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Exploration of Antimicrobial Property of Isolated Fungal Cultures from Soil of Northern Region of India

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Abstract: Soil of northern India was studied for fungal culture as therapeutic alternatives. Fifty fungal species belonging to different genera were isolated. Fungal species were further screened for antimicrobial activity using agar well diffusion and minimum inhibitory concentration method. Among the fifty fungal cultures, five species showed potential antimicrobial activity against test fungus C. albicans. So, there is high potential to discover useful antimicrobial agents producing soil fungi from the northern region of India.
Keywords: Northern region, Soil, antimicrobial activity, agar well diffusion and minimum inhibitory concentration.

I. INTRODUCTION

Microorganisms are ubiquitous in nature and form vital components of all known ecosystems on earth. Soil microorganisms are essential components of the biotic community in natural forests, and are responsible for the breakdown of organic materials, mobilization of nutrients, maintenance of soil-plant quality and ecosystem biogeochemistry [1]. They also known to influence physical, chemical and biological properties to the soil.[2]. Bacteria, fungi and action my cetes are three major groups of soil inhabiting microorganisms. Among all microorganisms, the soil heterotrophy- "fungi" represent a prime place in biodiversity that survive as saprotroph/ parasites/ biotroph or symbionts whereas numerous species also form mycorrhizal associations with plants or are plant pathogens [3]. They dwell in the majority part of this orbit including soil, air and water unlike other microorganisms. Soil is called the elixir of life as their biology is forever a fascinating and imperative milieu of study.

India is prosperous in fungal biodiversity which exceeds more than 27,000 species, the most widespread biotic community after insects [4]. Fungi are an important component of soil microbiota, contributing more soil biomass than bacteria [5]. They are present in soil both as actively growing organisms and dormant propagules [6]. Their role is extremely complex and is fundamental to the entire soil ecosystem [7]. Soil microfungi play a crucial role in the decomposition of plant structural polymers such as cellulose, hemi-cellulose and lignin, thereby contributing to the maintenance of the global nutrient cycle. Besides their praiseworthy significance, fungi execute the imperative job addressing well being of mankind in countless ways such as in industry, agriculture, pharmaceuticals, food industry, textiles, bioremediation, natural cycling, as biofertilizers, secondary metabolite production, industrial enzymes (e.g. amylases, cellulases, lipases, glucoamy lases, pectinases, phosphatases and proteases). In present scenario, my co biotechnology has occupied requisite position for betterment of humankind [8]. The spread of multidrug-resistant strains of fungus and the reduced number of drugs available makes it necessary to discover new classes of antimicrobials and compounds that inhibit these resistant mechanisms. This has led to a search for therapeutic alternatives. In the present study, we investigated the diversity and community dynamics of micro fungal population numbers from soil of Northern region of India for therapeutic alternatives. Northern Region of India is a huge potential area for bio-prospecting, since it is best known for its rich biodiversity and their varied climatic condition.

II. MATERIALS AND METHODS

A. Selection, climate and description of the study area

Fungi were cultured from different soil samples collected from temperate soils of Northern region of India (surrounding areas of Ganga River, Rishikesh, Dehradun, Roorkee, Neel Dhara, Kankhal and Bhel). These sites exhibited heterogeneity with regard to temperature, pH, moisture content, altitude and vegetation cover. These sites were selected because of their eurythermal conditions and dense vegetation which suggested a high fungal diversity and hence would be interesting to characterize.

B. Collection of soil samples

Soil samples were collected in sterile polyethylene bags with the help of a 5 cm diameter stainless steel corer pre-sterilized with 95%

ethanol and different depth (5, 8 and 15 cm soil cores). Ten soil cores were taken from a single site, mixed thoroughly and the composite sample was taken to the laboratory for isolation of fungi. The soil samples were kept in a refrigerator at 4±1°C till isolation procedure was completed.

C. Preparation of soil suspension

Soil samples were air dried and then crushed using mortar and pestle to get the powder form. From each sample, 1.0 g of soil sample was then added in different test tubes containing 10 ml demineralised water and shaken well using vortex mixer. These test tubes were considered as stock cultures for different soil sample sites.

D. Analysis of soil physico-chemical properties

After removing the aliquots for culturing, the pHs of the soil samples were measured with an electrical digital pH meter in 1:5 (w/v) soil-water suspensions. Soil temperature at different depths was recorded with a soil thermometer at the time of sampling. Soil moisture content was determined by drying 10 g of fresh soil in a hot air oven at 150°C for 24 h.

E. Isolation, growth and identification of fungi

From the stock cultures, a volume of 1 ml was transferred aseptically and added to a test tube containing 9.0 ml of demineralised water and mixed well. From this test tube, 1.0 ml of aliquot was again transferred and mixed with another 9.0 ml of demineralised water to make 10^{-2} dilution factor. Similarly, dilutions up to 10^{-4} were made using serial dilution technique for all soil samples. A volume of 0.1ml of suspension from 10^{-4} serially diluted tubes were taken and spread evenly with sterile L-shaped glass rod over the surface of sterile sabouraud dextrose Agar (SDA) plates aseptically using spread plating technique. Gentamycin (40ppm) and cyclohexamide (200ppm) were added in both media to inhibit bacterial contamination. The plates were incubated aerobically at 25°C up to 07 days and were observed intermittently during incubation [10]. After incubation, funguses on the plates were identified based on their morphological features such as color, dryness, rough, convex colony.

F. Isolation of pure fungal culture

The identified colonies were purified by repeated streak plate method [11,12]. After isolation of the pure colonies, each colony was further identified on the basis of its morphological characteristic odour, colony morphology, colour of hyphae and the presence or absence of aerial and substrate mycelium.

G. Preparation of Stock culture

Selected and identified colonies were picked and point inoculated on gentamycin containing SDA slants and incubated at 25°C and checked periodically. After incubation, the slants containing pure isolated fungus were documented and preserved for screening at 4 °C and maintained longer by periodic subculture on SDA.

H. Test Microorganisms

Two bacteria and one fungus were selected for studies. Gram positive bacteria; Staphylococcus aureus, Gram negative bacteria; Escherichia coli and Fungus; Candida albicanss were used as test organisms. All bacterial culture were maintained on Nutrient Agar (NA) at 37ºC and fungus on Potato Dextrose Agar (PDA) &Saboraud's dextrose agar (SDA) at 28ºC.

I. Primary screening for fungal isolates via agar overlay technique

Pure fungal isolates obtained from the preliminary selection were cultivated on PDA. After incubation at room temperature for 4 days, these fungal isolates were subjected to screening via agar overlay technique. Soft NA (0.75%) which seeded with test organisms (*E. coli, S. aureus, C. albicans*) were overlaid onto the growing fungal isolates. Four empty PDA plates overlaid with soft agar were used as controls. All the plates were incubated at room temperature for 24 hours for bacteria and 48 hrs for fungus and the zone of inhibition was observed.

J. Extraction of secondary metabolites from pure isolates

Fungal isolates; showing best antibacterial activities during the secondary screening, were selected and cultivated on PDA. The agar plates containing the selected fungal isolates were dried in a fume hood and grinded before they were immersed with methanol solvent. After 4 days of immersion, the solvent was filtered and then concentrated by using rotary evaporator at 40°C. The collected crude methanol extracts were kept at 4°C for further testing.

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K. Agar Well Diffusion Assay

Antimicrobial activities of different extracts were evaluated by the agar well diffusion method [13] modified by [14]. Nutrient agar (NA) and Sabouraud's Dextrose Agar (SDA) plates were poured inoculated with 24 hrs and 48 hrs old broth cultures of bacteria and fungi respectively under aseptic conditions. The plates were covered and allowed to cool. As soon as the agar was partly solidified, the plates were inverted and left for 2 hrs. Wells were made at the centre of the plate by using a 6 mm cork borer that was sterilized with alcohol and flame. About 50 µl of crude ethyl acetate extracts were added by sterile micropipettes into the wells and allowed to diffuse at room temperature for 2 hrs. Grisoflavin was used as control. The plates were incubated at 37°C for 18-24 hrs for bacterial pathogens and 28°C for 48 hours fungal pathogens. Diameter of the inhibition zone (mm) was measured, the readings were taken in three different fixed directions and the average values were recorded.

L. Minimum Inhibitory Concentration (MIC) values

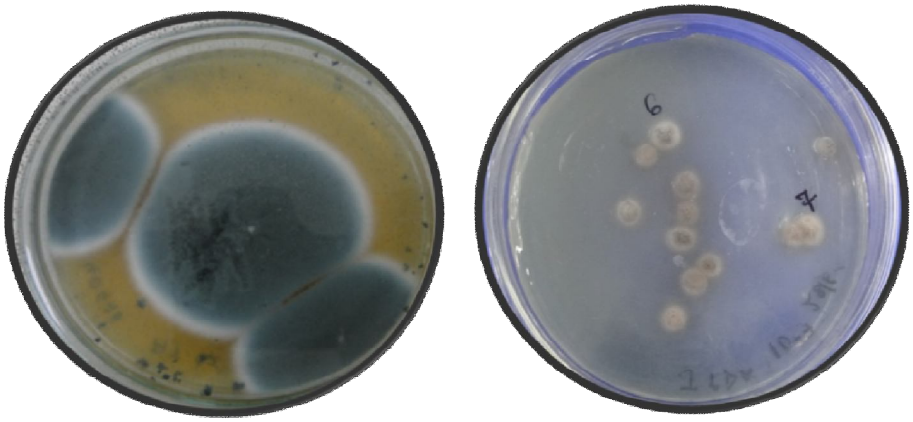
Minimum Inhibitory concentration of different extracts was evaluated modified by [15]. The minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates or in the tubes based on turbidity. This was determined from readings on the culture plates after incubation. The most commonly employed methods are the tube dilution method and agar dilution method. Serial dilutions are made of the products in bacterial and fungal growth media. The test organisms are then added to the dilutions of the products, incubated, and scored for growth. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against organisms. The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

III. RESULTS

A. Sampling and isolation of fungus

Fifty fungal species belonging to different genera were isolated from the study sites. Fungal counts were greater in surface soil at 5cm depth. The study sites also showed a marked variation in fungal species composition.

Fig. 1 (a): Mixed Fungal culture isolated from soil on SDA plates and Slants



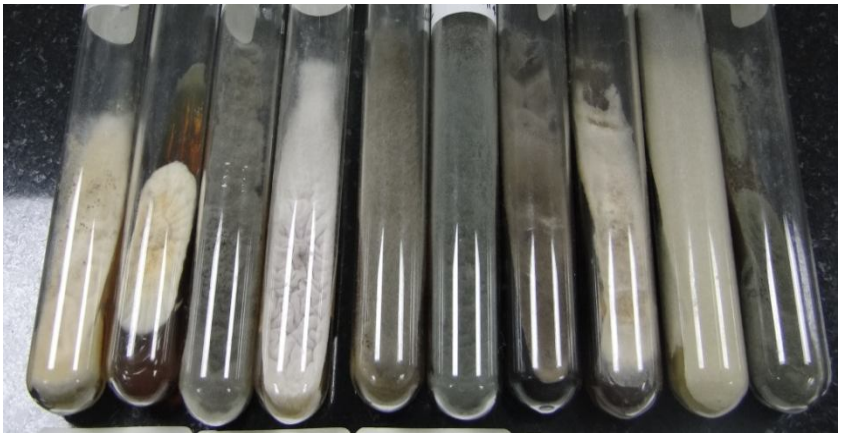


Fig. 1 (b): Fungal culture isolated from soil on SDA plates and Slants

B. Physico-chemical properties of soil

Soil properties of different depths are given as follow:

TABLE I: Physico-Chemical Characteristics Of Soil At Different Depths

Physico-chemical characteristics	Depth (cm)		
	5	8	15
pH	5.46	5.86	6.42
Moisture (%)	32.84	26.11	20.08
Temperature (°C)	35.4	28.7	20.1

C. Primary screening of isolated fungus for their antimicrobial activities

During the first round of preliminary selection, a total of 05 fungus isolates showed zone of inhibition. As the results of primary screening, 05 isolates showed antimicrobial activity against test fungus.

TABLE II: Comparison of The Antimicrobial Activity Of Isolates Against Test Organisms During Primary Screening

Isolates	Zone of Inhibitions (mm)		
	E. coli	S. aureus	C. albicans
1	00	00	17
2	00	00	23
3	02	08	17
4	00	05	18
5	00	00	25

D. Determination of minimum inhibitory concentration (MIC) values

Out of five isolates with greater antimicrobial activity in primary screening, the isolates also showed inhibitory action during secondary screening. The crude extracts have shown promising and encouraging antimicrobial activities against E.coli,S. aureus, C. albicans when compared to the standard antimicrobial drug Grieso flavin.

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TABLE III: Antimicrobial Activity of Ethyl Acetate Extract Of Promising Isolates Against Test Organisms

Isolates	Zone of Inhibitions (mm)		
	E. coli	S. aureus	C. albicans
1	00	00	19
2	00	00	25
3	02	06	14
4	03	05	11
5	00	00	25
Griesoflavin	05	08	10

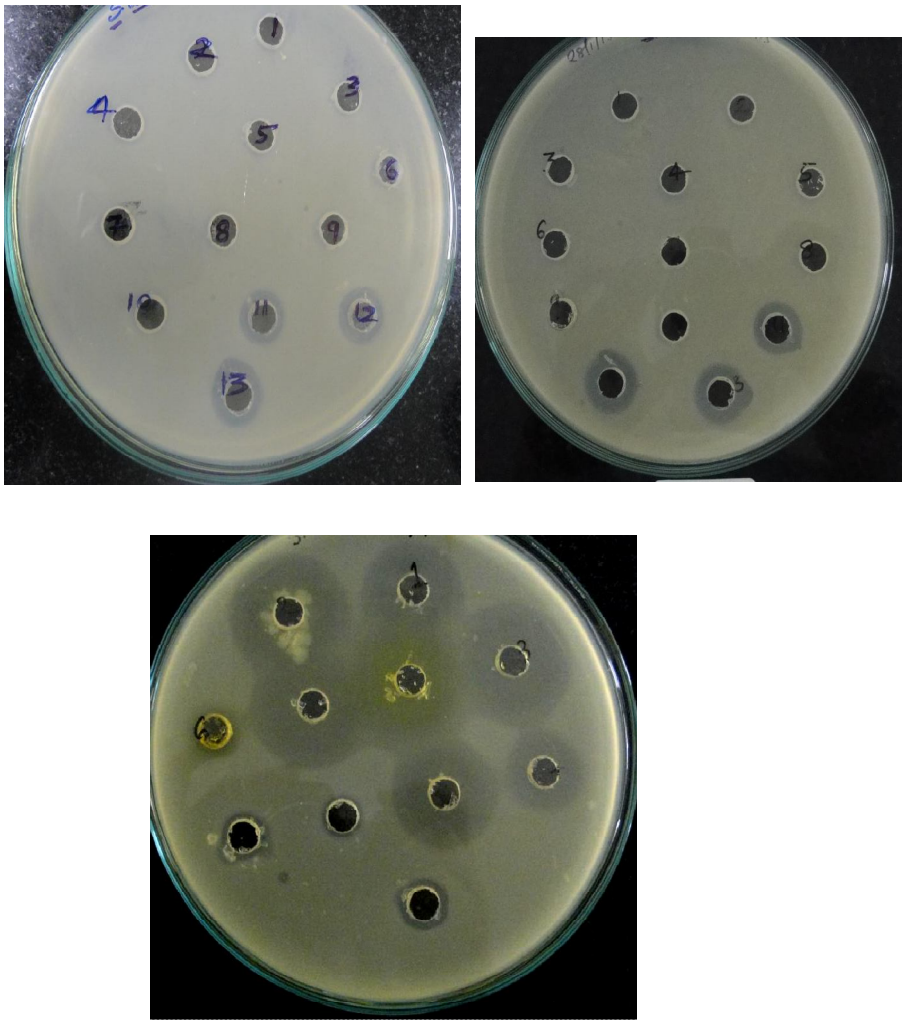

Fig. 2: Antimicrobial activity of ethyl acetate extract of promising isolates against test organisms: E. coli, S. aureusand C. albicans

Antimicrobial metabolites are the most important bioactive compounds for the treatment of infectious diseases. Because of the emergency of multi-drug resistant pathogens, there are basic challenges for effective treatment of infectious diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antimicrobial from soil fungi in diversified ecological niches[16].

In the present study, the randomly selected soil samples were taken from rhizosphere, industrial and public waste disposal areas for isolation of fungus. The successful isolation of fungi from environmental samples requires an understanding of the potential soil sample areas and environmental factors affecting their growth. Previous studies showed that selection of different potential areas such as rhizosphere soil samples were an important activity for isolation of different types of potent antibiotic producing soil fungi[17].

The present study of primary screening using single streak method indicated that, five out of 50 fungus isolates showed potential antimicrobial activity against test fungus. Observation of clear inhibition zones around the wells on the inoculated plates is an indication of antimicrobial activities of antibiotics. In this study, inhibition zone of crude extracts from five isolates against test fungus ranged from 0-20 mm. But, the crude extracts will show good inhibition zones like Griesoflavin after purification. Therefore, further purification process is significant to get pure antimicrobial substance for the application of treatment of different pathogenic microorganisms.

IV. CONCLUSION

In conclusion, there is high potential to discover useful antimicrobial agents producing soil fungi from the study site with some possibly novel strains that have not yet been studied by other researcher .

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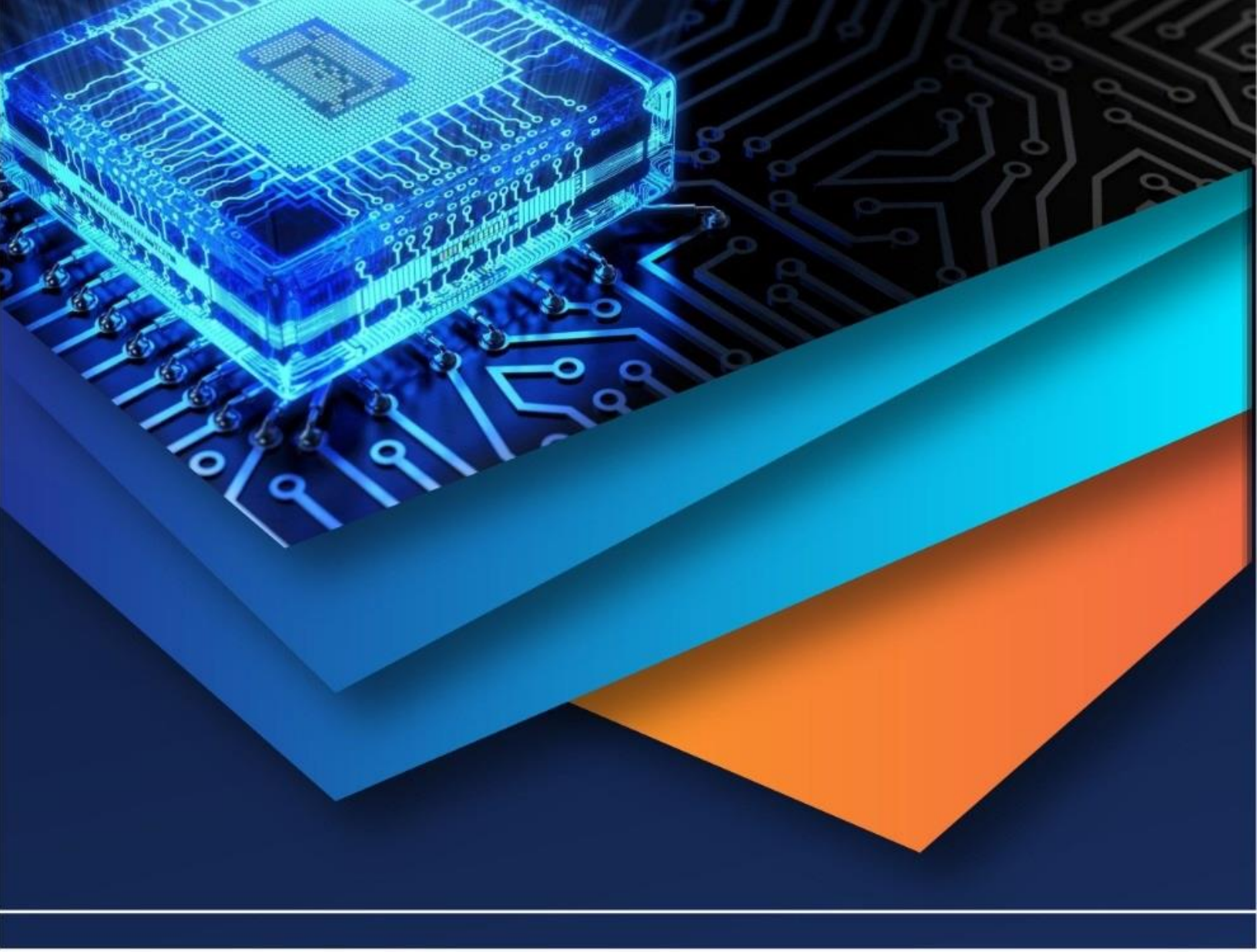

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