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In vitro activity of the hydroalcoholic extract of *Chenopodium ambrosioides* against engorged females of *Rhipicephalus (Boophilus) microplus*

Atividade **in vitro** *do extrato hidroalcoólico de* **Chenopodium ambrosioides** *diante de teleóginas de* **Rhipicephalus (Boophilus) microplus**

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ABSTRACT: *Rhipicephalus* (*Boophilus*) *microplus*, known as the cattle tick, is a cause of great economic losses for dairy cattle farming because of its high frequency of occurrence and the difficulty in controlling it. The aim of this study was to evaluate the *in vitro* activity of *Chenopodium ambrosioides* extract on *R. (B.) microplus*. For this purpose, 125 females were selected and classified into five groups according to their weight, in order to ensure that the females used presented homogeneous weight. The treatments comprised 40 and 60% extracts of *Chenopodium ambrosioides*, distilled water, ethanol (70ºGL) and 12.5% amitraz. The extracts of *C. ambrosioides* (40 and 60%) showed effectiveness of 99.7 and 100% and higher percentages of dead females than the other treatments: 64 and 96%, respectively ($p<0.001$). In the groups exposed to distilled water and ethanol (70º GL), 92 and 88% of the females maintained oviposition. In the females exposed to 40 and 60% extracts, oviposition of 36 and 4% occurred, respectively. It was concluded that the extract of *Chenopodium ambrosioides*, at both concentration evaluated, had high efficiency against engorged females of cattle ticks.

KEYWORDS: Santa Maria herb; phytotherapy; cattle tick.

RESUMO: O carrapato *Rhipicephalus* (*Boophilus*) *microplus*, popularmente conhecido como carrapato-do-boi, é responsável por grande prejuízo à pecuária leiteira, em razão da sua alta frequência e dificuldade em seu controle. O objetivo do estudo foi avaliar a atividade *in vitro* do extrato hidroalcóolico de *Chenopodium ambrosioides* sobre teleóginas de *R. (B.) microplus*. Para tal, foram selecionadas 125 teleóginas, classificadas em cinco tratamentos de acordo com o peso, de forma a manter os pesos das fêmeas homogêneos. Os tratamentos foram constituídos por: extrato hidroalcoólico da planta nas concentrações de 40 e 60% (v/v); água destilada; etanol 70º GL; e amitraz 12,5%. Os extratos de *C. ambrosioides* nas concentrações de 40 e 60% demonstraram eficácia de 99,7 e 100% e porcentagem de fêmeas mortas superior à dos demais tratamentos: 64 e 96%, respectivamente ($p<0,001$). Nos grupos tratados com água destilada e etanol 70º GL, 92 e 88% das fêmeas realizaram postura. Nas fêmeas expostas ao extrato 40 e 60%, a postura ocorreu em 36 e 4%, respectivamente. Conclui-se que o extrato hidroalcoólico de *C. ambrosioides*, nas concentrações avaliadas, possui alta eficiência contra teleóginas do carrapato bovino.

PALAVRAS-CHAVE: erva-de-santa-maria; fitoterapia; carrapato bovino.

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INTRODUCTION

A variety of factors influences the degree of success in cattle rearing. These may be genetic or environmental factors, technological advances applied to animal production, nutritional management or health management in the herd. Regarding animal health, problems with ectoparasites have gained prominence and are a matter of concern because of the production losses that it can lead to. It has been estimated that 80% of the worldwide cattle population is exposed to the tick *Rhipicephalus* (*Boophilus*) *microplus*. This ectoparasite is responsible for diminished leather quality, disease transmission (PATARROYO; LOMBANA, 2004), weight loss, worsened feed conversion and decreased milk production (SANTOS et al., 2013).

In Brazil, this tick is responsible for annual losses of 3.24 billion dollars (GRISI et al., 2014), and it presents resistance to the great majority of pesticides available on the market because of their uncontrolled use. Utilization of these pesticides leads to toxicity and losses of meat quality and production levels. Moreover, there is concern within society and government bodies regarding pesticide residues in milk and concern regarding the environmental damage caused by inappropriate use of these products (CHAGAS et al., 2002).

Thus, a need has arisen to seek alternative methods for tick control that would present high efficiency, low cost and absence of resistance. In seeking such methods, many plant species have already been used in the form of extracts. These include: *Copaifera reticulata* (FERNÁNDEZ-SALAS, 2011); *Petiveria alliacea* (ROSADO-AGUILAR et al., 2010); *Cuminum cyminum*, *Pimenta dioica* and *Ocimum basilicum* (MARTINEZ-VELAZQUEZ et al., 2011a); *Carapa guianensis*, *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Piper tuberculatum* (CHAGAS et al., 2012); *Manilkara zapota* (RAJAKUMAR; RAHUMAN, 2012); *Lippia graveolens*, *Rosmarinus officinalis* and *Allium sativum* (MARTINEZ-VELAZQUEZ et al., 2011b); and *Calea serrata* (RIBEIRO et al., 2008).

The plant *Chenopodium ambrosioides*, known as St. Mary's herb, is commonly found in southern Brazil. It has been studied because of its anthelmintic properties (PEREZGROVAS et al., 1994), fungicidal properties (KUMAR et al., 2007), anti-protozoan properties (MONZOTE et al., 2006), insecticidal properties (REIS et al., 2010) and even acaricidal properties (SANTOS et al., 2013; POZZATTI et al., 2012; ALMANÇA et al., 2013). Thus, the objective of this study was to evaluate the effect of extracts of *C. ambrosioides* on teleogynes (engorged females) of *Rhipicephalus (Boophilus) microplus*.

MATERIAL AND METHODS

Obtaining the extracts

C. ambrosioides plants (consisting of leaves, stalks and flowers) were gathered in the municipality of Canoinhas, Santa Catarina, and were subsequently identified in the laboratory at the University of Contestado. They were then dried in a heated chamber at 40ºC for 7 days and ground up in an electric milling machine. Following this, the material was mixed with ethanol (70º GL) in the proportions 1:12, and this mixture was left in a receptacle sealed using aluminum foil, to be macerated for 20 minutes. After this period, the material was filtered through a paper filter, and the liquid thus collected was placed in a water bath at 42ºC and was left there for 21 days. This yielded a crude extract, which was then diluted in distilled water to produce concentrations of 40 and 60%, for use in *in vitro* bioassays.

Qualitative phytochemical screening was performed at the Paraná Center for Scientific and Educational Research on Medicinal Plants (NUPPLAMED), in accordance with the methodology described by MATOS (1998). It was sought to ascertain which secondary metabolites were present: phenols, tannins, anthocyanins, anthocyanidins, leucoanthocyanidins, steroids, triterpenes, saponins, resins and alkaloids). Total phenols were assayed using the Folin-Ciocalteu test, following the adapted methodology of MCDONALD et al. (2001).

The total phenol concentration was determined through interpolation of the absorbance of the samples in comparison with a calibration curve constructed using Gallic acid standards. The results were determined from the regression equation of the calibration curve ($y = 0.02x - 0.0064$; $R^2 = 0.9911$) and were expressed in milligrams (mg) of Gallic acid equivalent per gram (g) of the sample. The toxic activity of the extract was evaluated by means of a lethality test against *Artemia salina* Leach, following the methodology of MEYER et al. (1982). The toxicity criteria for the extracts were established as follows: > 1,000 µg/mL (nontoxic); ≥ 500 ≤ 1,000 µg/mL (weakly toxic); and < 500 µg/mL (toxic). An antioxidant assay was performed using the reduction method on the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), in accordance with the methodology of Blois (1958), using ascorbic acid as the standard.

Collection of teleogynes (engorged females) and bioassays

Two hundred teleogynes of *Rhipicephalus (Boophilus) microplus* of length greater than 4 mm were collected from cattle of the breeds Friesian-Holstein, Girolando and Jersey that were naturally infected and had not been treated with acaricides for at least 60 days. From these 200 specimens, 125 that were free from malformations and lesions, and did not present remainders of skin on their buccal apparatus, were selected. These specimens were washed in distilled water, dried on absorbent paper and distributed into five homogenous treatment groups according to their weight (Table 1).

Each of the five treatments consisted of five repetitions, and repetition was composed of five teleogynes (i.e. n = 25 specimens

per group). The treatments were defined randomly between the five groups.

The first treatment group was exposed to distilled water (negative control), and this water was the same as used to dilute the plant extracts and the acaricide. The second treatment group was exposed to alcohol (70º GL). The third treatment group was exposed to 12.5% amitraz (amidine at a concentration of 0.025% after dilution), which was a contact acaricide that had previously been tested on the farm. The fourth and fifth treatment groups comprised 40 and 60% dilutions of the hydroalcoholic extract of *C. ambrosioides*, respectively.

The teleogynes were immersed for five minutes in the respective treatments. They were then dried on absorbent paper and were fixed to Petri dishes that had previously been labeled, using double-sided adhesive tape. They were kept in a chamber under biochemical oxygen demand (BOD) conditions at 27ºC (± 1) and 80% relative humidity (± 5). They were monitored regarding oviposition and mortality every day for the next 18 days.

On the 19th day after the start of the experiment, the egg masses were removed from the Petri dishes and were weighed and transferred to glass test tubes that were sealed using gauze and labeled. After an incubation period of 15 days, the percentage hatching of the eggs in each group was estimated.

Data collection

The following data were recorded and evaluated: mean weight of oviposition (g); weight of the teleogynes (g); percentage of dead females (positive or negative reaction to touching them with tweezers); percentage hatching after 15 days; percentage of the teleogynes that presented oviposition; reproductive efficiency (RE, %); and treatment efficiency (TE, %).

The reproductive efficiency (RE) and treatment efficiency (TE) were calculated using the methodology suggested by DRUMMOND et al. (1973): RE = ((weight of eggs (g)/weight of teleogynes (g)) × % hatching x 20,000*); and TE = ((RE of the control group – RE of the treated group) / RE of the control group) × 100.

Statistical analysis

The variables were subjected to the Shapiro-Wilk normality test. Since none of the variables presented normal distribution, the nonparametric Kruskal-Wallis test was used. Significant differences were deemed to exist between the treatments when $p \leq 0.05$.

RESULTS AND DISCUSSION

In a study conducted by ALMANÇA et al. (2013), extracts of *Chenopodium ambrosioides* at concentrations of 5, 10 and 25% were shown to be inefficient on teleogynes of *Rhipicephalus (Boophilus) microplus*. These authors therefore suggested that higher concentrations should be used.

In the present study, on the day after the teleogynes had been immersed in the treatments, 52% of the females treated with the 60% extract had died, and the mortality rate reached 96% on the sixth day (Fig. 1, Table 1). The females treated with 40% extract presented 24% mortality at the end of the first day and 64% on the sixth day. KOUAM et al. (2015) had already reported that, at a dose of 0.12 µL/g *in vitro*, the essential oil of *C. ambrosioides* had a toxic effect on *Rhipicephalus lunulatus*, with high mortality

Table 1. Mean ± standard deviation of egg weight, teleogyne weight, percentage mortality, percentage hatching, reproductive efficiency and treatment efficiency, relating to *in vitro* treatments of teleogynes of *Rhipicephalus (Boophilus) microplus* with distilled water, alcohol (70º), amitraz and 40 and 60% extracts of *C. ambrosioides*, which were maintained under biochemical oxygen demand (BOD) conditions at a temperature of 27ºC (± 1) and 80% relative humidity (± 5).

Treatment	Teleogyne weight (g)	Mean weight of oviposition (g)*	Mortality (%)*	Percentage hatching (%)*	Reproductive efficiency*	Treatment efficiency* (%)
Distilled water	0.748 ± 0.008	0.272 ± 0.040^{a}	8.00 ± 10.95^{a}	89.00 ± 4.18^{a}	651,159.0 ± 81,095.0^{a}	-
Ethanol (70º)	0.744 ± 0.015	0.232 ± 0.050^{b}	12.00 ± 17.88^{a}	85.99 ± 7.90^{a}	527,267.0 ± 93,680.0^{a}	19.02 ± 14.38^{a}
Amitraz	0.744 ± 0.011	0.003 ± 0.000^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.0 ± 0.0^{b}	100.00 ± 0.00^{b}
40% extract of *C. ambrosioides*	0.740 ± 0.007	0.024 ± 0.020^{cd}	64.00 ± 21.91^{b}	2.00 ± 2.74^{b}	1,637.0 ± 3,218.0^{b}	99.75 ± 0.50^{b}
60% extract of *C. ambrosioides*	0.738 ± 0.008	0.03 ± 0.00^{d}	96.00 ± 8.94^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	100.00 ± 0.00^{b}

*Different letters in the columns indicate significant differences (P < 0.05) according to the Kruskal-Wallis test.

rates of approximately 50% after 24 hours and 100% on the fourth day. In addition to the acaricidal effect of this plant, the mortality among the teleogynes exposed to the 60% extract may have been increased through the high viscosity of the solution, which may have occluded the peritreme, thus making it difficult for them to breathe.

The mortality in the control groups was low. On the 18th day, the distilled water, ethanol (70º GL) and amitraz groups showed mortality rates of 8, 12 and 0%, respectively. The lack of mortality due to amitraz (amidine) was expected, given that this substance has a high effect on oviposition and larval hatchability (ANDREOTTI, 2010; SPINOSA et al., 2006). These results suggest that six days is the time needed for the 40 and 60% extracts of *C. ambrosioides* to cause almost complete mortality among the teleogynes of *R. (Boophilus) microplus*.

Regarding efficiency, the groups treated with the extracts of *C. ambrosioides* and amitraz showed a significant difference in comparison with those ones treated with distilled water and ethanol (70º GL) (Fig. 2, Table 1). The hydroalcoholic extracts of *C. ambrosioides* presented very high efficiency: even at the lower concentration of 40%, efficiency of 99.75% was achieved, while the 60% extract showed 100% efficiency. Thus, these extracts can be considered for use as an alternative acaricide treatment, and not just as complementary therapy. Nonetheless, it would also be instructive to evaluate lyophilized extracts, to observe whether there might be any difference in the results.

Regarding the oviposition performed by the teleogynes in the different groups, the groups treated with the extracts and amitraz showed a significant difference ($p<0.05$) in the mean weight of oviposition (g) in comparison with those ones treated with distilled water and ethanol (70º GL). The percentage hatching and reproductive efficiency were lower in the groups treated with the 40 and 60% extracts and with amitraz ($p<0.05$).

These results demonstrate that, at both concentrations evaluated, the extracts were efficient for inhibiting oviposition and the hatchability of the eggs, with an effect similar to that of amitraz. Nonetheless, although amitraz demonstrated treatment efficiency, reproductive efficiency and percentage hatching equal to those of the two concentrations of the extract, the higher percentage mortality among the teleogynes that was observed with both the 40 and the 60% extract indicates that these extracts acted more efficiently on the teleogynes of *Rhipicephalus (Boophilus) microplus*.

Regarding the percentage of the teleogynes that presented oviposition, 92 and 88% of those ones that were treated with distilled water and ethanol (70º GL) respectively achieved oviposition with completely viable eggs. From the treatment with amitraz, only 4% of the teleogynes achieved oviposition, and 100% of these eggs were unviable. Among the engorged females treated with the 40% extract, 36% achieved oviposition; and among those treated with the 60% extract, only 4% achieved oviposition (Fig. 3). Regarding the appearance of the eggs, the females

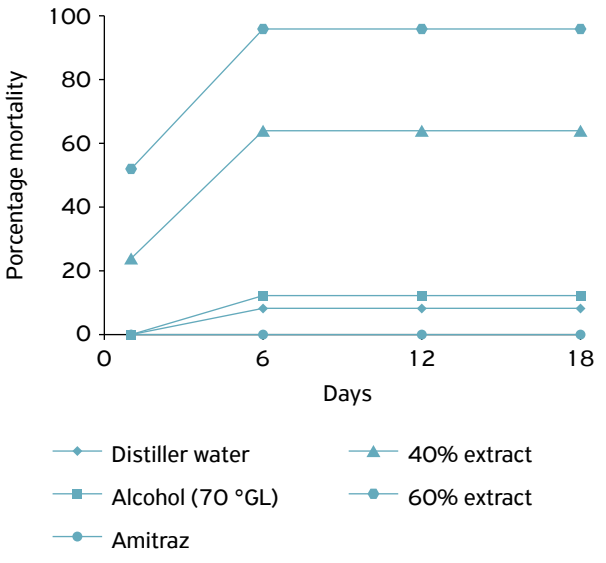


Figure 1. Mortality (%) on the 1st, 6th, 12th and 18th days among the teleogynes of *Rhipicephalus (Boophilus) microplus* that were treated *in vitro* with distilled water, alcohol (70º), amitraz and 40 and 60% extracts of *C. ambrosioides* and maintained under biochemical oxygen demand (BOD) conditions at a temperature of 27ºC (± 1) and 80% relative humidity (± 5).

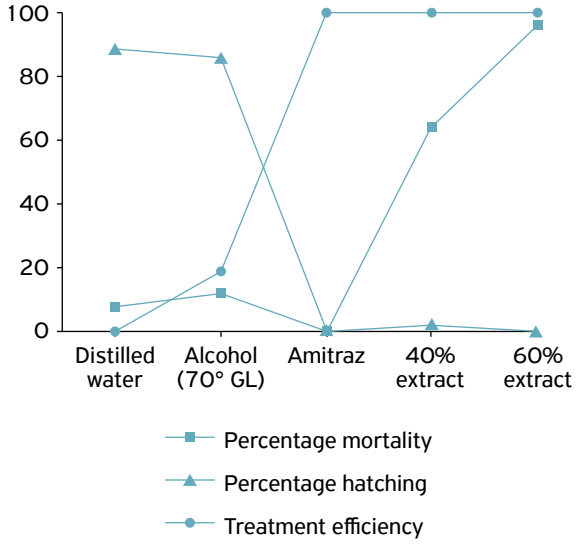
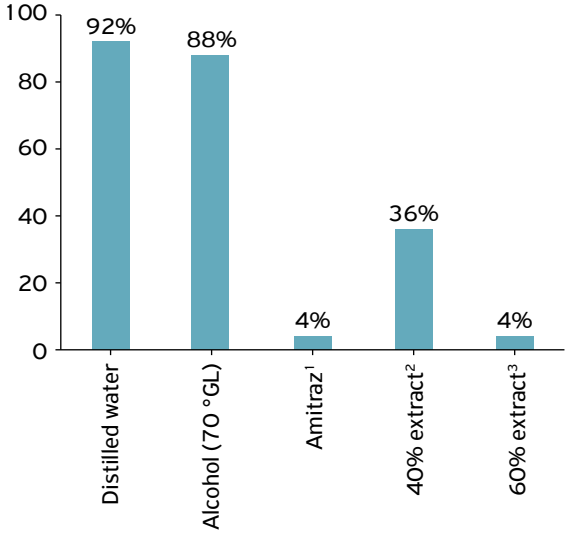


Figure 2. Percentage mortality, percentage hatching and treatment efficiency, relating to *in vitro* treatments of teleogynes of *Rhipicephalus (Boophilus) microplus* with distilled water, alcohol (70º), amitraz and 40 and 60% extracts of *C. ambrosioides*, which were maintained under biochemical oxygen demand (BOD) conditions at a temperature of 27ºC (± 1) and 80% relative humidity (± 5).

that performed oviposition after treatments with distilled water and ethanol (70º GL) produced shiny brown-colored eggs, thus indicating fertility. The teleogynes treated with the 40 and 60% extracts produced darker eggs of matt appearance, while those ones treated with amitraz only produced a yellow secretion.

Differing from what was observed in the present study, ALMANÇA et al. (2013) reported that the 5, 10 and 25% extracts of *C. ambrosioides* did not have any influence on the larval hatching of *R. (B.) microplus*, or consequently on reproductive efficiency. POZZATTI et al. (2012) evaluated the reproductive efficiency and treatment efficiency resulting from use of fluid extracts (5, 10 and 25%) and glycolic extracts (10, 20, 30 and 50%) on *C. ambrosioides*. They reported that there were high percentages of hatchability from all the treatments (between 93.5 and 99%) and low treatment efficiencies (between 1.5 and 34.82%), thus indicating that the extracts of *C. ambrosioides* only had a small effect. The difference in the results observed between the present study and those in the literature can be ascribed to the differences in extract preparation methods and in the concentrations of the product used.

For antiparasitic products to be licensed for veterinary use by the Brazilian agricultural protection bodies, the minimum criterion is that they need to present mean efficiency of 95% (BRASIL, 1997), which was seen in relation to the 40 and 60% extracts of *C. ambrosioides*. Nonetheless, although the extracts of *C. ambrosioides* presented an *in vitro* effect on the teleogynes of *R. (Boophilus) microplus* regarding oviposition and egg hatchability, studies evaluating the viability of using these extracts *in vivo* are necessary, given that the high concentration and viscosity of the product may cause difficulty in producing and applying it.



[1]4% of the teleogynes performed oviposition with unviable eggs; [2]36% performed partial oviposition; [3]4% performed unviable oviposition.

Figure 3. Percentage oviposition among the teleogynes of *Rhipicephalus (Boophilus) microplus* that were treated *in vitro* with distilled water, alcohol (70º), amitraz and 40 and 60% extracts of *C. ambrosioides* and maintained under biochemical oxygen demand (BOD) conditions at a temperature of 27ºC (± 1) and 80% relative humidity (± 5).

Phytochemical screening

The phytochemical screening demonstrated the presence of substances that may have positive effects in relation to combating ticks, such as phenols, tannins, catechins, steroids, triterpenes, resins and alkaloids (Table 2). Phenols are substances used in disinfectants, since they cause inactivation of the enzyme system and loss of essential metabolites through the cell wall, with a spectrum of bactericidal, fungicidal, viricidal and tuberculocidal action (GOVERNO DO ESTADO DA BAHIA, 2001). Catechins and steroids are considered to be potent allelopathic agents (RODRIGUES et al., 2009; LÔBO et al., 2008), *i.e.*, they produce secondary metabolites that may alter the growth and/or development of other biological systems. Triterpenes and alkaloids have been reported to have larvicidal and ovicidal effects on insects (MACIEL et al., 2006).

Tannins have been used for treating a variety of illnesses, including diarrhea, arterial hypertension, rheumatism, hemorrhages, wounds, burns, renal problems and inflammatory processes (MARTINS et al., 2007). The effects of

Table 2. Result from phytochemical screening of the hydroalcoholic extract obtained from the plant *Chenopodium ambrosioides*.

Test or reagent	Compound	Effect*
Ferric chloride	Phenols	+
	Tannins	+
Gelatin (2,5% w/v), NaCl 0.9% w/v	Tannins	+
Change of pH	Anthocyanins	-
	Anthocyanidins	-
Change of pH and heating	Catechins	-
	Leucocyanidins	-
Wood, HCl and heating	Catechins	+
Liebermann-Burchard	Steroids	+
H_2SO_4 and anisaldehyde	Triterpenes	+
Chloroform	Saponins	-
Ethanol	Resins	+
Dragendorff	Alkaloids	+
Folin-Ciocalteu	Total phenols	84.000 ± 0.689 µg/L
Toxicity	*Artemia salina*	1.058 µg/mL

*Effect: + positive reaction; - negative reaction.

tannin-rich plants (*Acacia pennatula*, *Piscidia piscipula*, *Leucaena leucocephala* and *Lysiloma latisiliquum*) were studied by FERNÁNDEZ-SALAS et al. (2011), who found that their aqueous extracts at a concentration of 1.92% presented efficiencies of 35, 39.21, 29 and 69.34%, respectively, on teleogynes of *R. (B.) microplus*.

In the present study, the presence of catechins, steroids, terpenes and tannins in *C. ambrosioides* may have been responsible for the low egg weight, low percentage hatching, high percentage mortality and low reproductive efficiency among the teleogynes.

CONCLUSIONS

The two concentrations of hydroalcoholic extract produced from the plant *Chenopodium ambrosioides* that were evaluated here demonstrated high efficiency against teleogynes of *Rhipicephalus (Boophilus) microplus*. They interfered with oviposition and egg hatchability and can thus be considered to be acaricidal products. They also caused changes to reproductive capacity. Therefore, it can be suggested that the 40 and 60% extracts from *Chenopodium ambrosioides* can be used as an alternative for controlling cattle ticks.

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