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Essential oils against *Candida* spp: *in vitro* antifungal activity of *Origanum vulgare*

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Experiments were conducted to evaluate the *in vitro* antifungal activity and determine chemical composition of *Origanum vulgare* essential oils. Nine *Candida albicans* (five clinical animal isolates and four ATCC strains), and three non-*albicans* species were studied; broth microdilution technique (CLSI) was used. Eight samples of commercial *O. vulgare* were submitted to hydrodistillation (Clevenger), and essential oils obtained were analyzed by gas chromatograph. Values of minimum inhibitory concentration (MIC) ranged from 1.0 to 0.065%, the average of MIC_{50} was 0.62 µL/mL while the average of MIC_{90} was 5.52 µL/mL and essential oils EO1 and EO8 were the ones with the best results. Results of thymol/carvacrol ratio were, for essential oils (EO) (EO1, 10.20/12.67; EO2, 7.88/0.50; EO3, 6.18/0.71; EO4, 0.58/21.58; EO5, 20.62/1.34; EO6, 21.95/4.71; EO7, 16.68/0.77; EO8, 8.42/9.44), demonstrated that differences between concentrations of thymol and carvacrol influenced MIC. These results indicate that the *O. vulgare* essential oils evaluated in this study presented growth inhibition activity over *Candida* spp.

Key words: *Origanum vulgare*, *Candida* spp., essential oil, thymol, carvacrol.

INTRODUCTION

Knowledge about medicinal plants was for a long time the only therapeutic resource of many communities and ethnic groups. The use of plants **as** treatment **to** cure illnesses has been since early times (Simões et al., 2003). Still today in poorer areas of the country and even in big Brazilian cities, medicinal plants are comercialized in popular markets and found in backyards of residences (Simões et al., 2003; Sartoratto et al., 2004). Regarding antimicrobial activity, plants of *Lamiaceae* family have been of interest due to their potential (Arango et al., 2004; Giordani et al., 2004; Rehder et al., 2004). Many species of this family, introduced in Brazil, are medicinal plants; they produce essential oils and are also used as seasoning or as ornamental flowers. Among them, *Origanum vulgare* stands out (Arcila-Lozano et al., 2004; Rehder et al., 2004; Rodrigues et al., 2004; Cleff et al.,

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2008a). Most important representatives of essential oils of this plant are terpenes, and among them monoterpenes and sesquiterpenes (Lambert et al., 2001; Arcila-Lozano et al., 2004; Rehder et al., 2004; Rodrigues et al., 2004; Cleff et al., 2008b). Studies with purpose of determining activity of *O. vulgare* essential oils have shown and demonstrated bacterial and fungal activity against different pathogens and this is attributed to the content of carvacrol and thymol that are major compounds in some oils (Lambert et al., 2001; Arango et al., 2004; Arcila-Lozano et al., 2004; Chami et al., 2004; Nostro et al., 2004; Rehder et al., 2004; Busatta et al., 2007, 2008; Cleff et al., 2008b).

Nowadays, due to higher frequency of patients susceptible to fungal infections, a larger number of reports of isolates resistant to antifungal have been observed as well as a higher rate of therapeutic failure, mainly in candidiasis (Chami et al., 2004; Santos Jr. et al., 2005; Magill et al., 2006). This mycosis has frequently been associated with hospital infections in immune-suppressed subjects, and in veterinary practice their importance is growing due to several reports of illness in animals (Mueller et al., 2002; Moretti et al., 2004; Magill et al., 2006; Cleff et al., 2007, 2008a).

The aim of this study was to evaluate *in vitro* antifungal activity and determine chemical composition of eight samples of *O. vulgare* essential oils, mainly in relation to thymol/carvacrol rate.

MATERIALS AND METHODS

O. vulgare samples

Commercial oregano samples with botanical certification were acquired in the market. Eight samples of oregano of Chilean and Uruguayan origin were evaluated considering the molar thymol/ carvacrol rate. Samples were numbered and named as: 1, Erechim; 2, Aves; 3, El Moncayo; 4, La Rosa; 5, Del Gaúcho; 6, Goes; 7, Treichel; 8, Torrenueva.

Isolation of essential oils

Oregano samples were subjected to hydrodistillation for 4 h using a modified Clevenger type apparatus, according to the Brazilian Pharmacopeia IV (1988). After extraction, oils were dried in anhydrous sodium sulphate, filtered, and concentrated under ultrapure N_2 and stored in amber flasks at 4°C. Essential oils obtained were named as EO1 (Erechim); EO2 (Aves); EO3 (El Moncayo); EO4 (La Rosa); EO5 (Del Gaúcho); EO6 (Góes); EO7 (Treichel) and EO8 (Torrenueva). The edium yield of essential oil (EO) in raw material was 1.282 mL (mL EO/100 g of oregano).

Chemicals

All chemicals (hexane, dichloromethane) were of analytical grade. Analytical standards such as α-pinene, β-pinene, myrcene, α-terpinene, p-cymene, camphene, limonene, 1.8-cineole, γ-terpinene, terpinolene, linalool, 4-terpineol, α-terpineol, thymol and carvacrol were supplied by Sigma. RPMI Media 1640 (Gibco BRL, Grand Island, NY, USA), Tween 80 (Sigma), Sabouraud dextrose agar (Oxoid, Basingstoke, UK), McFarland standard (bioMérieux, Marcy l'Etoile, France), and buffer 3-morpholinopropanesulfonic acid (MOPS) and phosphate-buffered saline (PBS) (Sigma) were also used in this research.

Chromatographic analyses

Essential oils were analyzed by gas chromatography with flame ionization detector (GC/FID - Schimadzu 17A) to identify main compounds. Chromatographic analyses were carried out in a chromatograph equipped with a DB-5 silica capillary column (methyl siloxane with 5% phenyl groups - 30 m x 0.25 mm ϕ, film thickness 0.25 μm). Nitrogen was used as a carrier gas with a flow rate of 1.0 mL min^{-1} and split ratio of 1:50. Injector and detector temperatures were both set at 280°C. Column temperature was programmed to 40°C, gradually increased to 145°C at 2°C min^{-1} and then increased to 280°C at 10°C min^{-1}, which was held for 10 min. Solution of each essential oil (5.000 µg mL^{-1}) and chromatographic standards (40 µg mL^{-1}) were prepared and 1 µL was injected at the same conditions. The compounds in the oregano samples were identified by comparison with retention time of standards and literature data (Rodrigues et al., 2004).

Candida spp. isolates

Twelve (12) isolates were used for *in vitro* tests: nine *C. albicans* (five of mucous membrane and cutaneous tegument of dogs and four standard strains - ATCC 44858, 4053, 18804 and IOC 3691), and three were non-albicans species: *C. parapsilosis* (ATCC 22019), *C. lusitanie* (ATCC 34449) and *C. krusei* (ATCC 34135). Regarding *Candida* spp. isolates, four isolates of *C. albicans* were from vaginal mucous membrane of canine and one isolate of cutaneous candidiasis was from a dog, from Pelotas, Rio Grande do Sul, Brazil. Isolated yeasts were maintained in subcultures in Sabouraud dextrose agar (SDA) and stored at 5°C, in Mycology Laboratory, Veterinary College, UFPel; Standard strains were kindly supplied by Osvaldo Cruz Foundation (Fiocruz, INCQS), Rio de Janeiro, Brazil.

Inoculums preparation

Yeast inoculum was prepared by growing *Candida* isolates on SDA for 24 h at 35°C and then suspended in 5 mL of sterile physiological saline solution and homogenized. The inoculum suspension had its turbidity adjusted according to 0.5 McFarland standards which corresponds to an initial inoculum with approximately 5 x 10^6 CFU mL^{-1}. Then, 1:50 dilution in sterile physiological saline solution was prepared, and another 1:20 dilution in RPMI Media 1640 was prepared in 96-well microtiter plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Adjusted inoculum suspension (100 µL) was dispensed in each well, resulting in desired final drug concentration and inoculum size between 1 and 5 x 10^3 CFU mL^{-1}.

Antifungal activity

Microdilution in broth technique was used to evaluate susceptibility of *Candida* spp. against oils, according to National Committee for Clinical Laboratory Standards, NCCLS, M 27-A2 (CLSI) with few modifications. A 1% solution of Tween 80 as dispersant of essential oil in RPMI Media was used, avoiding micellas formation and favoring dilution. Ten (10) serial dilutions from stock solutions of essential oil of *Origanum* were prepared in RPMI medium with L-

glutamine without bicarbonate and buffered with MOPS at pH 7.0. For yeasts, minimum inhibitory concentrations (MICs) were determined by subculturing 100 µL of those concentrations from each well (1 to 10) of microtiter plate. Positive control (inoculum/ media) and negative control (essential oil/media) were placed into wells 11 and 12. Plates were incubated aerobically at 35°C for 48 h with shaking. Susceptibility was expressed as MIC, defined as lowest concentration required to delay growth of fungi by 24 h of incubation in relation to the positive control. Also, minimum concentration were determined in µg/ml, capable to inhibit 50 and 90% from isolated, calculated as MIC_{50} and $MIC_{90.}$

RESULTS

Chromatographic analyses

Table 1 shows the results obtained from chromatographic analysis of eight *O. vulgare* essential oils where the difference between phenol constituent can be observed. Analyzed oils presented the following concentrations regarding thymol/carvacrol rate: EO1, 10.20/12.67; EO2, 7.88/0.50; EO3, 6.18/0.71; EO4, 0.58/21.58; EO5, 20.62/1.34; EO6, 21.95/4.71; EO7, 16.68/0.77; EO8, 8.42/9.44. In Table 1, other important compounds such as 4-terpineol, α-terpinene, *p*-cymene, γ-terpinene and α-terpineol can be observed. Figure 1 presents a graph with concentration variations (%) of *p*-cymene, 4-terpineol, thymol and carvacrol, in different essential oil samples of *O. vulgare* (origanum), where it was possible to observe great variation in tymol/carvacrol rate.

Antifungal activity

After incubation period, the microdilution plates were observed, by the technique of microdilution in broth, to detect the sensitivity of isolates of *Candida* spp. to *O. vulgare* essential oils. Table 2 shows the results of the MIC of eight *O. vulgare* essential oils using this technique. In this table, the MIC did not demonstrate significant variation among different isolates; what is observed when pure antifungal substances are used. MIC_{50} and MIC_{90} are indicative of a less concentration capable of growth inhibition of 50 and 90% of the isolates. This data is shown in Table 3, with an average of MIC_{50} of 0.62 µL/mL and the average of MIC_{90} of 5.52 µL/mL.

Results demonstrate susceptibility differences among field isolates, standards and different species of *Candida*. It was observed that the most active essential oils were one and eight (EO1 and EO8) which presented lower MIC_{90} values than other oils (EO2, EO3, EO4, EO5, EO6 and EO7).

DISCUSSION

Researchers have reported antimicrobial potentiality of essential oils, using different techniques of antifungigram as agar diffusion, disc diffusion testing and broth microdilution susceptibility assay, where the last used give good results (Lambert et al., 2001; Arango et al., 2004; Rehder et al., 2004; Busatta et al., 2007, 2008; Cleff et al., 2010).

These data are in accordance to what has been described in literature, where *O. vulgare* essential oil presents a great variation in composition, mainly in active compounds, where monoterpene phenols such as thymol and carvacrol reached 80.2 to 98% of total composition of this oil (Simões et al., 2003; Arcila-Lozano et al., 2004; Rehder et al., 2004; Rodrigues et al., 2004; Cleff et al., 2008). Geographic factors such as altitude, soil, climate, harvest, culture, drying process and storage conditions among others, have influence on composition, quality and amount of each compound present in essential oil from plants (Cox et al., 2000; Simões et al., 2003; Arango et al., 2004; Arcila-Lozano et al., 2004).

The higher values of MIC_{90} presented by six other essential oils (EO2 the EO7) can be justified by the variation in thymol/carvacrol compound rates (EO2-15.76; EO3-8.70; EO4-0.03; EO5-15.39; EO6-4.66 and EO2-21.66), showing always higher concentration of one in relation to the other.

Chromatographic analyses of oregano essential oil samples (EO1 and EO8) showed better result *in vitro* ($MIC_{90=}$ 3,54 and 3,87, respectively); presented mostly 4-terpineol, thymol and carvacrol components who deserves attention to concentration balance between thymol (10.20 and 8.42) and carvacrol (12.67 and 9.44) associated to a large concentration of 4-terpineol (21.32 and 47.95), respectively. It was reported that the composition 4-terpineol acts be inducing deformations in the cell membrane, modifying consequently its permeability (Cox et al., 2000). Another factor to be considered is that the use of commercial oregano could have leaf mixtures, flowers and stems of *O. vulgare* in the sample, as well as the presence of different oregano genus in the same sample. Although all organs of a plant can accumulate volatile oils, its composition can change according to part or species studied, since just *Origanum* genus presents 39 different species (Simões et al., 2003).

Compounds without aromatic ring and/or hydroxyl groups, such as γ- terpinene, α-terpinene and *p*-cymene, monoterpenes hydrocarbons, were found in significant amount in those samples. That could justify the MIC values achieved by these essential oils (Nostro et al., 2004). Presence of the *p*-cymene, has an antagonistic effect with carvacrol and thymol, which would explain lower antimicrobial activity of essential oils EO2 (20.38); EO4 (13.96) and EO6 (7.01).

High concentrations of these compounds and their proportion are of great importance for effectiveness of the product. However studies have demonstrated that these isolated compounds do not have same efficiency of essential oil. Bio-synthetic precursors γ-terpinene and *p*-cymene, as well as α-terpineol, 1.8-cineol and linalool, also showed antimicrobial activity, justifying higher efficiency

Table 1. Concentration (%) of compound in eight samples of *O. vulgare* essential oils analyzed by gas chromatography (GC/FID).

S/N	Compound**	EO1	EO2	EO3	EO4	EO5	EO6	EO7	EO8
1	α-thujene	0.63	2.15	0.71	0.63	0.90	0.12	0.31	0.25
2	α-pinene***	1.53	0.51	Nd	0.10	nd	nd	Nd	nd
3	Sabinene	1.53	5.47	0.93	1.60	1.24	0.18	0.47	0.33
4	β-pinene***	0.19	0.27	nd	0.13	nd	nd	Nd	nd
5	Myrcene***	1.22	1.71	0.12	0.70	0.28	0.20	0.30	0.18
6	α-phellandrene	1.47	2.27	3.54	1.80	2.28	1.73	2.26	2.47
7	α-terpinene***	3.46	7.00	4.64	4.27	5.60	2.43	2.83	2.83
8	p-cimene***	15.90	20.38	1.85	13.96	1.84	1.13	7.01	0.71
9	Limonene***	4.04	6.22	4.92	3.65	3.74	2.59	3.44	3.60
10	1,8-cineole***	2.75	0.09	nd	0.17	0.07	nd	0.07	0.53
11	cis/trans β-ocimene	0.56	0.62	0.04	0.43	0.26	0.13	0.24	0.08
12	γ-terpinene***	3.69	8.31	4.25	4.10	7.80	5.91	2.18	4.86
13	trans-sabinene hidrate	0.11	0.97	0.34	0.27	0.27	nd	0.13	0.07
14	Terpinolene***	1.61	3.41	1.89	2.10	2.78	1.63	1.29	1.69
15	cis-sabinene hidrate	0.26	2.36	0.54	0.55	0.61	nd	0.26	0.08
16	Linalool***	3.78	1.40	3.08	1.70	3.53	2.07	4.58	2.89
17	trans-*p*-menthenol	0.17	0.28	0.33	0.26	0.14	0.10	0.25	0.12
18	cis-*p*-menthenol	0.16	0.09	nd	0.10	nd	nd	Nd	0.05
19	Borneol	0.40	0.23	0.13	0.10	0.26	0.36	0.34	0.27
20	4-terpineol***	21.32	11.92	50.87	27.67	31.04	41.17	39.45	47.95
21	α-terpineol***	4.23	2.78	8.87	3.69	4.78	4.98	5.74	7.57
22	trans-piperitol	0.15	0.10	0.30	0.26	0.08	0.17	0.11	0.35
23	cis-piperitol	0.10	0.06	nd	0.09	nd	nd	0.05	nd
24	Methyl thymol eter	1.00	1.41	0.30	nd	1.03	0.58	1.73	0.10
25	Methyl carvacrol eter	1.92	2.60	0.57	5.78	2.41	3.04	2.80	0.71
26	Linalil acetate	0.31	0.21	nd	0.42	nd	nd	0.26	nd
27	Geraniol or nerol	1.35	5.30	0.68	0.29	2.21	0.51	1.25	0.70
28	Thymol***	10.20	7.88	6.18	0.58	20.62	21.95	16.68	8.42
29	Carvacrol***	12.67	0.50	0.71	21.58	1.34	4.71	0.77	9.44
30	Geranil or neril acetate	0.35	0.08	0.20	nd	0.51	0.21	0.47	0.20
31	β-caryophyllene	0.98	1.12	2.60	1.04	3.10	3.22	2.34	2.92
32	Germacrene	0.23	0.05	nd	0.64	nd	nd	0.07	nd
33	Spathulenol	1.13	1.25	1.18	0.78	1.06	0.81	1.83	0.59
34	Caryophylene oxide	0.60	0.98	0.23	0.56	0.24	0.07	0.51	0.05

EO1, Erechim; EO2, Aves; EO3, El Moncayo; EO4, La Rosa; EO5, Del Gaúcho; EO 6, Goes; EO7, Treichel; EO8, Terranueva.**C (%) = normalised peak areas without using correction factors; *** compounds identified by comparasion with standards ; nd = not detected. Other compounds were identified by literature data (Rodrigues et al., 2004; Busatta et al., 2008).

of essential oil when compared to isolated compounds (Simões et al., 2003). Action mechanism of essential oils is complex and it is not totally elucidated. Some studies suggested an alteration in activity of calcium canals, causing increase in permeability and liberation of vital intracellular constituents, promoting a decrease in intracellular ATP in cells. Simultaneously, it causes an increase in extracellular ATP, leading to rup-ture in cellular membrane of microorganism (Sikkema et al., 1994, 1995; Sartoratto et al., 2004). Antimicrobial activity can be due to damage caused to several enzymes, including those involved in production of energy and synthesis of structural components of microorganism (Lambert et al., 2001).

In the present work, samples analyzed, besides phenol compounds, 4-terpineol was sometimes in larger amount than thymol or carvacrol (phenolics compounds) and α-terpineol; both terpenes alcohol, monoterpenes with hydroxyl group (OH), were able to make polar hydrogen bonds, which justify the good result of MIC_{90}. For

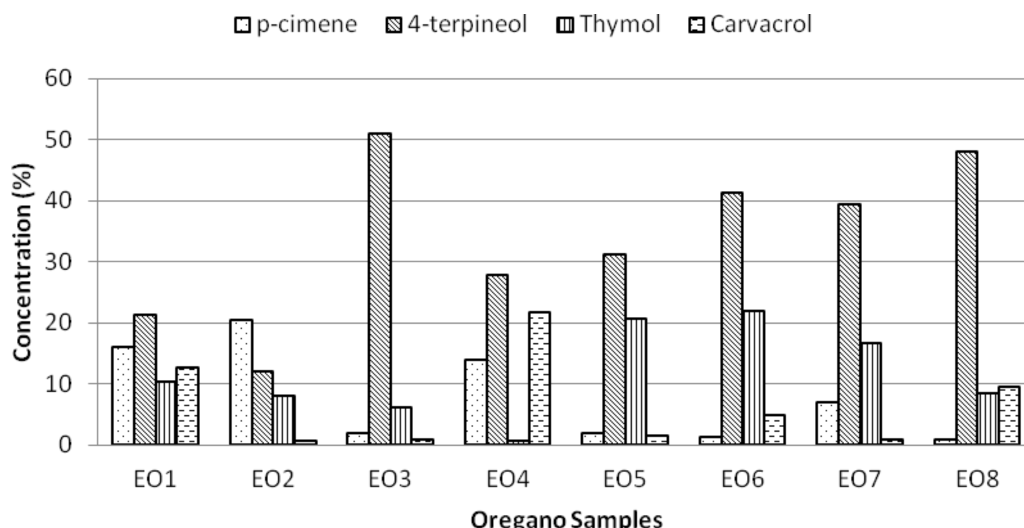

Figure 1. Graph of concentration (%) of compounds *p*-cymene, 4-terpineol, thymol and carvacrol, in oregano oil samples. EO1, Erechim; EO2, Aves; EO3, El Moncayo; EO4, La Rosa; EO5, Del Gaúcho; EO 6, Goes; EO7, Treichel; EO8, Terranueva.

Table 2. Minimum Inhibitory concentration (µL mL^{-1}) of the eight *O. vulgare* essential oils against *Candida* spp.

Isolate/essential oil	EO1	EO2	EO3	EO4	EO5	EO6	EO7	EO8
C. albicans cutaneous canine	2.5	5.0	10	10	5.0	2.5	5.0	2.5
C. albicans mucous canine	1.25	5.0	2.5	5.0	2.5	2.5	2.5	2.5
C. albicans mucous canine	2.5	2.5	10	5.0	2.5	5.0	2.5	2.5
C. albicans mucous canine	2.5	10	5.0	10	5.0	5.0	5.0	1.25
C. albicans mucous canine	2.5	10	5.0	5.0	2.5	2.5	10	2.5
C. albicans ATCC 18804	0.65	2.5	2.5	2.5	5.0	2.5	1.2	1.25
C. albicans ATCC 44858	1.25	2.5	5.0	5.0	2.5	2.5	2.5	2.5
C. albicans ATCC 4053	1.25	10	5.0	10	2.5	5.0	2.5	2.5
C. albicans IOC 3691	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
C. parapsilosis ATCC 22019	1.25	2.5	5.0	5.0	2.5	2.5	2.5	2.5
C. lusitanie ATCC 34449	1.25	5.0	2.5	1.2	1.2	5.0	2.5	1.25
C. krusei ATCC 34135	2.5	2.5	5.0	5.0	10	2.5	2.5	2.5

Table 3. MIC_{50} and MIC_{90} of eight oil extracts from *O. vulgare* against *Candida* spp isolates strains from regions of South Brazil and controls.

EO	Number (%) of strains inhibited					MIC_{50}	MIC_{90}	Thymol/Carvacrol	Rate
	0.62 µL/mL	1.25 µL/mL	2.5 µL/mL	5 µL/mL	10 µL/mL				
OE1	1 (8.3)	5 (4.16)	6 (50)	0	0	0.69	3.54	10.2/12.67	0.80
OE2	0	0	6 (50)	3 (25)	3 (25)	0.6	6.83	7.88/0.5	15.76
OE3	0	0	4 (33.3)	6 (50)	2 (16.6)	0.6	7.32	6.18/0.71	8.7
OE4	0	1 (8.3)	2 (16.6)	6 (50)	3 (25)	0.6	8.01	0.58/21.58	0.02
OE5	0	1 (8.3)	7 (58.3)	3 (25)	1 (8.3)	0.6	4.97	20.62/1.34	15.38
OE6	0	0	8 (66.6)	4 (33.3)	0	0.68	5.07	21.95/4.71	4.66
OE7	0	1 (8.3)	8 (66.6)	2(16.6)	1 (8.3)	0.6	4.6	16.68/0.77	21.66
OE8	0	3 (25)	9 (75)	0	0	0.6	3.87	8.42/9.44	0.89

example, in samples which showed lower MIC_{90} (EO1 and EO8), the rate of thymol/carvacrol was 0.80 and 0.89, respectively, that is, very close concentrations [EO1, (10.20/12.67) and EO8 (8.42/9.44)].

Several authors believe that aromatic ring with a polar functional group, is responsible for antimicrobial activity (Milos et al., 2000; Lambert et al., 2001). It is suggested that the presence of this group and hydroxyl group (-OH), would form hydrogen bond with the active microbial enzyme small sites. Therefore, it is possible to suppose that these groups (aromatic ring and hydroxyl group) are responsible for antimicrobial activity (Milos et al., 2000; Lambert et al., 2001; Ultee et al., 2002*).*

Conclusion

Results demonstrate that differences between concentrations of thymol and carvacrol influenced the MIC_{90} of *O. vulgare* oils against yeasts studied. Balance of concentration of phenolic compounds, thymol and carvacrol, seems to have synergic and positive effect on growth inhibition of different species of *Candida,* however the eight oils analyzed presented antifungal activity *in vitro.*

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