagonists/blockers failed to inhibit the effect of this hydantoin on the second phase of the formalin test. This could be explained by the involvement of anti-inflammatory mechanisms on the antinociceptive activity of IM-7.

Edema formation resulting from the increase in vascular permeability and vasodilatation of the arterioles (Szolcsányi, 1988) allows sensory neurons to release active mediators in the inflammatory process. These substances released by stimulated neurons are considered the neurogenic component of inflammation mediation (Fantini et al., 1995; Maggi and Meli, 1988). This means that the formation of edema is the major consequence of neurogenic inflammation (Bon et al., 2013). The obtained results demonstrated the significant inhibition of paw edema post-carrageenan injection by IM-7 at different times. One of the mechanisms by which this can happen is through the direct blocking of a receptor that contributes to the edema formation, such as transient receptor potential ankyrin 1 (Moilanen et al., 2012), a non-selective cation channel whose activation contributes to the increased excitability of nociceptors and neurogenic inflammation (Story et al., 2003). It is noteworthy that the inflammatory response induced by carrageenan in this model is biphasic. The first phase lasts approximately 6 h and is mediated by the release of histamine, serotonin, and kinins. The late phase involves the release of prostaglandins and is characterized by more pronounced edema that starts approximately 24 h after administration of carrageenan, with peak at 72 h (Henriques et al., 1987; Shibata et al., 1989). All the doses of IM-7 inhibited paw edema in the first 5 h of observation. Furthermore, treatment with doses of 150 mg/kg and 300 mg/kg of IM-7 inhibited paw edema by 53 and 83%, respectively, 24 h after the administration of carrageenan. After 48 h, the inhibitory effect of the 300 mg/kg dose could still be detected, with an inhibition of 60%. However, after 72 h, there was no significant inhibition resulting from IM-7 treatment at the tested doses. According to these results, it seems that IM-7 can inhibit the inflammatory process by reducing the release of mediators involved in both phases of carrageenan-induced paw edema.

Guerra et al. (2011) observed that some hydantoins reduce leukocyte migration and inhibit TNF-α and IL-1β release in air pouch and peritonitis models. At least in part, these anti-inflammatory effects may be attributed to the selective and potent inhibition of the TNF-α converting enzyme (TACE), responsible for the activation of TNF-α (Sheppeck et al., 2007; Yu et al., 2010) or inhibition of the nuclear factor (NF)-kappaB pathway (Wang et al., 2015).

In this study, IM-7 also reduced leukocyte migration and inhibited TNF-α and IL-1β release induced by carrageenan in a peritonitis model; therefore, reinforcing the anti-inflammatory activity of this hydantoin.

Conclusion

The results of this study strongly suggest that the acute intraperitoneal injection of the hydantoin derivative IM-7 in mice induces a dose-dependent antinociceptive response. This response is probably mediated by its interaction with opioid, dopaminergic, and adenosinergic receptors, along with its anti-inflammatory activity resulting in reduced leukocyte migration and TNF-α and IL1-β release.

Conflicts of Interests

The authors have not declared any conflict of interestS.

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Full Length Research Paper

Activity of red wine polyphenols on endothelial *no*-synthase (eNOS)

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Polyphenols (PPH) represent a group of chemical substances found in plants, characterized by the presence of more than one phenol unit. The largest and best studied polyphenols are the flavonoids. Polyphenols contained in red wine have beneficial effects on cardiovascular health. The aim of our research was a comparative and interactive study of four red wine polyphenols - resveratrol (R), quercetin (Q), kaempferol (K) and isorhamnetin (IH) which may have a long term properties as the increase of nitric oxide (NO) synthase expression, acting on the prometer activity when used alone or in equimolar mixtures. To determine their activity, we performed a luciferase reporter gene assay on EA.hy926 cells stably transfected with a luciferase reporter gene construct containing eNOS promoter. The Bradford assay was also performed to evaluate the cytotoxicity and the differences in cell number. The median effect equation, as an interaction analysis evaluating synergy or antagonism of the combinations was done according to the principle of mass-action law and the dose reduction index (DRI) was calculated for all mixtures. All single polyphenols activated eNOS promoter. The EC_{50} values were in micromolar concentrations ranging from 3.44 µM (R^2 = 0.96) for kaempferol to 9.89 µM for isorhamnetin (R^2 = 0.94). All mixtures activated eNOS promoter, but their interactions varied from synergy (Q+R, Q+IH+K, Q+R+K and Q+R+IH+K), through additive (R+IH+K) to antagonistic interaction (R+IH, R+K, Q+IH, Q+K, IH+K and R+Q+IH). In this study, we show for the first time that red wine polyphenols activated eNOS promoter when used alone and in mixtures with different types of interactions.

Key words: Polyphenols, eNOS promoter, luciferase reporter gene assay, interaction analysis.

INTRODUCTION

Growing interest in a polyphenol rich diet has been observed in recent years. It is believed that they can protect against various diseases, e.g. cancers, cardiovascular diseases, diabetes, and some immunological disorders (Haysteen, 2002; Xia et al., 2010). Polyphenols can counteract ROS as well as modulate signaling pathways

to enhance health outcomes in the realm of cardio-vascular disorders and aging. They play a key role of Reactive Oxygen Species in Disease (Sandhya et al., 2013).

Dietary polyphenols are widely distributed in vegetables, fruits and beverages such as tea and wine. Recent studies have demonstrated that polyphenols such as resveratrol, quercetin, epigallocatechin-3-gallate and delphinidin enhance NO output to improve endothelium-dependent vascular relaxation Moderate regular red wine consumption is associated with a reduced risk of cardiovascular diseases and is related with activation of eNOS system at different levels. Moderate ethanol intake from any type of beverage improves lipoprotein metabolism and, lowers cardiovascular mortality risk, but wine, particularly red wine with its abundant content of phenolic acids, polyphenols, and flavonoids seems to confer additional health benefits.

These include high-density lipoprotein cholesterol levels and oxidation of low-density lipoprotein (LDL) cholesterol, antioxidant activity, decreased platelet aggregation and adhesion, as well as improved endothelium-dependent vasodilatation. Many of these effects are compatible with the action of endothelium derived nitric oxide (NO) (Wallerath et al., 2003). In the development of atherosclerosis, reduced bioavailability of NO, formed by endothelial nitric oxide synthase (eNOS) precedes the appearance of visible vessel alterations (Li and Förstermann, 2000). Thus, improved NO bioavailability would be a promising step in the therapy and prevention of cardiovascular disorders (Räthel et al., 2007). As the long-term treatment of cultured endothelial cells with red wine polyphenols induces eNOS expression and causes a sustained increase in endothelial NO production. The up regulation of eNOS is probably based on synergistic mechanisms between the different polyphenolic components (Schmitt and Dirsch, 2009).

By contrast of the total contents of herbal product showing better effect than an equivalent dose of a single isolated active ingredient (Ma et al., 2009). There is an increasing awareness that analyses of single components are not always adequate to clearly assess the health benefits of natural product mixtures from dietary sources, since they involve interaction effects (Kurin et al., 2012). Interactions are generally described as being synergistic or antagonistic.

The aim of our research was an interaction study of four red wine polyphenols (Resveratrol: R, Quercetin: Q, Kaempferol: K, Isorhamnetin: IH) on eNOS promoter activation in endothelial EA.hy926 cells using median effect equations, where the effects of single compounds and their equimolar mixtures were determined and the interactions of combinations were evaluated according to general equation for the single drug effect extended to the multiple drug effect equation fo$_r$ n drugs. These equations provide the theoretical basis for the combination index (CI)-isobologram equation that allows quantitative determination of drug interactions, where CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively (Chou, 2006).

MATERIALS AND METHODS

Cell culture

The human endothelial cell line EA.hy926 (Edgell et al., 1983), stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo (Li et al., 1998) containing 3600 base pairs of the human eNOS promoter driving a luciferase reporter gene (EA.hy926-heNOS-Luc) were used for measuring the eNOS promoter activity.

Luciferase reporter gene assay

Stably transfected EA.hy926-heNOS-Luc cells were grown in Dulbecco's modified Eagle's medium without phenol red supplemented with 584 mg/ml glutamine, 100 U/ml benzyl penicillin, 100 mg/ml, streptomycin (Lonza, Belgium), HAT supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine) (Biochrom, Germany) and 10% heat-inactivated foetal bovine serum (Gibco via Invitrogen, UK) until passage 15. For the experiments, the cells were seeded for 24 h in 96-well plates at a density of 4×10^5 cells/well and were stimulated with polyphenols - resveratrol (99% purity), quercetin (98% purity) (Sigma-Aldrich, USA), kaempferol (99% purity) and isorhamnetin (99% purity) (Carl Roth, Germany) dissolved in dimethyl sulfoxide (DMSO). Phorbol-12-myristate-13-acetate (PMA) (Alexis Biochemicals, Austria) was used as a reference (positive control) and the final DMSO concentrations in all treatment did not exceed 0.1%. Control cells were always treated with an equal volume of solvent. The concentration of single polyphenols used was 3 to 100 µM and their equimolar combinations final mixture concentrations were 1 to 30 µM (e.g., the 30 µM final equimolar combination Q+R was composed of 15 µM of R and 15 µM of Q). After 18 h incubation with the respective compounds, the cells were washed with PBS and lysed with lysis buffer (Promega, Germany). To determine eNOS promoter activity, the luminescence generated from the luciferase activity was measured using Tecan Genios Pro (Tecan, Austria) plate reader. The values were then normalized to the protein level determined by the Bradford assay as described by Bradford with slight modifications (Bradford, 1976).

Statistical and interaction analysis

All data were obtained in three independent experiments performed in quadruplets. Data are expressed as mean ±SD. Differences between groups for statistical significance were evaluated by ANOVA with *Bonferroni post hoc test* using GraphPad Prism

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Table 1. Activation of eNOS promoter with single wine polyphenols.

Compound	EC_{50} (µM)	R^2
Resveratrol (R)	4.07	0.93
Quercetin (Q)	6.92	0.93
Isorhamnetin (IH)	9.89	0.94
Kaempferol (K)	3.44	0.96

software (version 5.01, GraphPad Software, Inc., La Jolla, CA, USA). P-values < 0.05 were considered significant.

The concentration of sample leading to 50% effect (EC_{50}) was calculated from the dose-effect relationship of polyphenols effect on eNOS promoter activation using GraphPad Prism software. The interaction analysis evaluating synergy or antagonism of the combinations was done according to mass - action law principle (Chou, 2006), described by Equation (1) for n-drug combination at x% inhibition, using combination index (CI) for interaction interpretation

$$^{n}(\mathrm{CI})_x = \sum_{j=1}^{n} (D)_j / (D_x)_j \quad (1)$$

^{n} (CI)x: is the sum of the dose of n drugs that exerts x% inhibition in a combination.

In the denominator (Dx): is for D "alone" that inhibits a system x%. If CI value is =, > or < 1, an additive, synergistic or antagonistic effect is indicated.

The *dose-reduction index* (DRI) means how many-fold the dose of each drug in a synergic combination could be reduced at a given effect level compared with the doses of each drug alone. The DRI value for each corresponding drug was given for n-drug combinations:

$$(\mathrm{DRI})_1 = \frac{(D_x)_1}{(D)_1};\ (\mathrm{DRI})_2 = \frac{(D_x)_2}{(D)_2} \quad (2)$$

Value of DRI > 1 indicates a favorable dose reduction, and the higher DRI value indicates the higher dose reduction for a given therapeutic effect, but does not necessarily always indicate synergism. Both CI and DRI were calculated using a median-effect analysis by CompuSyn software (version 1.0.1, ComboSyn, Inc., Paramus, NJ, USA).

RESULTS

All polyphenols activated eNOS promoter, the EC_{50} values were in micromolar concentration ranging from 3.44 µM (R^2 = 0.95) for kaempferol to 9.89 µM for isorhamnetin (R^2 = 0.94) (Table 1). EC_{50} (effective concentration) means the concentration (in µM) of the compound leading to the half maximal effect. EC_{50} and R^2 (value quantifying the goodness of fit) were calculated using Graph Pad Prism (version 5.01, USA).

The ability of resveratrol to activate eNOS promoter described in two previous studies (Wallerath et al., 2002, 2005) and our results (Table 1) are in accordance with them.

The results of Q+R and IH+K isobolograms analysis were in concordance with the CI analysis where the CI for Q+R was 0.65 (By the definition CI < 1 indicates synergy) and CI for IH+K was 2.17 (By the definition CI > 1 indicates antagonism). We can see in Table 2, that four mixtures acted synergic (Q+R, Q+IH+K, R+Q+K and Q+R+IH+K) where the CI vary from 0.65 to 0.87. One mixture effect was nearly additive: R+IH+K with CI = 1.08 and other six mixtures were antagonistic with CI from 1.16 to 2.01.

DISCUSSION

NO is one of the main mediators of vasodilatation. Its decreased level plays a central role in endothelial dysfunction. In mammalians, endothelial NO is produced by the enzyme eNOS, which converts L-arginine in the presence of O_2 and NADPH into L-citrulline and NO (Appeldoorn et al., 2009). The generation of NO plays a major role in maintaining cardiovascular homeostasis by governing blood pressure, improving endothelial function, suppressing vascular smooth muscle mitogenesis, inhibiting leukocyte adhesion and platelet aggregation.

Dietary polyphenols are widely distributed in vegetables, fruits and beverages such as tea and wine. Average total of polyphenol content measured by the Fooling method is 216 mg/100 ml for red wine and 32 mg/100 ml for white wine. The content of phenols in rosé wine (82 mg/100 ml) is intermediate between that in red and white wines.

Recent studies have demonstrated that polyphenols such as resveratrol, quercetin, epigallocatechin-3-gallate and delphinidin enhance NO output to improve endothelium-dependent vascular relaxation (Xu et al., 2004).

Moderate regular red wine consumption or the consumption of red grapes, fruit, cereals, several vegetables such as red onions, chocolate, tea, and coffee with different polyphenolic composition (Bravo, 1998; Tsao, 2010) is associated with a reduced risk of cardiovascular diseases and is related with activation of eNOS system at different levels (Wallerath et al., 2005).

In this study, we investigated the influence of resveratrol, quercetin, kaempferol and isorhamnetin: Polyphenols present in red wines, on the eNOS promoter activity. Both individual substance or in their equimolar mixtures were investigated. Further, we evaluated their interactions when used in combinations.

As it was described in Materials and Methods, first of all we explored activity of single polyphenols on eNOS promoter activation in four different concentrations of polyphenols (3-10-30-100 µM) and from the dose-effect relationship the EC_{50} values using GraphPad Prism software were determined.

As it is seen in Table 1, all polyphenols activated eNOS promoter, where the EC_{50} values were in micromolar concentration ranging from 3.44 µM (R^2 = 0.96) for kaempferol to 9.89 µM for isorhamnetin (R^2 = 0.94).

Table 2. EC_{50} , CI, and DRI values of polyphenol mixtures at 50% effect dose level.

Polyphenol mixture	EC_{50} (µM)	R^2	CI	Interaction	DRI
R+Q	3.30 (1.65 : 1.65)	0.97	0.65	Synergy	2.5 : 4.2
R+IH	6.75 (3.38 : 3.38)	0.99	1.16	Slight antagonism	1.2 : 3.0
R+K	4.50 (2.27 : 2.27)	0.95	1.22	Moderate antagonism	1.8 : 1.5
Q+K	5.41 (2.71 : 2.71)	0.89	1.18	Slight antagonism	2.6 : 1.3
Q+IH	11.39 (5.74 : 5.74)	0.96	1.41	Moderate antagonism	1.2 : 1.7
IH+K	11.09 (5.54 : 5.54)	0.95	2.17	Antagonism	1.8 : 0.6
R+Q+IH	7.30 (2.43 : 2.43 : 2.43)	0.99	1.20	Slight antagonism	1.7 : 2.8 : 4.1
Q+IH+K	4.88 (1.63 : 1.63 : 1.63)	0.95	0.87	Slight synergy	4.2 : 6.1 : 2.1
R+IH+K	5.04 (1.68 : 1.68 : 1.68)	0.97	1.08	Additivity	2.4 : 5.9 : 2.0
R+Q+K	3.50 (1.16 : 1.16 : 1.16)	0.97	0.79	Moderate synergy	3.5 : 5.9 : 2.9
Q+R+IH+K	3.60 (0.91 : 0.91 : 0.91 : 0.91)	0.97	0.71	Moderate synergy	7.6 : 4.5 : 10.8 : 3.8

Polyphenols equimolar mixtures: R - resveratrol; Q - quercetin, IH - isorhamnetin, K - kaempferol. EC_{50} (effective concentration) means the concentration (in µM) of the compound leading to the half maximal effect. **EC_{50}** and **R^2** (value quantifying the goodness of fit) were calculated using Graph Pad Prism (version 5.01., USA). **CI** - combination index, based on the mass-action law is quantifying drug interaction in terms of synergy (CI <1), additivity (CI =1) or antagonism (CI >1). **DRI** represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone. **CI** and **DRI** were calculated using CompuSyn software (version 1.0.1, USA). Interactions are determined according to Chou (2006).

Wallerath et al. (2005) demonstrated that quercetin has no effect on eNOS promoter activity up to 33 µM. However, in our experiment, we found out that quercetin activated eNOS promoter (EC_{50} 6.92 µM; R^2 = 0.93). We also demonstrated that quercetin activates eNOS promoter not only alone but also in mixtures with other red wine polyphenols (Tables 1 and 2).

It is known that kaempferol significantly induces NO production in endothelial cells (Chen et al., 2010) and isorhamnetin has shown inhibitory effect on ox-LDL induced eNOS down regulation (Bao and Lou, 2006), but for the first time we described that kaempferol and isorhamnetin activate eNOS promoter (Table 1).

Resveratrol has been shown to enhance the expression of eNOS modulate the deacetylation of eNOS and increase the plasma NO levels (Wang et al., 2012). The ability of resveratrol to activate eNOS promoter was described in the two previous studies (Wallerath et al., 2002, 2005) and our results (Table 1) are in accordance with them.

It is also known that resveratrol has protective effects on multi-targets related to cardiovascular diseases. It seems that a drug targeting multiple points may exhibit better therapeutic efficacy than one target blocking or activating in complex conditions. Common disorders such as cardiovascular diseases tend to result from multiple molecular abnormalities (Wang et al., 2012), thus multi-targeting drugs or combinations of drugs seem to bring much more efficiency into therapy or prevention. We prepared binary, tertiary and quaternary mixtures of tested polyphenols in four concentrations (1-3-10-30 µM), where the contribution of each part was always equimolar (e.g. the 30 µM final equimolar combination Q+R was composed of 15 µM of R and 15 µM of Q) and gave the same final molar concentration of the mixture as the single compound samples. As it is shown in Table 2, EC_{50} values of polyphenols mixtures ranged from 3.31 µM (R^2 = 0.97) for R+Q to 11.09 µM (R^2 = 0.95) for IH+K.

When we used the combination index analysis based on the mass-action law for quantifying drug interactions, we were able to determine not only binary mixtures interactions but also perform n>2-drug combinations interactions analysis.

The interaction studies, which determine synergy or antagonism of substances, are relatively well known for at least three decades amongst antioxidants. Nevertheless, there is no information about red wine polyphenols interactions related to eNOS pathway.

Räthel et al. (2007) investigated apart from resveratrol also red wine polyphenol extracts (RWPE) from 180 wine types. Using luciferase reporter gene expression (Kurin et al., 2013) as an indicator for eNOS promoter activity they found out that all RWPE under investigation increased eNOS promoter activity, but the biological activity was dependent on an individual polyphenol pattern.

When they compared the RWPE results with resveratrol, they discovered that resveratrol mimics the effects of RWPE at concentrations higher than that calculated to be present in analyzed wines and thus, resveratrol alone does not account for the observed effects of RWPE. Thus, synergy with other compounds in red wine is suggested (Räthel et al., 2007). Chan et al. have shown that the effects of ethanol on NO production and inducible nitric oxide synthase (iNOS) gene expression in murine macrophage cells (RAW 264.7) was synergistically increased when combined with quercetin and resveratrol in reducing NO production by both scavenging NO and reducing iNOS gene expression (Chan et al., 2000). We found out that quercetin with resveratrol act synergistically in eNOS promoter activation (Table 2). This is in

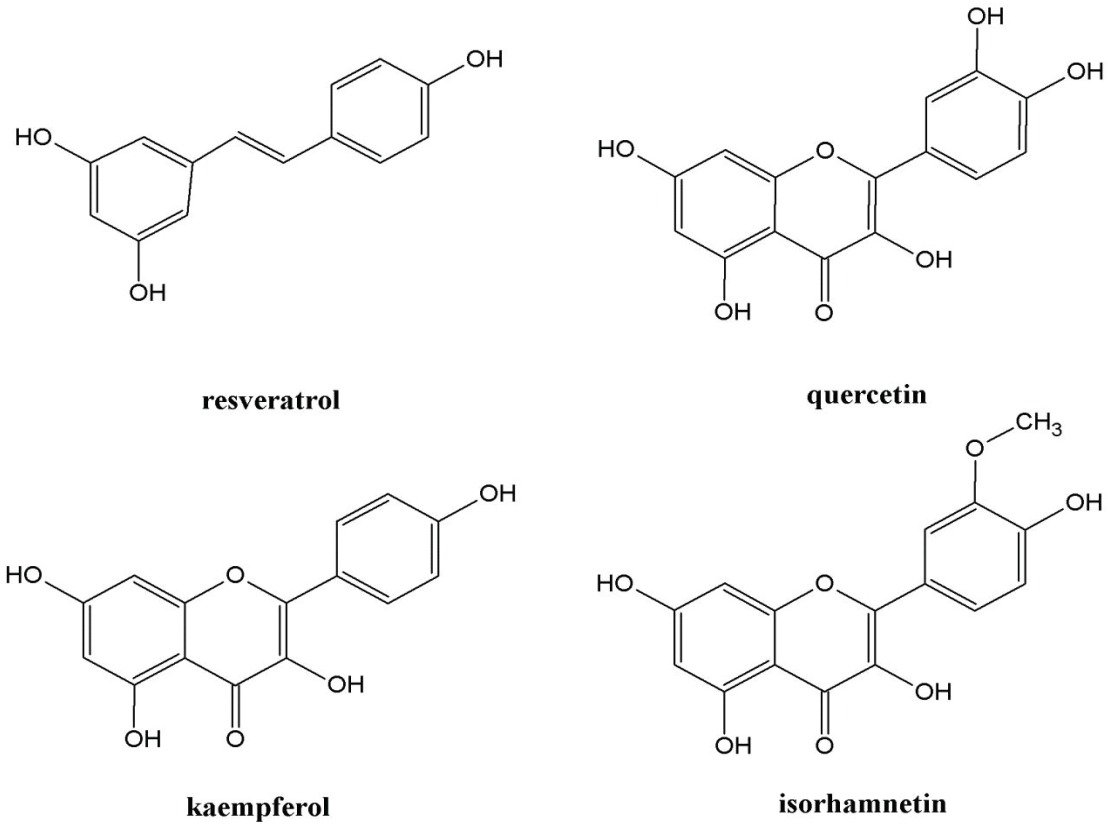

Figure 1. Structures of resveratrol (R), quercetin (Q), kaempferol (K) and isorhamnetin (IH).

accordance with the results where resveratrol and quercetin, synergistically inhibited vascular smooth muscle cell proliferation when used in a mixture (Kurin et al., 2012).

Besides CI we determined the DRI as well. DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared lowed in combination for a given degree of effect as compared with the dose of each drug alone, or in other words it indicates to what extent the concentration of drug can be reduced in a mixture in order to achieve a given effect level compared with a single drug treatment. DRI values higher than 1 are desirable, but they do not necessarily indicate synergy. As seen in Table 2, in the Q+R mixture are DRI values 2.5 for quercetin and 4.2 for resveratrol, what means that in Q+R mixture we needed 2.5 times lower dose of quercetin and 4.2 times lower dose of resveratrol to achieve the same effect that would be reached by the single compound treatment.

Despite we are not able to explain the inner mechanism of interactions among tested red wine polyphenols in eNOS promoter activation, we've take into account that as the eNOS promoter activation involves multiple processes, the interference with multiple different targets is needed. Herbal drugs as complexes of substances or prepared mixtures of natural compounds open the possibility of novel multicomponent treatment or prevention approach development through synergistic interactions, which could impact multiple targets simultaneously, thus being better suitable for controlling complex diseases or biochemical pathways such as eNOS (Zimmermann et al., 2007).

In a small experimental model, we have shown that red wine polyphenols when used in mixtures are needed in a smaller amount and reach many times higher effects that is single molecule able to. "French paradox" until today has not been explained by a single effective molecule, our work suggests that the positive effects of red wine on cardiovascular system should be explained by the synergy of polyphenols mixtures present in red wine, thus despite their low concentration, their effects could be given by their cooperation in multiple system.

Conclusion

Resveratrol, quercetin, kaempferol and isorhamnetin, the substances present in red wine, can activate eNOS promoter when used alone or in equimolar mixtures Figure 1. The interaction study of red wine polyphenols indicated that in eNOS promoter activation, the final effects of mixtures vary from synergistic to antagonistic.

Currently, the mechanism of their interaction is not known. However, when they are used together in a quaternary mixture, the final effect is synergic. In summary, the presented data support the idea that red wine contains unique polyphenolic constituents that may increase eNOS expression and thus endothelial NO output. More work is needed, however, to determine the bioavailability and pharmacokinetics of polyphenols and to identify metabolites of red wine components that may mediate red wine activity.

Conflict of interest

The authors declare no conflict of interest.

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Full Length Research Paper

In vitro acaricidal activity of crude extracts of *Schinus molle (L.)* leaves against field population of *Bophilus decoloratus* and *Rhipicephalus pulchellus* ticks

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Plant products are a rich source of bioactive organic chemicals and offer an advantage over synthetic pesticides as these are less toxic, less prone to development of resistance and easily biodegradable. The present study aimed at screening the acaricidal potential of crude methanolic and aqueous extracts of *Schinus molle (L.)* leaves on the adult *Bophilus decoloratus* and *Rhipicephalus pulchellus* cattle ticks, using *in vitro* immersion method. Freshly collected adult ticks were exposed to three graded concentrations of the crude extracts; 1% (1g/100ml), 2% (1g/50ml), and 4% (1g/25ml) for 24 h and mortality rates were recorded post exposure for each concentration every 3 h. Diazinon (0.2%) and distilled water were used as positive and negative controls respectively. Acaricidal activities of each concentration were measured by mean number of ticks died and antiparasitic efficacy (%) relative to the negative control. Analysis result indicated that highest (4%) and middle (2%) concentrations of both extracts caused a statistically significant (P<0.05) killing effect on *R. pulchellus* and *B. decoloratus* for most of the observation hours as compared to the extract unloaded *in vitro* groups. The relative antiparasitic efficacy (%) was highest for 4% concentration of both extracts (100%). The standard acaricide failed to completely eliminate the parasites after 24 h of exposure although it showed a slightly better effect against *B. decoloratus* (96.7%) compared to *R. pulchellus* (93.3%). Put together, this finding showed that the crude extracts of the plant have promising acaricidal properties and warrant further investigation.

Key words: Acaricidal, crude extracts, *Boophilus decoloratus, Rhipicephalus pulchellus, Schinus molle.*

INTRODUCTION

Ticks are destructive blood sucking ecto-parasites of livestock and wild animals species causing huge economic losses, thus creating food insecurity (Habeeb, 2010), with an estimated global cost of control and productivity losses of 7 billion US-Dollar annually (Nchu et al., 2012). Their effects are diverse, including reduced growth, milk production, paralysis/toxicosis, and trans-mission of tick-borne pathogens that reduce production or cause mortality, extensive damage to body surfaces exposing animals to secondary attacks from other

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parasites and microbial infections (Walker et al., 2003). In Ethiopia, ticks and tick borne diseases cause considerable losses to the livestock economy (Bayu, 2005).

Tick control worldwide is based mainly on the repeated use of acaricides, which have resulted in problems related to environmental pollution, milk and meat contamination, and the development of resistance leading to increased cost of control (Habeeb, 2010; Pirali-Kheirabadi and Teixeira da Silva, 2011). Synthetic chemicals have also been reported to have genotoxic and cytotoxic effects on human target cells (Pirali- Kheirabadi and Razzaghi-Abyaneh, 2007). Thus, there is an urgent need for new parasitic control strategies to overcome the drawback associated with the use of synthetic drugs. One alternative control strategy could be phyto-therapy, an important component of ethno-veterinary medicine (Madzimure et al., 2011). The use of ethno-veterinary botanicals is sustainable and ecologically sound because the plants are locally available, potentially easy to be produced, locally processed and used by farmers themselves (Habeeb, 2010).

The use of botanicals for the control of ticks is compatible with traditional practices in Africa, where most resource poor-farmers use plant materials to treat endoparasites and ectoparasites of livestock (Nchu et al*.,* 2012). Acaricidal activity of crude extracts from stem and leaves of different plants against cattle ticks has been reported (Habeeb, 2010; Rosado-Aguilar et al., 2010; Madzimure et al., 2011; Ravindran et al., 2011; Kalume et al., 2012; Vongkhamchanh et al., 2013; Dehghani-Samani et al., 2015).

Schinus molle L., commonly known as pink pepper or American pepper is a tree belonging to the Anacardiaceae family which is native to subtropical regions of South America. It is a short tree with thin, long leaves often used in subtropical climates for landscaping (Taylor, 2005). *Schinus* species has been traditionally used as medicine by indigenous people throughout the tropics (Erazo et al., 2006). In traditional cuisine, *S. molle* fruits (berries) have been used as a replacement for black pepper and also to prepare alcoholic drinks and beverages (Marongiu et al., 2004).

In folk medicine, *S. molle* has been used due to its antibacterial, antiviral, topical antiseptic, antifungal, antioxidant, anti-inflammatory, anti-tumoural, anti-spasmodic, analgesic properties, as well as a stimulant and an antidepressant (Alanís-Garza et al., 2007; Machado et al., 2007; Molina-Salinas et al., 2007; Guala et al., 2009). Pharmacological studies carried out with extracts from the plant validated its therapeutic properties for different ailments (Erazo et al., 2006; Machado et al., 2007; Kasimala, 2012), but there is no evidence of any effect against ticks.

In Somali Regional State of Ethiopia, *S. molle (*Qundo berbere-Amaharic and Mirmir-Somali) is well used against ticks by pastoralists and agro-pastoralists. Based on this traditional claim, this preliminary work aimed at evaluating the *in vitro* acaricidal activity of crude methanolic and aqueous extracts of leaves of *S. molle (L.)* against adult *Rhipicephalus pulchellus* and *Boophilus decoloratus* ticks.

MATERIALS AND METHODS

Study design

This investigation employed an experimental study design; a laboratory based *in vitro* acaricidal activity test of crude methanolic and aqueous extracts of leaves of *S. molle* using an *in vitro* immersion method (IIM) as described by Vongkhamchanh et al. (2013).

Plant extracts preparation

Fresh leaves of the plant collected from Jigjiga university botanical garden was cleaned, shade dried, mechanically grinded and coarsely powdered using laboratory mortar and pestle. The powdered specimen was then subjected to extraction using two solvents; methanol and distilled water to obtain the crude methanolic and aqueous extracts respectively. The extracts were prepared by cold maceration technique. A total of 250 g of the coarsely powdered plant materials were separately soaked in each extraction solvents (100 g of powder in 1000 ml of methanol or distilled water) followed by shaking periodically for three days and then filtered. This was repeated three times to allow the solvents extract substantial quantities of the chemical constituents from the pounded plant materials. The mixture was first filtered using gauze and then the filtrate was passed through sterile filter paper (Whatman No. 3, Whatman Ltd. England). The filtered extracts were then dried in hot air oven. Finally, the resulting extracts were transferred into well labeled vials and kept in a refrigerator until required for use.

In vitro acaricidal activity test

The *in vitro* acaricidal efficacy study was conducted using IIM on two dominant adult tick species infesting cattle in and around Jigjiga. Accordingly, unattached adult *R. pulchellus* and *B. decoloratus* were collected from cattle extensively reared in the vicinity of Jigjiga city, Eastern Ethiopia. All collected ticks were examined under stereomicroscope and identified to the species level using the taxonomic key described by Kaiser (2000). Adult ticks of equal size were then divided into 3 replicates of 10 each and immersed into extract concentrations of 1 (1g/100ml), 2 (1g/50ml), and 4% (1g/25ml) and incubated in petri dishes, kept at room temperature and 75% relative humidity to observe for acaricidal activity. Diazinon (0.2%) and distilled water were used as positive and negative controls respectively. The number of ticks alive or dead was counted every 3 h after exposure for 12 h and finally after 24 h. The experiment was repeated three times for precision and mean value was taken for the analysis. The antiparasitic acaricidal efficacy of each treatment was calculated using the following equation (Wang et al., 2009):

$$AE = [B - T] \times 100\%/B$$

Where AE is the antiparasitic efficacy, B is the mean number of surviving ticks in the untreated control, and T is the mean number

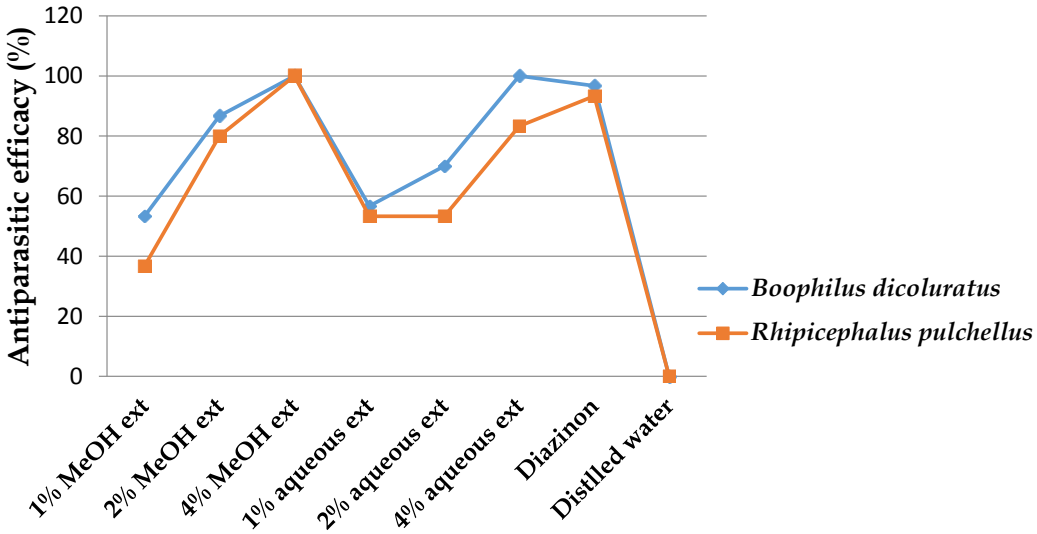

Figure 1. Relative antiparasitic efficacy (%) of graded concentrations of crude methanolic and aqueous extracts of leaves of *Schinus molle* against adult *Rhipicephalus pulchellus* and *Boophilus decoloratus* ticks. MeOH= methanol; ext= extract.

of surviving ticks in treatment group.

Statistical analysis

Data were organized, edited and analyzed using statistical package for social sciences (SPSS) Version 20. Results generated from the investigation were expressed using descriptive statistics (mean ± standard error of mean, percentage, and graph). One way analysis of variance (ANOVA) was employed for analysis of differences between the *in vitro* groups. Results were deemed statistically significant if p≤0.05 at 95% confidence intervals.

RESULTS

The results of this study are shown in Tables 1 and 2 as mean ± SEM of dead ticks at graded concentrations of crude extracts of the plant. The analysis result indicated that both extracts of leaves of *S. molle* produced a relatively comparable acaricidal effects against both species of ticks when compared with the conventional acaricide, diazinon. The activity increased with concentration and time. After 3 h of exposure, only the highest concentration of the methanolic extract produced a significant tick killing effect as compared to the negative and positive controls (*P<0.05*). As measured by mean number of ticks died and antiparasitic efficacy (%), the methanolic extract appeared to be superior to the aqueous extract in eliminating both *R. pulchellus* and *B. decoloratus* ticks under the employed *in vitro* condition (Figure 1). This was particularly true at higher methanolic extract concentrations, 2 (1g/50ml) and 4% (1g/25ml). The standard acaricide failed to completely eliminate the parasites after 24 h of exposure. It, however, showed a slightly better effect against *B. decoloratus (*96.7%) compared to *R. pulchellus* (93.3%). None of the extract unloaded ticks, that is, those exposed only to distilled water, died after 24 h of *in vitro* exposure.

In vitro acaricidal activity of the crude extracts against *R. pulchellus*

All concentrations of methanolic extract and only the highest concentration of aqueous extract elicited mortality after 3 h of exposure but progressively the ticks started dying at 6 h (Table 1). The analysis result indicated that the 4% methanolic extract (from 3 through 24 h of exposure) caused a significant acaricidal effect (P<0.05) as compared to the negative control and diazinon. Whereas, the highest (4%) and middle (2%) concentrations of aqueous extract elicited a considerable level of *R. pulchellus* mortality post 9 h of exposure as against the negative control (P<0.05). Mean number of surviving ticks (6.33±1.15) was highest in the *in vitro* groups which were exposed to 1% methanolic extract, as compared to groups loaded with other concentrations of both extracts at the end of observation period.

In vitro acaricidal activity of the crude extracts against *B. decoloratus*

Table 2 shows the *in vitro* acaricidal effect of graded

Table 1. *In vitro* tick killing effect of crude leaf extracts of *S. molle* against *R. pulchellus.*

Treatment	Concentration	Time (hour) post exposure/Mean number of ticks died (Mean±SEM)						
		3	6	9	12	24		
						MND	MNS	AE (%)
Methanol extract	1%	0.67±0.57	1.00±0.00	1.33±0.57	2.33±0.57^{b}	3.67±1.15^{ab}	6.33±1.15^{a}	36.7
	2%	0.67±0.57	1.33±0.57	2.33±0.57	5.33±1.52^{acd}	8.00±1.00^{a}	2.00±1.00^{acd}	80
	4%	1.67±0.57^{abd}	5.00±1.00^{abcd}	5.00±1.00^{abc}	7.33±1.53^{acd}	10.00±0.00^{a}	0.00±0.00^{bcd}	100
Aqueous extract	1%	0.00±0.00	1.67±1.15	3.00±1.00	5.00±1.00^{a}	5.33±0.57^{ab}	4.67±0.57^{ad}	53.3
	2%	0.00±0.00	0.67±0.57	2.00±1.00^{a}	5.00±1.00^{acd}	8.00±1.00^{a}	4.67±3.78^{acd}	53.3
	4%	0.33±0.57	1.67±1.15	3.67±1.53^{a}	6.00±1.00^{acd}	8.33±1.15^{a}	1.67±1.15^{acd}	83.3
Diazinon	2ml	0.33±0.57	1.00±1.00	2.33±0.57	6.00±1.00^{acd}	9.33±0.57^{a}	0.67± 0.57^{ac}	93.3
Distilled water	2ml	0.00±0.00	0.00±0.00	0.00±0.00^{d}	0.00±0.00^{bcd}	0.00±0.00	10.00±0.00^{bcd}	0

Values are mean ± SD; NE= Number of ticks exposed; MNS=Mean number of ticks survived; MND= Mean number of ticks died; AE= Antiparasitic Efficacy. All superscripts indicate significance at $p < 0.05$ (^{a} compared to untreated; ^{b} compared to diazinon group; ^{c} compared to lowest methanolic extract concentration; ^{d} compared to lowest aqueous extract concentration).

Table 2. *In vitro* tick killing effect of crude leaf extracts of *S. molle* against *B. decoloratus.*

Treatment	Concentration	Time (hour) post exposure/Mean number of ticks died (Mean± SEM)						
		3	6	9	12	24		
						MND	MNS	AE (%)
Methanol extract	1%	1.00±1.00	1.00±1.00	1.67±1.15^{b}	4.00±1.00^{ab}	4.67±1.15	4.67±1.54^{a}	53.3
	2%	1.33±0.57	1.67±0.57	4.00±1.00^{a}	6.00±1.00^{a}	8.67±1.52^{acd}	1.33±1.52^{acd}	86.7
	4%	2.00±0.00^{a}	4.67±1.5^{acd}	5.67±0.57^{ac}	8.00±2.00^{acd}	10.00±0.00^{acd}	0.00±0.00^{acd}	100
Aqueous extract	1%	0.00±0.00	0.33±0.57	2.33±1.52	3.33±1.15^{bc}	5.67±0.57	4.33±0.57^{a}	56.7
	2%	0.00±0.00	1.67±0.57	2.67±0.57	6.00±1.00^{a}	7.00±1.00^{a}	3.00±1.00^{a}	70
	4%	0.33±0.57	3.67±0.57^{ad}	5.33±1.15^{a}	8.67±1.52^{acd}	10.00±0.00^{acd}	0.00±0.00^{acd}	100
Diazinon	2ml	1.00±1.00	2.00±2.00	5.67±2.51^{a}	9.00±1.00^{acd}	9.67±0.57^{a}	0.33±0.57^{acd}	96.7
Distilled water	2ml	0.00±0.00	0.00±0.00	0.00±0.00^{b}	0.00±0.00^{bc}	0.00±0.00^{b}	10.00±0.00^{cd}	0

Values are mean ± SD; NE= Number of ticks exposed; MNS=Mean number of ticks survived; MND= Mean number of ticks died; AE= Antiparasitic Efficacy. All superscripts indicate significance at $p < 0.05$ (^{a} compared to untreated, ^{b} compared to diazinon group, ^{c} compared to lowest methanolic extract concentration, ^{d} compared to lowest aqueous extract concentration).

concentrations of crude extracts of *S. molle* leaves against *B. decoloratus*.

It was noted that both extracts produced a concentration dependent tickicidal effect on this species showing a strong concentration–effect relationship. Both extracts produced maximum

efficacy only at their highest concentration as suggested by mean number of ticks died after 24 h of exposure (10.00±0.00).

For the *B. decoloratus,* the highest concentration of methanolic extract seemed to be superior to the highest concentration of aqueous extract at different time duration*.* ANOVA results indicated that highest concentration of both extracts caused a statistically significant (*P<0.05*) *B. decoloratus* killing effect for most of the observation hours as compared to the negative controls. The relative antiparasitic efficacy (%) was highest for 4% concentration of both extracts (100%) against *B. decoloratus*. However, only the methanolic extract (4%) caused 100% mortality of *R. pulchellus.* The conventional acaricide, diazinon, appeared to show slightly better efficacy against *B. decoloratus* (96.7%) compared to *R. pulchellus* (93.3%) at the end of the observation period.

DISCUSSION

The use of natural products, mainly acaricides from the botanical source used for the control of ticks has been the focus of research in many countries, principally to withstand the noticeable increasing frequency of acaricides resistant tick strains. In line with this trend, this preliminary work evidenced that crude methanolic and aqueous extracts of *S. molle* leaves have acaricidal effects comparable to diazinon, a conventional acaricide, justifying the traditional use of this plant against ticks. This was true especially at the highest concentration of the extracts (4%) where 100% mortality was observed after 24 h of exposure. In addition, more than 50% of tick mortality was observed as early as 12 h post exposure to the extracts. Generally, a positive correlation was noted between graded concentrations of the extracts, the exposure test-time interval and ticks mortality. This observation, in one hand, proved that crude extracts of some medicinal plants have acaricidal activities as reported by various workers (Deore and Khadabadi, 2009; Magadum et al., 2009; Ribeiro et al., 2007; Ribeiro et al., 2008) and are a promising alternative for the control of ticks.

In the folk medicine, *S. molle* is an extensively studied medicinal plant throughout the world and has been reported to be used against wide ranges of human and livestock ailments *(*Erazo et al., 2006; Machado et al., 2007; Kasimala, 2012). In Ethiopia, its leaves are used as natural repellents against insects such as flies (Abdel-Sattar et al., 2010). To the study knowledge, there are no published studies on the acaricidal effect of *S. molle* against ticks but the results are comparable with those obtained using different medicinal plants. Most *in vitro* studies on medicinal plants often use different concentrations of extracts (varying from μgml^{-1} to $mgml^{-1}$) for both adultcidal and larvicidal activities. This preliminary assay showed toxic effects against adult *B. decoloratus* and *R. pulchellus* ticks with low doses of extracts suggesting that the crude extracts have excellent potency against ticks.

The results are in line with the work of Vongkhamchanh et al. (2013), who have reported that crude extract derived from *Annona squamosa* Linnaeus leaves produced 100% adultcidal activity against cattle tick, *R. microplus* after 24 h of exposure. Borges et al. (2011) also reported Boophilus larval mortality rate of 100% for chloroformic extracts and 98% for hexamic extracts of *Azadirachta indica*. Similarly, Chagas et al. (2002) indicated that essential oils of *Eucalyptus citrodoro* and *Eucalyptus staigeriana* (Myrtaceae) killed 100% of the Boophilus tick larvae at 10% concentration. With regards to dose dependent activity, inconsonance with our observation, Ribeiro et al. (2007) also recorded that the crude extract of *Hypericum polyanthemum* produced a 100, 96.7, 84.7 and 52.7%, respectively for Boophilus larval mortality rates at concentrations of 50, 25, 12.5, and 6.25 mg/ml respectively after 48 h of exposure.

The extracts of the plant were more effective against *B. decoloratus* than *R. pulchellus* at different duration of time and concentration; it could be speculated that there might be variation in sensitivity to phyto-acaricides between different tick species. Furthermore, as measured by mean number of ticks died and antiparasitic efficacy (%), the methanolic extract appeared to be superior to the aqueous extract in eliminating adult ticks under the employed *in vitro* condition. This might be ascribed to the fact that difference in solvent of extraction may reveal different phytochemicals in the same plant contributing to differential activity between extracts. Plant derived compounds such as saponins, tannins, polyphenols and essential oils were reported to have acaricidal properties against ticks such as Boophilus (Deore and Khadabade, 2009; Magadum et al., 2009; Ribeiro et al., 2008). The anti-tick activities of the crude extracts of *S. molle* in the present study might, thus, be attributed to the presence of such biologically active acaricidal compounds. Several mechanisms have been speculated for the acaricidal activities of medicinal plants and herbal extracts: producing viscous fluids that poison and kill ticks directly; repelling ticks from individuals and populations at high risk for tick bites; attracting to the larvae as a trap to control ticks; reducing attachment of tick introduced to animals fed on diet mixed with plants; killing ticks exposed to powder or diluted extracts; reducing feeding, moulting, fecundity and viability of eggs (Abdel-Shafy et al., 2006).

CONCLUSION

The present study is the first report which investigated the *in vitro* acaricidal effect of the crude methanolic and aqueous extracts of *S. molle* leaves against field

population of parasitic ticks infesting cattle in and around Jigjiga, Ethiopian Somali Regional State. It was noted that both extracts of *S. molle* leaves have a promising acaricidal properties justifying the ethno-veterinary usage of the plant. This data is potentially helpful for further experimentation that encompasses larval stages, *in vivo* protocols and biological-activity-guided characterization of bio-active ingredients responsible for anti-tick activities.

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Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analysis on effects of diets on antiretroviral therapy (ART) immune response among HIV/AIDS Patients in Eastern Ethiopia: Particular emphasis to camel dairy consumption

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Diets and nutrition have critical effect on immune status of human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) patients and on recovery of immune competence after antiretroviral therapy (ART). A pilot study was conducted to explore associations between the dietary pattern of HIV/AIDS patients and their immune (CD4+ cell recovery) response following ART. A recall interview survey of dietary patterns (staple diets and consumption of dairy, other animal proteins, vegetables and fruits) and ART and CD4+ cell count record analysis was conducted on 92 participants visiting the Karamara hospital HIV/ART clinic between February and March, 2015. The staple diet of participants during ART comprised different cereal products and pulse stews. Dairy products, other animal proteins and vegetable/fruit diets were consumed by 88, 46.7 and 40.2% of the participants, respectively. CD4+ count increased more rapidly (p = 0.003) during the first 6 months of ART (144 ± 13.7 cells / mm3) compared to subsequent intervals (74±15.7 to 76.9±20.3 cells / mm^3). ART CD4+ counts were consistently higher (p < 0.01) in participants having baseline values > 200 cells / mm3. CD4+ cell recoveries tend to be lower in participants aged > 40 years and in those interrupting ART (p < 0.05). Consumption of camel milk and fermented dairy was associated with relatively higher CD4+ cell recovery after 1 year on ART (p > 0.050). In particular, participants who take soured camel milk demonstrate CD4+ cell counts close to > 500 cell / mm^3 after 1 year on ART. Long term CD4+ cell recovery was similarly improved (p = 0.051) in participants consuming fruit/vegetable diets. In contrast, consumption of other animal products had minimal impact on ART CD4+ cell count changes. Fermented or sour camel milk intake could enhance long-term ART immunological response. Deeper, systematic investigation is recommended to verify and establish potential ART-complimenting therapeutic benefits of camel dairy intake in HIV/AIDS patients.

Key words: Antiretroviral therapy (ART), camel, CD4+cell count, diets, HIV/AIDS, pastoralists.

INTRODUCTION

Despite persisting absence of a curative treatment, introduction of effective antiretroviral therapy (ART) has

enabled substantial reduction in morbidity and mortality associated with human immunodeficiency virus (HIV) infection worldwide (Palella et al., 1998; WHO, 2006). There are currently over 30 different ARV drugs used in various combinations (Misgena, 2011). CD4 cell count and HIV viral RNA load estimation are key measures for gauging ART response, with former offering a more practical approach in resource poor settings (Hirigo et al., 2015; WHO, 2006). The success of ART in enhancing clinical wellbeing and longevity is predicated on a wide range of factors including; patient characteristics (age, sex, education, income, etc), ART program (accessibility, ARV type, regimen, timing, shifting, etc), CD4+ cell count at initiation of ART, viral load, clinical stage of AIDS, body mass index (BMI), strict adherence and monitoring of ART etc. (Hirigo et al., 2015; Misgena, 2011; Nash et al., 2008; Sieleunou et al., 2008).

Malnutrition constitutes a major contributor to the persistent burden of immune status of human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) in resource poor settings. AIDS patients who enroll in ART without adequate nutritional support have lower survival rates (Paton et al., 2006). Meanwhile, different diets and nutrients have a direct interaction with ARVs which can alter ART response (Raiten et al., 2005).

Micronutrients play important roles in maintaining immune function and neutralizing the reactive oxygen intermediates produced by activated macrophages and neutrophils in their response to microorganisms. Serum and plasma measurements of vitamins and trace elements, which are imperfect indicators of body stores, have shown that deficiencies are common among HIV infected persons, especially those who are underprivileged, such as women in developing countries, and injection-drug users. Furthermore, micronutrient supplementation strategies have shown promising potential in enhancing response to ART (Marston and De Cock, 2004). Adequate protein, energy and micronutrient intake and absorption are essential for achieving the full benefits of antiretroviral therapy (ART) in boosting immune recovery. Moreover, different diets have a direct interaction with antiretroviral drugs (ARVs) which can alter ART response. Enhanced understanding of potential interactions between nutrition and ART is critical for promulgating contextual evidence-based practice guidelines (Raiten et al., 2005).

Camel milk represents an important dietary resource for dry-land pastoralist societies. Moreover, pastoralist communities in Ethiopia traditionally prescribe the product as remedy to a range of human ailments including; gastritis, asthma, abdominal discomfort, HIV/AIDS, liver disease, tuberculosis, fever, urinary problems, common cold, diarrhea, nausea and diabetics (Asresie and Yusuf, 2014). In fact, emerging scientific evidences supports broad antimicrobial and immune modulating activities (Wernery et al., 2012) which could be relevant to traditional indication of camel milk against HIV/AIDS. However, the nutritional and therapeutic values of camel dairy diets for HIV/AIDS patients have not been properly explored. Moreover, research on interactions between nutritional and HIV/AIDS challenges in Ethiopia's pastoralist areas is still limited.

Therefore, this study attempted to explore potential associations between the dietary trends and immunological response of HIV/AIDS positives undergoing ART at Karamara hospital in eastern Ethiopia. Accordingly, CD4+ cell count variability was tested according to the consumption pattern of different local diets (including camel dairy products) among ART patients.

METHODOLOGY

A pilot study was conducted at the Karamara hospital HIV clinic - in eastern Ethiopia between February and March, 2015. The study adopted a retrospective-exploratory design involving recall interview survey of ART patients and analysis of corresponding clinical records. Recovery of CD4+ cell count (speed and magnitude) and immunological response quality after ART represented dependent study variables. Basic demographic characteristics, ART history and dietary intake patterns of ART patients comprised the major independent study variables.

Study participants were selected from a sampling frame comprising HIV positives having a regular check-up and ART refill schedule at Karamara HIV/ART clinic during the study period. Patients older than 15 years and those with ART duration of 6 months or less were excluded from the sampling frame. A total of 100 potential participants were identified by a random lottery sampling method using the unique clinical ID of ART patients. Subsequently, 8 selected ART patients expressed unwillingness to take part in the study leaving a total of 92 participants.

A simple, structured format was prepared and tested for gathering relevant data pertaining to individual participants. An experienced counseling nurse well-known to the participants was trained on purpose and procedures of the study. On their next visit to the HIV/ART clinic, participants were interviewed by the counseling nurse to give recall account of their dietary intake patterns as summary of average trends for covering their entire ART period. Major parameters addressed included regular staple diets and consumption pattern of other diets including; dairy products (animal source, frequency and type of dairy), other animal proteins, vegetables and fruits. Demographic data, ART history (duration, interruption, etc) and serial CD4+ cell counts (baseline, 6 moth, 1 year and most recent ART intervals) of participant was also registered from clinical records. A total of 7 CD4+cell count records were missing including 2 (1st year) and 5 (recent) ART interval records.

Baseline CD4+ counts were categorized in to three ranges that is, < 100 cells/mm^3 (too low), 100 to 200 cells/mm^3 (low) and > 200 cells/mm^3 (optimum) for comparative analysis against recommended standards. Meanwhile, difference of CD4+ count between

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Table 1. Dietary pattern according to participant's demographic characteristics (n (%)).

Variable	Age group			Gender	
	<20Yrs	20-40Yrs	>40yrs	Male	Female
Fruit/vegetable intake (37/92)	2 (25)	22 (46.8)	13 (35.1)	14 (38.9)	23 (41.1)
Meat egg of fish intake (43/92)	1 (12.5)*	27 (57.4)	15 (40.5)	19 (52.8)	24 (42.9)
Dairy intake (_/92)					
None	0	6 (12.8)	5 (13.5)	5 (13.9)	6 (10.7)
Non-camel dairy	7 (87.5)	32 (68.1)	24 (64.9)	21 (58.30	42 (75)
Camel dairy	1 (12.5)	9 (19.1)	8 (21.6)	10 (27.8)	8 (14.3)
Dairy consumption frequency (_/81)					
Daily	4 (50)	14 (34.1)	12 (37.5)	15 (48.4)	15 (30)
Regular(≥ 2 days/week)	4 (50)	12 (29.3)	9 (28.1)	5 (16.1)	20 (40)
Occasional	0	15 (36.6)	11 (34.4)	11(35.5)	15 (30)
Dairy products (_/81)					
Milk only	8 (100)	38 (992.7)	25 (78.1)	28 (90.3)	43 (86)
Milk + Fermented dairy	0	3 (7.3)	7 (21.9)	3 (9.7)	7 (14)

Superscript * indicates significant variation at $P < 0.05$.

successive ART intervals was calculated to analyze speed and magnitude of immunological recovery. According to WHO (2006), ART immunologic failure was determined when CD4+ count falls to below the pre-therapy baseline, or below 50% of the on-peak value, or is persistently < 100 cells/mm.

Statistical analysis

Statistical analysis was conducted using statistical package for social sciences (SPSS-20). Chi-square test was used to evaluate degree of associations between; demographic characteristics, dietary trends, ART history, baseline CD4+ count groups and ART immunological response quality. Independent effect of latter categorical variables on CD4+ count was performed by comparison of means using students't' test and one way ANOVA. Statistical significance was determined at $p < 0.05$.

RESULTS

The proportion of female participants (60.9%) was higher than ($p = 0.037$) that of males (39.1%). Meanwhile, fewer ($p = 0.000$) participants under 20 years (8.7%) were involved as compared to those aged 20 to 40 years (51.1%) or > 40 years (40.2%). The duration of ART was > 3 years, 1 to 3 years and < year for 72.8%, 21.7% and 5.4% participants, respectively ($p = 0.000$). History of treatment interruption was documented in 6 (6.5%) participants (4 females and 2 male aged > 20 years).

The staple diets comprised of cereal (Teff, Wheat, Rice, Sorghum and/or Maize) products and pulses (Beans and Peas). Majority ($p = 0.000$) of participants consumed dairy diets > 2 times/ week (59.8%) or at less frequent intervals (28.3%). Dairy products from other animals (68.5%) was consumed more commonly ($p = 0.000$) than camel dairy (19.6%). All dairy consumers took pasteurized/boiled milk but few (10.9%) also took fermented dairy products including soured camel milk (4.4%). Other animal product (meat, egg or fish) and fruit/vegetable intake was reported by 46.1% and 40.2% participants, respectively. Dairy consumption trend did not vary across age and gender groups. However, intake of meat, egg or fish was higher ($p = 0.039$) in participants aged > 20 years (Table 1).

A total of 350 CD4+ count records were documented and 7 records were missing for 1st year (2) and recent (5) ART intervals. CD4+ count of participants ranged from 5 to 1592 cells / mm^3. Mean CD4+ count (cells / mm^3) increased from baseline (192.7 12.9) to the 6 month (336.8 18.3), 1 year (396.5 21.8) and recent (462.4 23.7) ART intervals.

Baseline CD4+ count was too low (< 100 cells / mm^3), low (100 to 200 cells / mm^3) and optimum (> 200 cells / mm^3) in 26.1, 31.5 and 42.4% participants, respectively ($p = 0.149$). Overall, CD4+ count recovery in 1st 6 months of ART (144 ± 13.7 cells / mm^3) was higher ($p = 0.003$) than changes in the 6 to 12 month (74 ± 15.7 cells / mm^3) or 12 month to recent (76.9±20.3 cells / mm^3) ART intervals. Optimum baseline CD4+ count was associated with improved immune cell recovery on treatment (Figure 1).

CD4+ counts did not vary significantly between male and female participants. Participants > 40 years had lower baseline ($p = 0.012$), 6 month ART ($p = 0.008$) and 1 year ART ($p = 0.025$) mean CD4+ cell counts. Mean baseline CD4+ count was lower ($p = 0.015$) in advanced

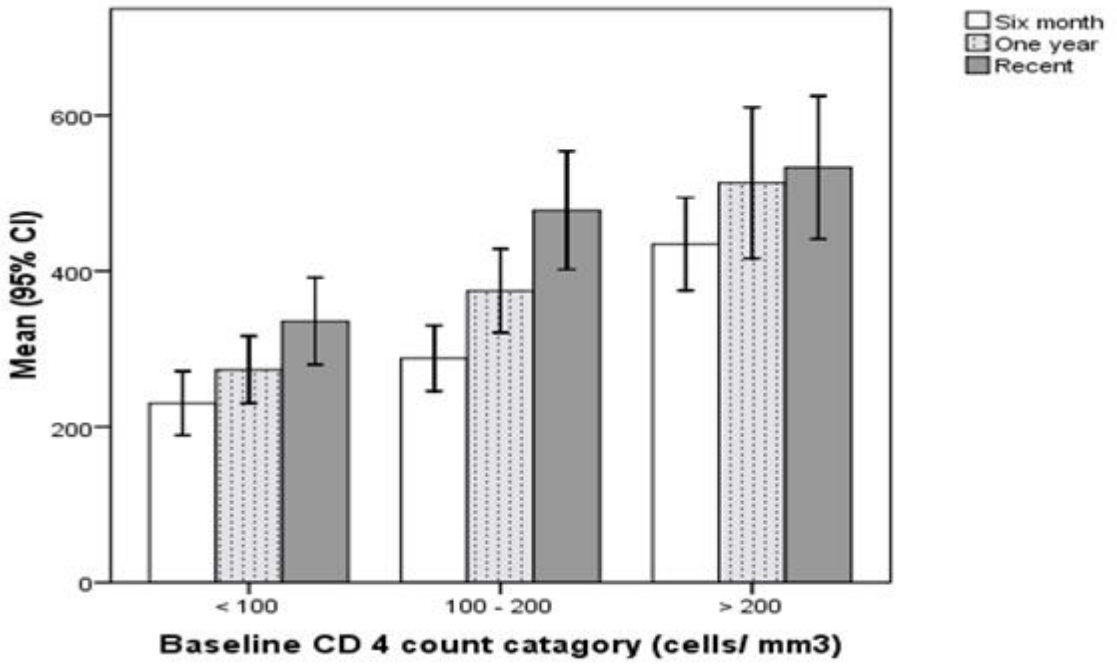

Figure 1. Effect of baseline CD+ count on systemic immune cell recovery under ART.

Table 2. CD4+ cell count profile of participants according to demographic and ART features.

Variable	CD4+ count (Mean ± SE (Median)			
	Baseline (92)	6 Months (92)	1 Year (84)	Recent (82)
Gender				
Male	180.7±22.8 (168)	321.9±29.6(257	400.1±45.1(325)	429.7±41.8(364)
Female	200.4±15.3(191.5)	346.4±23.5(342)	394.1±20.4(400.5)	484.4±22.8(484)
Age Group (Years)				
<20	271.4±65.1(225.5)	397.6±71.3(370)	638.1±147(467)*	632.6±124.29480)
20-40	209.9±17.1(209)	377.5±28.1(350)	393.9±23.8(398.5)	461.9±30.7(485)
>40	153.8±17.3(125)*	272±20.4(245)*	342.9±22.2(307)	428±33.8(4070
ART stage (Years)				
<1	284.4±61.3 (237)	398.2±93.9 (299)	-	-
1-3	249.3±33.2 (234.5)	409.3±52.1(364.5)	399.9±43.8(394.5)	519.3±57.9 (509)
>3	168.9±13 (163)*	310.6±18 (289)	395.6±25.2(377)	449.7±25.9 (463)
ART interruption				
Absent	193.4±12.6 (1780)	340.9±19 (310)	402.9±22.4 (388)	468.6±923.6 (478.5)*
Present	182.7±86.6 (95.5)	277.5±72.5 (228.5)	298±87.59180)	384±131.3 (222.5)

Superscript * indicates significant variation at P < 0.05.

(> 3 years) than recent ART stages. Treatment interruption reduced (p = 0.050) mean CD4+ count on recent (> 1 year) ART interval (Table 2).

Consumption of non-dairy animal products was associated with lower baseline and higher ART mean CD4+ count, but these variations lacked statistical significance. Intake of fruit/vegetable diets was associated with somewhat higher (p = 0.051) mean CD4+ count on the > 1 year ART interval. Camel dairy consumers demonstrated relatively higher mean CD4+ counts under

Table 3. CD4+ cell count variations related to dietary patterns.

Dietary pattern		CD4+ count (Mean ± SE (Median)			
Category	**Intake**	**Baseline (92)**	**6 Months (92)**	**1 Year (84)**	**Recent (82)**
Fruit/	No	185.3±16.8(174)	319.8±20.7(308)	390.7±30.4(339)	435.6±(445)
Vegetable	Yes	203.7±20.1(188)	362.2±33.6(334)	405.6±29.8(429)	504.3±36.1(525)
Meat, Egg	No	216.4±20.1	320.8±22.3	409.7±33	456.6±32.9
or Fish	Yes	165.6±14.5	355±29.9	382.7±28.3	468.2±34.4
	None	232.4±49.2(171)	346.6±42.9(310)	348.2±33.8(322)	437.6±70(421)
Dairy	No-Camel	186.6±14.1(181)	334.4±24.4(310)	395.6±29.6(370)	454.3±29.7(459)
	Camel Dairy	189.8±32.9(168)	339.1±30.9(315)	423.7±36(416)	503.2±47.7(554)

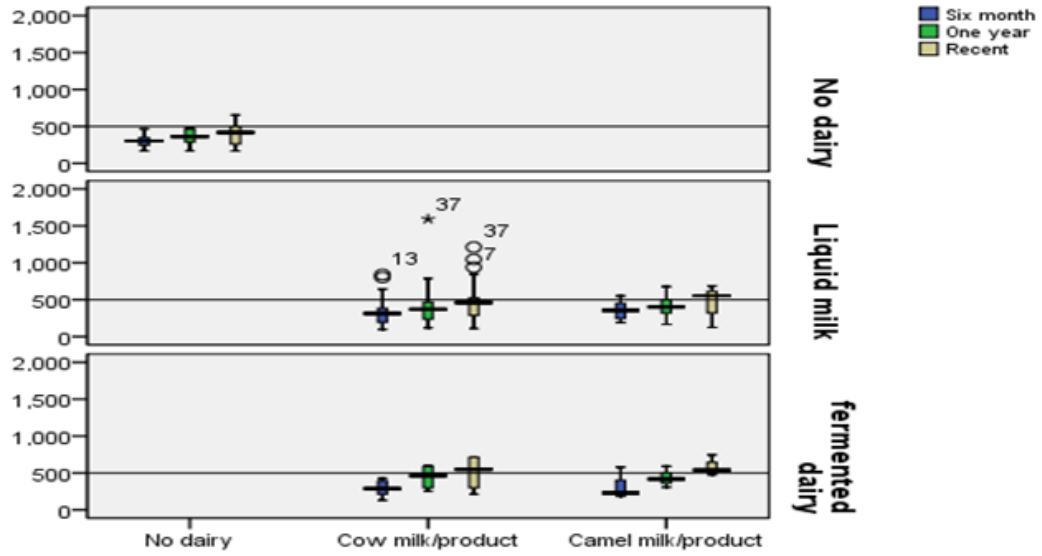

Figure 2. ART – CD4+ count changes relative to source and type of dairy diets (box plot).

ART (p > 0.050). Likewise, long term (> 1 year ART interval) immune cell recovery was relatively higher (p > 0.050) among fermented dairy consumers (Table 3). In particular, participants who consumed soured camel milk demonstrate recent (> 1 year) ART interval CD4+ counts close to normal (> 500 cell / mm3) levels (Figure 2).

According to standard world health organization (WHO) definitions, 5 (6.1%) participants had encountered long term (> 1 year) ART immunological failure. Long term ART immunological failure was associated with limited CD4+ count recovery during 1st 6 months of ART and dropping CD4+ counts thereafter. Higher (p = 0.024) treatment failure risk was also noted in participants that interrupted treatment (33.3%) compared to strictly adherent patients (6.6%). None of the participants that reportedly consumed fermented dairy products encountered ART immunological failure (p > 0.050).

DISCUSSION

Higher female ART enrollment was previously noted at same clinic by Damtew et al. (2014). Improving maternal – child health care services could facilitate better HIV detection and treatment in females. Low ART coverage among children (< 15 years) as well as ART non-adherence and follow-up loss are recognized challenges in Ethiopia (HAPCO, 2014). Current observation of higher recent (< 3 years) baseline CD4+ count agrees with global trends (Nash et al., 2008). This could reflect impact of the revised integrated HIV/AIDS treatment guideline encouraging ART enrolment at higher CD4+ count cut points (WHO, 2006) which was adopted by Ethiopia in December 2013 (HAPCO,2014).

An important ART target is the recovery of immune competence (CD4+ cell count). This results from

enhanced redistribution of reserve cells, recruitment of naïve T cells, and/or suppression of immune activation mediated apoptotic loss (Kaufmann et al., 2005). ART CD4+ recovery generally occurs in two phases, including a rapid increase of cells during the first two months of therapy followed by slower but sustained increment thereafter (Autran et al., 1997). In agreement, this study found significantly higher CD4+ recovery during the first 6 months of ART compared to subsequent intervals. Higher CD4+ recovery during first 6 months of ART was also reported in Ethiopia (Damtew et al., 2014; Alemu and Sebastián, 2010) and sub Saharan Africa (Lawn et al., 2006).

In this study, patients with low (< 100 cells/mm^3) baseline CD4+ count were unable to regain a normal cell count (> 500 cells/mm3) after ART. In agreement, Nash et al. (2008) indicated that only patients initiating ART at baseline CD4+ count of > 200 cells / mm3 could be expected to achieve normal immune cell levels (near or above 500 cells/ mm3) and reduced risk of AIDS related morbidity and mortality. The study noted lower baseline, 6 months and one year CD4+ counts in ART patients over 40 years of age. A similar trend was reported in over 50 years ART recipients in southern Ethiopia (Hirigo et al., 2015). Other had implicated such variations to aging related impairment of thymic function (Douek *et al.,* 1998) and changes in CD4+ reconstitution (Stuart et al., 2002). Another current observation involved significantly lowered recent (> 1 year) ART CD4+ count among patients with history of treatment interruption. ART non-adherence was associated to poor control of viral load (Misgena, 2011; Rougemont et al., 2009) which could foster destruction of immune cells.

In this study, intake of fruit/vegetable and non-dairy animal diets was noted in less than half of the participants. Whereas majority of participants did indicate dairy consumption, intake of camel dairy and fermented dairy products was limited. Consumption of camel dairy and fruit/vegetable diets was associated with relatively higher ART CD4+ recovery. Meanwhile, long term (> 1 year) ART CD4+ count was substantially better (>500 cells/ mm3) among participants consuming fermented camel dairy diets. Chronic malnutrition and nutrient deficiencies represent major contributors to elevated early mortality under ART in resource poor settings. Micronutrients (especially Vitamins A, B6 and B12, Iron and Zinc) play crucial roles in maintaining immune function and neutralizing reactive oxygen intermediates produced by activated macrophages and neutrophils in their response to microorganisms (Marston and De Cock, 2004).

Therefore, the high vitamin (C, B1, B12 and A) and mineral (Na, Cl, Ca, Fe, Mg, etc) contents of camel milk (Al-Humaid et al., 2010) could enhance immunity and tissue protection in HIV/AIDS patients. Moreover, antibacterial and virocidal components (including; lysozyme, highly neutralizing immunoglobulins, lactoferin, lactoperoxidase, peptidoglycan recognition protein (PPR), and N-acetyl glucosmainidase (NA Gase)) which abound in camel milk could facilitate control of infections and immune hyper-activation (Yagil, 2004). Generally, the scientific world (Perdigon *et al.,* 1995) and pastoralist communities (Asresie and Yusuf, 2014; Gizachew *et al.,* 2014) have recognized fermented dairy products to be more nutritious and health promoting than fresh milk.

CONCLUSIONS

This study evidenced that consumption of camel milk, fermented dairy and vegetable/fruit diets tend to enhance long term ART immune response. In particular, soured camel milk consumption appears to enhance recovery of normal systemic CD4+ cell levels (>500 cells/ mm3) after > 1 year of ART. Further study is recommended to validate current observations giving better account to all potential confounders as well as in collaborating immunological findings with strong virological and clinical evidences.

Conflict of Interests

The authors have not declared any conflict of interests.

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