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Searching for Algaecide or Algaestatic Effects of Several Plant Extracts on Phytoplankton: Preliminary Results

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CF and AMG designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors CF and SB did the lab work and author VG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Evaluate the *in vitro* effects of essential oils and water extracts of *Laurus nobilis*, *Rosmarinus officinalis*, *Mentha suaveolens* and *Fraxinus angustifolia* on the growth of *Anabaena cylindrica* and *Chlorella vulgaris.*

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Study Design: Experimental research.
Place and Duration of Study: The cyanobacterium *Anabaena cylindrica* and the green alga *Chlorella vulgaris* were used as test strains to evaluate the effects of plant extracts on algal growth. All experiments were undertaken in the Agricultural School of Bragança - Polytechnic Institute, from September 2010 to July 2011.
Methodology: Essential oils were obtained by means of hydrodistillation of the plants. The oils and the water that remained, after the hydrodistillation, were further used for the growth screening of *Anabaena cylindrica* and *Chlorella vulgaris* under axenic cultures. Both types of extracts were tested at different concentrations. The essential oil effects were evaluated by disc diffusion method and water extracts effects were evaluated in batch cultures.
Results: Essential oils had an algaecide effect in all tested concentrations (1:1; 1:3; 1:4 and 1:10) for both algal strains. Contrarily, none of the water extracts evidenced a complete algaecide effect. Nevertheless, promising results were obtained with rosemary water extract since the highest concentrations (1:4) had an algaestatic effect on *C. vulgaris*. Conversely, the observed effects on *A. cylindrica* varied from cellular density decrease to an algaestatic effect. Therefore, the tested algal strains presented distinct responses to both extract types and concentrations.
Conclusions: Comparing the different extracts' activity, it can be concluded that essential oils mostly influenced algal growth.

Keywords: Plant extracts; algaecide/algaestatic effect; phytoplankton; eutrophication; ecosystems management.

1. INTRODUCTION

Algal blooms are increasingly frequent in lakes and in reservoirs as a consequence of eutrophication. The ultimate consequence of this phenomenon is the degradation of the ecological, economical and aesthetic value of the affected water bodies [1].

Mitigating the eutrophication consequences became an increasing concern of the aquatic ecosystem managers. The long-term goal is reducing nutrient inputs but even if this is achieved continued management will often be necessary to maintain lakes and other water bodies in the 'water clear' state. Whilst nutrient levels are being reduced or in situations where the chances of doing so are extremely remote, emergency measures may be required to deal with algal growth problems. There are several methods to control the excessive algal growth (e.g. [2]). One of the most common direct methods of controlling the phytoplankton growth is the application of conventional industrial algaecides such as copper-based algaecides. However, the use of these substances has several limitations such as toxicity towards non-target organisms, short term effects and environmental prevalence [3]. Field evidence and laboratory studies indicated that several active compounds produced by plants can influence phytoplankton growth [4-10].

Therefore, the determination of the effects of natural products, such as plant extracts, on algal growth is of paramount importance to allow the development of safe non-pollutant methods to control excessive algal growth. In this context, the present study aims to evaluate the *in vitro* effects of essential oils and the water extracts of *Laurus nobilis*, *Rosmarinus officinalis*, *Mentha suaveolens* and *Fraxinus angustifolia* on the growth of *Anabaena cylindrica* and *Chlorella vulgaris*. Literature concerning antibacterial and fungicide activity of essential oils of the plants that are used in the present research is very abundant

[11-17]. However, research on screening the effects of plant essential oils on algal growth is much less abundant. In fact, the present study seems to be the first to report effects of these essential oils on microalgae growth. Concomitantly, the effect of the water extracts (remained water from hydrodistillation process) on both phytoplankton species growth was also assessed. Data concerning the effects of these water extracts on phytoplankton growth are, until the present time, inexistent.

2. MATERIALS AND METHODS

2.1 Plants and Extracts

The laurel (*Laurus nobilis* L.), rosemary (*Rosmarinus officinalis* L.), mint (*Mentha suaveolens* Ehrh.) and ash (*Fraxinus angustifolia* Vahl.) plants were collected in the Trás-os-Montes region, Portugal (41°47'54.14" N; 6°45'35.49" W) in September of 2010. Plants were stored at –20°C before the extraction. Essential oils were extracted from 100 g of plant material (stem and leaves) by hydrodistillation during 3 h using a Clevenger-type apparatus at < 100°C and ultrapure water (Milli-Q50). Water extracts were prepared from the previous process by filtering the remaining water where plant material was boiled. Essential oils and water extracts were stored at –20°C in the dark prior to experiments.

2.2 Test Algal Strains

The cyanobacterium *Anabaena cylindrica* and the green alga *Chlorella vulgaris* were used as test strains to evaluate the effects of plants extracts on algal growth. *Anabaena cylindrica* UTAD- A212 was provided by the University of Trás-os-Montes e Alto Douro and *Chlorella vulgaris* CBSC 15-2075 (Carolina Biological Supply Company, USA) was provided by the College of Biotechnology of the Portuguese Catholic University. *C. vulgaris* and *A. cylindrica* were separately grown in 250 mL flasks with 100 mL sterilized Walne modified medium and BG_{11} medium, respectively. Microalgae strains were pre-cultured in a controlled chamber under the following conditions: temperature of 22±1°C, light intensity of 4500 lux (Gro-Lux fluorescent lamps), 16:8 h light: dark photoperiod, with agitation, until they reached exponential growth phase and were able to be used for experimental purposes.

2.3 Assessment of Essential Oils Effects

The effect of essential oils on both algal species was evaluated by modifying the disc diffusion method. Shortly, Petri dishes (60x15mm) with solified agar-growth medium (Walne Modified or BG_{11}) were plating with 150 µL of each test microalgae culture. Immediately after, sterile discs (6 mm, Whatman N° 2017-006 AA) were placed on the agar medium and impregnated with 30 µL of essential oils solution. This later consisted of essential oils dissolved into DMSO (dimethylsulfoxide, Merck K40270552 936). The concentration gradients tested were 1:1; 1:3; 1:4 and 1:10 in a total volume of 30 µL. Discs alone as well as those impregnated with DMSO were also placed in the inoculated plates with agar-growth medium, for control. Control algal grow, plated only with test microalgae culture, was also performed. One disk per Petri dish was used. All Petri dishes were incubated in the controlled growth chamber under the same conditions for 10 days. All the assays were performed in duplicate. The degree of algal growth inhibition was assessed by the size of inhibitory zones formed around the leaf disk.

2.4 Assessment of Water Extracts Effects

The effect of water extracts on the growth of microalgae was evaluated in batch cultures. All culture experiments were carried out in Erlenmeyer flasks filled with culture medium (Walne modified for *C. vulgaris* and BG_{11} for *A cylindrica*) and supplemented with water extracts. Two concentrations of water extracts were tested: 1:4 and 1:10 in a total volume of 100 mL (25 mL and 10 mL of water extracts, respectively). Control cultures with culture medium alone were also carrying out. All the cultures, after inoculation, were incubated in the controlled growth chamber, at the same previous conditions for several days. Growth assessment for *C. vulgaris* was made by means of cell density by cell counting, using Improved Neubauer haemocytometer. Growth of *A. cylindrica* was assessed by chlorophyll *a* content, estimated spectrophotometrically according to [18].

Results were expressed as specific growth rate (μ), maximum cell density (MCD) and maximum chlorophyll a content (MCC). Specific growth rate was determined during exponential growth phase as:

$$\mu = \frac{(LogNt - LogN0)}{t}$$

Nt = cells number at time t; *N*0 = cells number at start; t = time.

Maximum cell density (MCD) and maximum chlorophyll content (MCC) were calculated as the ratio between maximum and initial concentration, using results of cell density and chlorophyll *a* content, respectively.

3. RESULTS

3.1 Assessment of Essential Oils Effects

The plants for essential oil effect assessment were chosen considering their essential oil extraction recovery since a very low volume of extract limits the viability of plant use. Consequently, only laurel, rosemary and mint essential oils were tested.

All the essential oils and concentrations tested had a negative effect on algal growth when compared with control plates. After incubating the plates for 10 d a generalized inhibitory zone was observed in the surface of all the dishes. As expected, the control, i.e. discs alone, as well as those impregnated with DMSO exhibited the same growth aspect as the control growing, i.e. Petri dishes only with test microalgae culture (Fig. 1). Even with the extension time of the incubation for more additional 7days no growth was observed for both strains.

3.2 Assessment of Water Extracts Effects

Results of *C. vulgaris* growing in batch cultures supplemented with water extracts were followed during 9 days until control algal cultures reached their exponential growth phases. Results of daily counting are presented on Table 1.

The experimental set of 1:4 concentrations of water extracts showed, for control cultures, an exponential growth phase from 2d to 8d, reaching a μ of 0.6 day^{-1} and a MCD of $2800x10^6$

cells/mL. Comparing cultures supplemented with mint water extract showed an exponential growth phase between 3d to 9d with a lower μ and lower MCD, of 0.47 day^{-1} and 1630x10^6 cells/mL, respectively. Interesting results were obtained for rosemary and laurel water extracts. In fact, cultures of *C. vulgaris* exposed to these extracts showed quite the same cellular density after incubation time and did not achieve the exponential growth phase. Despite higher initial cell density in these both assays, the MCD obtained in the presence of rosemary extract was 2x10^6 cells/mL and 7x10^6 cells/mL for the laurel extract (Table 1).

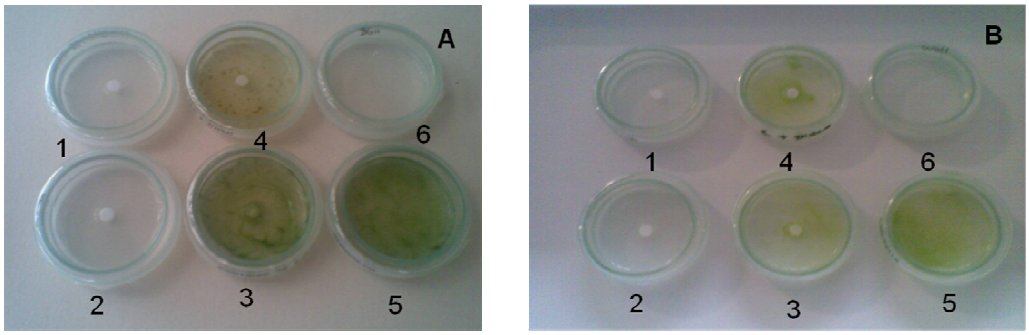

Fig. 1. Example of algae growth response with essential oil, by disc diffusion assay: (A) *A. cylindrica*; (B) *C. vulgaris*: 1 and 2 - growth assessment with rosemary essential oil (1:4); 3 – control disc + DMSO; 4 – control disc; 5 – growth control; 6 – axenic control

Table 1. Initial cell concentration (IC), specific growth rate (μ) and maximum cell density (MCD) for *C. vulgaris* cultured in batch, under two concentrations of water extracts (1:4 and 1:10)

	1:4			1:10		
	IC (x10^4/mL)	μ (day^{-1})	MCD (x10^6/mL)	IC (x10^4/mL)	μ (day^{-1})	MCD (x10^6/mL)
Control	35±4.95	0.60±0.11	2800±283	120±19.09	0.50±0.03	250±26.16
Mint	54±5.24	0.47±0.02	1630±131	43±6.36	0.54±0	1256±31.11
Rosemery	89±12.73	a	2±0.49	140±8.49	0.54±0.02	250±36.77
Laurel	150±16.26	a	7±0.94	81±17.09	0.53±0.02	642±29.70
Ash	59±2.83	n.c.	339±47	33±4.95	0.45±0.05	1000±141

M±DP, n=2; a - absence of exponential growth phase; n.c.– not calculated

The *C. vulgaris* cultured with ash extract did not show a true exponential growth phase and therefore μ was not calculated, yet exhibiting a slightly cell increase with a MCD of 339 x10^6 cells/mL, however lower than the control.

The experimental set of 1:10 concentrations of water extracts showed, for control cultures, an exponential growth phase from 3d to 8d leading to a μ of 0.50 day^{-1} and MCD of 250x10^6 cells/mL. Cultures supplemented with water extracts generally showed both, μ and MCD, higher than control cultures. Cultures supplemented with mint, rosemary and laurel water extracts showed a little increase of μ. However, high MCD was observed just for cultures supplemented with mint and laurel. Only cultures supplemented with ash water extracts exhibited a slightly lower μ (0.45 day^{-1}) but with higher MCD of 1000x10^6 cells/mL than the control (Table 1).

Batch cultures of *A. cylindrica*, supplemented with 1:4 and 1:10 concentrations of water extracts, were followed during 23 and 19 days, respectively, until control algal cultures reached their exponential growth phase. Results of chlorophyll *a* (Chl *a*) content measured 5 times along the experiments are presented in Table 2.

For the experimental set of 1:4 concentrations of water extracts, control cultures showed a gradual Chl *a* increase with a MCC of 5.34 µg/mL. Conversely, all the cultures supplemented with water extracts exhibited a general Chl *a* content decrease (Table 2, Fig. 2A and B), not allowing MCC calculation. Compared with control cultures, all the supplemented ones showed a negative net Chl *a* variation with rosemary and laurel water extracts showing the highest reduction (Fig. 2B). Cultures supplemented with mint and ash water extract showed a similar net Chl *a* decrease.

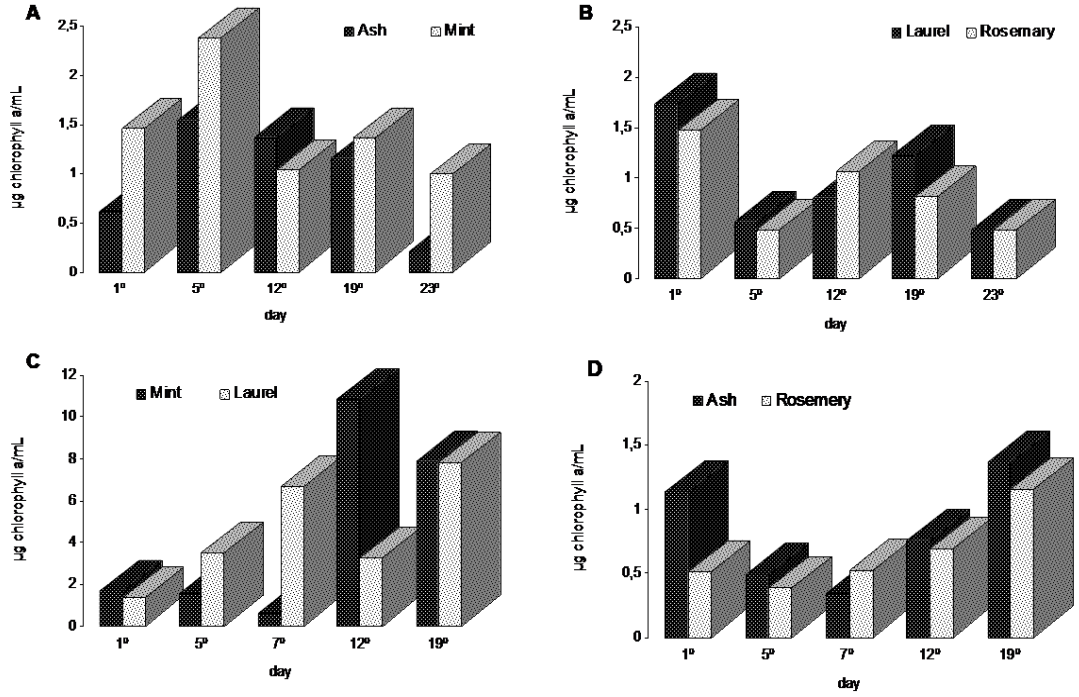

Fig. 2. Chlorophyll *a* variation of *A. cylindrica* cultures, supplemented with 1:4 concentrations (A, B) and 1:10 concentrations (C, D) of water extracts

For the experimental set of 1:10 concentrations of water extracts, control cultures showed a MCC of 5.45 µg/mL. Mint and laurel water extracts were the cultures exhibiting the highest growth (Fig. 2C), with a MCC of 4.64 µg/mL and 5.69 µg/mL, respectively. Both treatments presented also the highest net Chl a increase (Table 2). On the other hand, Chl *a* content of *A. cylindrica,* exposed to rosemary and particularly ash water extract, although with an high value for both noticed at 19d (leading to a MCC of 2.25 µg/mL and 1.21 µg/mL, respectively), generally shows small Chl *a* increase (Table 2, Fig. 2D). In fact, when time of incubation was extended until to 23d it revealed Chl *a* values slightly lower than initial values for these cultures (µg/mL): for rosemary 1d=0.51 and 23d= 0.41; for ash 1d=1.13 and 23d=0.91.

Table 2. Initial chlorophyll a content (IC), maximum chlorophyll a content (MCC) and net variation of chlorophyll a content (NV) for *A. cylindrica* cultured in batch, under two concentrations of water extracts (1:4 and 1:10)

	1:4 (µg/mL)			1:10 (µg/mL)		
	IC	**MCC**	**NV***	**IC**	**MCC**	**NV***
Control	1.63±0.83	5.34±2.99	7.07±1.95	1.28±0.23	5.45±1.01	5.70±2.28
Mint	1.45±0.09	n.c.	-0.46±0.09	1.70±0.06	4.64±0.90	6.19±1.77
Rosemery	1.47±0.11	n.c.	-1.00±0.26	0.51±0.41	2.25±2.06	0.64±0.48
Laurel	1.72±0.76	n.c.	-1.24±0.17	1.37±0.53	5.69±3.97	6.41±2.53
Ash	0.61±0.19	n.c.	-0.41±0.17	1.13±0.08	1.21±0.46	0.23±0.53

M±DP, n=2; NV = (final chlorophyll a content – initial chlorophyll a content); n.c. – not calculated*

4. DISCUSSION

During a comparison of the different extract activities, it can be concluded that essential oils were the most active extracts inhibiting algal growth, thus showing algaecide effect. This evidence certainly arises from the different composition of these extracts. Chemical characteristics of essential oils could have been decisive for these results since no plaque zone diameter was observed. In fact, volatility of the essential oils compounds could have been the cause for the absence of algal growth in all Petri dish areas.

The observed algaecide effect could have arisen from the presence of strong active allelopathic substances with effects against *C. vulgaris* and *A. cylindrica.* Despite the absence of studies concerning the composition of these plant ecotypes, it is plausible that compounds such as 1,8-cineole methyleugenol and α-terpinyl acetate could be present in the studied plant ecotypes. According to [19,20], these compounds are mainly present on essential oils of stems and leaves of laurel. In the same way, rosemary essential oils are composed by large amounts of pinene, 1 and 8-cineole [21-24] and mint by pulegone, menthenolide, piperitenone and piperitone oxides [11,15]. Some of the mentioned compounds are known for having a wide spectrum of antifungal and antibacterial activity. The 1,8-cineole tested alone showed moderate antimicrobial potential [12,25-27]. Microbiological experiments revealed that methyleugenol was active against fungi and bacteria [28,29] and α-terpinyl acetate showed a reduction in viable spores of *Bacillus subtilis* [30] and a strong activity against *Staphylococcus aureus* [27]. Pulegone showed some antimicrobial activity against several microorganisms [31,11,15] and menthenolide exhibited a fair antibacterial activity [15], as well as piperitenone and piperitone oxides [11]. In contrast, pinene did not show any significant inhibitory activity against spoiling and pathogenic microorganisms [27]. The fact that these compounds alone usually did not show the same level of antimicrobial activity as the entire essential oils suggested that minor components may also be involved or synergistic interactions may occur. Indeed, the strong algaecide effect of these essential oils, observed in the present research, could have been a consequence of this issue.

The antibacterial properties of essential oils are associated with their lipophilic character [23]. According to [32,14]; essential oil fractions sensitise the cell membrane, causing an increase in its permeability and the leakage of vital intracellular constituents as well as the impairment of bacterial enzyme system and cell respiration. Therefore, the observed algaecide effect could have been caused by a similar effect on algal membrane cells.

The most promising results of cultures supplemented with the concentration of 1:4 water extracts were obtained with rosemary and laurel water extracts. Batch cultures of *C. vulgaris* growing with these water extracts showed an absence of exponential growth phase and almost the same cellular density after incubation time. These results suggest that rosemary and laurel water extracts, at this concentration, had an algaestatic effect on *C. vulgaris,* leading to a null μ. All of *A. cylindrica* cultures supplemented with 1:4 concentrations of water extracts exhibited a Chl *a* net content decrease. However, rosemary and laurel water extracts induced the highest reduction, suggesting an algaecide effect.

On the other hand, *C. vulgaris* supplemented with the 1:10 concentrations of water extracts did not show any algaestatic or algaecide effect. Quite the opposite, revealed when exposed to rosemary and laurel water extracts and a slight increase of μ occurred. These results could reflect an action-effect depending on concentration. At low concentrations, rosemary and laurel water extracts might act as increasing nutrient supplementation leading to higher μ. Conversely, the highest concentrations of these water extracts induced an algaestatic effect on *C. vulgaris* growth. The Chl *a* content of *A. cylindrica* when exposed to 1:10 concentrations of rosemary water extracts indicated a slightly decrease when incubation time was extended to 23 days. Thus, changes in terms of Chl *a* content of *A. cylindrica* under rosemary water extract suggested an algaecide effect for the highest concentration (1:4) and an algaestatic effect for the lowest concentration (1:10).

The results of ash water extract were interesting. For high concentration for both cultures, *C. vulgaris* and *A. cylindrica*, a decrease on growth, comparing with control cultures, was observed, suggesting an effect on cell numbers. Lower concentration still had an effect on *A. cylindrica* since with the extension of incubation time a slightly lower value of Chl *a* content, than the initial, was observed.

Although none of the tested water extracts revealed complete algaecide action i.e. total microalgae death, results obtained with rosemary pointed out its potential algistatic effect. The results obtained were not surprising since water extracts of this plant prepared by boiling whole plant for 1 h and tested on a biological system (*Allium cepa)* showed a significant capacity to inhibition of cell division [33]. According to these authors, a gradual decrease in protein synthesis occurred in the consequence of exposition which causes a deleterious effect.

However, it seems that rosemary water extract action differed between *A. cylindrica* and *C. vulgaris*, being more pronounced on the first alga. The *C. vulgaris* exposure to the highest concentration brought on an algaestatic effect and exposure to the lowest concentration did not show any promising effect. On the other hand, *A. cylindrica* cultures when incubated in the highest concentration of rosemary water extract presented a significant cellular decrease whereas the lowest concentration induced an algaestatic effect. These results could correspond to differences in physiological/biochemical properties. In fact, Cyanobacteria are prokaryotic organisms and Chlorophyta are part of eukaryotic organisms, thus phylogenetic being more related to higher plants. The huge susceptibility of *A. cylindrica* to rosemary water extracts could be additionally related to the absence of chloroplasts in the cells. Therefore, as thylakoids are not protected, they might be easily damaged, jeopardizing photosynthetic activity and consequently the cyanobacteria growth was affected.

5. CONCLUSION

During the comparison of the different extract activities, it can be concluded that essential oils mostly influenced algal growth, showing algaecide effect. This fact certainly arises from the different compositions of these extracts. Essential oils are mainly composed of terpenes, terpenoids and aromatic compounds that are known to possess antimicrobial activity. Conversely, plant water extracts certainly include a greater variety of chemical compounds such as phenols, polysaccharides and resins among others. Some of these compounds when present at low concentrations can promote instead of inhibiting algal growth.

However, the present study is only an early step towards the assessment of the plant algaecide and algistatic potential. Therefore, future research will focus on the: (1) search the lowest observed effect concentration; (2) chemical characterization of oils and plant water extracts; (3) identification of the compounds responsible for algal growth effects and their evaluation on algae physiology; (4) assessment of the effects of plant extracts against non-target organisms**;** (5) evaluation of algaecide/ algistatic potential of other plant species, namely aquatic macrophytes. These data will be valuable to develop new environmentally friendly and healthier safe alternatives to the conventional algaecides/ algistatics application.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

ETHICAL APPROVAL

Not applicable.

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