

1 **Antifungal activity of *Conocephalum conicum*(L) Dumort. (Marchantiophyta) against**
2 ***Fusarium oxysporum* f. sp. *lycopersici***

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9 **Abstract**

10 Tomato, a high value vegetable crop, suffers huge production losses in tropics due to a wilt
11 disease caused by *Fusarium oxysporum* f. sp. *lycopersici*. Present study was undertaken to find
12 an effective biocontrol method to check fusarium wilt in order to curb the losses suffered by
13 the crop growers. Organic extracts (acetone, methanol/ethanol) of thalloid bryophytes
14 (*Conocephalum conicum* (L.) Dumort. and *Marchantiapapillata* Raddi subsp.
15 *grossibarba* (Steph.) Bischl.) were tested against *F. oxysporum* f. sp. *lycopersici* using disc
16 diffusion and micro broth dilution assay. Methanol extract of *C. conicum* (L.) Dumort.
17 (CCDM) showed significantly high antifungal activity (85.5% mycelial inhibition;
18 31.25 µg/mL MIC and 125 µg/mL MFC). Potential of methanol extract was tested in a
19 glasshouse experiment on tomato, which illustrated the efficacy of the plant extract to control
20 the fusarial wilt. Morphological and ultrastructural alterations in CCDM treated fusarium
21 mycelia were observed in scanning electron microscopy. GC-MS analysis of CCDM extract
22 showed the presence of 51 constituents, and the dominant compounds were bis (bibenzyl),
23 acyclic alkanes, fatty acids, sesquiterpenoids and steroids. The study suggested that *C.*
24 *conicum* being an efficient source of Riccardin C like antifungal compounds provides a potent
25 and eco-friendly alternative to conventional fungicides in vegetables.

26 **Keywords:** Bryophytes, Biocontrol, *Conocephalum conicum*, *Fusarium oxysporum* f.
27 *sp. lycopersici*, Antifungal, SEM and GC-MS.

28 1. Introduction

29 Tomato (*Solanum lycopersicum* L.) is used around the world due to its flavour, colour, anti-
30 oxidative and anticancer properties (Gerszberg and Konka 2017). It is the world's largest
31 consumed vegetable crop after potato, sweet potato, and onion and, it also tops the list of
32 canned vegetables (Olaniyi et al. 2010). Presence of lycopene in tomato gives specific
33 benefits against number of ailments.

34 Fusarium wilt, one of the most prevalent and widespread diseases of tomato, severely
35 damages the crop as *Fusarium oxysporum* f. *slycopersici* (FOL) starts growing within the
36 vascular tissues of the plant and impedes water supply to different parts of the plant (Ignjatov
37 et al. 2012, Moretti et al. 2008). Singh and Kamal (2012) reported 10-90% loss in tomato
38 yield due to this wilt. Significant losses in tomato production in greenhouse condition and in
39 fields have been reported by Amini and Sidovich (2010).

40 Carbendazime and propiconazole are some common fungicides which are highly effective
41 against fusarium wilt (Manasa et al. 2017). Fungicides are synthetic in nature and may affect
42 environment and human health adversely due to their toxicity, carcinogenicity and persistent
43 nature (Fisher et al. 2018). Therefore, biocontrol measures seem to be a suitable and
44 sustainable solution to control fusarium wilt. Using botanicals in controlling fusarium wilt
45 provides an ecofriendly, cost-effective and non-toxic biocontrol method (Trda et al. 2019).
46 Botanicals belonging to different plant groups viz., lichens and angiosperms have been
47 reported to possess antifungal efficacy against *F. oxysporum* (Basile et al. 2015; Yeole et al.
48 2016). Antifungal activities have also been reported in some bryophytes (Tadesse 2002;
49 Kirisanth et al. 2020; Commisso et al. 2021).

50 Bryophyta, a very primitive and simplest group of the embryophytes, have acquired unique
51 survival strategies. They lack protection shields like bark but possess variety of bioactive
52 compounds against inhospitable environments. Rich repository of these secondary

53 metabolites viz.,benzyls, bis benzyl derivatives, sesquiterpenes, phenols etc. in bryophytes
54 have protected them against microbes and, hence, enabled their survival in diverse habitats
55 (Asakawa, 2017). In view of the above, an attempt was made in the present study to assess
56 the efficacy of thalloidbryophytes for an effective control of *F. oxysporum* f.
57 *splycopersici*employing *in vitro* and *in vivo* approaches.

58 2. Material and Methods

59 2.1 Collection of plant material

60 Based on the ethnomedicinal value reported by Glime (2017) and Negi et al(2018), two
61 species of bryophytes (liverworts),*Conocephalumconicum* (L.) Dumort(Conocephalaceae)
62 and *Marchantiapapillata*Raddi subsp. *grossibarba*(Steph.) Bischl.(Marchantiaceae) were
63 collected from an altitudinal range of 200-2100m from Uttarakhand, India.*C. conicum* was
64 collected from Mukteshwar (Altitude: 2100m, shady wall) and Dwarahat (Altitude: 1400m,
65 moist wall). *M. papillata*was collected from Dwarahat (Altitude: 1400m, river basin) and
66 Pantnagar (Altitude: 213m, moist wall).Voucher specimens of the collected species (NC501,
67 NC502) were submitted to G.B. Pant University Herbarium, Pantnagar.

68 2.2 Preparation of plant extract

69 Collected plant samples were thoroughly washed under running tap water, shade dried,
70 pulverized and extracted in 80 % ethanol/methanol and acetone (Analytical Grade, Sigma
71 Aldrich). Extraction was performed using hot (Soxhlet) and cold percolation methods (10
72 g/100 mL). The extracts were filtered through Whatman No. 1 filter paper and concentrated
73 using a rotary evaporator(Biogen Scientific). The crude extract was dissolved in the
74 respective solventfor the preparation of stock solution of 1mg/mL. The dilutions of different
75 concentrations (100, 400, 700, 1000 µg/mL) were prepared from the stock solution and used
76 for further study.

77 2.3Antifungal Assay

78

79 *Fusarium oxysporum*f. sp. *lycopersici*(FOL) was obtained from the Rhizosphere laboratory of
80 Department of Biological Sciences, G.B. Pant University of Agriculture & Technology,
81 Pantnagar(**Joshi et al. 2013**). The culture was revived on Potato dextrose agar(Himedia) at 27
82 °C and the same was used for disc diffusion assay. For further experiments, inoculum was
83 prepared by culturing FOL in potato dextrose broth in shake culture at same temperature for
84 5-6 days.

85 For disc diffusion assay, Potato Dextrose Agar (PDA) medium was poured aseptically in the
86 Petri plates (90 mm) and plates were allowed to solidify.Extract (20 µL) of varying
87 concentrations in different solvents was pipetted out into the discs (Hi Media). Four discs (5
88 mm), two treated (T) with plant extract (20µl) and two control (C) (20µl) along with the test
89 fungus(as shown in **Fig 1**) were kept in the same Petri plate following a standard protocol of
90 **Bauer (1966)** with minor modifications as suggested by **Negi and Chaturvedi**
91 **(2016a)**.Carbendazim (Bavistin 50 %) was used as a positive control while the solvents were
92 used as negative control in different plates. Inoculated fungal plates were incubated for 5
93 days at 28± 2° C. Inhibition (%) of fungal growth was calculated.

94
$$\% \text{ Inhibition} = \frac{\text{Mycelial growth (control)} - \text{Mycelial growth (treatment)}}{\text{Mycelial growth (control)}} \times 100$$

95

96

97 **FIGURE 1** |Plate of disc diffusion Assay. C = Control disc; T = Treatment disc; F=*Fusarium*
98 *oxysporum*

99 **2.4 Determination of Minimum Inhibitory/Fungicidal concentration**

100 Micro broth dilution assay was performed to determine the inhibitory and fungicidal
101 concentration of bryophyte extracts using freshly prepared potato dextrose broth (PDB) as
102 diluents (**Janovska et al. 2003**). Fresh and revived culture of the test fungus was diluted
103 upto 100 folds in broth (100 µl of microorganism in 10 mL broth). Colony forming units
104 (CFU) of the fungus were determined (1×10^9 CFU/mL) by taking OD at 620 nm using a UV-
105 Visible spectrophotometer (Thermo Fisher Scientific)(**Sutton 2011**). Plant extracts, with
106 1000 to 0.98 µg/mL concentration in two-fold dilution series were added to the test tubes
107 containing fresh fungal cultures. All the tubes with fungal cultures were incubated at 28°C for
108 72 h. The visible turbidity and optical density of the cultures were determined at 620 nm
109 using spectrophotometer. The lowest concentration of the extract that inhibited the visible
110 growth of the test microorganisms was recorded as minimum inhibitory concentration (MIC)
111 and the test culture without any visible microbial growth was considered as minimum
112 fungicidal concentration (MFC).

113 **2.5 Soil collection and preparation of experimental pots**

114 Autoclaved loamy soil and river bed sand were used (sand: soil= 3:1) in the pot experiment.
115 The pH and EC of this mixture were 8.15 and 65.7 μS , respectively. Tomato seeds were
116 surface sterilized in 1% HgCl_2 for 2-3 min, rinsed three times in sterile distilled water and
117 then dipped in sterile water for 1 day for imbibition. After drying in towel paper, the seeds
118 were sown in the sterilized soil mixture in the Departmental glasshouse. Twenty days old
119 tomato seedlings (four leaf stage) grown in autoclaved sand: soil (3:1) were transplanted into
120 500g pot containing similar ratio of sand and soil. Plants were irrigated with tap water and
121 left for 1-2 days for equilibration before setting up the experiment. The fungal inoculum was
122 taken from PDB as it contained a large number of macroconidia. The population of FOL in
123 the substrate was estimated as the number of CFU per gram of soil. Approximately 2×10^8
124 CFU/g of the fungal inoculum was used for pathogenesis in tomato roots. 1 ml of fungal
125 inoculum suspension (made in water) was used for inoculating the seedlings by soil
126 drenching method. The infection of FOL was confirmed by the symptoms of wilting in tomato
127 plants within 15 days of the experiment.

128 Pot experiment was conducted in a completely randomized block design in the glass
129 house with 5 replicates each using two different treatments viz., 125 $\mu\text{g}/\text{mL}$ and 31.25 $\mu\text{g}/\text{mL}$
130 of aqueous solution of CCDM extract (EC1, EC2 respectively). All the treatments were
131 applied in form of fine solution to the roots of tomato plants per pot until the roots absorbed
132 the solution completely. The treatments were given one day prior and 15 days after FOL
133 treatment. Three control(s) used were fusarium infested negative control (C+F), non-infested
134 water control, and positive control (carbendazim). Pots were irrigated with deionised water
135 as and when required.

136 Pots were maintained in the glasshouse under the following growth conditions: Temperature-
137 27°C, Photoperiod- 16/8 hour day/night cycle, Light intensity- 400 $\text{Em}^{-2}\text{s}^{-1}$, (400-700 nm),
138 Relative humidity- 60%.

139 **2.6 Observations**

140 Observations related to shoot length, root length, fresh weight and dry weight of tomato
141 plants were taken for a period of 30 days after planting. After harvesting the plants, roots were
142 washed thoroughly with tap water followed by washing with deionised water. Shoot and root
143 length were measured from the soil base to the tip of the fully expanded leaf and soil base to
144 the tip of the root. Shoot and root fresh weight (g) were weighed separately immediately after
145 harvesting. Dry weight of shoot and roots were taken (g) separately after drying the samples
146 at 60°C in an electric oven for 48 h.

147 **2.7 Gas Chromatography-Mass Spectroscopy (GC-MS)**

148 The crude methanol extract of *C. conicum* (CCDM) was filtered using 0.45 µm syringe filters.
149 GC-MS analysis of the extract was carried out using a GC-MS System (Shimadzu) QP 2010
150 equipped, with Rxi ®-5Sil MS capillary GC column (5 % phenyl 95 % dimethyl
151 polysiloxane) with 30 m length, 0.25 mm diameter and 0.25 µm film thickness at Central
152 Instrumentation Facility, Jawaharlal Nehru University, New Delhi. Helium was the carrier
153 gas and used at a flow rate of 16.3 mL/min. Sampling time was maintained at 1 min. at a
154 column pressure of 81.7 kPa. Column oven and injector temperatures were maintained at 80 °
155 C and 270 ° C respectively. Samples (1 µl) were injected into the column with a split ratio of
156 10:1. Names, molecular weight and structures of the individual compounds were identified by
157 matching their mass fragmentation pattern with the National Institute of Standard Technology
158 (NIST) Library.

159 **2.8 Scanning electron microscopic (SEM) study**

160 Effect of methanol extract of *C. conicum* on FOL was observed by SEM following a standard
161 protocol of **Plodpai et al. (2013)** with minor modifications as reported by **Negi and**
162 **Chaturvedi (2016a)**. SEM (JEOL6610LV) facility was provided by Department of Anatomy,

163 College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture &
164 Technology, Pantnagar, India.

165 **2.9 Statistical Analysis**

166 Analysis of variance was calculated using standard statistical methods (**Snedecor and**
167 **Cochran 1994**). Disc diffusion assay was performed in triplicate while pot experiment was
168 conducted using five replicates. Values were represented as mean \pm SEm. Mean value
169 comparison was computed using four factorial ANOVA (for antifungal activity through disc
170 diffusion assay). It revealed level of significance at $P < 0.05$ and $P < 0.01$ among different
171 bryophyte species, solvents, concentrations and days. The analysis was carried out using IBM
172 SPSS statistical software, version 15.0 (IBM, New York, USA).

173 **3. Results**

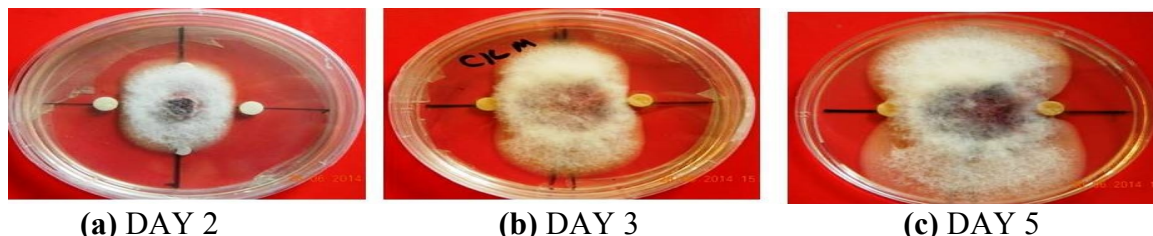
174 **3.1 Bioactivity of plant extract against *Fusarium oxysporum* f. sp. *lycopersici***

175 Organic extracts (alcohol and acetone) of *Conocephalum conicum* and *Marchantia papillata*
176 showed significant antifusarium activity (Fig 2). Organic extracts (100-1000 μ g/mL) of
177 bryophytes revealed dose - dependent inhibition of FOL (using disc diffusion assay) at
178 different time intervals (2-5 days) (Table 1 and Fig 2). The methanol extract of *C. conicum*
179 (CCDM) showed maximum per cent inhibition of FOL mycelial growth, values ranging from
180 25.31 ± 0.005 (after 2 days) to 85.5 ± 0.57 (after 5 days) at the concentration of 100 and 1000
181 μ g/mL respectively (Table 1). Similarly, acetone extract of *C. conicum* (CCDA) showed
182 second highest per cent inhibition from 31.4 ± 0.57 (after 2 days) to 70.16 ± 0.5 (after 5 days) at
183 100 and 1000 μ g/mL respectively. Minimum inhibitory concentration (MIC) and minimum
184 fungicidal concentration (MFC) of different organic extracts of bryophytes ranged from 31.25
185 to 500 μ g/mL and 125 to 500 μ g/mL respectively (Table 2). Again, CCDM extract was found
186 most potent against FOL with lowest MIC (31.25 μ g/mL) and MFC (125 μ g/mL). Hence, the

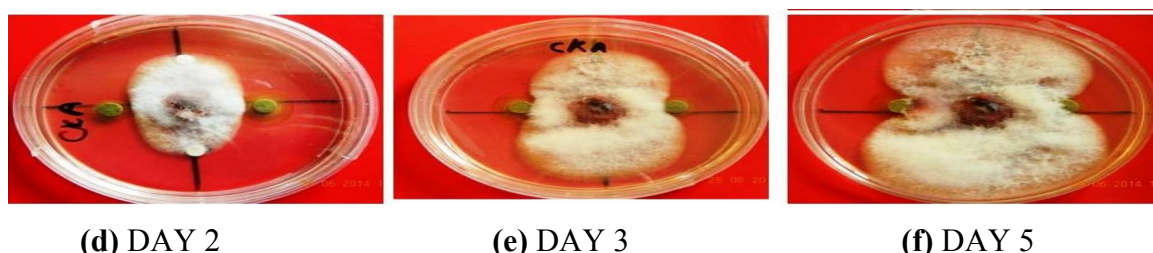
187 effectiveness of CCDM extract was further assessed by chemical characterization and *in vivo*
188 pot experiment.

189

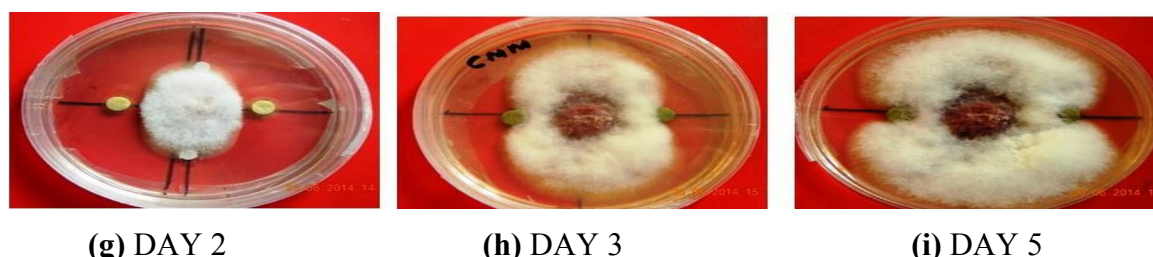
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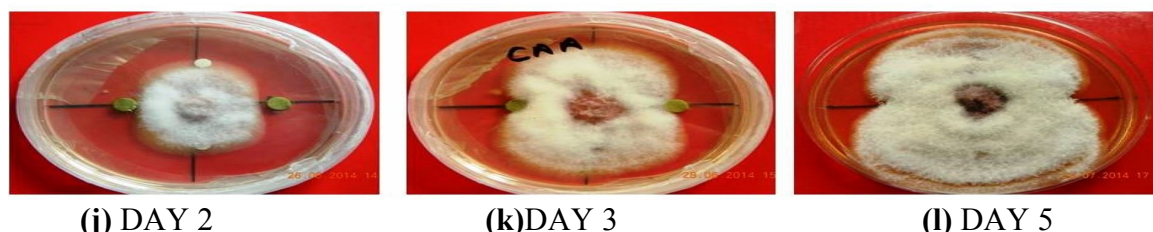
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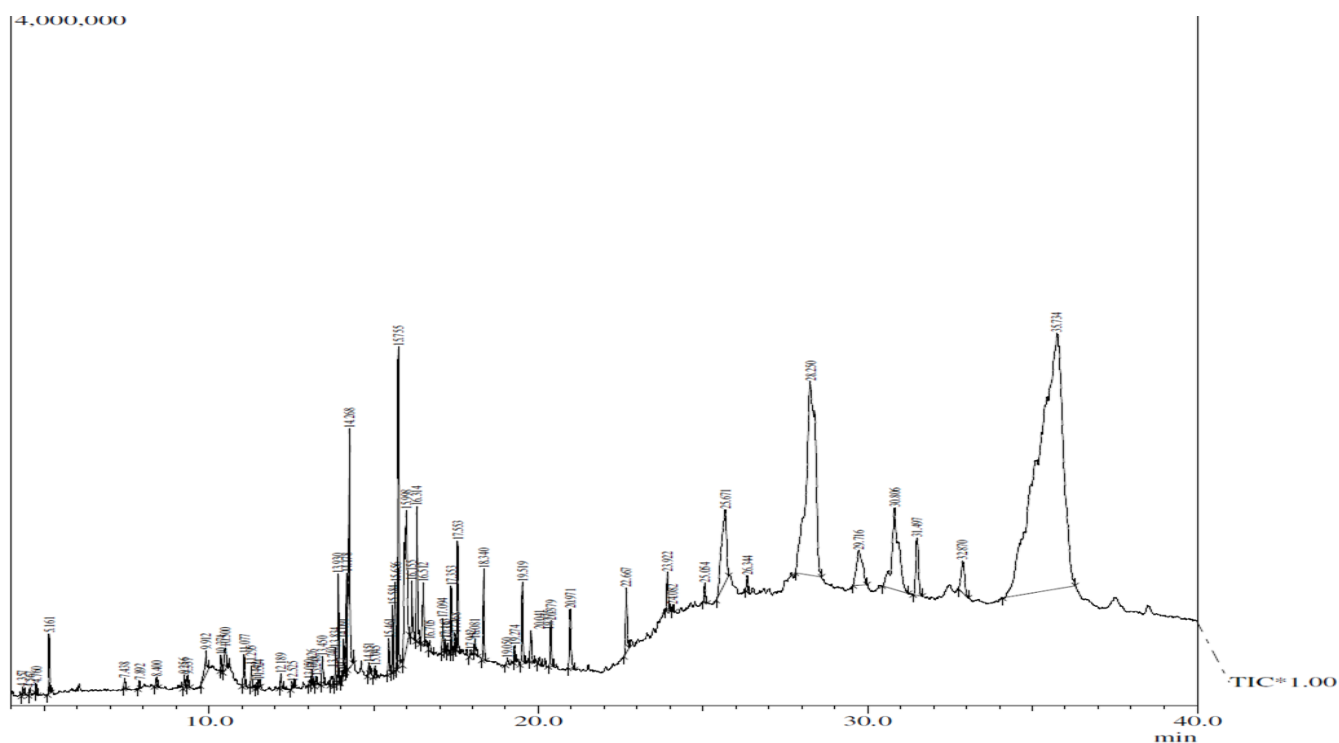


197 **FIGURE 2** | Disc diffusion plates showing growth inhibition of *Fusarium oxysporum* sp.
198 *lycopersici* under different treatments. (a, b, c) Mycelia treated with CCDM extract, (d, e, f)
199 Mycelia treated with CCDA extract, (g, h, i) Mycelia treated with CCMM extract, (j, k, l)
200 Mycelia treated with CCMA extract (CCDM-methanol extract of *Conocephalum conicum*
201 collected from Dwarahat, CCDA- acetone extract of *C. conicum* from Dwarahat, CCMM-
202 methanol extract of *C. conicum* from Mukteshwar, CCMA-acetone extract of *C. conicum*
203 from Mukteshwar).

204

205 3.2 Chemical Characterization

206 The most potent extract (CCDM) was chemically characterized using gas chromatography
207 and mass spectrophotometry. Percentage and the retention timings of the major components

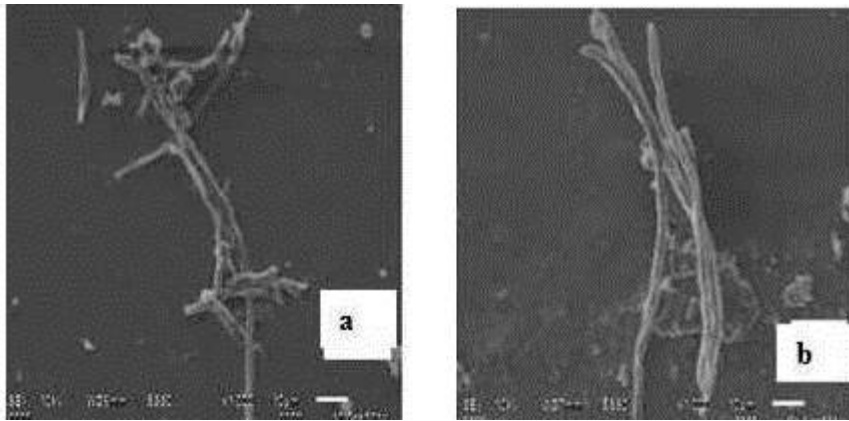


208
209 **FIGURE 3| GC-MS chromatogram of methanol extract of *Conocephalum conicum* Dwarahat (CCDM)**

210 are given in Table 3 and Fig 3. It showed the presence of 51 constituents contributing 100%
211 of the total extract. The results revealed that the extract was dominated by bis (bibenzyl) =
212 riccardin c (64%), acyclic alkanes (eicosane = 5%), fatty acids (n-hexadecanoic acid =
213 3.43%), sesquiterpenoids (10-epi- α -eudesmol = 1.6%) and steroids (δ .5-ergosterol = 4.3%,
214 stigmasterol = 1.2%).

215 **3.3 Effect of CCDM extract on hyphal and conidial morphology**

216 Scanning electron micrographs (SEM) demonstrated that 125 μ g/ml (EC1) of CCDM extract
217 caused profound changes in hyphal morphology of FOL (Fig 4). Treated mycelia appeared
218 wrinkled and ruptured. In contrast, SEM image of untreated fungus mycelia displayed smooth
219 hyphal surface and intact conidia with typically tapered apices.



220

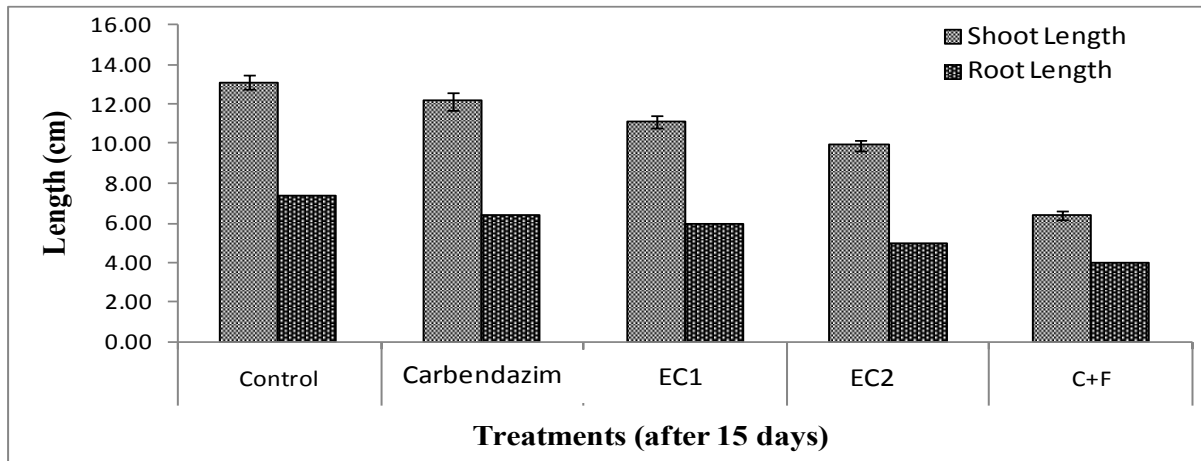
221 **FIGURE 4** | SEM images of (a) ruptured mycelia under EC1 treatment of CCDM extract (b)
222 mycelia in control

223 **3.4 Effect of CCDM extract on growth parameters of tomato plants**

224 CCDM extract was tested for *in vivo* antifungal efficacy against FOL in a pot experiment
225 using two most effective concentrations, EC1 (125 $\mu\text{g}/\text{mL}$) and EC2 (31.25 $\mu\text{g}/\text{mL}$) besides
226 keeping a positive control (carbendazim treated plants + Fusarium inoculated), a negative
227 control (Fusarium inoculated without any treatment) and a water control in the pot
228 experiment (30 days). Results of the experiment are given in Fig 5, Fig 8.

229 **3.4.1 Observations of shoot and root length**

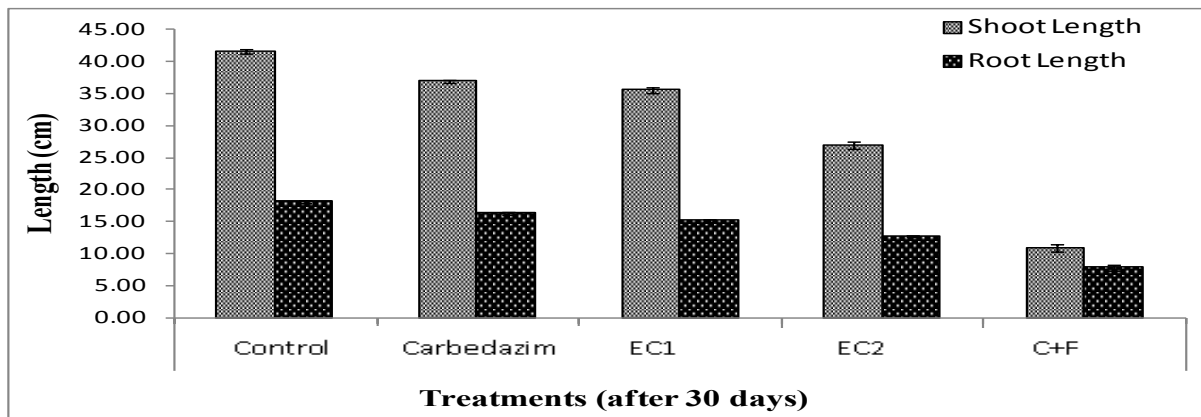
230 Both EC1 and EC2 treated plants showed significantly higher shoot/root length compared to
231 the negative control (Fusarium inoculated without any treatment) after 15 and 30 days of the
232 treatment (Fig 5a, 5b). After 30 days, the EC1 treated plants showed comparable shoot length
233 (35.50 \pm 0.36cm) and root length (15.17 \pm 0.27cm) as that of the positive control
234 (36.77 \pm 0.22cm; 16.27 \pm 0.12cm). Shoot and root length was, however, significantly low in all
235 other inoculated treatments compared to water control.



236

237 **FIGURE 5a** | Effect of different doses of CCDM extract on shoot/ root length of tomato
238 plants(after 15 days). Control= water; Carbendazim = positive control; EC1 = 125 μ g/mL;
239 EC2 = 31.25 μ g/mL; C+F = negative control (*Fusarium* infested without treatment). Data
240 represents mean \pm SE from five replicates

241

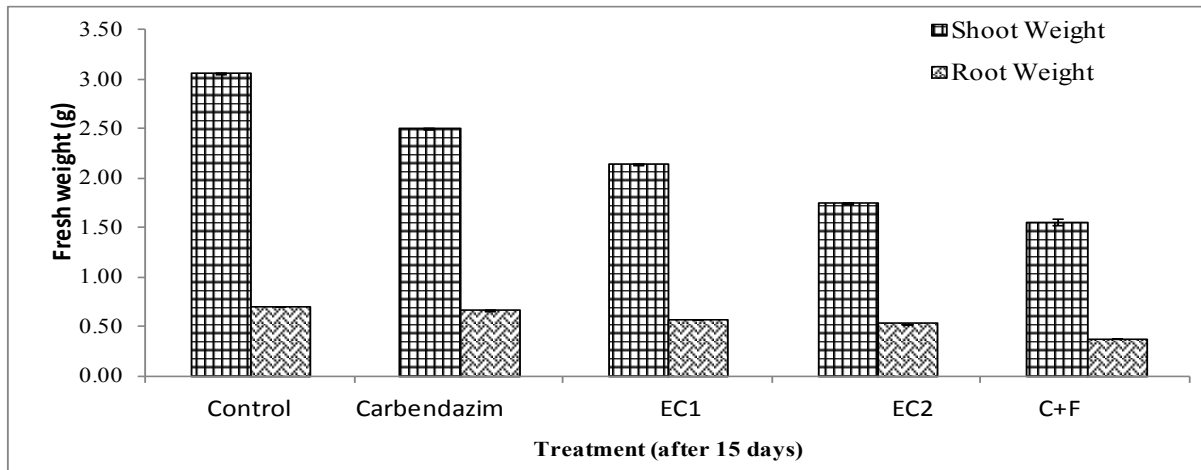


242

243 **FIGURE 5b** |Effect of different doses of CCDM extract on shoot/root length of tomato
244 plants (after 30 days). Control=water, Carbendazim = positive control; EC1 = 125 μ g/mL;
245 EC2 = 31.25 μ g/mL; C+F = negative control (*Fusarium* infested plant without treatment).
246 Data represents mean \pm SE from five replicates

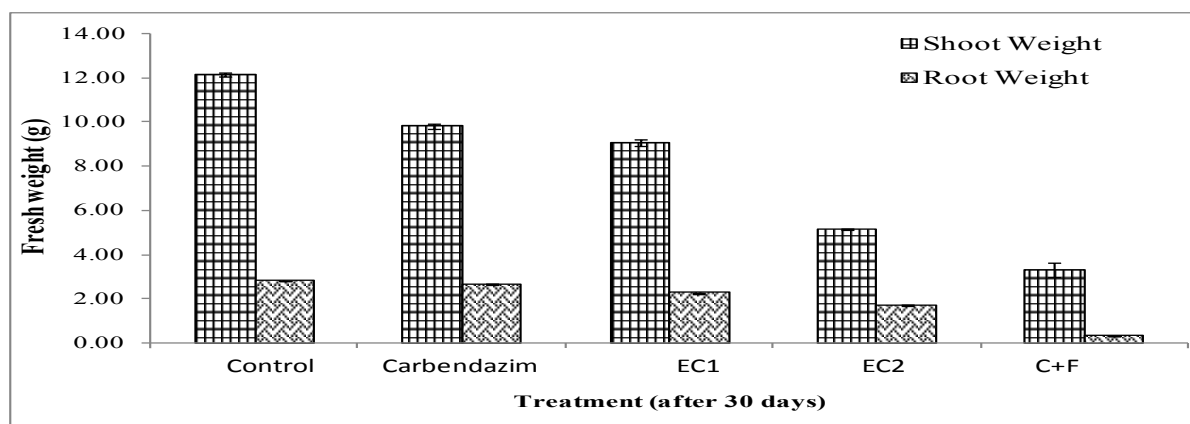
247 3.4.2 Shoot and root fresh weight

248 A similar trend of shoot and root fresh weight (with different treatments) was observed in
249 the pot experiment after 15 and 30 days of setting up the experiment (Fig 6a, Fig 6b). After
250 30 days, EC1 treated plants showed shoot and root fresh weight in EC1 treated plant was
251 4.62 \pm 0.18 g and 2.26 \pm 0.03grespectively after 30 days, where as inpositive control it was
252 4.91 \pm 0.20g and 2.67 \pm 0.01g after same time. Reduction of wilt in EC2 treated plants was
253 significantly higher than the negative control.



254

255 **FIGURE 6a** | Effect of different doses of CCDM extract on shoot and root fresh weight of
256 tomato plants after 15 days. Control= water; Carbendazim= positive control; EC1=Extract
257 concentration($125\mu\text{g/mL}$); EC2= Extract concentration($31.25\mu\text{g/mL}$); C+F =negative control
258 (*Fusarium* infested plant without any treatment). Data represents mean \pm SE from
259 fivereplicates



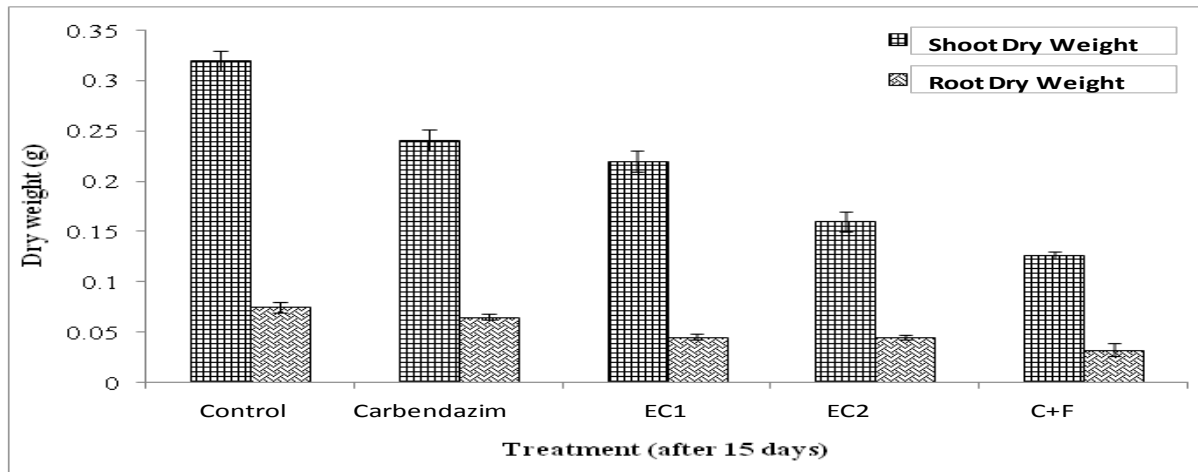
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261 **FIGURE 6b** | Effect of different doses of CCDM extract on shoot and root fresh weight of
262 tomato plants after 30 days. Control= water; Carbendazim= positive control; EC1=Extract
263 concentration ($125\mu\text{g/mL}$); EC2=Extract concentration($31.25\mu\text{g/mL}$); C+F =negative control
264 (*Fusarium* infested plant without any treatment). Data represents mean \pm SE from five
265 replicates

266

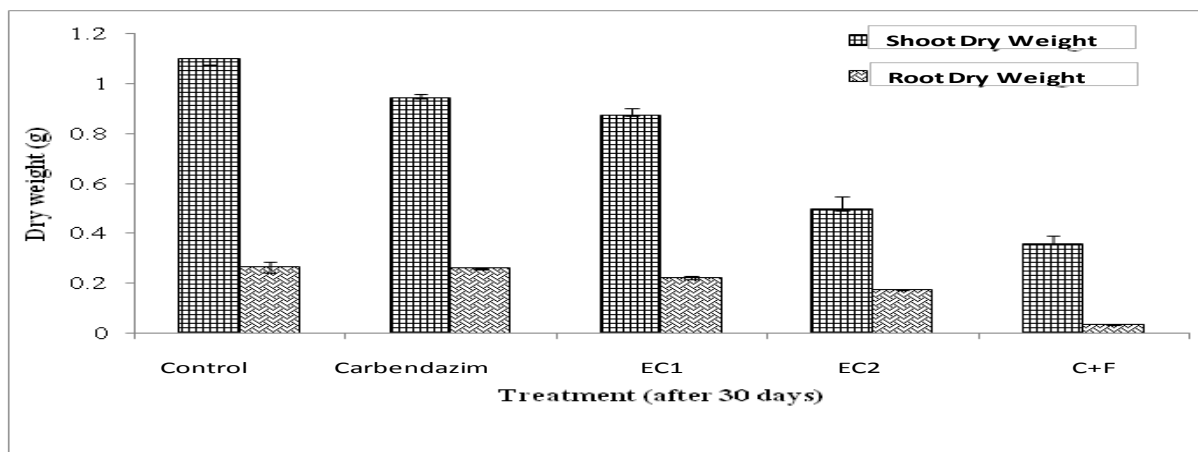
267 3.4.3 Shoot and root dry weight

268 Shoot and root dry weight was significantly higher in both EC1 and EC2 treated plants as
269 compared to negative control at all time intervals (Fig 7a, Fig 7b). After 30 days, dry weight
270 of shoot and root was $0.87\pm 0.22\text{g}$ and $0.22\pm 0.005\text{g}$ respectively in EC1 treated plants whereas
271 it was $0.94\pm 0.25\text{g}$ and $0.25\pm 0.002\text{g}$ respectively in positive control.



272

273 **FIGURE 7a** |Effect of different doses of CCDM extract on shoot and root dry weight of
274 tomato plants after 15 days. Control=water; Carbendazim= positive control; EC1=Extract
275 concentration($125\mu\text{g}/\text{mL}$); EC2=Extract concentration ($31.25\mu\text{g}/\text{mL}$); C+F =negative control
276 (*Fusarium* infested plant without any treatment). Data represents mean \pm SE from five
277 replicates



278

279 **FIGURE 7b** |Effect of different doses of CCDM extract on shoot and root dry weight of
280 tomato plants after 30 days. Control=water, Carbendazim= positive control; EC1=Extract
281 concentration ($125\mu\text{g}/\text{mL}$); EC2=Extract concentration($31.25\mu\text{g}/\text{mL}$); C+F =negative control
282 (*Fusarium* infested plant without any treatment). Data represents mean \pm SE from five
283 replicates

284 3.5 Discussion

285 To the best of our knowledge, no such study is available on the bioefficacyof
286 bryophyte extracts against *Fusarium oxysporum* f. sp.*lycopersici*(FOL) using *in vitro*and *in*
287 *vivo* approaches. However, reports on *in vitro* antifungal activity of bryophytes against other
288 fungi have been givenbyMewariet al. (2007), Deora and Guhil(2015), Negi et al. (2016a),
289 Negi et al. (2020).In the present study, alcoholic extracts of bryophytes showed higher

290 antifungal activity than acetone extracts. Methanol extract of *C. conicum*(CCDM) showed
291 highest mycelial inhibition followed by acetone extract (CCDA) as shown in **Table 1**. Other
292 studies have also shown higher antimicrobial efficacy of alcoholic extract over acetone
293 extract against different microorganisms (**Singh et al. 2011; Kandpal et al. 2016; Negi et al.**
294 **2018**). Variable efficacies of crude organic extracts may be due to the differences in the
295 polarities of the solvents which affect the effective extraction of antifungal compounds in
296 different solvents (**Negi and Chaturvedi, 2016 b**).

297 In the present study, significantly higher antifungal activity was exhibited by CCDM
298 extract (plant collected from Dwarahaat, lower altitude) in comparison to CCMM extract
299 (plant collected from Mukteshwar, higher altitude). Similarly, higher antimicrobial activity
300 was also reported in *Dumortierahirсутa* (Sw) Nees collected from lower altitude (**Mukherjee**
301 **et al. 2012**).Difference in the bioactivity of the plants growing at different altitudes might be
302 due to variation in accumulation of diverse secondary metabolites influenced by temperature
303 and duration of exposure to UV radiation prevailing at those altitudes (**Zhang and Bjorn**
304 **2009**). Habitat conditions and microniche also govern the responses of different ecotypes
305 occupying diverse habitats.

306 Since maximum fungal growth inhibition (85.5%) was shown by CCDM extract, hence the
307 same extract was chemically characterized to search for its important chemical constituents
308 responsible for antifungal efficacy against FOL. Chemical characterization of CCDM extract
309 showedthe presence of different bioactive compounds. viz., steroids, fatty acids,
310 sesquiterpenoids, bibenzyls etc. Most of these compounds,like hexadecanoic acid,
311 stigmasteroland δ .5-ergostenolare antifungal in nature (**Ahmed et al. 2010; Mujeeb et al.**
312 **2014; Abubakar and Majinda,2016**). Presence of hexadecanoic acid in organic extract of *C.*
313 *conicum* was also reported by **Ludwiczuk et al. (2013)**. Liverworts also contain lipophilic oil
314 bodies which possessed most characteristic antifungal compounds like bis (bibenzyl)

315 derivatives (riccardin C, riccardin F, isoriccardin C and marchantin A) (**Xie et al. 2010;**
316 **Asakawa 2013**). Riccardin D isolated from Chinese *Marchantiapolyomorpha* showed
317 antifungal activity against fluconazole – resistant *Candida albicans* strains (**Asakawa and**
318 **Ludwiczuk 2018**). Riccardin C is one of the most important compounds of liverworts which
319 shows strong antifungal activity (**Asakawa et al. 2013**). Interestingly, GC-MS analysis of
320 CCDM extract in the present study, revealed the presence of very high concentration of
321 riccardin C (64%), which is a major biomarker compound of liverworts having significant
322 antifungal activity. Presence of high concentration of antifungal compounds like riccardin C,
323 fatty acids and steroids in CCDM extract can be directly correlated with its significantly high
324 antifungal activity. Cell membrane damage is the most possible mechanism responsible for
325 antifungal nature of bioactive compounds (**Parvuet al. 2010; Plodpai et al. 2013**). In the
326 present study, SEM images showed alteration in hyphal morphology which confirmed the
327 fungistatic nature of CCDM extract. Scanning electron microimages clearly showed that
328 EC1 concentration of CCDM extract as well as carbendazim treatment (positive control) caused
329 substantial damage to the hyphal structure and hence controlled the growth of FOL mycelia.
330 *In vitro* antifungal testing of CCDM extract was followed by an *in vivo* glasshouse
331 experiment on tomato plants. The first *in vivo* greenhouse experiment using bryophyte
332 extracts was performed on tomato plants against *Phytophthora infestans* (**Frahm 2004**). In the
333 present study, different effective dosages of *C. conicum* extract were used to control
334 FOL infection in tomato. Plants grown in EC1 amended pots attained significantly higher
335 shoot and root height, shoot and root fresh weight and dry weight as compared to EC2 and
336 the negative control. All these parameters were quite close to the positive control and
337 indicated significant biocontrol potential of CCDM. Fusarium wilt caused blockage inside the
338 xylem vessels by mycelia producing microconidia, which travel upward in the transpiration
339 stream (**Okungbowa and Shittu 2012**). Interruption of the xylem vessels and transpiration

340 stream caused wilting in fusarium infested plants. It also caused poor development of lateral
341 roots because of high infection rate (**Loganathan et al. 2009**). Poor root and shoot growth was
342 also seen in fusarium infested plants in the present study. However, tomato treated plants
343 with EC1 and EC2 dosage of CCDM could overcome the biological stress caused by the
344 fungus and showed significantly higher root and shoot growth parameters compared to
345 fusarium control (C+F). Here, in the study, EC1 concentration of CCDM extract was found to
346 be more effective than EC2. Chemical characterization of CCDM extract revealed presence
347 of large number of antifungal compounds. One of the major bioactive compounds of CCDM
348 extract was riccardin C which is a well known antifungal compound and contributes
349 significantly to the antifungal potential of *C. conicum*.

350 **3.6 Conclusion**

351 The present study was aimed to find an ecofriendly biological control of *Fusarium*
352 *oxysporum* f. sp. *lycopersici* (FOL) from lesser known cryptogamic plants viz., bryophytes.
353 The first step to meet the objective was finding a potent bryophyte viz.,
354 *Conocephalum conicum*, which inhibited the mycelia growth of FOL (85 % per cent
355 inhibition) in a laboratory test. The second step was to find out the effective dosage of the
356 biocontrolling botanical. The effective concentration (125 µg/ml) of the methanolic extract of
357 *C. conicum* disrupted the hyphal structure and emerged as the most potent dosage to control
358 fusarium wilt under glasshouse conditions as well. To the best of our knowledge, this is the
359 first study to report the use of organic extracts of *C. conicum* to control *F. oxysporum* f. sp.
360 *lycopersici* using *in vitro* and *in vivo* approaches. Present study proved the antifungal potential
361 of crude methanol extract of *C. conicum* (owing to the presence of riccardin C and other
362 antimicrobial compounds). Based on the present study, *C. conicum* can be utilized as an
363 environment friendly botanical fungicide providing a promising alternative to chemicals.
364 However, still extensive field and advanced ultra structural studies are needed to understand the

365 molecular mode of interaction between the bioactive compound and the pathogen. Procuring
366 enough quantity of the material is another challenge that can be tackled by scaling up their
367 propagation in bioreactors.

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Table 1 |Growth inhibition (%) of *Fusarium oxysporum*f. sp. *lycopersici*(FOL)with different extracts of bryophytes

Name of Bryophyte extracts	Percent Inhibition (%)														
	Conc. (100µg/mL)			Conc. (400µg/mL)			Conc. (700 µg/mL)			Conc. (1000 µg/mL)					
	Day 2	Day 3	Day 5	Day 2	Day 3	Day 5	Day 2	Day 3	Day 5	Day 2	Day 3	Day 5			
CCDM	25.31±0.05	26.86±0.05	47.84±0.57	50.84±0.57	44.13±0.56	51.35±0.55	66.10±0.28	67.58±1.14	75.12±1.1	82.03±0.5	84.4±1.1	85.5±0.57			
CCDA	31.4±0.57	49.34±0.57	42.86±0.57	49.58±0.57	41.09±0.57	49.72±1.15	60.95±0.57	66.42±0.66	64.91±0.55	65.28±0.57	66.43±0.57	70.16±0.57			
CCMM	22.43±0.88	27.23±0.57	27.45±0.57	53.84±0.57	61.63±0.33	43.88±0.57	58.08±1.1	59.56±0.28	61.36±0.57	56.25±0.57	62.08±1.11	62.79±0.57			
CCMA	23.63±0.33	25.54±0.57	24.89±0.57	32.5±0.57	30.95±0.66	41.07±0.33	17.18±0.33	43.43±0.57	49.10±0.66	47.5±0.57	35.71±0.57	58.23±0.57			
MPDE	20.6±0.57	23.33±0.57	21.54±0.57	46.80±0.57	50.88±0.88	49.15±0.66	36.09±0.66	51.6±0.33	49.74±0.57	31.66±0.57	45.61±0.57	55.97±0.33			
MPDA	12.56±0.66	14.71±0.57	14.34±0.57	12.54±0.57	13.63±0.28	34.73±0.57	29.26±0.33	32.63±0.33	41.02±0.33	46.58±0.33	49.22±0.57	54.02±0.33			
MPPE	12.48±1.1	14.48±0.66	14.48±1.15	18.64±0.33	56.25±0.33	43.95±0.33	24±0.66	40.47±0.66	44.96±0.57	42.27±1.15	40.88±0.66	53.97±0.33			
MPPA	11.23±0.33	13.56±0.66	14.67±1.15	16.45±0.66	51±0.88	41.89±0.33	20±0.66	36.89±0.66	41.96±0.5	40.27±1.1	39±0.66	51.89±0.33			
Carbendazim	72.0±0.66	78.48±0.33	88.87±1.1	ND	ND	ND	ND	ND	ND	ND	ND	ND			
	Bryophyte species (A)	Organic solvent (B)	Conc (C)	Days (D)	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	ABCD
CD at 1%	0.39	0.27	0.39	0.34	0.55	0.79	0.68	0.96	0.48	0.68	1.11	0.96	1.37	0.96	1.9
CD at 5%	0.30	0.21	0.30	0.26	0.42	0.60	0.52	0.73	0.36	0.52	0.85	0.73	1.04	0.73	1.47
SEm	0.10	0.07	0.10	0.09	0.15	0.21	0.18	0.26	0.13	0.18	0.30	0.26	0.37	0.26	0.53

CCDM-methanol extract of Conocephalum conicum from Dwarahat, CCDA- acetone extract of C. conicum from Dwarahat, CCMM-methanol extract of C. conicum from Mukteshwar, CCMA-acetone extract of C. conicum from Mukteshwar, MPDE- ethanol extract of Marchantiapapillata from Dwarahat, MPDA- acetone extract of M. papillata from Dwarahat,MPPE-ethanol extract of M. papillata from Pantnagar, MPPA-acetone extract of M. papillata from Pantnagar, Conc.-Concentration, ND-not determined. All the experiments were performed in triplicates. Mean value (±SEm) with four factorial ANOVA revealed level of significance at P<0.05 and P<0.01 among different bryophyte species, solvents, concentrations and days.

Table 2 | Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentrations (MFC) of different extracts of bryophytes against *Fusarium oxysporum* f. sp. *lycopersici*.

Name of Bryophyte extracts	Minimum Inhibitory Concentration MIC ($\mu\text{g/mL}$)	Minimum Fungicidal Concentration MFC ($\mu\text{g/mL}$)
CCDM	31.25	125
CCDA	62.5	250
CCMM	62.5	250
CCMA	125	500
MPDE	125	500
MPDA	125	-
MPPE	250	-
MPPA	500	-

CCDM-methanol extract of *Conocephalum conicum* from Dwarahat, *CCDA*- acetone extract of *C. conicum* from Dwarahat, *CCMM*-methanol extract of *C. conicum* from Mukteshwar, *CCMA*-acetone extract of *C. conicum* from Mukteshwar, *MPDE*- ethanol extract of *Marchantiapapillata* from Dwarahat, *MPDA*- acetone extract of *M. papillata* from Dwarahat, *MPPE*-ethanol extract of *M. papillata* from Pantnagar, *MPPA*-acetone extract of *M. papillata* from Pantnagar, (-)=did not show fungicidal concentration.

Table 3 |Chemical characterization (Gas Chromatography- Mass Spectrometry) of CCDM extracts (methanol extract of *Conocephalum conicum* collected from Dwarahat).

S.No.	Retention time	Name of compound	Area %
1	4.357	1,2,3-propanetriol	0.1
2	5.161	2,3-dimethyl-2-pentanol,	0.4
3	7.438	4-hydroxy, benzaldehyde,	0.1
4	7.892	2,7,10-trimethyl-dodecane,	0.1
5	9.256	humulen-(v1)	0.1
6	9.357	α .-selinene	0.1
7	9.912	dodecanoic acid	0.4
8	10.374	limonen-6-ol, pivalate	0.1

9	10.500	α -methyl mannofuranoside	0.3
10	11.077	alloaromadendrene oxide-(1)	0.3
11	11.295	Globulol	0.2
12	12.189	tetradecanoic acid	0.1
13	12.525	(-)-loliolide	0.1
14	13.126	6,10,14-trimethyl-2-pentadecanone	0.1
15	13.248	tetradecanoic acid	0.1
16	13.450	diisobutyl phthalate	0.3
17	13.740	methyl (9z)-9-octadecenoate	0.1
18	13.834	methyl ester, (z)-9-hexadecenoic acid	0.2
19	13.930	hexadecanoic acid, methyl ester	0.7
20	14.091	(z)-7-tetradecenal	0.3
21	14.268	n-hexadecanoic acid	3.43
22	14.851	2,2'-dihydroxydiphenylmethane	3.5
23	15.045	azuleno[4,5-b]furan-2(3h)-one,3a,4,6a,7,8,9,9a,9b-octahydr	0.1
24	15.461	fumaric acid, pentadecyl 2,2,2-trifluoroethyl ester	0.3
25	15.584	9,12-octadecadienoic acid (z,z)-, methyl ester	0.5
26	15.656	(z,z,z)-9,12,15-octadecatrienoic acid, methyl ester	0.9
27	15.755	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[r	2.3
28	15.998	(z)6,(z)9-pentadecadien-1-ol	3.0
29	16.155	octadecanoic acid	0.6
30	16.314	2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-	1.1
31	17.094	5,8,11,14-eicosatetraenoic acid, ethyl ester	0.3
32	17.163	cis-5,8,11,14,17-eicosapentaenoic acid, methyl ester	0.1
33	17.233	tert-butyldimethylsilyl ester, hexadecanoic acid,	0.1
34	17.353	Eicosane	5.0
35	17.553	eudesma-4(14),11-diene	0.9
36	18.081	9-octadecenamide	0.1
37	19.050	17-octadecynoic acid, tert-butyldimethylsilyl ester	0.1
38	19.274	(z)-longipinane	0.2
39	19.775	hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	0.3

40	20.041	Cignolin	0.1
41	20.379	isooctyl phthalate	0.5
42	22.667	Nonadecane	0.8
43	24.082	dioctylsebacate	0.1
44	25.054	Dotriacontane	2.9
45	65.671	5h-naphtho[2,3]carbazole	2.9
46	26.344	2-bromo dodecane	0.2
47	29.716	10-epi- α -eudesmol	1.6
48	30.806	delta.5-ergosterol	4.3
49	31.497	Stigmasterol	1.2
50	32.870	(3 β) stigmast-5-en-3-ol	0.8
51	35.734	riccardin c	64.4
		Total =	100%
