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EVALUATION OF ANTI-MICROBIAL ACTIVITY OF *IN VITRO* AND *IN VIVO* PLANT PARTS OF *MERREMIA DISSECTA* AND *MERREMIA AEGYPTIA*

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Tetrazolium Chloride, *in vitro*, *in vivo*, Antimicrobial activity, *Merremia dissecta*, *Merremia aegyptia*

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ABSTRACT: The present study deals with the evaluation of the antimicrobial activity in methanolic extracts of *in vivo* (leaf, stem and seed) and *in vitro* (callus) plant parts of *Merremia dissecta* and *Merremia aegyptia* belonging to family Convolvulaceae. The antimicrobial activity against six different microbial strains, four bacterial viz. *Bacillus thuringiensis*, *Bacillus subtilis*, *Eschrechia coli* and *Pseudomonas putida* and two fungal strains viz. *Candida albicans* and *Aspergillus niger* was studied. Initially, plant extracts prepared in four different solvents (Aqueous, Methanol, Ethanol, and Petroleum Ether) were screened for their antimicrobial activity through TTZ test (Tetrazolium Chloride Test). It was assessed that alcoholic sample extracts possessed highest antimicrobial activity amongst all. Later the antimicrobial activity of the methanolic extracts was confirmed using Disc-Agar Diffusion technique. The inhibition zone observed was representative of the antimicrobial potency of that particular sample extract. Zones of inhibition ranging from 6mm to 14mm were observed. The largest zones of inhibition were observed from the leaf extracts of these plants and the leaf extract of *Merremia aegyptia* also showed high antifungal activity as compared to other extracts.

INTRODUCTION: India is a vast land with storehouse of a large variety of plants. A lot of medicinal plants have been identified and are being used for centuries for their medicinal property by the inhabitants. Medicinal plants produce bioactive compounds which are used mainly for medicinal purposes. These compounds either act on different systems of animals including man, and/or act through interfering in the metabolism of microbes infecting them. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals present in them [1].

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One major problem that seeks our attention is that the disease producing organisms are also continuously evolving and speedily becoming resistant to the medicinal options that are available, so there is an urgent need to give attention and search for the diverse options for the above mentioned problem. Now-a-days multiple drug resistance has also become prominent due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases [2]. In India, in addition to this problem antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune suppression and allergic reactions. This situation has forced scientists to search for new and safer antimicrobial substances.

Major classes of antimicrobial compounds obtained from plants include phenolics, terpenoids, essential oils, alkaloids, lectins, polypeptides and

polyacetylenes [3, 4, 5]. This paper focuses on identification of antimicrobial properties of plant extracts of *Merremia aegyptia* and *Merremia dissecta* through agar disc diffusion technique and to suggest the possible use of these two plants as herbal medicines.

Merremia is a genus of flowering plants in morning glory family, Convolvulaceae. *Merremia aegyptia* (Linn.) Urban. is a twinning, annual herb, whose stem is used for binding, for instance for frames in house building in Ghana. Some stocks also graze on the plant. It causes diarrhoea in small stock when its large quantities are eaten. In Nigeria, the dried leaves are used as a dressing for burns. Leaves are ground and 30 ml of the extracted juice is taken once a day in jaundice till cure [6]. Seeds are edible, and seed oil is suitable to be used as biodiesel[7].

Plant is attractive and used as ornamental plant. It is an elegant creeper for fences and garden walls. Fruits are used for day flower arrangement. Chlorogenic and benzoic acids, as well as a metabolite of the latter (the glycoside 1,2,3,4-tetrahydro-β-carboline-3β-carboxylic acid), have been isolated from *Merremia aegyptia.*

Merremia dissecta (Jacq.) Hallier f. is been used as a condiment, medicine and ornamental by an array of cultures. Although the plant has escaped in several areas to become a weed, it continues being sold as an ornament in even those regions. The white flowers with red center are attractive, as are the finely lobed and toothed leaves. Fruits are 3-seeded. The leaves smell like bitter almonds and are used in India for making liquor. In Africa, an infusion of its leaves is taken as a sedative for chest complaints, and a poultice of fresh, crushed leaves is applied as a resolutive [8].

The aim of screening was to correlate and confirm the antimicrobial activity of *in vivo* and *in vitro* plant parts of *Merremia* species.

MATERIAL AND METHODS:

Source of microorganisms:

The following six strains were collected from the microbial type culture collection and gene bank (MTCC), Institute of microbial technology (IMTECH), Chandigarh viz. two gram positive bacteria *E.coli* (MTCC 40) and *Bacillus subtilis* (MTCC 121), two gram negative bacteria *Bacillus thuringiensis(*MTCC 1953) *Pseudomonas putida* (MTCC 102) and two fungi *Candida albicans* (MTCC 7315) *Aspergillus niger* (MTCC 10180).

Culture and maintenance of microorganisms: Pure bacterial cultures were activated and maintained on Mueller Hinton broth medium and fungal cultures on Sabouraud media. Each bacterial and fungal cultures were further maintained by subculturing regularly on the respective media and storing at 4 ºc. 24 hours incubated active microbial cultures were used for inoculations.

Plant material:

The leaves, stems, seeds of *Merremia aegyptia* and *Merremia dissecta* were air dried and crushed to powder using mortar pestle and electric grinder. The powders were successively extracted using four different solvents i.e. distilled water, ethanol, methanol and petroleum ether. The plant extracts were prepared in soxhlet extractor which were collected and stored in vials for further use [9, 10, 11].

Eight Samples from two plants (*M. aegyptia* and *M. dissecta*) viz. root, stem, seed and callus from both the plant species (*in vivo*) and mature callus (*in vitro*) were taken and their antimicrobial activity against six different strains of microbes was detected through two sets of antimicrobial evaluation methods; the first was the Tetrazolium/ formazan test (TTC) which was evaluated as a qualitative antimicrobial test method. The second set contained the standard method that is, zone of inhibition or disc diffusion assay method which was used to analyze the specific concentration of plant extract from the range where it shows the maximum inhibition of microbial growth [12, 13]

Tetrazolium/Formazan Test (TTC):

The Tetrazolium/ formazan couple is a special redox system acting as proton acceptor or oxidant.In the presence of microbes, TTC is reduced to red formazan which is directly proportional to the viable active cells. In TTC assay, less than 1hr is required to attain susceptibility results. Therefore, the TTC test method is considered as a comparatively fast

method for evaluating the antibacterial/antifungal activity of antimicrobial agents [14, 15].

This test was performed at final concentration i.e. (1000µg/ml, 750µg/ml, 500µg/ml and 250µg/ml) of extract/ml. In the presence of microbes, TTC is reduced to red formazan. The red formazan obtained indicated the activity and viability of the cells. A set of both growth control (broth) and sterility control (Ampicillin for bacteria (1mg/5ml in methanol)/ Fluconazole for fungi (1mg/ 5 ml methanol) and a blank with methanol only were also prepared. The vials were incubated at 37 ºc for 24 hours for bacteria and at 30 ºc for 48 hours for fungi.

TTC powder was dissolved in sterile distilled water at a concentration of 2mg/ml at room temperature then filtered through 0.22µ Whatman filter paper and stored at −20ºC until used. 50µl of .2% 2, 3, 5-triphenyl tetrazolium chloride (TTC) was added in these vials and incubated for an hour at 37 ºc. The colour change was then assessed visually.

Agar Disc Diffusion method:

Disc Preparation:

The zone of growth inhibition test was carried out with a modified agar diffusion assay. Whatman papers No. 4 were cut into 6 mm diameter discs and sterilized by autoclaving at 15Psi and 121°C. After the sterilization the moistured discs were dried on hot air oven at 50°C. The discs were soaked in 8 different methanolic extracts of *Merremia aegyptia* and *Merremia dissecta* plant each having three different concentrations (0.75, 0.25, 1.00mg/ml). Discs dipped in standard antibiotics i.e. antibacterial (ampicillin) and antifungal (fluconazole) were also prepared.

Preparation of Agar Plates:

The sterile nutrient agar (1000 ml) and potato dextrose agar (500ml) were prepared in flask at appropriate pH for bacteria and fungi respectively. The sterilized media was cooled and the flasks were shaken gently to avoid the formation of air bubbles. 25ml portions of this media were transferred to petri dishes of 9cm diameter each so as to obtain 3-4 mm thickness of the media layer. The media in the plates was allowed to solidify at room temperature. All these procedures were conducted aseptically in laminar air flow workstation.

The test pathogenic microorganisms: *E. coli* (MTCC 40), *Pseudomonas putida* (MTCC 102), *Bacillus thuringiensis* (MTCC 1953), *Bacillus subtilis* (MTCC 121), *Candida albicans* (MTCC 7315), *Aspergillus niger* (MTCC 10180) were spread over the nutrient agar and PDA plates accordingly and resistant plates for each strain were also produced. After the microbial lawn preparation, discs soaked in eight different methanolic plant extracts of different concentration (0.25, 0.50, 1mg/ml) were placed on these microorganism inoculated plates for each strain with equal distance and antibiotic/antifungal disc were placed on resistant plates.

The plates were then inverted and incubated at 37°c for 24hrs for optimum growth of organisms. The test materials having antimicrobial property inhibit bacterial/fungal growth in the media surrounding the discs & thereby yielded a clear distinct area that are defined as the zone of inhibition which were expressed in millimeter.

The inhibtion zones found were then compared with the standards Ampicillin /Fluconazole to find out degree of antimicrobial activity. Activity index was measured as Inhibition zone of the sample-/Inhibition zone of the standard.

RESULTS AND DISCUSSION: The leaf, stem, seed and callus extracts of the *Merremia aegyptia* and *Merremia dissecta* have been tested for their antimicrobial and antifungal activities against *E. coli*, *Pseudomonas putida*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*. All the extracts showed sustained activity against all bacteria and fungi tested.

In TTC Test, any colour change from purple to pink showed the growth of microorganisms and no colour or only a slight tinge of pink colour in the vials showed the potent antimicrobial activity of that particular plant sample extract. Inhibitory concentration does not exhibit reduction of TTC into formazan, so MIC was defined as the lowest concentration of the plant extracts contained in the vial in which the absence of visual colour change

(colourless) was first observed. The different concentrations of the methanolic and ethanolic (alcoholic) extracts were found to have more antimicrobial activity as compared to petroleum ether and distilled water extracts and MIC ranging from 0.5-1mg/ml has been evaluated for all the tested organisms.

The inhibition zone assay revealed primarily two types of observations, namely, discs without any surrounded clear or inhibition zones which could be attributed to the absence of any inhibitory activity and clear inhibition zone representing the bacteriostatic and antifungal actions of plant extracts.

This method is the universal qualitative assay. Previous studies have shown that leaf extracts of *Glycyrrhiza glabra, Colocasia esculenta* and *Cassia fistula* showed antibacterial activity [16, 17, 18,]. Antimicrobial activity of *Cannabis sativa* and various other plants and plant parts with different solvent extracts has also been reported [19, 20].

Antimicrobial screening of 250µg/ml, 500µg/ml and 1000µg/ml concentrations of methanolic extract from stem, leaf, seed and callus of *M. aegyptia* and *M. dissecta* revealed that the zone of inhibition (IZ) increased along with increasing extract concentration [21].

The largest inhibition zone and activity index (AI) were found with *M. aegyptia* leaf against *P. putida* (IZ=14±0.2mm, AI=0.780), *C. albicans* (IZ=14±0.3mm, AI=0.722) and *B. thuringiensis* (IZ=11±0.5mm, AI=0.696); with *M. aegyptia* stem against *B. Subtilis* (IZ=12mm, AI=0.710) and *C. albicans* (IZ= 12±0.5mm, AI=0.631(**Table 1**); and with *M. dissecta* stem against *B. subtilis* (IZ= 12±0.1mm, AI=0.715) and *B. thuringiensis* (IZ= 10±0.3mm, AI=0.624) (**Table 2**).

The maximum antibacterial and antifungal activity were observed in methanol extracts of *Merremia aegyptia* leaf (14±0.2, 14±0.3) for *Pseudomonas putida* and *Candida albicans* at 1mg/ml concentration and 0.50 and 1mg/ml concentration of *Merremia aegyptia* leaf extract was found effective against both of the tested fungi.(**Table 1).**

TABLE 1: REPRESENTING ZONE OF INHIBITION AT VARIOUS CONCENTRATIONS OF DIFFERENT PLANT PART EXTRACTS OF *MERREMIA AEGYPTIA*

		Zone of Inhibition(mm)					
Plant exract	**Conc (µg/ml)**	**Ec**	**Pp**	**Bt**	**Bs**	**Ca**	**An**
M. a. Leaf	250 µg/ml	8	6.8	8	6.1	8	6.1
	500 µg/ml	9	9	10	6.2	8.2	8.1
	1000 µg/ml	13	14.2	11.5	6.3	14.3	9.5
M. a. Stem	250 µg/ml	8	8	8.2	6.1	7.5	6.1
	500 µg/ml	9	8.2	8.5	6.2	8	6.8
	1000 µg/ml	10.5	10	10	12	12.5	8
M.a. Seed	250 µg/ml	8	6.5	8	6.2	6.1	6.1
	500 µg/ml	9	8	8.1	6.5	6.3	6.7
	1000 µg/ml	9.2	9.5	10	8	7	8.2
M.a. Callus	250 µg/ml	8	6	6.5	6.1	6.3	6.3
	500 µg/ml	8.5	8.2	7.3	7.5	6.5	6.89
	1000 µg/ml	9	10	9.1	7.8	9.2	7.1
Standard antibiotic							
Ampicillin	1000 µg/ml	33.1	18.2	16.5	16.9	-	-
Fluconazole	1000 µg/ml	-	-	-	-	19.8	19.4
Methanol	1ml/disc	11.1	10.2	11.4	8	11.1	8

TABLE 2: REPRESENTING ZONE OF INHIBITION AT VARIOUS CONCENTRATIONS OF DIFFERENT PLANT PART EXTRACTS OF *MERREMIA DISSECTA*

		Zone of Inhibition(mm)					
Plant extract	**Conc(µg/ml)**	**Ec**	**Pp**	**Bt**	**Bs**	**Ca**	**An**
M. d. Leaf	250 µg/ml	7.6	6.2	7	6.1	6.1	6.1
	500 µg/ml	9	6.7	8	7.2	6.9	6.3
	1000 µg/ml	10	7.2	9	8	8.5	6.7
M. d. Stem	250 µg/ml	6.8	6.5	7	6.1	6.5	6.2
	500 µg/ml	7.3	6.8	8.2	9.2	7.2	6.3
	1000 µg/ml	8.1	8.2	10.3	12.1	8	9
M.d. Seed	250 µg/ml	8.5	6.5	7.5	7	6.2	6.1
	500 µg/ml	9.5	7.5	8.5	8	7.5	6.3
	1000 µg/ml	11	9	10	10	8.2	6.9
M.d. Callus	250 µg/ml	7	7.1	7.5	6.5	6.4	6.5
	500 µg/ml	8	8	8.1	6.8	6.6	6.6
	1000 µg/ml	8.8	9	9.2	7.6	7.5	7.1
Standard antibiotic							
Ampicillin	1000 µg/ml	33.1	18.2	16.5	16.9	-	-
Fluconazole	1000 µg/ml	-	-	-	-	19.8	19.4
Methanol	1ml/disc	11.1	10.2	11.4	8	11.1	8

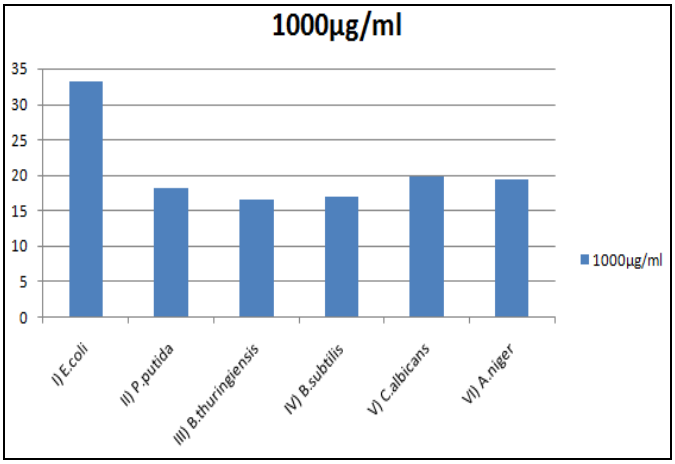

FIG. 1: ZONE OF INHIBITION OBSERVED FOR DIFFERENT MICROBES WITH STANDARD ANTIBIOTICS (AMPICILLIN FOR BACTERIA (I-IV) AND FLUCONAZOLE FOR FUNGI (V-VI))

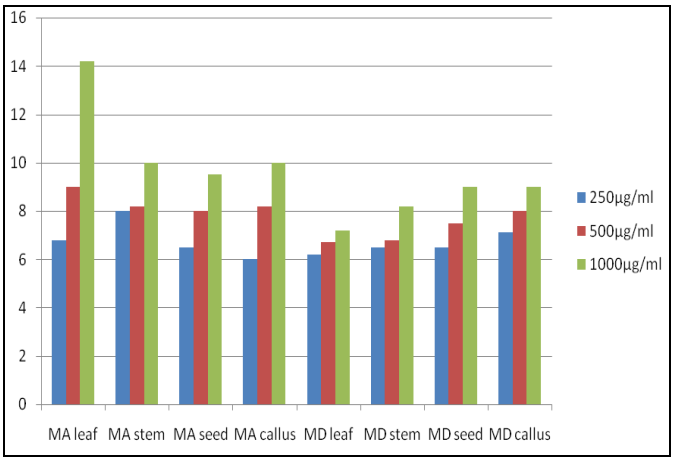

FIG. 3: GRAPH SHOWING ZONE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *P. PUTIDA*

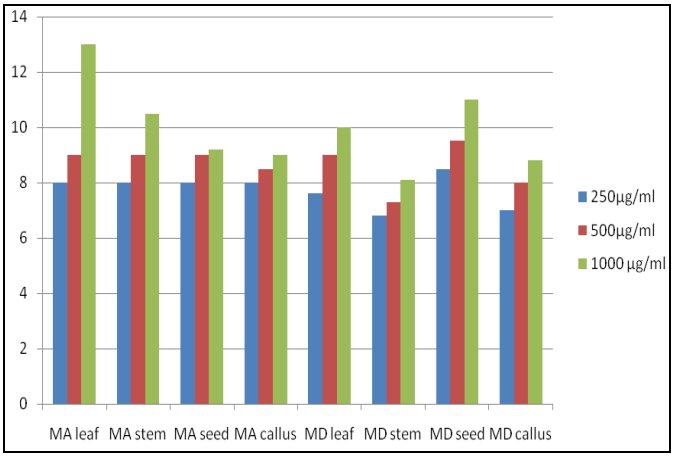

FIG. 2: GRAPH SHOWING ZONE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *E. COLI*

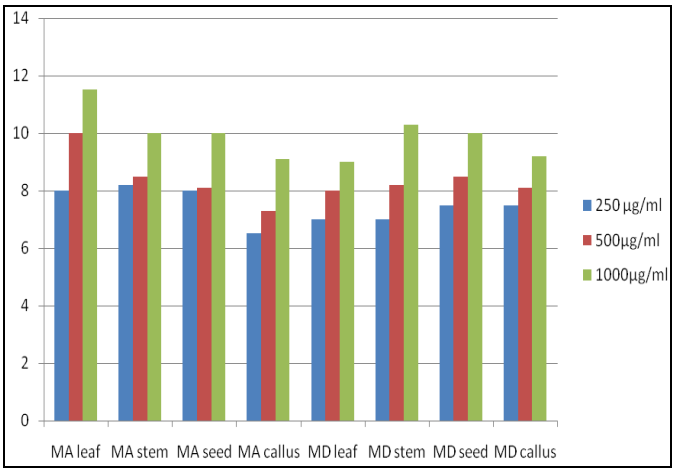

FIG. 4: GRAPH SHOWING ZONE OF INHIBITIONS AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *B. THURINGIENSIS*

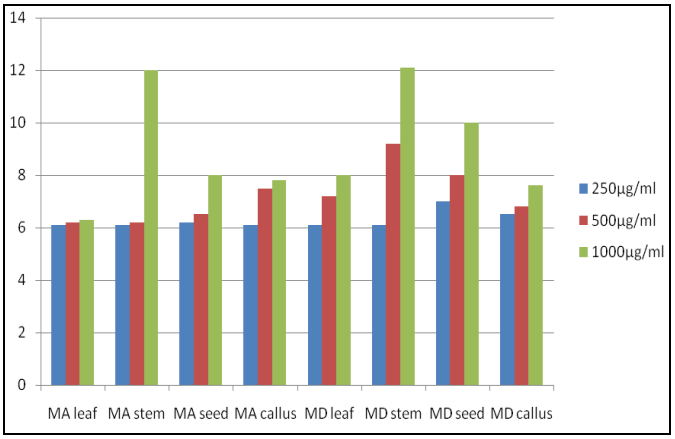

FIG. 5: GRAPH SHOWING ZONE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *B. SUBTILIS*

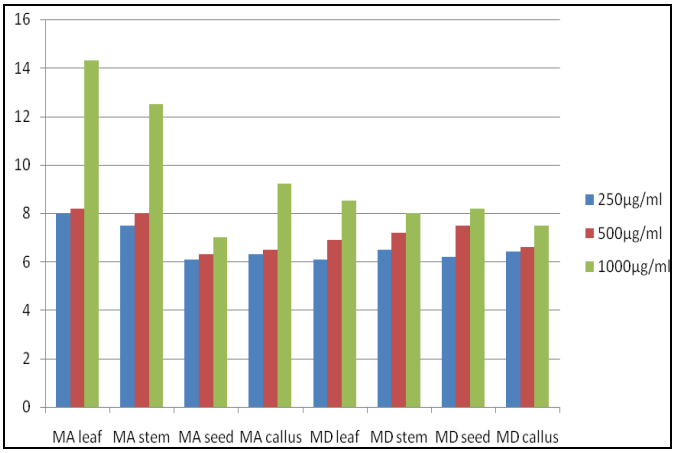

FIG. 6: GRAPH SHOWING ZONE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *C. ALBICANS*

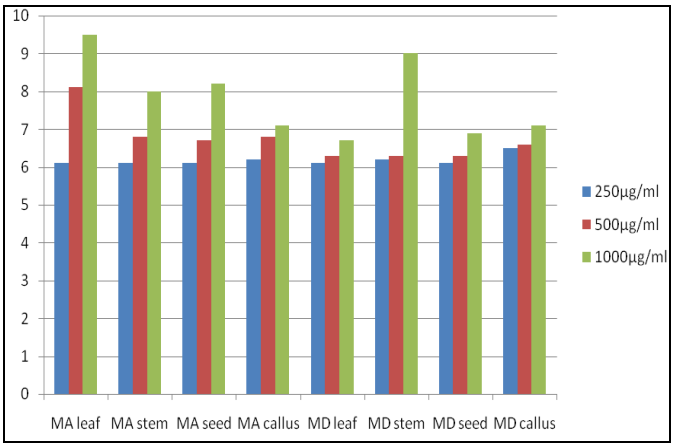

FIG. 7: GRAPH SHOWING ZONE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF PLANT EXTRACTS AGAINST *A. NIGER*

CONCLUSION: From the above set of experiments, it could be concluded that both of the plant species namely *Merremia aegyptia* and *Merremia dissecta* showed good antimicrobial activity and it could be stated that 1mg/ml concentration of each extract was effective against all tested microorganisms(**Table 1-2).** *Bacillus subtillis* was found to be more susceptible to stem and seed extracts as compared to leaf extracts of both the plant species **Fig. 5.** 500µg/ml and 1000µg/ml concentrations of *Merremia aegyptia* leaf extracts were found effective against both the fungal strains (**Table 1** and **Fig. 6-7**). The significance can be made on the basis of this comprehensive perusal of literature of the plant *Merremia aegyptia* and *Merremia dissecta* belonging to the family convolvulaceae being used traditionally due to their immense therapeutic potential to treat/cure various diseases.

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