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Antimicrobial activity of *Diospyros chloroxylon* Roxb leaf methanolic extract

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Abstract
The Ebenaceae family has 500 species in this genus, making it the most dominant. Traditional therapeutic systems have been using these for thousands of years. An agar well diffusion method was used to assess the antibacterial activity of the isolated compounds. One fungus and three human pathogenic bacterial strains were tested for the antibacterial activity of *Diospyros chloroxylon* leaf methanol crude extracts. Nearly all bacterial strains exhibit possible antibacterial activity against the methanolic leaf extracts of *Diospyros chloroxylon*. Gentamycin was utilized as the antibacterial control, and fluconazole was utilized as the antifungal control. The concentration of the examined zone of inhabitation (1000 ug/ml to 1 ug/ml) is the zone of inhibition of *Diospyros chloroxylon* methanol leaf extract against some pathogenic bacteria.

Keywords: Antimicrobial activity, methanolic leaf; *Diospyros chloroxylon*

1. Introduction
Phytomedicines have been an essential part of customary health care system in most parts of the world for thousands of years. According to World Health Organisation, greater than 80% of world population depends on traditional remedy for their most important healthcare desires [4]. Recently, it has become more important to research medicinal plants with folkloric reputation and ethno botanical value in order to support the use of herbal remedies and demonstrate their potential as a source of novel pharmaceuticals [11]. As the pharmaceutical industry produces more innovative antibiotics, the fight against these medications has also intensified [10]. Antimicrobial drug use in the treatment of communicable illnesses has produced various drug confrontations in recent years (Service, 1995) [19]. In order to discover fresh leads for improved medications against microbial diseases, scientists are now focusing on traditional medicine. New antibacterial medications with plant origins have been discovered via research, since plant resources are widely recognized as a source of novel antimicrobial compounds. Numerous substances with significant medicinal potential have been extracted from plants, including vincristine, quinine, salicylic acid, eligitalis, morphine, and codeine [11]. Many plants still have therapeutic qualities that need to be explored in terms of phytochemistry and pharmacognosy, and it is critical to identify lead compounds that are active against infections [16].

2. Diospyros species
It is the leading genus of Ebenaceae family with 500 species. These have been used over thousands of years in traditional therapy system. [12] Reported 11 species of *Diospyros* from Andhra Pradesh. In this eleven species one of the species *Diospyros choloroxylon* collected in Krishna district of Andhra Pradesh.
Previous studies have been found about *Diospyros choloroxylon* antiviral activity reported [9, 21, 22] reported anti-dermatophytic activity leaf of petroleum ether and methanol extract, [21] reported antibacterial activity of siliver nano particles from ripened fruit pulp. Traditionally, this plant is used to cure different ailments include boils, body pains, swellings, skin diseases etc. [17].
The present study investigated antimicrobial activity of methanol leaf extract concentration i.e (1000 ug/ ml to 1 ug/ ml) of *Diospyros Choloroxylon* plant due to its pharmacological importance.

3. Material and methods

3.1 Collection of plant material

The *Diospyros Choloroxylon* was collected from their natural habitat from Kondapalli Reserve forest in Krishna district of Andhra Pradesh. The plant was scientifically identified at the Botanical survey of India Deccan Regional centre Hyderabad they give voucher no BSI/DRC/2019-20/Tech./173.

3.2 Drying and grinding of plant material

The studied and cleaned plant organs were then air dried at room temperature. The dried plant substance was then finely ground into powder form and packed in labelled neat airtight small plastic bottles at room temperature for further experiments.

3.3 Chemicals and Reagents

- **Peptone:** Microbiology grade, Merck Chemicals, Mumbai.
- **Beef extract:** Microbiology grade, Merck Chemicals, Mumbai.
- **Agar:** Bacteriological grade, Merck Chemicals, Mumbai
- **Sodium Chloride:** Laboratory reagent, Merck Chemicals, Mumbai.
- **Distilled water:** EMPLURA double distilled water, Merck Chemicals, Mumbai.

3.4 Composition for nutrient Agar medium (g/lit)

- Peptone - 0.5
- Beef extract - 0.3
- Agar - 1.5
- Sodium Chloride -0.5
- Distilled water – 1000 mL

3.5 Composition for Czapek Dox agar medium (g/lit)

- Sodium nitrate - 2.0.
- Potassium chloride - 0.5.
- Magnesium glycerophosphate - 0.5.
- Ferrous sulphate - 0.01.
- Potassium sulphate - 0.35.
- Sucrose - 30.0.
- Agar - 12.0.
- pH 6.8 ± 0.2 @ 25 °C.

3.6 Microbial strains studied

Standards: Antibacterial standard: Gentamycin.
Anti-fungal standard: Fluconazole.

3.7 Samples studied

Diospyros choloroxylon plant samples were studied for anti-bacterial fungal activity

Concentrations studied

1000 µg/ml = 1 mg/ml; 500 µg/ml = 0.5 mg/ml; 250 µg/ml = 0.25 mg/ml; 100 µg/ml = 0.1 mg/ml; 10 µg/ml = 0.01 mg/ml; 1 µg/ml = 0.001 mg/ml

Anti-microbial activity Procedure (Farjana *et al.* 2014) [5]

The antimicrobial activity of the *Diospyros chloroxylon* leaf extract was evaluated by agar well diffusion method. Bacteria were grown in Nutrient Agar media to match the turbidity of 0.5 McFarland standards to be inoculated on Nutrient Agar media agar. After inoculation, plates were dried for 15 min, and the wells were punched using sterile cork borers. Once wells were formed, they were filled with 50 µL of isolates and blank (water). Commercially available Gentamycin (10 µg) discs were used as a positive control in this study. Plates were incubated for 24 h at 37 °C to allow leaf extracts to diffuse through the agar media to form zones of inhibition. The diameters of the zone of inhibition for different isolates against different bacteria were measured in millimetre for further analysis. An agar well (6 mm) showing no zone of inhibition was considered as no antimicrobial activity.

4. Results

The indiscriminate continuous and increased use of antibiotics for the treatment of various microbial diseases led to the development of antibiotic resistance and multidrug resistance in the pathogenic microbes. Therefore, the search for new natural active biomolecules particularly from plants has been intensified in recent times. Although many plant species have been subjected to antimicrobial activity assessment and identification of active phyto-molecules, a vast majority of plant species have not been adequately evaluated. *Diospyros* is the principal genus of the family Ebenaceae with more than 500 species, majority of which are with active biomolecules of enormous importance. Hence, in the present study the antimicrobial activity evaluation of *Diospyros chloroxylon* methanolic leaf extracts is carried out.

The antimicrobial activity of methanolic leaf extracts (1µg, 10 µg, 100 µg, 250 µg, 500 µg and 1000 µg/ml) of different concentrations was evaluated against three test bacteria (*Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*) and one fungal strain (*Candida albicans*) table 1. The growth inhibition of microbial strain observed in the culture medium in terms of inhibition zone that it has produced is taken as measure of antibacterial activity. The size of inhibition zones produced by test microbial strains with the introduction of different concentrations of plant extract are measured and presented in Table 2 & 3.

Methanolic leaf extract of 1 µg/ml concentration was in effective in producing significant inhibition against test organisms. *Bacillus cereus* was significantly effected by 250 µg/ml extract and the zone produced was 19.6 ± 0.50 mm and it was larger than the zone produced by standard drug Gentamicin (17.6±0.58 mm), Fig 1 A-H. The higher concentrations (500 & 1000 µg/ml) also produced inhibition zones greater in size than Gentamicin. Gram negative bacterial strains (*E. coli* and *Salmonella typi*) were also affected by the leaf extract of 500 & 1000 µg/ml concentrations (Fig 2, 3 A-H). The fungal strain i.e. *Candida albicans* was also inhibited by 250µg/ml leaf extract equally (10.30±1.54) to that of Fluconazole the standard drug (10.6±0.58). The higher concentrations (500 & 1000 µg/ml) further resulted in enhanced growth inhibition of the fungus.

The present study highest that *Diospyros chloroxylon* methanolic leaf extracts i.e. 250, 500 & 1000 µg/ml are very effective in preventing the growth of bacteria and fungi.

The inhibition zone produced by the 1000 µg/ml leaf extract against *Bacillus cereus* is 23.3±0.58 mm and against *Escherichia coli* is 20.30±0.58. These inhibition zones are more or less equal in size with the inhibition zone produced by standard drug (Gentamicin) against the said bacteria. 500 µg/ml leaf extract produced 20 mm zone equal to that of drug Gentamicin. The fungus (*Candoda albicans*) was inhibited by 250 mg/ml extract effectively on par with standard drug Fucanozole (Table & Fig.4 A-H).

5. Discussion

In the folklore medicinal system the leaves of *Diospyros*

chloroxylon were reported to have used in the treatment of boils, body pains, swellings and skin diseases [17]. Anumber of important plants of traditional medicine have been phyto-chemically screened to report valuable compounds that control many biological activities including antimicrobial activity [7, 24]. Therefore, in recent years many research investigators are inclined to screen folklore medicinal plants for phytochemicals and to validate their biological activities. The genus *Diospyros* is an under exploited genus with more than 500 species of proved potentiality in traditional medicine. The different species were reported that they contain multitude of bioactive compounds having potent therapeutic value. These species contain naphthoquinones (Diospyrin & plumbagin), anthraquinones, terpenoids, phenolic acids and flavonoids. Hence, the phytochemical screening and biological activities evaluation of *Diospyros* species became the target topics of investigation. The antibacterial activity evaluation of *Diospyros chloroxylon* comes under the above priority, since the research studies conducted on this species are very few.

The antimicrobial activity of methanolic leaf extracts has been evaluated against three bacterial species (*Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*) and one fungal strain (*Candida albicans*) in the present study. The leaf extracts (250, 500 & 1000 µg/ml) are found to be very effective against test bacterial strains and fungal strain. The inhibition zone produced by 1 mg/ml (1000 µg/ml) leaf extract against *Bacillus cereus* is 23.3±0.58 mm and against *Escherichia coli* is 20.30±0.58. The activity of leaf extracts is almost equal with the activity of standard drug (Gentamicin). 500 µg/ml leaf extract effectively inhibited *Salmonella typhi* (20 mm) equal in activity to that of standard drug. The fungal strain (*Candida albicans*) was effectively inhibited by the 250 µg/ml leaf extract of *Diospyros chloroxylon* (10.66 mm) and the activity is equal to that of Fucanozole the standard drug.

Shivakumar & Vidyasagar (2016) [21] studied the antibacterial effect of *Diospyros chloroxylon* and reported that 40 mg/ml petroleum ether leaf extract produced 12.33 mm and 16.66 mm inhibition zones against *Escherichia coli* and *Candida albicans* respectively. The methanolic leaf extracts (40 mg/ml) against *Candida albicans* and *Escherichia coli* produced the inhibition zones of size 5.30 and 4.66 mm respectively. But the results of our present study differ greatly in that the methanolic leaf extract of far less in concentration i.e. 1000 µg/ml (or 1 mg/ml) is highly effective against *Escherichia coli* (20.30 mm). Similarly 1000 µg/ml methanolic leaf extracts produced significant inhibition (17.60 mm) against fungal strain *Candida albicans*. It implies that the antibacterial activity of plant many vary significantly based on the occurrence of species in different habitats. The present study results on antibacterial activity of *Diospyros chloroxylon* methanolic leaf extracts emphasize that the activity is greater and comparable with *Diospyros melanoxylon* and *Diospyros mespiliformis* species.

The antibacterial activity of *Diospyros chloroxylon* evaluated now is compared with *Diospyros melanoxylon* the mostly used Indian species and *Diospyros mespiliformis* the most widely distributed African tree species. Rath *et al.* (2009) [14] reported the antibacterial activity of *Diospyros melanoxylon* and noticed methanolic bark extract (50 mg/ml) inhibiting the growth of *Escherichia coli* to produce the inhibition zone of size 22. 33 mm. The ethanolic leaf extract of *Diospyros melanoxylon* according to [2] also inhibited *Escherichia coli* by producing the inhibition zone of 24 mm [1]. Also evaluated the antibacterial activity of stem extract of *Diospyros melanoxylon*. They reported that 500 mg/ml stem extract inhibited the growth of *Escherichia coli* and *Candida albicans* by producing 14.10 mm and 15.40 mm inhibition zones. The antibacterial activity of the *Diospyros melanoxylon* as evidenced by the above studies is almost equal to the antibacterial activity of *Diospyros chloroxylon* against *Escherichia coli* and *Candida albicans*. The antibacterial activity of *Diospyros mespiliformis* was evaluated by [20, 3, 18, 20] Found that the ethanolic leaf, bark and root extract of *Diospyros mespiliformis* produced inhibition zones of size 4, 8 and 11 mm size against *Escherichia coli.* [3] Reported that the aqueous leaf extracts of *Diospyros mespiliformis* produced inhibition zones of size 20 mm against *Escherichia coli* and 16 mm against *Candida albicans* [18]. Observed that the inhibition zone size of 11 mm was produced by *Diospyros mespiliformis* bark & leaf extract (5 mg/ml) of n-hexane against *Candida albicans*. The above reported activity of *Diospyros mespiliformis* extracts against *Candida albicans* and *Escherichia coli* can also be considered as equal to the antibacterial activity of *Diospyros chloroxylon* methanolic leaf extracts evaluated now through present study. The superior antibacterial activity of methanolic leaf extracts of *Diospyros chloroxylon* can be ascribed to the presence of different bioactive chemical compounds [8]. Reported that quercetin is a strong antibacterial agent [23, 15]. Stated that saponins are indicators of antibacterial activity [13]. Claimed that terpenoids possess antibacterial, anti-viral, anti-inflammatory and anti-hyperglycemic activity [6]. Stated that flavonoids besides their antioxidant activity also possess antibacterial, anti-fungal and anti-viral activities. The methanolic leaf extract of *Diospyros chloroxylon* revealed the presence of anthraquinones, glycosides, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins, terpenoids and phlobatanins. In addition, HPLC analysis of methanolic leaf extracts revealed the presence of gallic acid, quercetin, rutin, kaempferol, epigallocatechin and epicatechin gallate. The same compounds isolated from other plant species by earlier investigators were ascribed them to the antibacterial activity along with other biological activities. Hence, the present study discloses the discernable phytochemical profile of *Diospyros chloroxylon* that has lot of potential in the assay and validation of many biological activities.

Table 1: Determined against three human pathogenic bacterial strains and one fungal strain

S. No.	Name of The organism	Bacteria/Fungi	MTCC
1.	*Bacillus cereus*	Gram positive Bacteria	MTCC - 1307
2.	*Escherichia coli*	Gram negative Bacteria	MTCC - 294
3.	*Salmonella typhi*	Gram negative Bacteria	MTCC - 3224
4.	*Candida albicans*	Fungi	MTCC - 3017

Table 2: Zone inhibition of methanol leaf extract *Diospyros choloroxylon*

S. No.	Microorganism studied	Inhabitation zone observed (mm)					
		Extracts concentration (mg/ml)					
		1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	10 µg/ml	1 µg/ml
1.	*Bacillus cereus* (+)	23.3±0.58	23 ± 1.00	19.6±0.5	14.3±0.58	14±1.00	3.6±0.58
	Gentamicin controle	22.6±0.58	19.6±0.58	17.6±0.58	15.3±0.58	9.6±0.58	4.6±0.58
2.	*Escherichia coli* (-)	20.3±0.58	17.6±0.58	15.6±0.58	9.3±1.52	4.3±1.5	2 ± 1.00
	Gentamicin controle	21.3±0.58	19.6±0.58	17.6±0.58	12.6±0.58	8.6±0.58	5.6±0.58
3.	*Salmonella typhi* (-)	21±1.00	20.00	15.6±1.15	12.00	10.6±0.58	8 ± 1.00
	Gentamicin controle	23.3±0.58	20.6±0.58	17.6±0.58	16.3±0.58	12.6±0.58	9.6±0.58
4.	*Candida albicans*	14.6±0.58	13.6±1.15	10.3±1.54	3 ± 1.00	2.6±0.58	-
	Fluconazole controle	17.6±0.58	14.3±0.58	10.6±0.58	8.3±0.58	6.3±0.58	2.6±0.58

Table 3: Determination of minimum inhibitory concentration of *Diospyros choloroxylon* extract

S. No.	Microorganism studied	Minimum inhibitory concentration from
1.	*Bacillus cereus*	4
2.	*Escherichia coli*	2
3.	*Salmonella typhi*	9
4.	*Candida albicans*	3

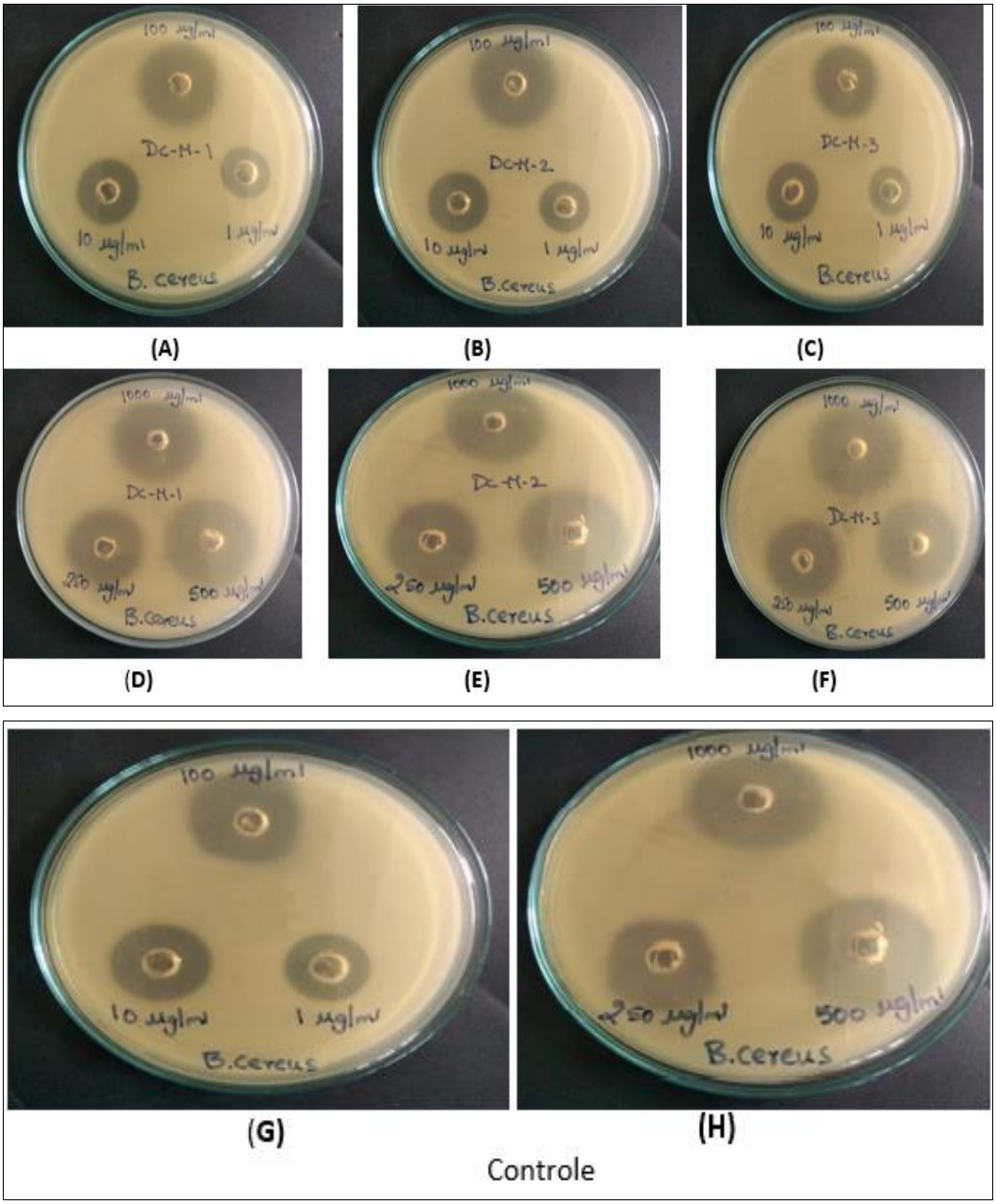

Fig 1 (A-H): Antimicrobial activity of *Diospyros chloroxylon* methanolic leaf extract Bacteria - *Bacillus cereus*

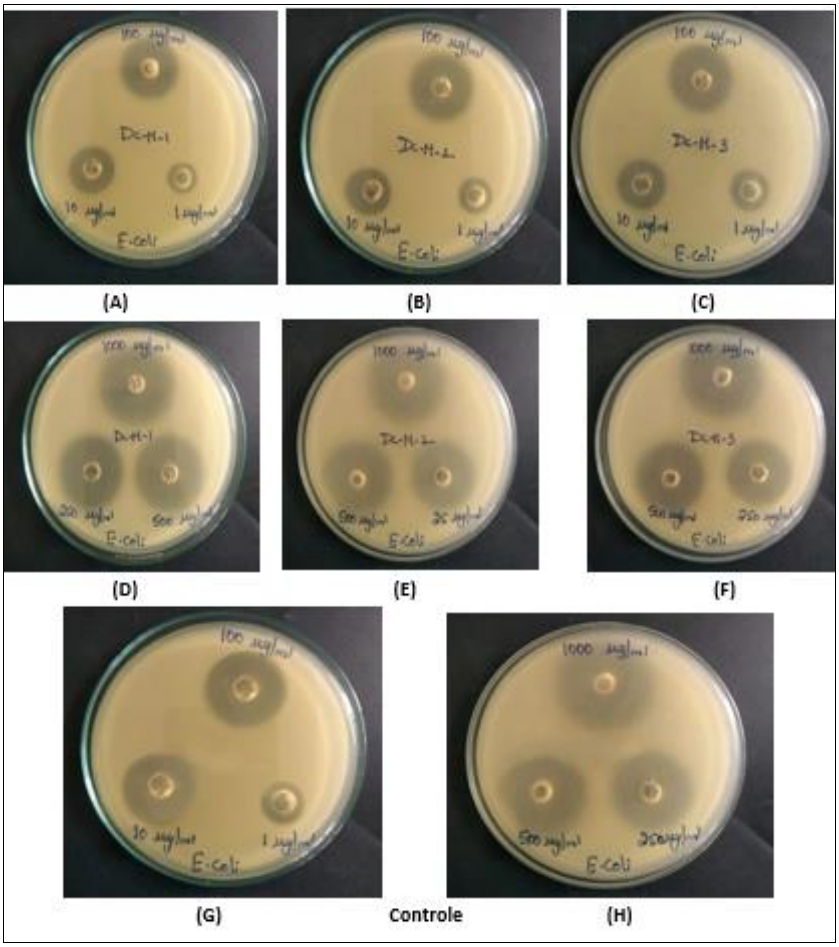

Fig 2 (A-H): Antimicrobial activity of *Diospyros chloroxylon* methanolic leaf extract Bacteria - *Escherichia coli*

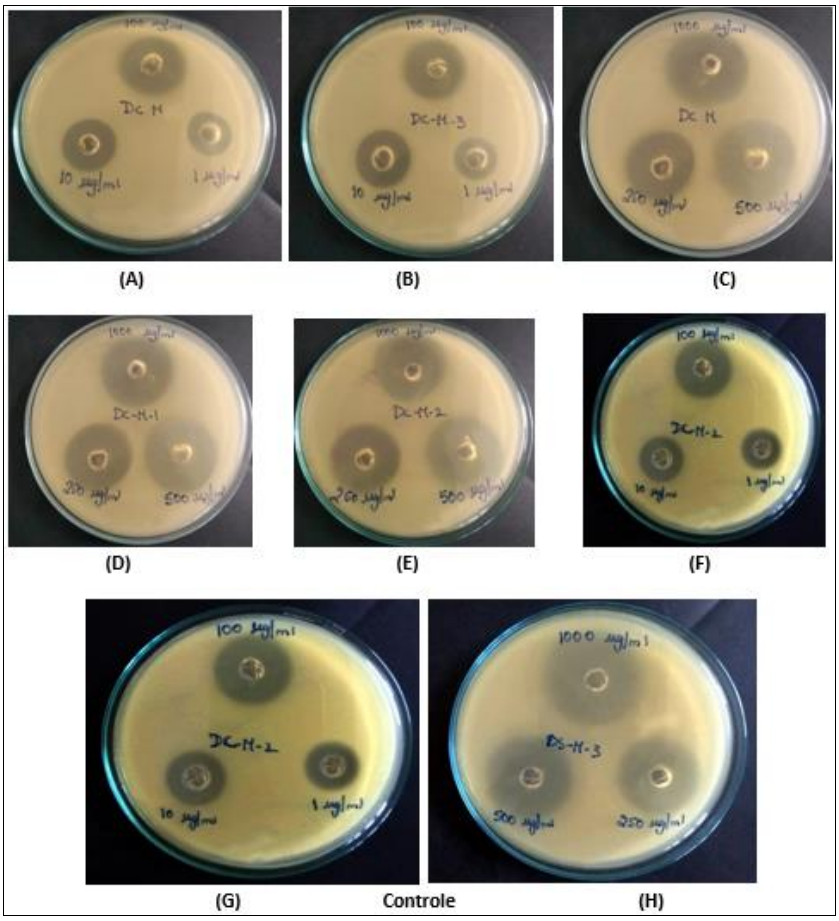

Fig 3 (A-H): Antimicrobial activity of *Diospyros chloroxylon* methanolic leaf extract Bacteria - *Salmonella typhi*

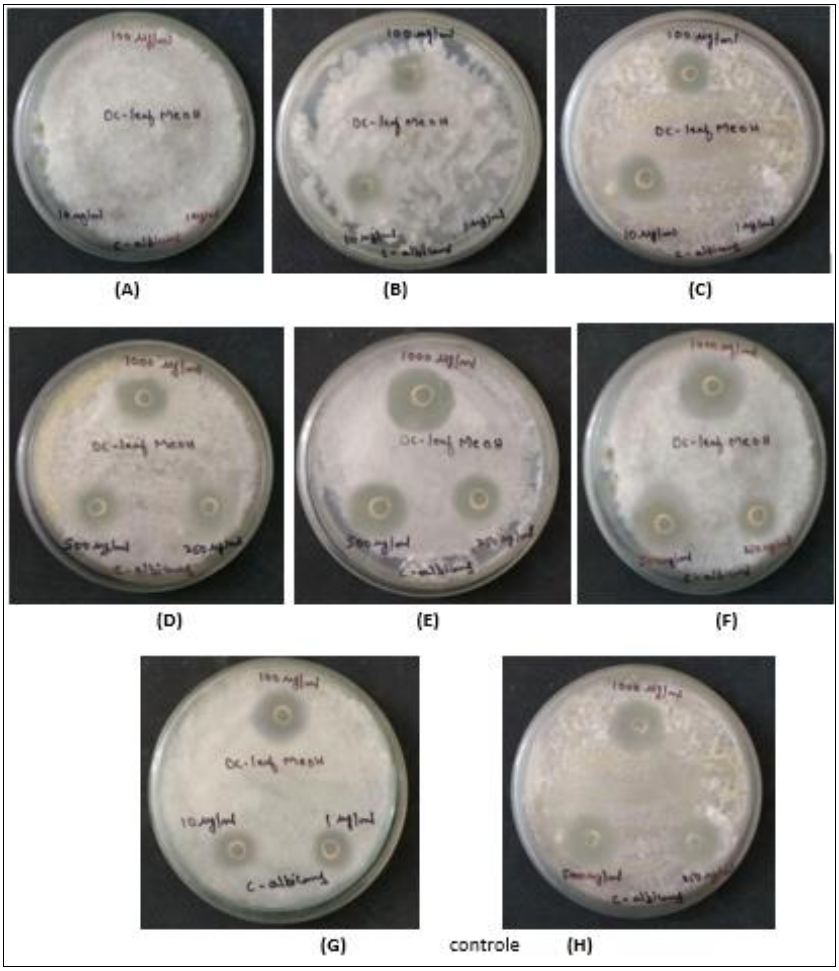

Fig 4 (A-H): Antimicrobial activity of *Diospyros chloroxylon* methanolic leaf extract Fungai - *Candida albicans*

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