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Physicochemical properties, toxicity and larvicidal activity of the essential oil of cymbopogon winterianus in front of Aedes Aegypti

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ABSTRACT

Due to the increasing rate of morbidity and mortality caused by vector diseases in the current context, especially by Aedes aegypti, substances of plants have been studied as alternatives to chemical insecticides, among them, the essential oil (EO) of the species Cymbopogon winterianus. Thus, this study evaluated the larvicidal activity of the EO of C. winterianus against the A. aegypti larvae. EO was extracted through the hydro-distillation technique and physicochemical properties were determined. To evaluate larvicidal activity, tests were performed with larvae in the third instar at the final concentrations of 19.54; 26.50; 55.59; 138.98; 208.47 and 277.97 mg L^{-1} of C. winterianus EO. In addition, Artemia salina Leach bioassay was used to verify toxicity effect. EO obtained presented satisfactory results in 2.64%. In the larvicidal assay, 100% mortality of larvae was observed after 24 hours at concentrations of 208.47 mg L^{-1} and 277.97 mg L^{-1} of the EO, showed effective in the other concentrations and with LC50 of 46.18 mg L^{-1}, considered highly active. In the toxicity assay, the EO presented LC50 at 532.34 mg L^{-1}, showed considered nontoxic. These results reinforce the use of EO front A. aegypt larvae control.

Keywords: Arboviruses control; Hydro-distillation; Biological assays

RESUMO

Devido a crescente taxa de morbimortalidade causada por doenças vetoriais no contexto atual, sobretudo pelo Aedes aegypti, substâncias de plantas vêm sendo estudadas como alternativas aos inseticidas químicos, entre elas, o óleo essencial (OE) da espécie Cymbopogon winterianus. Desta forma, este estudo avaliou a atividade larvicida do OE de C. winterianus frente as larvas do mosquito Aedes aegypti. O OE foi extraído através da técnica de hidrodestilação e parâmetros físico-químicos foram determinados. Para avaliação da atividade larvicida foram realizados testes com larvas no terceiro estágio nas concentrações finais de 19.54; 26.50; 55.59; 138.98; 208.47 e 277.97 mg L^{-1} do óleo essencial de C. winterianus. Além disso, o bioensaio de Artemia salina Leach foi utilizado para verificação da toxicidade. O OE obtido apresentou rendimento satisfatório em 2,64%. Nos ensaios larvicidas observou-se mortalidade de 100% das larvas após 24 horas nas concentrações 208,47 mg L^{-1} e 277,97 mg L^{-1} do OE, sendo eficazes nas demais concentrações e com CL50 de 46,18 mg L^{-1}, considerado altamente ativo. No ensaio de toxicidade, o OE apresentou CL50 em 532,34 mg L^{-1}, sendo considerado atóxico. Estes resultados reforçam o uso do óleo essencial frente ao controle de larvas de A. aegypti.

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Palavras-chaves: Controle de arboviroses; Hidro-destilação; Ensaios biológicos

1 INTRODUCTION

Aedes aegypti is a vector of the most widespread urban arboviruses worldwide, causing high morbidity and mortality such as dengue, chikungunya, zika and yellow fever, especially in countries where environmental, climatic and sanitary conditions favor its reproduction (LIMA-CAMARA, 2016). Transmission of vector diseases occurs when the infected female of A. aegypti encounters, during daytime and low flights, a susceptible host and transmits the arbovirus through saliva while sucking up the blood, the latter it is essential for egg maturation. After this, the pregnant female seeks reservoirs with clean water and stops to begin oviposition; laying between 150 to 200 eggs which will start the larval phase of the mosquito upon hatching, consisting of 4 instars over a duration of 10 days. Upon entering adulthood, adult mosquitos mate and to trigger a new cycle period with an interval of 30 to 35 days of adulthood (CANTANE et al., 2015).

The Ministry of Health from Brazil adopts different measures to prevent vector-borne diseases. The practice of chemical control mechanisms is widely used to combat the mosquito A. aegypti. It occurs mainly through the use of synthetic insecticides that release neurotoxins, analogous juvenile hormones and chitin synthesis inhibitors capable of killing mosquitoes in their larval and adult stages (ZARA et al., 2016). However, the frequent and intensive use of these chemicals causes the emergence of resistant A. aegypti populations, in addition to the undesirable effects on the ecosystem, and supporting species for environmental balance due to the lack of selectivity and long-term effects of those products on the environment (CORRÊA, 2011; VELOSO et al., 2015).

The application of plants compounds as an alternative method to the use of insecticides has numerous advantages such as rapid degradation, low resistance potential, high availability and high economic viability (CORRÊA, 2011). Essential oil from plants is obtained by releasing volatile compounds encapsulated in "pouches"

called trichomes, which are produced for self-defense and attraction of pollinators and are found in various parts of the plant, as leaves, flowers and stalks (WOLFFENBUTTEL, 2007). Natural compounds have been investigated by several researchers who had proven the potential larvicide of plants against different species of insects, including A. aegypti (CARVALHO et al., 2003; CAVALCANTI et al., 2004; CHENG et al., 2004; MORAIS et al., 2006; PUSHPANATHAN et al., 2006).

Cymbopogon spp, popularly known as citronella grass, is an aromatic and perennial plant with long, flat leaves and curved appearance, which is grown in tropical regions for use in preparation of teas and for its known antifungal, antibacterial and insect repellent properties that are obtained by extracting its essential oils (VELOSO et al., 2015). Essential oil (EO) from citronella grass (C. winterianus) demonstrates good performance as a natural repellent and larvicide, as its chemical constitution is integrated with major components such as aldehyde, citronellal, geraniol and citronellol, to which therapeutic, pharmaceutical and cosmetic efficiency characteristics are attributed (CASSEL et al., 2009; KAKARAPARTHI et al., 2014). Studies developed on the efficacy of the use of C. winterianus essential oil has been recommended as an alternative in arboviruses control, as a way to improve its possibility of use (MACEDO et al., 2010). Thus, this study evaluating the toxicity and larvicidal activity of the EO of C. winterianus (citronella grass) against the larvae of Aedes aegypti.

2 METHODOLOGY

C. winterianus leaves were harvested on 9th January, 2020 between 10 a.m. and 12 p.m. at the Berta Lange Morretes Medicinal Plants Garden, located at Federal University of Maranhão (UFMA), São Luís, Maranhão, Brazil and identified by specialists Prof. Dr. Ana Zélia Silva. The leaves was rinsed, ground (cut into small parts) and dried in greenhouse at 45° C for 24 h.

Leaves was subjected to the hydro-distillation technique using a glass Clevenger extractor coupled with a round bottom flask packed in an electric blanket as a heat

source. 300 g of C. winterianus leaves were used, adding distilled water (1:10). Hydro-distillation was conducted at 100°C for 3 hours and the extracted EO was collected. The oil was dried over anhydrous sodium sulfate (Na2SO4), and was then stored in glass bottles covered with aluminum foil and stored in a refrigerator (4°C) until further investigation. The yield of essential oil was expressed in percentage w/w.

Physicochemical properties such as density, refractive index, solubility, color and odor were determined according to the Brazilian Pharmacopoeia (2019).

A. aegypti trap and ovitrap, adapted from FAPERJ (2013), were used to capture the larvae. These systems consisted of a 1 gallon PET bottle, cut in half allowing to reach a larger opening area, where 1mL of an aqueous solution containing a concentration of 12% (6 g / 50 mL) brewer's yeast was aspirated and added to 300 mL of running water and black polyethylene pails (50 mL) filled with chlorinated water and two eucatex vanes to oviposition.

The traps installed were sheltered from the sun and rain, in neighborhoods with a high occurrence of the vector in the capital São Luís of State of Maranhão, were inspected weekly and analyzed in the Laboratory of Research and Application of Essential Oils (LOEPAV/UFMA).

The larvae collected were kept in plastic trays containing clean water with pH 6.9, and remained for a photoperiod of 14 hours of light and 10 hours of darkness in laboratory conditions (29 ± 1 °C), being fed with finely ground cat food until they reached the 3rd instar (L3).

The biological assays were performed according to the methodology recommended by the WHO (1970) adapted by Veloso et al. (2015). The larvae that reached the 3rd instar were separated by means of a pipette, placed on filter paper for the removal of excess water and then 10 larvae were deposited in beakers containing 30 mL of distilled water, where EO aliquots diluted in 500 µL of dimethyl sulfoxide (DMSO) were added to formalize the final concentrations of 19.54; 26.50; 55.59; 138.98; 208.47 and 277.97 mg L^{-1} corresponding to the aliquots of 0.70µL/500 DMSO, 0.95 µL/500 DMSO, 2.0 µL/500 DMSO, 5.0 µL/500 DMSO, 7.5 µL/500 DMSO e

10 µL/500 DMSO of C. winterianus essential oil, respectively. The bioassays were performed in triplicate.

Mortality was verified after 24 h of exposure concentrations in EO. The larvae's death was declared by absence of muscle contraction when stimulated with a pipette.

Considering the percentage of mortality for each concentration, the median lethal concentration (LC50) was submitted to Probit analysis (log concentrations), with a 95% confidence interval, using the software R version 3.6.3 or Excel version 2010.

The toxicity test was carried out according to the methodology described by Meyer et al. (1982). In a rectangular container, with a partition containing holes of approximately 0.02 cm in diameter, spaced by 0.5 cm and evenly distributed, artificial saline solutions (60 g L^{-1} of distilled water) (60 g of sea salt/liter of distilled water) were added. The container was placed inside an incubator illuminated by a fluorescent lamp, with aeration. On one side of this container, about 64 mg of A. salina cysts were added, given that they did not cross the partition. The part of the system containing A. salina cysts was covered with aluminum foil, so that the organisms, at birth, were attracted by light on the other side of the system, forcing them to cross the partition. This procedure aims to homogenize the physical conditions of the test organisms. Incubation was performed for a period of 48 hours. The temperature was monitored during all bioassay.

For the evaluation of the lethality of A. salina, a stock saline solution of EO was prepared at a concentration of 10,000 mg L^{-1} and 0.02 mg of Tween 80 (surfactant). From this, aliquots of 5, 50 and 500 µL were transferred to test tubes and completed by adding saline solution previously prepared up to 5 mL, obtaining concentrations of 10, 100 and 1000 mg L^{-1}, respectively. All tests were carried out in triplicates, where 10 larvae in the nauplii phase were transferred to each of the test tubes.

For control 5 mL of saline solution was used, for positive control K2Cr2O7 and for negative control 5 mL of a solution 4 mg L^{-1} of Tween 80. After 24 hours of exposure, the live larvae were counted by considering exclusively those that did not move the during observation period or only with the slight agitation of the vial. In order to classify the EO toxicity, the criterion established by Dolabela (1997) was

adopted; being considered a highly toxic product when LC50 ≤ 80 mg L^{-1}, moderately toxic to 80 mg L^{-1} ≤ LC50 ≥ 250 mg L^{-1} and mildly toxic or nontoxic when LC50 ≥ 250 mg L^{-1}.

3 RESULTS AND DISCUSSION

The physicochemical properties obtained for the C. winterianus EO are presented in Table 1.

Table 1 – Physicochemical properties of the essential oil of C. winterianus leaves.

Physicochemicals properties	**C. winterianus EO**
Density (g mL^{-1})	0,85
Solubiility in ethanol 70 % (v/v)	1:6
Solubiility in ethano 90 % (v/v)	1:1
Yield (w/w %)	2,64
Refractive index at 25° C	1,475
Color	Yellow
Odor	Characteristic

The density of the extracted EO is less than water (1 g mL^{-1}). The extracted EO presented a yellowish color, with a clear appearance and strong odor. It was found that the EO in question has better solubility in interaction with 90% ethanol, based on proportionate comparison to a concentration of 70%, although both are considered easily soluble according to the Brazilian Pharmacopoeia. The percentage yield obtained through hydro-distillation was 2.64%, expressing a mass of 2.64 g EO extracted for every 100 g of dried leaves of the species (Table 1).

According to the study by Pereira et al. (2016), in which the result in Cymbopogon flexuosus leaves collected in January was 3.73%, the EO yield is subject to monthly influence of the region temperature and soil moisture. According to Rocha et al. (2000), changes are due to variations in drying temperature, which, in comparison to their research, resulted in 0.987% drying at 40°C of leaves of C. winterianus.

The EO of the leaves of C. winterianus obtained in the same place of collection of the present study was investigated by Rodrigues et al. (2013), and presented yield of 1.3%, without coloration and distinct odor: This distinction between the physicochemical properties analyzed may have been influenced by the harvest time. Based on the work developed by Blank et al. (2007), the yield of volatile citronella oils produced at 9:00 am by season with a value corresponding to 2.71, 2.36 and 4.24% for summer, winter and spring, respectively, as the volatile oil content generally peaked at that time.

The results of the larvicidal test for the L3 Aedes aegypti larvae in 6 distinct concentrations of 19.54; 26.50; 55.59; 138.98; 208.47 and 277.97 mg L^{-1} of the C. winterianus EO are shown in Table 2.

Table 2 – Average mortality of Aedes aegypti larvae against the action of Cymbopogon winterianus essential oil

EO mg L^{-1}	**Nº dead**	**Nº alive**	**Deaths accumulated**	**Alives accumulated**	**Mortality (%)**
277.97	10	0	39	0	100
208.47	10	0	29	0	100
138.98	9	1	19	1	90
55.59	5	5	10	6	50
26.50	3	7	5	13	30
19.54	2	8	2	21	20

Nº = average number of larvae per replicate

The EO was shown to be efficient in the control of larvae in all concentrations tested, being more promising in the concentrations of 208.47 mg L^{-1} and 277.97 mg L^{-1} where after 24 hours 100% of the larvae had died (Table 2). In the other concentrations, the mortality rate is below 90% in the 24-hour reading. Larvae in water and 2% DMSO showed nullmortality during the test, indicating that the DMSO used for dilution of EO did not influence the larval death and biological activity in the treated groups. Considering that all EO concentrations of C. winterianus produced mortality data after 24 hours of exposure, it is possible to state that the plants show a high larvicidal effect in the larval stage of Aedes aegypti.

According to studies carried out by Amer and Mehlhorn (2006), the EO of C. winterianus caused 60% mortality in A. aegypti larvae after 24 hours of exposure to a concentration of 50 mg L^{-1} similar to the action of EO at the concentration of 55.59 mg L^{-1} presented in this study. On the other hand, the larvicidal assays developed by Falcão (2018) also reached the maximum mortality percentage (100%) of A. aegypti with an esterified (250 mg L^{-1}) and crude (250 mg L^{-1}) EO of C. winterianus, however, are higher concentration values than those observed in this study. This fact may have occurred due to several factors such as origin, seasonality, extraction method, collection and synergism of the major chemical compounds of the species as citronellal, citronelol and geraniol that are the main responsible for larvicidal activity (GOMES et al., 2019; LEITE et al., 2011; SINHA et al., 2014).

In the study developed by Veloso et al. (2015), the larvicidal effect of citronella grass EO caused 100% of the mortality of A. aegypti larvae from the aliquot of 10 µl, while the aliquots of 5,0 µl and 7,5 µl presented the same result after 6 hours of exposure from the beginning of the experiment.

Cymbopogon nardus belonging to the same genus of plant evaluated in this work and its EO was investigated by Phasomkusolsil and Soonwera (2010). The authors found that EO presented 100% mortality in 3rd instar larvae of Culex quinquefasciatus and Anopheles minimus species after 5 and 10 minutes of exposure, respectively. The results of this study corroborate the efficacy of plant species of the genus Cymbopogon against vector insects, proving to be a promising strategy for moderating the indiscriminate use of synthetic insecticides that has promoted the emergence of resistant populations (Veloso et al., 2015).

Statistical analysis indicated that the lethal concentration capable of reducing 50% of the larvae with the EO was 46.18 (41.62 - 50.74) mg L^{-1} (Table 3).

Table 3 - Larvicidal activity of Cymbopogon winterianus essential oil against cultivated larvae of Aedes aegypti.

Exposure time	**Concentration mg L^{-1}**	**Mortality (%)**	**LC50[a](mg L^{-1}) (IC 95%)**
24 hours	DMSO	0	46,18 (41,62 – 50,74)[b]
	19,57	20	
	26,50	30	
	55,59	50	
	138,98	90	
	208,47	100	
	277,97	100	

a = The LC_{50} was calculated by Probit analysis using the software R version 3.6.3

b = 95% confidence interval; no larvae killed in the negative control, composed of 2% DMSO solution; the positive control, 1 mg L^{-1} temefos, exhibited 100% larval mortality

According to Cheng et al. (2003), the values of LC50 < 50 mg L^{-1} are considered highly active. Thus, from this classification, the EO extracted from the leaves of C. winterianus is considered of highly efficient potential, encouraging its application capacity.

The observed potential is justified by the s C. winterianus being known to have insect repellent properties, being widely commercialized for this purpose (MENDONÇA et al., 2005). It is possible that the larvicidal potential of the EO against A. aegypti can be attributed to the presence of terpenic, alcohol and aldehyde components (LEE, 2006; GOMES et al., 2016).

Among the chemical larvicides used by the Ministry of Health from Brazil, pyriproxyfen is a field-applied development inhibitor for the treatment of domestic A. aegypti breeding sites and water for human consumption. In a bioassay recently perfomed by Fiaz et al. (2019), a LC50 was estimated for pyriproxyfen equal to 8.20 mg L^{-1}, where there were 6 concentrations of pyriproxyfen with triplicates containing 20 larvae (L3) of A. aegypti in 25 mL of distilled water. In addition to pyriproxyfen, when compared to other commercial products such as BTI (0.026 mg L^{-1}) (FANSIRI et al., 2006), the efficacy of the EO obtained is considerably low, limiting its application in practice.

In comparison with the previous investigations of Mendonça et al. (2005), where the ethanol extract of C. winterianus leaves has a LC50 value equal to 38.8 mg L^{-1} against A. aegypti larvae, the EO proved to be less efficient than the reported extract. However, for the others EOs that proposed the same bioassay in the literature, the EO exhibited higher larvicide action against A. aegypti larvae, such as EOs of C. argyrophyllus (LC50 = 310 mg L^{-1}) (Cruz et al., 2017), leaves of Mentha spicata (LC50 = 56.08 mg L^{-1}) (Govindarajan et al., 2011), roots of Acorus calamus L (LC50 = 99.41 mg L^{-1}) (Manzoor et al., 2013), aerial parts of Anethum graveolens L. (LC50 = 50 mg L^{-1}) (Amer and Mehlhorn, 2006), seeds of Carum carvi L (LC50 = 54.62 mg L^{-1}) (Pitasawat et al., 2007), flowers of Dendropanax morbifera Leveille (LC50 = 62.32 mg L^{-1}) (Chung et al., 2009) and fruits of Heracleum pastinacifolium subsp. Transcaucasicum (LC50 = 69.72 mg L^{-1}) (Tabanca et al., 2012).

In previous studies, other biological activities of EO of C. winterianus were found, such as its acaricidal action investigated in larvae and adult females engorged with tick Boophilus microplus (Agnolin et al., 2014) and also molluscicide against adult snails of the genus Biomphalaria, being reported for the first time by studies conducted by Rodrigues et al., 2013. The insect repellent action has already been reported by several authors (MENDONÇA et al., 2005; EDEN et al., 2018), among these investigations the repellency of citronella oil associated with 5% vanilla to three species of mosquitoes, Aedes aegypti, Culex quinquefasciatus and Anopheles sp. who were exposed to EO for more than 8 hours (TAWATSIN et al., 2001). Regarding fungicidal activity, a study developed by Cruz et al., (2015) indicated a reduction in the mycelial growth of F. solani according to the addition of the oil under analysis.

In the United States of America or USA, the Federal Insecticide, Fungicide, and Rodenticide (FIFRA) is the federal law that establishes the basic system of pesticide regulation in the United States for registration, distribution, sale and use (EPA, 2020). According to ISMAN (2016), Section 25(b) of FIFRA, which contains chemicals with active and inert ingredients considered to be of minimal risk, includes a dozen essential oils, among them citronella oil (C. winterianus). In the European Union, citronella oil is being considered for approval for use as insect repellents.

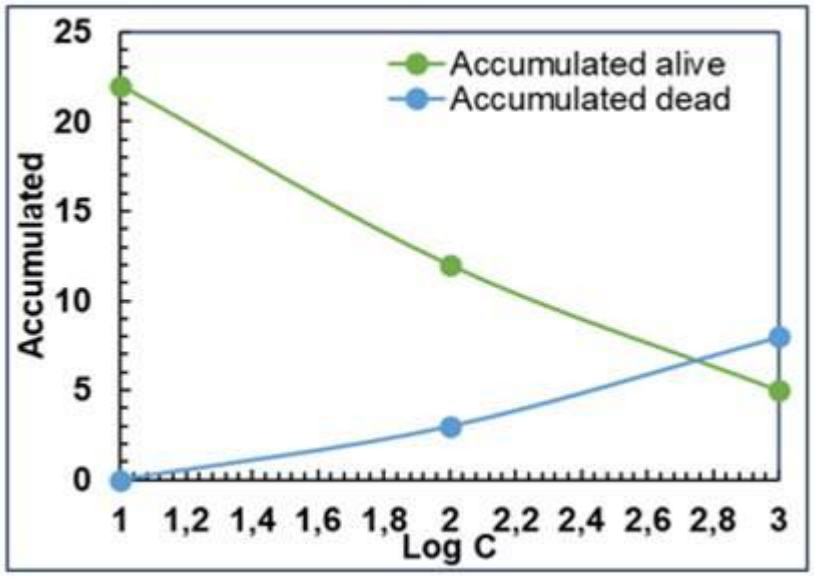
Table 4 shows that the EO solution extracted from C. winterianus causes a 50% mortality rate in the Artemia salina Leach in concentrations 1000 mg L^{-1}, represented by the Log of dose 3.00.

Table 4 - Mortality of the species of Artemia salina Leach due to the action of essential oil

Log C	N° dead	N° alive	Deaths accumulated	Alives accumulated	Mortality (%)
3	5	5	5	8	50
2	3	7	12	3	30
1	0	10	22	0	0

The LC50 calculated by the intersection of the accumulated curve alive and dead presented in Figure 1 resulted in LC50 of 532.34 mg L^{-1} and by the Dolabela criterion (1997) this classifies the EO as non-toxic. This result is contrasting with that obtained by Brasileiro et al. (2006), who identified the EO of Cymbopogon nardus as toxic (LC50 118.92 mg L^{-1}) for brine shrimp larvae. In addition, Pertiwi et al. (2014) investigated citronellal, one of the main chemical constituents of C. winterianus, performing its toxicity test against A. salina before and after 2 and 4 weeks of storage and found initial LC50 equal to 71,1 mg L^{-1}, showing to be highly toxic.

Figure 1- Curve of living and dead accumulations per concentration log



According to Ferreira et al. (2017), plants classified as toxic produce secondary metabolites that by inhalation, ingestion or contact can cause pathological changes in men and animals and, in certain situations, can lead to serious disorders in the body and even death.

Veletovac (2016) mentioned the importance of knowing the composition and concentration of the constituents of an EO, since its toxic potential is directly attributed. The author also reports that EOs of the genus Cymbopogon have a high range of application and have useful and popular substances, and after screening some EO of the family Poaceae, oils with large amounts of geraniol compared to those with citral as the main component can be classified as safe.

Thus, the obtained result is considered important, since the non-toxicity of the EO of C. winterianus proves its safety against non-target organisms in the regions of occurrence of insect vectors. No analyses were found to corroborate the non-toxicity of this oil in scientific productions, presenting an interesting characteristic for the use of EO studied in urban environments, where there is a higher incidence of water reservoirs that favor the proliferation of A. aegypti.

4 CONCLUSION

The essential oil of Cymbopogon winterianus presented larvicidal action against Aedes aegypti, proving to be more efficient for the aliquots of 7.5 µl and 10.0 µl that caused 100% mortality of the insect larvae studied after 24 hours of exposure. The extracted essential oil was classified as highly active with a lethal concentration of 46.18 mg L^{-1}. Biological assays with nauplii of Artemia salina were performed at different concentrations of the oil to evaluate toxicity. The calculated LC50 resulted in 532.34 mg L^{-1} configuring the non-toxic character, which demonstrates the safety of this essential oil for non-target organisms that coexist with the vector.

For future studies, the chemical composition of the essential oil and assays in endemic areas are necessary to identify the major metabolic species present in the botanical species, to explore its biological potential and to make viable the

development of natural larvicidal insecticides derived from the essential oil of citronella grass.

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