



## Original article

Antifungal potential, chemical composition of *Chlorella vulgaris* and SEM analysis of morphological changes in *Fusarium oxysporum*Kahkashan Perveen<sup>a,\*</sup>, Najat A. Bukhari<sup>a</sup>, Luluah M. Al Masoudi<sup>b</sup>, Amara Naser Alqahtani<sup>a,c</sup>, Mashael W. Alruways<sup>d</sup>, Fatimah S. Alkhattaf<sup>a</sup><sup>a</sup> Department of Botany & Microbiology, College of Science, King Saud University, Riyadh 11495, Saudi Arabia<sup>b</sup> Department of Biology, Faculty of Science, Taif University, Saudi Arabia<sup>c</sup> Biological Sciences Department, Faculty of Science, University of Jeddah, Saudi Arabia<sup>d</sup> Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Shaqra, Saudi Arabia

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## ABSTRACT

In pursuit of an environmentally benign fungicide alternative, the current study explored the antifungal activity of *Chlorella vulgaris* extracts against six plant pathogenic fungi (*in vitro*). The well diffusion agar method was used to investigate the growth inhibition of *Fusarium oxysporum*, *Fusarium sp.*, *Fusarium solani*, *A. flavus*, *A. niger*, and *A. alternata* using the three *C. vulgaris* extracts viz. methanol (CvME), acetone (CvAE), and diethyl ether (CvDE). Different concentrations of CvDE were also investigated against *F. oxysporum*. The morphological modifications in *F. oxysporum* treated with CvDE (5 mg/kg) were studied using SEM and the chemical composition of CvDE was also determined by GC-MS analysis. All extracts, with the exception of *A. alternata*, were found to be effective in inhibiting the growth of plant pathogenic fungi. The CvDE extract, followed by CvME and CvAE, was found to be efficient against tested fungi. The CvDE was most effective against *F. oxysporum* with a 73.3% growth inhibition. The effects of various CvDE concentrations on *F. oxysporum* were found to be dosage dependent. The SEM micrograph revealed that CvDE-treated *F. oxysporum* had substantially less conidia than the control. The CvDE treatment damaged the mycelial structure as well. Major chemical components detected in CvDE were Heptaldehyde (15.7%), Octadecenoic acid, methyl ester (12.6%), Hexadecanoic acid (12%), 3-Decyn-2-ol (10.98%), (E)-3,7,11,15-tetramethylhexadec-2-ene (9.76%), heptadecane-1,2,3,4,5-pentol (8.7%), Docosane, 4-methyl (7.28%).

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## 1. Introduction

Microalgae are widely distributed and can cultivate practically any environment. A few of them are well-known for producing bioactive chemicals that have various applications, including in drugs and medicine, bioremediation, and the food industry (Ranglová et al., 2021; Santhosh et al., 2016; Vaz et al., 2016). How-

**Abbreviations:** DMSO, Dimethyl sulfoxide (); CvME, *C. vulgaris* methanol extract; CvAE, *C. vulgaris* acetone extract; CvDE, *C. vulgaris* diethyl ether extract; SEM, Scanning Electron Microscopy; GC/MS, Gas Chromatography/Mass Spectrometry.

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ever, a large number of bioactive compounds remain in these microalgae that haven't been identified yet. Microalgae have been actively investigated in recent decades for new chemicals that could be used as medicinal agents, antimicrobial, antiprotozoal, and antiplasmodial agents (Balaji et al., 2017; Dinev et al., 2021; Shaima et al., 2022). *Chlorella* is a green alga that is widely utilized in pharmaceuticals and cosmetics, as well as a food supplement due to its high nutritional content. Protein, vitamins, lipids, polysaccharides, minerals, carotenoids and immune stimulator chemicals are the primary components of *Chlorella* (Vaz et al., 2016). *Chlorella* extracts have been shown to have antimicrobial effects. This genus has been linked to the antibiotic Chlorellin (Pratt et al., 1944).

Plant pathogenic fungi cause numerous diseases to plants, causing yield as well as economic losses (Fletcher et al., 2006). Species of *Fusarium*, *Aspergillus*, and *Alternaria* cause various plant diseases such as blight, fruit rot, root crown rot, and wilt. The genus *Fusarium* is among the most economically important genera of

fungi in the world. Its species such as *Fusarium oxysporum*, *F. solani*, *F. equiseti*, *F. chlamydosporum* etc. cause root and stem rot, vascular wilt, and/or fruit rot in a number of economically crop species (Dean et al., 2012). The members of the genus *Alternaria* causes many pre and postharvest diseases and losses to plants, fruits and vegetables (Roy et al., 2019). *Alternaria alternata* infect more than 400 plant species with leaf spots, rots and blights. The genus *Aspergillus* cause plant and fruit rot with production of mycotoxins such as aflatoxin that contaminate several oil bearing seeds (Perrone et al., 2007; Wilson and Payne, 1994).

Chemical fungicides are the most effective strategy at the moment to treat fungal diseases, albeit they are not environmentally friendly. Consequently, natural chemicals that can substitute these hazardous fungicides are in high demand. Several types of algae have been reported to inhibit soil-borne fungus, including *Anabaena* spp., *Scytonema* spp., and *Nostoc* spp.. The mycelial growth of plant pathogenic fungus was entirely suppressed by extracts from certain algae (Galal et al., 2011; Kulik, 1995).

*C. vulgaris* extracts were tested against a variety of key plant pathogenic fungi in this investigation, and the fungus that was found to be most susceptible to the extract was examined under a scanning electron microscope for morphological changes, as well as the chemical constituents of *C. vulgaris* extract was examined using GC–MS.

## 2. Materials and methods

### 2.1. Cultivation of *Chlorella vulgaris*

*C. vulgaris* was cultivated for 4 weeks at 25 °C under constant light on BG 11 nutrition media. Filtration through Whatman no. 1 filter paper yielded the active cells. The harvested biomass was utilized to make crude extracts with various solvents (Perveen and Alwathnani, 2013).

### 2.2. Preparation of the extracts

Crude extracts of *C. vulgaris* were prepared using the solvents methanol, acetone, and diethyl ether. In the conical flask, five grams of *C. vulgaris* biomass and 100 mL of the aforementioned solvents were mixed separately and placed at 20 °C for three days on an incubator shaker. Whatman no. 1 filter paper was used to filter the mixture. The resultant filtrate was dried thoroughly in a hot air oven at 40 °C. The dried residue was dissolved in 10% dimethyl sulfoxide (DMSO) to make a stock solution (@ 5 mg/ml).

### 2.3. Plant pathogenic fungi

The plant pathogenic fungi; *F. oxysporum* (MT151384), *Fusarium* sp. (ITCC 8189.11), *F. solani* (MW265001), *A. flavus* (Identified at the Department of Botany and Microbiology), *A. niger* (Department of the Botany and Microbiology), and *A. alternata* (ITCC 7912.10) were procured from the Department of Botany and Microbiology. The fungal cultures were stored at 4 °C on Potato Dextrose Agar (PDA) slants. Prior to the antifungal assay the fungal cultures were grown on the PDA plates at 27 °C for five days.

### 2.4. Antifungal assay

By using the well diffusion method, the antifungal activity of *C. vulgaris* extracts such as methanol (CvME), acetone (CvAE), and diethyl ether (CvDE) against six fungi (*F. oxysporum*, *Fusarium* sp., *F. solani*, *A. flavus*, *A. niger*, and *A. alternata*) was evaluated (Balouiri et al., 2016). A well was made 10 mm inside the plate's edge, and a 100 µl aliquot of the *C. vulgaris* extract was transferred into

it, a disc (5 mm) of actively growing fungi (5 days old culture) was placed and it kept on the opposite side of the well. In the control treatment, instead of extracts, the well was filled with 10% DMSO. Fungal growth was measured after 5 days of incubation at 25 °C. Fungal growth inhibition was determined with the help of following formula

$$GI(\%) = \frac{GC - GT}{GC} \times 100$$

Where, I = percent growth inhibition, GC = growth of fungi in control plate, GT = growth of fungi in treated plate.

### 2.5. Effect of different concentration of CvDE on *F. oxysporum*

The influence of various concentrations of most potential extracts (CvDE) against the most susceptible fungus (*F. oxysporum*) was also evaluated in a similar way as mentioned in the antifungal assay. Seven different concentrations of the CvDE were prepared by double dilution in 10% DMSO (5 mg/ml–0.0785 mg/ml) and different concentrations of the extract were poured into wells.

### 2.6. Observation of morphological changes in *F. oxysporum* by CvDE

Scanning electron microscopy (SEM) was utilized to examine the morphological changes in the fungus (*F. oxysporum*) treated with CvDE (5 mg/ml). The samples were taken from the margin of the inhibition area adjacent to the well of CvDE or control. The samples for SEM observation were prepared according to the method described earlier (Alwathnani and Perveen, 2017). The samples were first fixed for two hours at room temperature with 3% glutaraldehyde. After washing twice with 0.1 M phosphate buffer, the samples were fixed in osmium tetroxide (1%) for 2 h at room temperature. Next step was dehydration by transferring samples through a graded series ethanol (10 to 100%) for 15 min each. The samples were critical point dried with CO<sub>2</sub> in a drying apparatus (EMS 850, USA). With the help of Sputter Coater, samples were coated with gold. A scanning electron microscope (JEOL, JSM-5500LV) at an accelerating voltage of between 18 and 20 kV was used to observe the prepared samples.

### 2.7. GC–MS analysis of *Chlorella vulgaris* extract

In order to find the chemical components of *C. vulgaris*, the extract that yielded promising results was subjected to GC–MS analysis. The methodology by Perveen and Alwathnani (2013) was employed. Gas chromatography (Perkin Elmer) was utilized with a mass spectrometer. It was equipped with an RTX-DB5 column with a dimension of 30 × 0.32 mm for GCMS analysis. The oven temperature was set at 75 °C for 2 min, then increased to 175 °C at a rate of 50 °C/min for 7 min. Helium (3 mL/min) was used as the carrier gas. The NIST database was used for chromatographic analysis and interpretation of the resulting mass spectra.

### 2.8. Statistical analysis

For each treatment there were four replicates. The gathered data was statistically analyzed using ANOVA and Tukey (HSD) post hoc through XLSTAT (version. 2021.3.1.1162).

## 3. Results

The extracts of *C. vulgaris* prepared using solvents such as methanol (CvME), acetone (CvAE), and diethyl ether (CvDE) were tested against *F. oxysporum*, *Fusarium* sp., *F. solani*, *A. flavus*, *A. niger*, and *A. alternata*. Well diffusion assay was employed to assess the

inhibition potential of the extracts. The outcomes are depicted in Fig. 1. It reveals that all of the extracts had different impacts on the fungus that were examined. CvDE was significantly effective in inhibiting the growth of all fungi tested except *A. alternata*. CvDE inhibited *F. oxysporum*, *Fusarium* sp., *F. solani*, *A. flavus*, and *A. niger* to a high degree with percent inhibition of 73.3%, 63.3%, 60.0%, 56.75%, and 50.0% respectively (Fig. 1). CvME, on the other hand, was proven to be effective against *F. oxysporum* (66.7%), *Fusarium* sp. (60.0%), *A. flavus* (53.3%), and *A. niger* (54.3%). Whereas, CvAE was discovered to be efficient in hindering the growth of *Fusarium* sp. (54.3%), *F. oxysporum* (41.0%), *A. flavus* (33.3%), and *A. niger* (32.3%). The positive control, carbendazim (2%) prevented the growth of all fungus completely.

Data is mean ± Sd for n = 4. Bars showing different letters are significantly different (P ≤ 0.05) according to Tukey's HSD tests.

Among all extracts, CvDE was most effective and caused maximum growth inhibition of *F. oxysporum* (73.3%). Therefore, different concentrations of CvDE were evaluated against *F. oxysporum*. The findings revealed that the inhibition of fungal growth was concentration dependent; it ranged between 74% and 16.7%. The minimum reduction in the growth of fungus by CvDE, was 0.156 mg/ml. While a concentration of 0.078 mg/ml was failed to prevent the fungus from growing (Fig. 2).

The morphological changes in *F. oxysporum* treated with CvDE were observed under SEM (Fig. 3). Variation in the number of conidia was the apparent difference observed in the *F. oxysporum* treated with CvDE (Fig. 3 b,c) and untreated control (Fig. 3a). In the control sample, the micro and macroconidia of *F. oxysporum* were observed in abundance while they were sparse in the treated sample. The difference in the morphology of the mycelium between *F. oxysporum* treated with CvDE and untreated control was also visible. The mycelium of the treated fungus was slender, distorted, and rough as compared to the control where mycelium was well formed and smooth (Fig. 3a).

The chemical constituents of CvDE was identified by GCMS analysis, and the results are reported in Table 1. Fatty acids and esters were discovered to be abundant in the extract. A substantial amount of various alcohols were also found. Investigation of CvDE for chemical composition, identified 14 components. Major components were Heptaldehyde (15.7%), Octadecenoic acid, methyl ester (12.6%), Hexadecanoic acid (12%), 3-Decyn-2-ol (10.9%), (E)-3,7,11,15-tetramethylhexadec-2-ene (9.7%), heptadecane-1,2,3,4,5-pentol (8.7%), Docosane, 4-methyl (7.2%).

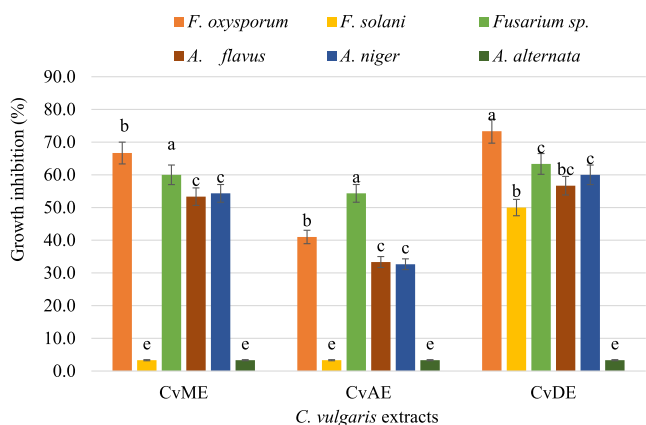


Fig. 1. Percent growth inhibition of *F. oxysporum*, *Fusarium* sp., *F. solani*, *A. flavus*, *A. niger*, and *A. alternata* by the methanol (CvME), acetone (CvAE) and diethyl ether (CvDE) extracts of *C. vulgaris*.

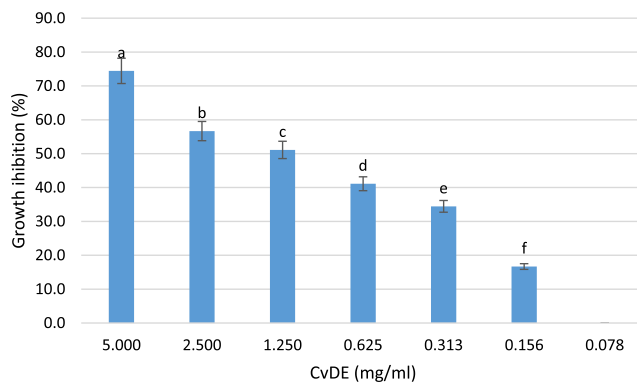


Fig. 2. Percent growth inhibition of *F. oxysporum* by different concentration of diethyl ether extract of *C. vulgaris* (CvDE). Data is mean ± Sd for n = 4. Bars showing different letters are significantly different (P ≤ 0.05) according to Tukey's HSD tests.

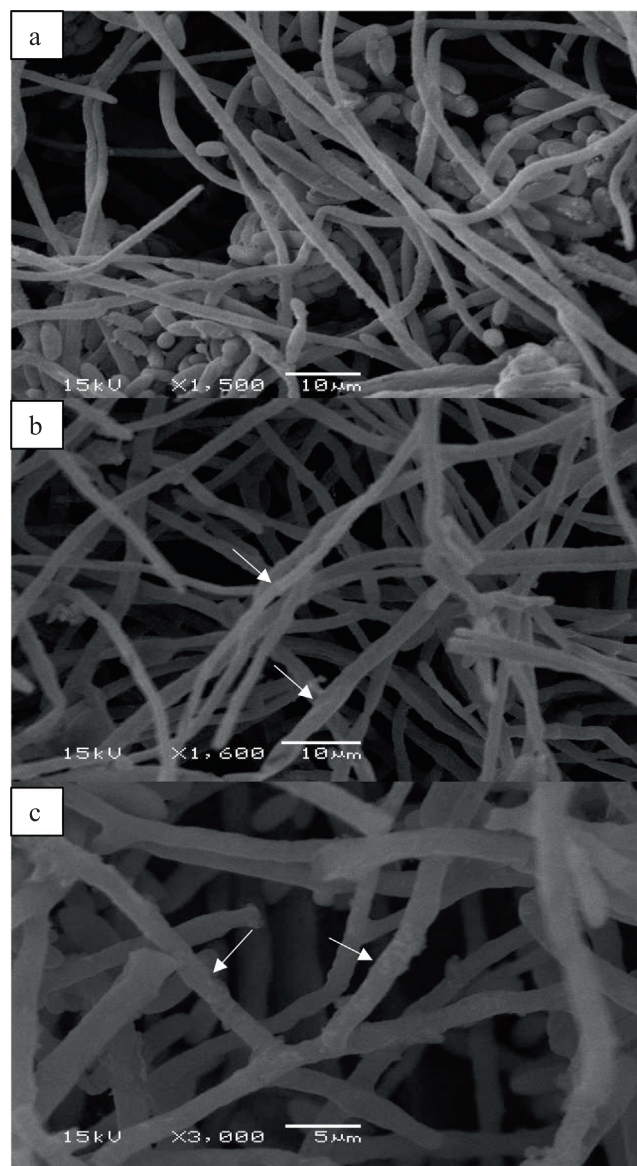


Fig. 3. Morphological changes in *F. oxysporum*. The untreated control has a large number of conidia and smooth mycelium (a). The treated with (5 mg/ml) of CvDE has sparse conidia and deformed mycelium (b, c).

**Table 1**  
Chemical composition of *C. vulgaris* extract (CvDE) analyzed by GCMS.

S. No.	Compound	Formula	Area (%)
1	3-methylbutan-2-ol	C <sub>5</sub> H <sub>12</sub> O	1.1
2	(S)-(+)-2-Hexanol	C <sub>6</sub> H <sub>14</sub> O	1.6
3	Heptaldehyde	C <sub>7</sub> H <sub>14</sub> O	15.7
4	N-Isopentylacetamide	C <sub>7</sub> H <sub>15</sub> NO	1.1
5	3-Decyn-2-ol	C <sub>10</sub> H <sub>18</sub> O	10.98
6	2-isopropyl-5-methylhexan-1-ol	C <sub>10</sub> H <sub>22</sub> O	3.4
7	5,9-Dodecadien-2-one, 6,10-dimethyl-	C <sub>14</sub> H <sub>24</sub> O	4.7
8	Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	12.0
9	Octadecenoic acid, methyl ester	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	12.6
10	(E)-3,7,11,15-tetramethylhexadec-2-ene	C <sub>20</sub> H <sub>40</sub> O	9.76
11	Heptadecane-1,2,3,4,5-pentol	C <sub>17</sub> H <sub>36</sub> O <sub>5</sub>	8.7
12	Docosane, 4-methyl	C <sub>23</sub> H <sub>48</sub>	7.28
13	propyl (9Z,12Z)-octadeca-9,12-dienoate	C <sub>21</sub> H <sub>38</sub> O <sub>2</sub>	3.7
14	(3Z)-3-hexadec-15-ynylidene-4-hydroxy-5-methylxolan-2-one	C <sub>21</sub> H <sub>34</sub> O <sub>3</sub>	6.2
Total			98.82

#### 4. Discussion

Micro-algae produce a variety of metabolites to protect themselves from adverse conditions. These metabolites include polysaccharides, amino acids, enzymes, antibiotics, and toxic substances. Antimicrobial properties have been observed in several of these compounds. Present results show that the extracts of *C. vulgaris* have antifungal properties. The current findings corroborate previous observations made by a number of researchers. Extract of *C. vulgaris* reduced the growth of plant pathogenic fungi such as *A. niger*, *A. alternata*, and *P. expansum* (Vehapi et al., 2020). A study discovered that a methanol extract of *C. vulgaris* inhibited significantly the growth of human pathogenic fungi (Ghasemi et al., 2003). While, another study reported that among four microalgae evaluated for antimicrobial activity, *C. vulgaris* was superior in inhibiting the growth of bacteria and pathogenic fungi than other tested microalgae (Ahmed, 2016). The current study's findings prove unequivocally that the *C. vulgaris* extracts were more effective against species of *Fusarium* than against other species. Whereas, none of the extracts could inhibit the growth of *A. alternata*. The phylogenetic differences between the fungal species could explain this variation (Philip et al., 2009). The fact that antifungal activity varies depending on the extract reveals that extract bioactivity is solvent dependent (Mian et al., 2003; Salvador et al., 2007; Stramarkou et al., 2017). A growth inhibition of 53.8% in *R. solani* by 5% methanolic extract of *C. vulgaris* has been recorded (Al-Nazwani et al., 2021). Further, they have recorded that the fungal inhibition was concentration dependent. Present study noticed that the maximum zone of inhibition by *C. vulgaris* extracts was against *F. oxysporum*. A report has demonstrated that the lowest concentration (1 gL<sup>-1</sup>) of the extract of *C. vulgaris* could inhibit 40% growth of *F. oxysporum* (Ferreira et al., 2021). Earlier researchers have also reported a reduction in *F. oxysporum* growth due to an extract of *C. vulgaris* (Vehapi et al., 2020, 2018).

SEM analysis showed morphological changes in the *F. oxysporum* treated with CvDE. The bioactive compounds could be responsible for the morphological changes in the fungus. Recently, the morphological changes and a reduction in *R. solani* spore production due to *C. vulgaris* methanolic extract has been reported. Fatty acids with C10 or more have been shown to trigger protoplast lysis (Al-Nazwani et al., 2021). Damage to the cell wall, breakdown of the cellular membrane or its proteins, which could lead to leaking of cell contents and cytoplasm coagulation (Jyotirmayee et al., 2014). Present study show the presence of several such fatty acids. Various studies have reported the potential of algal and cyanobac-

teria extracts in inhibiting the plant pathogenic fungi as well as controlling plant diseases caused by *Fusarium* spp. (Al-Nazwani et al., 2021; Kim, 2006; Kim and Kim, 2008; Omar, 2012; Vehapi et al., 2020, 2018). However, no one has observed morphological changes in *F. oxysporum* as a result of *C. vulgaris* extracts.

Fatty acids, esters, and alcohols were detected in CvDE when analyzed by GCMS. Previous studies have recorded the presence of many of these compounds in the extracts of *Chlorella* sp. (Al-Wathnani et al., 2012; Alwathnani and Perveen, 2017; Pantami et al., 2020). A study identified the chemical content of methanol extract of *C. vulgaris* by GC-MS probe. It demonstrated the existence of n-hexadecanoic acid, eicosanoic acid, octadecenoic acid, oleic acid, and pentadecanone, among other fatty acids. Several fatty acids, methyl esters, and carotenoids were identified in *C. vulgaris* extracts after a detailed analysis (Al-Nazwani et al., 2021). Compounds from various chemical families have been associated with microalgae's antimicrobial activity. Chlorellin is a combination of fatty acids that is a known antibiotic extracted from *Chlorella* (Pratt et al., 1944). The antimicrobial activity of bioactive chemicals found in the present extract, such as 3-Decyn-2-ol, heptanal, hexadecanoic acid, and heptadecane-1,2,3,4,5-pentol, has previously been reported (Costa et al., 2019; Dinev et al., 2021; Shaima et al., 2022). In the present study, these chemicals may have aided in the suppression of fungal growth.

#### 5. Conclusions

The results of this investigation clearly demonstrated that plant pathogenic fungi could be inhibited by the extracts of *C. vulgaris*. The damaging potential of the CvDE, as revealed by SEM analysis of CvDE treated *F. oxysporum*, and the presence of bioactive compounds in the extract of *C. vulgaris*, proves its potential as an effective component for the control of plant pathogenic fungi. Since the plant pathogenic *F. oxysporum* is difficult to control, the findings of this study will aid in the development of strategies for managing the pathogen in an environmentally friendly manner.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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