

Antimicrobial Screening of Solvent Extracts of *Marchantia Polymorpha* L. from Kumaon Himalaya

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Abstract - The present study was carried out to evaluate antimicrobial activity of *Marchantia polymorpha*- a liverwort (bryophyte) against five pathogenic bacteria (*Agrobacterium tumefaciens*, *Bacillus subtilis*, *Escherichia coli*, *Erwinia chrysanthemi*, *Xanthomonas phaseoli*) and five pathogenic fungi (*Alternaria alternata*, *Fusarium oxysporum*, *Pyricularia oryzae*, *Sclerotinia sclerotiorum*, and *Tilletia indica*). The extract was prepared in ethanol, methanol, acetone, chloroform and water. All the crude extracts of *M. polymorpha* showed significant antibacterial activity except aqueous extract while antifungal activity was observed only against three fungal pathogens.

The maximum antibacterial activity was observed in ethanol extract against *Bacillus subtilis* (ZOI=21±0.66mm) followed by acetone extract against *A. tumefaciens* (ZOI= 20 ±0.88 mm). The highest antifungal activity was showed by ethanol extract against *S. sclerotiorum* (PI= 34 ±3.47). The antibacterial and antifungal activity of *M. polymorpha* was also compared with standard antibiotics. The good antimicrobial potency of this liverwort indicates the presence of some active principles in the extracts which can be isolated and employed in the manufacture of alternative antibiotic therapeutics.

Keywords: Antibacterial activity, antifungal, bryophyte, ampicillin, clotrimazole.

INTRODUCTION

Bryophyta is a unique division in plant kingdom, phylogenetically placed between vascular plants and algae (Matsuo 1991). They are generally represented by about 21,000 species (Scofield 1985) with a worldwide distribution. Bryophytes in natural environment are linked with culture, beliefs and ethics of mankind. They are traditionally used in North America, Europe, China and Indian herbal medicine. The doctrine of signature has dictated the use of a variety of bryophytes, especially liverworts in herbal medicine. The members of marchantiaceae are well known in traditional Chinese herbal medicine to treat skin tumefaction, to protect the liver and to treat hepatitis also used as antipyretics (Chobot *et al.*, 2006; Harris 2008). Large number of members of Marchantiaceae occur in Chinese Guangxi Zhuang district such as *Marchantia paleacea*, *M. polymorpha* and *M. convoluta* and are used by local people (Sabovljevic *et al.*, 2010). The European used *Marchantia polymorpha* to treat the jaundice of hepatitis and an external cure to treat pulmonary tuberculosis as the similarity of the thallus to the texture of lung tissue (Bland 1971). The appearance of *M. polymorpha* resembles the cross section of liver so it is used to treat liver and other ailments (Miller and Miller 1979) and as an external cure to reduce inflammation (Hu 1987). The Himalayan Indians have used *M. polymorpha* to treat boils and abscesses (Pant and Tewari 1989). In France, *M. polymorpha* was used to enhance diuresis (Basile 1998a). Asakawa (1990) suggested the use of *M. polymorpha* as antipyretic, antihepatic, antidotal, diuretic, used to cure cuts, fracture, poisonous snake bite, scalds and open wounds, show antifungal and antibacterial activity. Pande *et al.*, (2011) summarized the findings of the researches on the antimicrobial activity of the plants (liverworts and mosses) extracts (crude) isolated in different organic solvents and the chemical compounds exhibiting the inhibitory effect on the growth of microorganisms.

Evidences are also available in literature that confirms the antibiotic activity of *M. polymorpha* against fungi and bacteria (Baneerjee and Sen 1979, Asakawa 1990). Its pharmaceutical potency however was only recognized during the last three decades. The phytochemical investigation showed that the *Marchantia* sps. contain terpenoids, flavonoids, steroids and bis bibenzyls (Asakawa *et al.*, 1990; Friederich *et al.*, 1999; So *et al.*, 2002; Xiao *et al.*, 2006; Qu *et al.*, 2007). The most prominent examples of the group of bibenzyls are lunularic acid (Pryce 1971a), its decarboxylation product lunularine (Pryce 1972) and “prearomatic” precursor prelunularic acid (Ohta *et al.*, 1977).

MATERIAL AND METHODS

The aim of the present study was to study the antimicrobial activity of *M. polymorpha* extracts in different solvents like chloroform, acetone, ethanol, methanol and water against both plant pathogenic bacteria and fungi. The liverwort (*M. polymorpha*) was collected from various localities of Nainital.

PREPARATION OF EXTRACTS

The thoroughly washed plant material was blotted dry to remove the extra moisture. The sample was crushed in mortar and pestle along with a pinch of sterilized sand for the preparation of the extract in water and organic solvents.

To prepare stock solution 50g of the crushed material was added to 200 ml of solvents (w/v 50g/ 200 ml). Solvents used for extraction were ethanol, methanol, acetone, chloroform and water. Each extract was shaken for at least 6 hour and after that each extract was filtered with whatman filter paper no. 1.

MICROORGANISMS USED

Five (gram +ve and - ve) bacteria *Agrobacterium tumefaciens* (gram -ve) MTCC No. 609, *Escherichia coli* (gram -ve) MTCC No. 40, *Bacillus subtilis* (gram +ve) MTCC No. 121, were procured from institute of microbial technology, Chandigarh, India and *Xanthomonas phaseoli* (gram -ve) and *Erwinia chrysanthemi* (gram -ve) obtained from Plant pathology department, G. B. Pant University, Pantnagar, India were used in this investigation.

SCREENING OF ANTIMICROBIAL ACTIVITY

Antibacterial tests of selected microorganisms were carried out by using disc-diffusion method (Bauer *et al.*, 1966). Nutrient agar (Hi Media, Laboratories, Mumbai, India) was poured in sterilized petri plates (90 mm size) and cooled at room temperature ($20 \pm 2^\circ\text{C}$). A small sterile cotton swab was dipped into the 24 h old culture of bacteria and was inoculated by streaking the swab over the entire agar surface. This process was repeated by streaking the swab two or more times rotating the plates approximately 60° each time to ensure even distribution of inoculum. After inoculation the plates were allowed to dry at room temperature ($20 \pm 2^\circ\text{C}$) for 20 minute in laminar chamber for settling down of inoculums. The whatman no.1 filter paper discs (5 mm) loaded with 40 μl of extract were placed onto the bacteria seeded agar plates and it was allowed to diffuse for 5 minutes then these plates were incubated at $37 \pm 1^\circ\text{C}$ for 24 h.

The three antibiotics, Gentamycin (10mcg), Azithromycin (15mcg) and Ampicillin (10mcg) used as positive control were also placed onto the agar plates while filter paper discs (5mm) loaded with respective solvents were used as negative control. After 24 h of incubation, the diameter of inhibition zone was measured in millimeter (including the disc size). Each extract was tested in

triplicates and was performed twice. The values of zone of inhibition (ZOI) were expressed as near value with standard error of mean (SEM).

For antifungal activity agar well method (Perez *et al.*, 1990) was employed. Potato dextrose agar plates (Hi Media Laboratories, Mumbai India) (90mm size) were prepared and cooled at room temperature ($20 \pm 2^\circ\text{C}$). Thereafter a well of 7mm diameter was made with the help of a borer. In this well the extract was filled. Then the mycelial disc (diameter 7mm) from seven days old culture of fungal strains (test fungi) was cut from the periphery of the culture plate and aseptically placed in the centre of the treatment (extract) and control (clotrimazole) plates. The fungal plates were incubated at $27 \pm 1^\circ\text{C}$ for 4 days. On fourth day of incubation the radius of the growing colony of test fungi was measured. First towards the well (filled with the extract) and second in opposite direction, depicted as R2 and R1. The percent inhibition of radial growth was calculated by the formula given by Fokkema (1973), Shear and Zane Maivan (1988)

$$\text{PI} = \frac{R_1 - R_2}{R_1} \times 100$$

where, R_1 = the radius in opposite direction

R_2 = radius in the direction of well filled with respective

RESULTS

The results of the present study for antibacterial activity of *M. polymorpha* using disc diffusion method showed that all the bacterial strains are sensitive to the tested organic extracts (ethanol, methanol, acetone, chloroform) except aqueous extract (Table 1). The order of inhibitory activity of different extracts of *M. polymorpha* was ethanol > acetone > methanol > chloroform. In ethanol extract, the maximum inhibition zone was 21 ± 0.66 mm for *B. subtilis*, 19 ± 0.88 mm for *A. tumefaciens* and *X. phaseoli*, 18 ± 1.2 mm for *E. chrysanthemi* and 15 ± 0.57 mm for *E. coli*. All the bacterial strains were found more sensitive to the tested extracts in comparison to the commercially available susceptibility discs of ampicillin (10mcg) used as positive control.

The antifungal activity of *M. polymorpha* using agar well method showed that the lipophilic extracts (acetone, ethanol and methanol) were active against three fungal strains namely, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Tilletia indica*. Their activity order was ethanol > acetone > methanol. The percent inhibition was 34 ± 3.47 for *S. sclerotiorum* and 26 ± 5.04 for *T. indica* (Table-2). *F. oxysporum* was more sensitive to the ethanol extract while *S. sclerotiorum* was equally sensitive to acetone and methanol extract. Meanwhile, the ethanol extract was more effective in controlling the growth of *F. oxysporum* than the commercial antifungal-clotrimazole.

The analysis of variance (ANOVA) values indicated that the effect of extract from *M. polymorpha* showed significant difference between solvent and bacteria as well as their interaction ($p < 0.01$). For antifungal test the ANOVA indicated that the percent inhibition of the test fungi by *M. polymorpha* extracts was significant ($p < 0.01$). However the effect of solvent was insignificant.

DISCUSSION

It is known that one of the characteristic features of Marchantiophyta (Hepatics), in difference from Bryophyta (Mosses) and Anthocerotophyta (Hornworts) is the presence of cellular oil bodies which contain mainly lipophilic mono-, sesqui-, and diterpenoids, aromatic compounds (bibenzyls, bis-bibenzyls, benzoates, cinnamates, long-chain alkyl phenols, naphthalenes, phthalides, isocoumarins) and acetogenins. The biological activities of liverworts are due to these substances (Asakawa 1982, 1990a, 1990b, 1993, 1995, 2001, 2007). These oil bodies can be extracted with organic solvents.

The results of the test showed that the lipophilic extracts (ethanol, methanol, acetone and chloroform) of *M. polymorpha* were active against all the selected bacteria and some pathogenic fungi. The ethanolic extract showed highest antibacterial effect against *Bacillus subtilis* (ZOI=21±0.66mm) while the chloroformic extract showed lowest level of antibacterial effect against *A. tumefaciens* (ZOI=8±0.66mm). Dhondiyal *et al.*, (2013) investigated the antibacterial activity of *Lunularia cruciata* from the Kumaon Himalaya and found that the ethanol, methanol, acetone and chloroform extract of this liverwort were significantly effective against a wide range of bacterial pathogens.

The acetonic extract was significantly active against *A. tumefaciens* with 20±0.88 mm inhibition zone and the methanol extract was highly effective against *B. subtilis* with 14 ±0.66 mm zone of inhibition. Although some workers reported that ethanol, acetone and chloroform extracts of bryophytes were found to be effective than methanol extract (Bodade *et al.*, 2008, Rusell 2010). In the present study the methanol extract of *M. polymorpha* was quite effective against all the selected bacterial pathogens and against some fungal pathogens (*F. oxysporum*, *S. sclerotiorum* and *T. indica*). The methanol extract showed significant activity ZOI= 13±1.66 mm) against *E. coli*. The similar results were also observed by Mewari and Kumar (2008) with *M. polymorpha*. Sabovljevic *et al.*, (2010) also obtained the similar results when DMSO extract of *M. polymorpha* was tested against *E. coli*.

This species is also evaluated by Lakshmi and Rao (2023) for antimicrobial activity against four bacterial strains such as *Bacillus subtilis* and *Streptococcus mutans* (Gram positive), *Klebsiella pneumonia* and *Salmonella enterica* (Gram negative) and two fungal pathogens *Candida albicans*, *Rhizopus oryzae*).

Pavletic and Stilinovic (1963) reported the antibacterial activity of *M. polymorpha* against *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*. In our study too, the ethanol extract of *M. polymorpha* also showed promising activity against *Bacillus subtilis*. The aqueous extract may be ineffective due to the insolubility of organic compounds in water. Banerjee and Sen (1979) tested 52 species of bryophyte against nine bacteria (both gram-ve and gram +ve) and three fungi. Water, ethanol, methanol, ether, acetone and chloroform were used for the isolation of active principles. The alcoholic extracts of liverworts exhibited most of the antibacterial activity as compared to other solvents. The aqueous extracts of the selected bryophytes were found inactive in inhibiting the growth of either type of microorganisms. The liverwort *Marchantia polymorpha* along with another liverwort *Asterella sanguinea* and moss *Brachythecium procumbens* showed the broadest spectrum of antibacterial activity.

It is known that the majority of the bacteria are gram negative and conventional antibiotics are generally more active against the gram positive bacteria than gram negative bacteria (Elibol *et al.*, 2011). Besides some researchers reported antimicrobial activities of bryophyte samples against gram negative bacteria (Basile *et al.*, 1998a, Ilhan *et al.*, 2006; Bodade *et al.*, 2008). Furthermore, the antibacterial results of this study showed that *M. polymorpha* had an inhibition effect against gram positive bacteria as well as gram negative bacteria.

In addition, this study showed that the ethanol, acetone and methanol extract from this liverwort have an antifungal activity against three selected fungi *F. oxysporum*, *S. sclerotiorum* and *T. indica*. Asakawa (1990) reported the antifungal activity of *Marchantia* sps. against a wide range of fungi namely *Alternaria kikuchiana*, *Aspergillus fumigates*, *A. niger*, *Candida albicans*, *Penicillium chrysogenum*, *Rhizoctonia solani* and *Microsporium gypseum* etc. The results obtained in this study are incongruent with the results of some researchers who reported that extract from liverworts displayed antifungal activities (Castaldo *et al.*, 1988, Lahlou *et al.*, 2000, Scher *et al.*, 2004, Bodade *et al.*, 2008).

Lastly, the effectiveness of different extracts of *M. polymorpha* differed largely due to the relative solubility of various secondary metabolites in different solvents and perhaps the antibacterial and antifungal activity of *M. polymorpha* observed in our study may be due to the presence of the secondary compounds present in *M. polymorpha*.

The antimicrobial test results revealed that *M. polymorpha* had a potential activity against plant as well as animal pathogens. Therefore, this liverwort may be advantageous as antimicrobial agent and this study will help for the discovery of new wide spectrum antimicrobial formulation in future.

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Table 1: Antibacterial activity of <i>Marchantia polymorpha</i> extracts on pathogenic bacteria								
Diameter of inhibition zone (mm)*								
Microorganisms	C	A	E	M	W	G	Az	A
<i>A. tumefaciens</i>	8±0.66	20±0.88	19±0.88	13±0.66	na	33±1.11	39±0.85	na
<i>B. subtilis</i>	11±0.33	14±1.33	21±0.66	14±0.66	na	23±2.44	30±1.22	na
<i>E. coli</i>	10±0.66	15±0.57	14±0.66	13±1.66	na	24±2.92	32±1.65	na
<i>E. chrysanthemi</i>	13±1	15±1.2	18±1.2	12±0.57	na	30±1.15	33±0.88	na
<i>X. phaseoli</i>	9±0.66	11±1.33	19±0.88	11±1	na	25±3.1	34±2.05	na

*All the values are mean of three determinations, C, A, E, M, W- Chloroform, Acetone, Ethanol, Methanol, Aqueous extract, G-, Az, A - Gentamycin, Azithromycin, Ampicillin (+ve control), na-not active

Table 2: Percent inhibition in the growth of pathogenic fungi with different extract of <i>Marchantia polymorpha</i>				
Microorganisms	E	M	A	C
<i>Fusarium oxysporum</i>	30 ±4.40	14±0.88	22±4.05	29 ±4.7
<i>Tilletia indica</i>	26±5.04	13±0.88	19±1.85	29±0.66
<i>Sclerotinia sclertiorum</i>	34±3.47	20±2.9	20±2.84	46±4.04

C, A, E, M, W- Ethanol, Methanol, Acetone, Chloroform

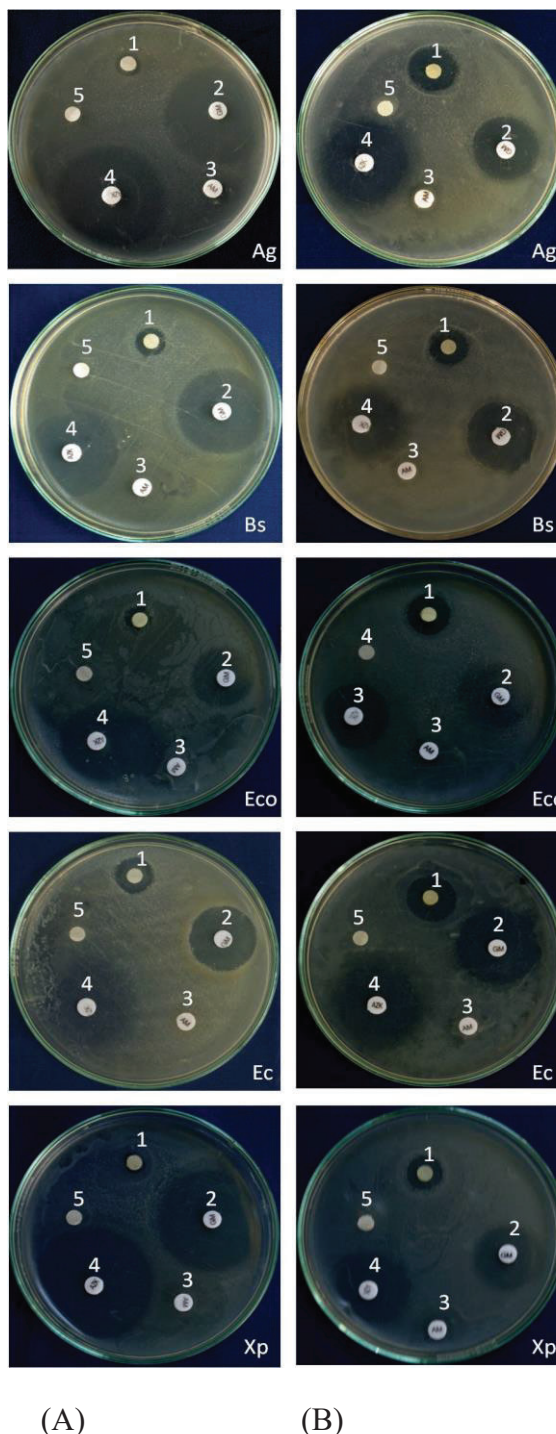


Plate 1a: Antibacterial activity of *Marchantia polymorpha* extracts against some pathogenic bacteria. (A)-chloroform extract, (B)-acetone extract, Ag-*Agrobacterium tumefaciens*, Bs- *Bacillus subtilis*, Eco- *Escherichia coli*, Ec- *Erwinia chrysanthemi*, Xp- *Xanthomonas phaseoli*, 1- extract, 2, 3, 4- positive controls (gentamycin, ampicillin, azithromycin), 5- negative control (solvent).

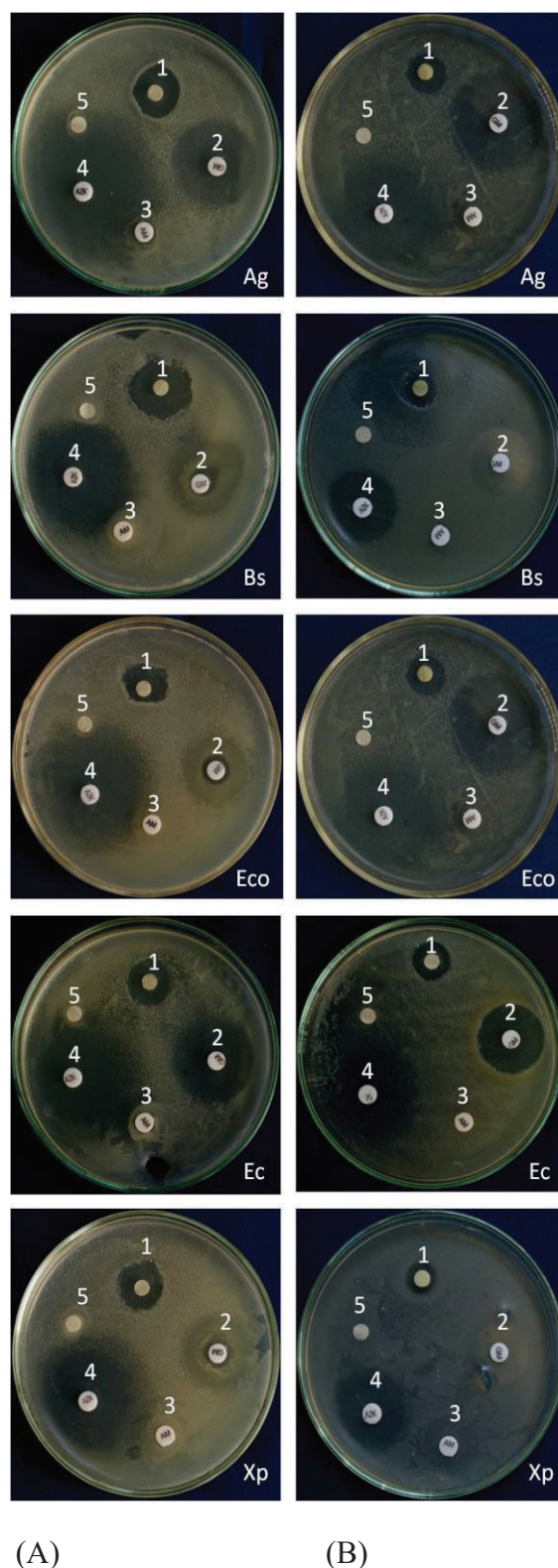


Plate 1b: Antibacterial activity of *Marchantia polymorpha* extracts against some pathogenic bacteria. (A)-ethanol extract, (B)-methanol extract, Ag-*Agrobacterium tumefaciens*, Bs- *Bacillus subtilis*, Eco- *Escherichia coli*, Ec- *Erwinia chrysanthemi*, Xp- *Xanthomonas phaseoli*, 1- extract, 2, 3, 4- positive controls (gentamycin, ampicillin, azithromycin), 5- negative control (solvent).

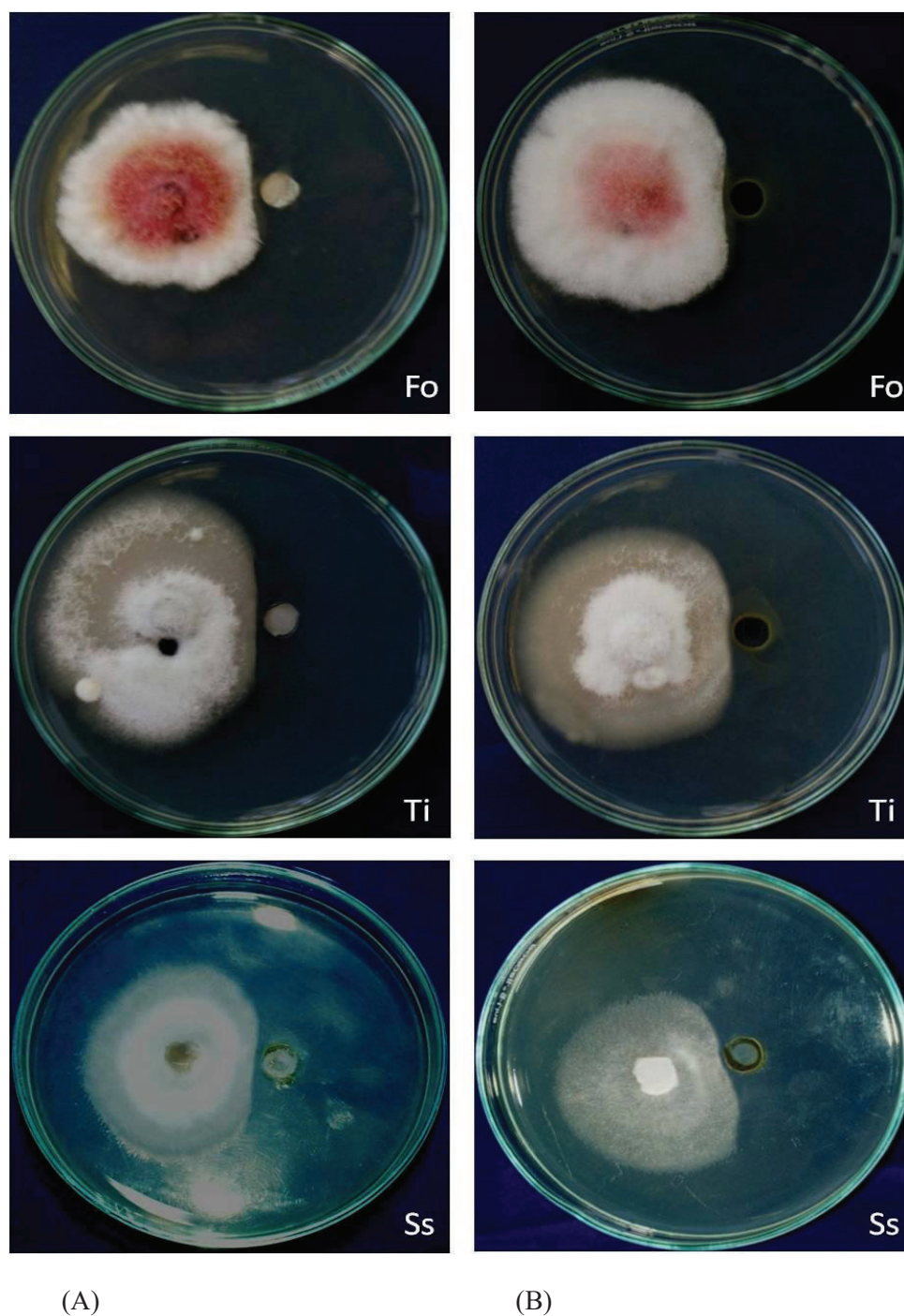


Plate 2a: Antifungal activity of *Marchantia polymorpha* against some pathogenic fungi. (A) Control (Clotrimazole), (B) Acetone extract, Fo- *Fusarium oxysporum*, Ti-*Tilletia indica*, Ss- *Sclerotinia sclerotiorum*.

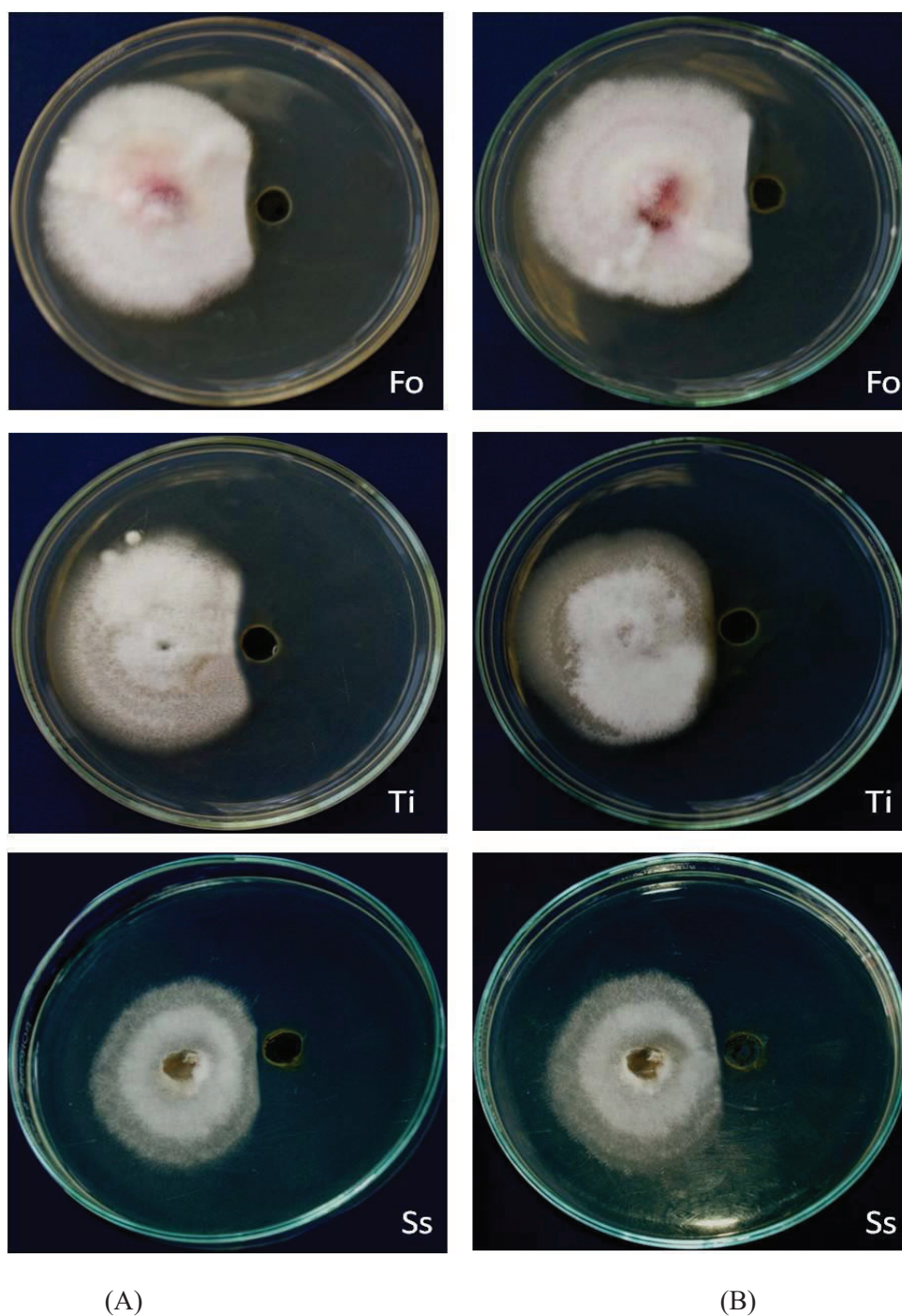


Plate 2b: Antifungal activity of *Marchantia polymorpha* against some pathogenic fungi. (A) Ethanol extract, (B) Methanol extract, Fo- *Fusarium oxysporum*, Ti-*Tilletia indica*, Ss- *Sclerotinia sclerotiorum*.