Alternative control of early blight of tomato using plant extracts from *Acacia nilotica*, *Achillea fragrantissima* and *Calotropis procera*

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**Summary.** The *in vitro* and *in vivo* antifungal potential of extracts of three wild medicinal plants, (*Acacia nilotica* (L.) Delile, *Achillea fragrantissima* (Forssk.) Sch.Bip. and *Calotropis procera* (Aiton) W. T. Aiton) was examined against *Alternaria solani*, the causal agent of the early blight of tomato. *Aqueous or ethanol extracts of all tested plants reduced the mycelial growth and conidium germination of *A. solani in vitro*. Ethanol extracts were more effective against the pathogen than the aqueous extracts. Extract of *C. procera* exhibited more antifungal potential against the pathogen than other plant extracts. Observations by scanning and transmission electron microscopy showed dramatic alterations in the morphology and ultrastructure of *A. solani* when treated with the ethanol extract of *C. procera* at a concentration of 20%. Phytochemical screening confirmed the presence of many bioactive constituents in the extracts which were in greater amounts in *C. procera* than the other two plants. In a plot experiment, both types of extracts from *C. procera* reduced disease severity. Tomato fruit yield was increased after the treatment with the plant extracts.

**Key words:** *Alternaria solani*, antifungal activity, electron microscopy.

**Introduction**

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetables in the world, including Egypt. Early blight, caused by *Alternaria solani* (Ell. and Mart.) Jones and Grout., is one of the common foliar diseases which results in yield losses up to 70% (Glala et al., 2005). Control of this disease is heavily reliant on multiple applications of chemical fungicides during flowering and fruiting (Akila et al., 2012). Furthermore, the use of synthetic pesticides to control fungal plant diseases of food commodities is restricted by their possible carcinogenicity, high and acute toxicity, long degradation periods, and potential environmental pollution.

The use of compounds in plant extracts may be an alternative to currently use chemical fungicides for controlling phytopathogenic fungi, because they constitute a rich source of bioactive chemicals such as phenols, flavonoids, quinones, tannins, alkaloids, saponins and sterols (Isman, 2000). Since these extracts can be active against fungal phytopathogens, and are biodegradable to nontoxic products, they are potentially suitable for use in integrated pest management programmes (Al-Askar and Rashad, 2010; Al-Askar et al., 2014; Khafari et al., 2014).

Egypt has about 384 different species of medicinal plants found in the Mediterranean coastal region, deserts, oases and Sinai Peninsula. The Egyptian flora is rich in its diversity, with hundreds of plants used in traditional medicine since antiquity, when the ancient Egyptians recorded thousands of plants and their uses in the Ebers papyrus (Saleh, 2003; Shafik and Elseesy, 2003).

*Acacia nilotica* (L.) Delile, (Egyptian thorn; gum Arabic tree), is a multipurpose tree legume. It is widespread in subtropical and tropical Africa and in
Asia. The plant is traditionally and widely used for the treatment of various ailments. Satish et al. (2007) recorded antifungal activities of methanol and aqueous extracts of *A. nilotica* against important seedborne *Aspergillus* sp. *Achillea fragrantissima* (Forssk.) Sch.Bip. (Lavender cotton; Qaysūm) is a medicinal plant that has been used in folk medicine and for pharmaceutical purposes since ancient times. Alsohaili and Al-fawwaz (2014) recorded varied antifungal activities of *A. fragrantissima* essential oil against three food spoilage fungi (i.e. *A. nigar*, *Penicillium* sp. and *Rhizopus* sp.). *Calotropis procera* (Aiton) W. T. Aiton (milk weed; osher), is a glabrous laticiferous shrub or small tree, which has been used in Egypt as a folk medicine (Shafik and Elseesy, 2003). Leaves, flowers and methanol extracts from latex of *C. procera* showed fungicidal activities against *Candida albicans*, *C. tropicalis*, *P. chrysogenum* and *Saccharomyces cerevisiae* (Nenaah and Ahmed, 2011).

The search for plant extracts from medicinal plants to control plant pathogens has increased during the last few years, and efficacy of plant extracts against *A. solani* has been demonstrated in many countries (Suleiman, 2010; Sallam, 2011; Sallam and Abo-Elyoussr, 2012; Maya and Thippanna, 2013; Ravikumar and Garampalli, 2013; Khafari et al., 2014).

The aim of present study was to evaluate aqueous and ethanol extracts from three Egyptian wild medicinal plants (*A. nilotica*, *A. fragrantissima* and *C. procera*) collected from the Sinai Peninsula, for the management of *A. solani*, the causal agent of the early blight of tomato.

**Materials and methods**

**The pathogen**

*Alternaria solani* was isolated from naturally infected tomato leaves showing the early blight symptoms. The pathogen was isolated using potato dextrose agar (PDA) and V8 media, and identified in the Botany Department, Faculty of Science, Damietta University, Egypt.

**Plant materials and extract preparation**

Three wild medicinal plants (*A. nilotica*, *A. fragrantissima* and *C. procera*) were collected from different locations in the Sinai Peninsula, Egypt. Leaves were extracted using water or ethanol. The methods of extraction were applied according to Rivillas-Acevedo and Soriano-García (2007). For aqueous extraction; 100 g of each air-dried sample of plant material was added to 1 L of distilled water, and extracted under cold conditions for 24 h. Plant extracts were filtered and this concentration (10%) was used as a crude aqueous extract. For ethanol extraction, a prepared fine powder (40 g) of dried plant material was macerated with 200 mL of ethanol (80%) for 24 h in a 500 mL capacity flask, and treated in an ultrasonic cleaner for 10 min. The mixtures were then centrifuged at 4000 g for 20 min. The supernatant was evaporated under reduced pressure at a temperature below 45°C. The residues were dissolved in 40 mL ethanol (50%) as original extracts and stored at 5°C in darkness.

**Effects of plant extracts on linear mycelial growth**

Crude leaf extracts of the three medicinal plants were tested *in vitro* for efficacy against *A. solani* mycelial growth using agar plate assay (Schmitz, 1930). The plant extracts were added to conical flasks containing sterilized PDA before solidification to obtain the final concentrations of 2.5, 5, 10 and 20% (v/v). After solidification, the plates were each inoculated with a 6 mm agar piece containing *A. solani* mycelia. For each crude leaf extract, the experiments were performed in three replicates. Plates containing mancozeb (1 mL at of concentration of 0.2%) and sterile distilled water were used, respectively, as positive and negative controls. The inoculated plates were incubated at 25°C, and the diameters of fungal colonies were measured each day for 5 d. The antifungal activity of the extracts was measured as the linear mycelial growth of the colonies of the pathogenic fungus.

**Effects of plant extracts on conidium germination**

Antifungal activity of aqueous and ethanol extracts on conidium germination of *A. solani* was tested using a microscope slide technique (Nair and Llingboe, 1962). The plant extracts (at concentrations of 2.5, 5, 10 and 20%) were added as films to dried clean slides. Then, conidia suspensions (0.1 mL containing 5 × 10^6 conidia) was spreaded over these films. Control treatments were prepared as films with sterilized distilled water. Three slides were used as replicates for each concentration. Each slide was placed on a glass rod in a Petri dish under moist conditions and incubated for 24 h at 25°C. Four microscopic slides (at 400x magnifica-
tion) were used for each slide. Mancozeb (commercial fungicide at 0.2%) and sterile distilled water were used, respectively, as positive and negative controls. The percentage of germinated conidia was calculated.

**Electron microscopy**

Because the ethanol extract of *C. procera* was the most effective treatment for reduction of mycelial growth of *A. solani*, a culture of the pathogen, of the same age as used in the control and extract treatments (20%) was prepared for Scanning (SEM) and transmission (TEM) electron microscopy. For the SEM preparation, mycelial plugs (1 × 1 × 3 mm) were fixed in phosphate-buffered 3% glutaraldehyde at pH 6.8, and then dehydrated in a crescent acetone series (30, 50, 70, 85, 95 and 100%). The samples were then critical point dried (Polaron CPD 7501, UK). The specimens were sputter-coated with plutonium using a JFC-1600 autofine coater (Polaron SC7620). Fungal hyphae were examined using a Joel SEM-6400JSM-6360LV microscope (JEOL Ltd., Japan). For TEM preparation, samples were fixed in phosphate-buffered 3% glutaraldehyde at pH 6.8, post-fixed in phosphate-buffered 1% Osmium tetroxide and then dehydrated in a crescent ethanol series following the procedure described by Alberto et al. (1997). Mycelial plugs were embedded in plastic epoxy resin. Ultra-thin sections were cut with a Reichert ultramicrotome, stained with uranyl acetate followed by lead citrate, and examined with JEOL model JEM-1230 TEM.

**Determination of Minimum Inhibitory Concentration (MIC)**

Different concentrations (0.01, 0.02, 0.03, 0.06 and 0.08 μg mL⁻¹) of the extracts were prepared. Each of these was added to 18 mL of potato dextrose broth in test tubes. Each tube was then inoculated with 0.1 mL of conidium suspension of *A. solani* diluted to give a final conidium suspension of 10⁶ mL⁻¹. The tubes were incubated at 28 ± 2°C and examined for growth after 7 d. The least concentration of the plant extracts that permitted visible growth of the inoculated test organism in the broth medium was regarded as the MIC in each case. Control experiments were performed without the plant extracts.

**Qualitative analysis of phytochemical constituents**

Qualitative studies for tannins, saponins, flavonoids, cardiac glycosides, alkaloids and phenols were carried out according to Mir et al. (2013). Based on the intensity of colouration or the precipitates formed during the test, bioactive constituents were characterized as strongly present (+++), present (++), weakly present (+), and absent (-) when the test result was negative.

**Field experiments**

Egyptian tomato seeds (type Heirloom), used in this study were obtained from the Ministry of Agriculture, Egypt. The field trials were conducted twice over two growing seasons (2013 and 2014) at the Experimental Farm of the Faculty of Science, Damietta University, Damietta, Egypt. Field plots (3 × 3.5 m) each comprised two rows and five plants per row, and were arranged in a completely randomised block design. Three plots were used as replications for each treatment. Fifty-d-old plants were separately sprayed four times at 15 d intervals with the aqueous or ethanol plant extracts (30 mL at 20% concentration). Three other plots were sprayed with the chemical fungicide (Mancozeb, 30 mL, 0.2% concentration) as positive controls. Another three plots were sprayed with water to serve as negative controls. Two days after the second spraying, tomato seedlings were inoculated with a conidium suspension of *A. solani* (20 mL at concentration of 5 × 10⁶ conidia mL⁻¹). All plants were sprayed until run-off.

**Disease severity**

Disease severity was estimated 7 d after the final spray. Ten plants were taken from each treatment, and leaves of each plant were used to determine the disease severity. Infected leaves were classified into five categories (0, 1, 2, 3 and 4) according to the blighted area of leaves (Cohen et al., 1991), i.e. 0 = no infected leaves, 1 = 25% or less, 2 = 26–50%, 3 = 51–75%, or 4 = 76–100% leaf area affected. Disease severity percentage was calculated according to the following formula:

\[
\text{Disease severity (\%)} = \frac{\Sigma (n \times r_i) - (n \times r_4)}{4N} \times 100
\]

Where \( n = \text{Number of infected leaves}, \ r_1 - r_4 = \text{disease severity category and} \ N = \text{Total number of examined leaves.}
Determination of fruit yield

After harvest, total weight of fruit per plant (g) following the different treatments of aqueous and ethanol plant extract applications were determined.

Statistical analyses

Data were analyzed with the statistical analysis software CoStat (version 6.4), for one way completely randomized block design. Duncan’s multiple range test was used to compare means at a probability (P) level of ≤ 0.01.

Results

Effect of plant extracts on mycelial growth of Alternaria solani

The effects of aqueous and ethanol extracts of C. procera, A. nilotica and A. fragrantissima in vitro tests on linear mycelial growth of A. solani is shown in Table 1. All of the treatments reduced linear mycelial growth of the pathogen compared with the experimental controls. The proportional reductions of mycelial growth increased with increasing concentrations of the extracts from 2.5 to 20%. Greater inhibition of growth was obtained with the ethanol extracts than the aqueous extracts. At concentration of 20%, ethanol extracts from C. procera, A. nilotica and A. fragrantissima gave, respectively, mean mycelial growth of 17.3, 18 and 21.3 mm. Aqueous extracts from the plants gave, respectively, mean mycelial growth of 27.3, 28 and 31.7 mm.

Effects of plant extracts on conidium germination

All treatments significantly decreased the conidium germination (%) of A. solani compared with the control (Table 2). Ethanol extracts were more effective against reducing the conidium germination of the pathogen than aqueous extracts. The values of conidium germination were decreased with increasing the concentrations of extracts from 2.5 to 20%. The greatest reduction of germination (%) with aqueous or ethanol extracts was for those from C. procera followed by A. nilotica and A. fragrantissima. At concentration of 20% water extract, mean conidium germination was, respectively for extracts from the three plants, 13.5, 15.4, and 18.5%. For the ethanol extracts, germination was less, at means, respectively, of 11.1, 13.2 and 16.2 %.

Electron microscopy

SEM revealed that the hyphae of the pathogen exposed to plant extract were collapsed and curling, and short branching, whereas no such changes were noticed in the mycelia developed in the control treatment (Figure 1a,b). TEM observations revealed that the sub-cellular components of the pathogen in the control treatment were enclosed by a distinct electron-dense wall and a normal plasma lemma. Many

Table 1. Mean linear mycelial growth (mm) of Alternaria solani after 7 d incubation at 25°C, when exposed to aqueous (A) or ethanol (E) extracts from three medicinal plants at different concentrations.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Plant extracts concentration (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>E</td>
<td>A</td>
<td>E</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>66.3 a</td>
<td>66.3 a</td>
<td>66.3 a</td>
<td>66.3 a</td>
<td>66.3 a</td>
<td>66.3 a</td>
</tr>
<tr>
<td>Mancozeb (0.2%)</td>
<td></td>
<td>15.6 e</td>
<td>15.6 d</td>
<td>15.6 d</td>
<td>15.6 d</td>
<td>15.6 d</td>
<td>15.6 d</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td></td>
<td>36.3 d</td>
<td>24.0 c</td>
<td>33.2 c</td>
<td>22.2 c</td>
<td>29.0 c</td>
<td>19.0 c</td>
</tr>
<tr>
<td>Acacia nilotica</td>
<td></td>
<td>39.2 c</td>
<td>25.1 c</td>
<td>34.7 bc</td>
<td>22.3 c</td>
<td>31.0 c</td>
<td>20.3 c</td>
</tr>
<tr>
<td>Achillea fragrantissima</td>
<td></td>
<td>41.7 b</td>
<td>28.5 b</td>
<td>37.3 b</td>
<td>26.6 b</td>
<td>33.7 b</td>
<td>24.3 b</td>
</tr>
</tbody>
</table>

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter(s) are not significantly different according to Duncan’s multiple range test (P=0.01).
Alternative control of early blight of tomato

Organelles are observed in each cell, such as a nucleus surrounded by a double membrane and an endoplasmic reticulum (ER) associated with the nucleus, vacuoles, lipid droplets, Golgi bodies and mitochondria (Figure 2a). When the extract of C. procera was added to the growth medium of the pathogen, many ultrastructural changes of the mycelium occurred. These included electron-transparency of the cell walls, degeneration of cytoplasm and an increase in numbers of lipid droplets and vacuoles (Figure 2b).

Minimum Inhibitory Concentration (MIC)

MICs of ethanol plant extracts from the tested plants were less than those of aqueous extracts (Figure 1).
ure 3). MICs for the ethanol extracts from the three different plants were, respectively, about 6, 8, and 10 mg mL$^{-1}$ for *C. procera*, *A. nilotica* and *A. fragrantissima*. MICs of aqueous extracts were, respectively, about 11, 12, and 16 mg mL$^{-1}$ (Figure 3).

**Phytochemical constituents of plant extracts**

Phytochemical screening for aqueous leaf extracts from the tested plants revealed the presence of flavonoids, saponins, tannins, alkaloids, cardiac glycosides and phenol. The ethanol leaf extracts contained also these phytochemicals, except that saponins were not detected in the extract from *C. procera* (Table 3). Flavonoids, alkaloids, and cardiac glycosides were present in high amounts in the extracts from *C. procera* compared with the other plants.

**Effects of plant extracts on disease severity in the field**

Early blight severities (%) on tomato plants in the field, when treated with the tested plant extracts are shown in Table 4. All of the treatments reduced the disease severity compared with the control treatment. Mean early blight severity ranged from 13.2–23.7% for the aqueous plant extract treatments and 10.5–20.6% for the ethanol extracts, while in the control treatment gave mean severity of 53.2%. The aqueous and ethanol extracts of *C. procera* were the most effective against early blight, followed by the extracts from *A. nilotica* and *A. fragrantissima*. Aqueous extracts from the three plants, respectively, reduced the disease severity by 75, 70, and 56%, and equivalent reductions for the ethanol extracts were 80, 70, and 60%.

**Effects of plant extracts on fruit yield**

The effects of extracts from the three medicinal plants on fruit yield of tomato are shown in Table 5. Mean tomato fruit yields ranged from 374 to 481 g from the aqueous plant extract treatments, and 391 to 496 g from the ethanol extracts, compared with 258 g for the untreated plants. The aqueous and ethanol extracts plants increased fruit yield per plant compared with the control. The greatest yields were obtained with extracts from *C. procera* followed by the extracts from *A. nilotica* and *A. fragrantissima*. Aqueous extracts from the three plants, respectively, increased fruit yield per plant about 86, 67 and 45%, while the ethanol extracts increased the yield per plant about 92, 77 and 54%. The yields followed a

| Table 3. Qualitative analysis of phytochemical constituents of aqueous (A) or ethanol (E) extracts from leaves of three medicinal plants. |
| Phytochemical constituents | Plant extract | Calotropis procera | Acacia nilotica | Achillea fragrantissima |
| Flavonoids | A | +++* | ++ | + |
| | E | + | + | + |
| Saponins | A | + | + | + |
| | E | - | + | + |
| Tannins | A | + | + | + |
| Alkaloids | A | +++ | ++ | + |
| | E | ++ | + | + |
| Cardiac glycosides | A | +++ | ++ | + |
| | E | + | + | + |
| Phenols | A | ++ | ++ | ++ |
| | E | + | + | + |

* Strongly present (+++), present (++), weakly present (+) and absent (-)

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similar trend to that observed for the reduction of early blight severity.

**Discussion**

The present study has demonstrated that fungitoxic compounds were found in the three medicinal plants under study, since the plant extracts inhibited growth and conidium germination of *A. solani*. These results are in agreement with other reports where different plant extracts have been assessed (Aslam *et al.*, 2010; Goussous *et al.*, 2010; Suleiman, 2010; Sal-lam, 2011; Sallam and Abo-Elyousr, 2012; Ravikumar and Garampalli, 2013; Khafari *et al.*, 2014).

The potency of plant extracts differed with the concentration and extractions solvent. Ethanol extracts were more effective than aqueous extracts, indicating that water was did not dissolve all the bioactive compounds present in the plants, while these could have been present in the ethanol extracts. Ethanol is an organic solvent and dissolves organic compounds more completely than water, and hence is likely to have liberated the active antifungal phytochemical compounds (Ekwenye and Elegalam, 2005). Stephan *et al.* (2005) suggested that aqueous extracts showed a poorer yield of active compounds than organic solvent extracts, and the fungicidal materials are mostly lipophilic.

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**Table 4.** Mean early blight severity and proportional (%) disease reduction for tomato plants treated with fungicide, or aqueous or ethanol extracts from three medicinal plants, in a filed trial.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aqueous extract</th>
<th>Ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DS (%)</td>
<td>DR (%)</td>
</tr>
<tr>
<td>Inoculated and not treated</td>
<td>53.2b a</td>
<td>0.0 d</td>
</tr>
<tr>
<td>Mancozeb (0.2%)</td>
<td>9.4 e</td>
<td>82.7 a</td>
</tr>
<tr>
<td><em>Calotropis procera</em></td>
<td>13.2 d</td>
<td>75.1 b</td>
</tr>
<tr>
<td><em>Acacia nilotica</em></td>
<td>15.7 c</td>
<td>70.4 b</td>
</tr>
<tr>
<td><em>Achillea fragrantissima</em></td>
<td>23.7 b</td>
<td>55.5 c</td>
</tr>
</tbody>
</table>

* DS = Disease severity and DR = Disease reduction.
  b Each value represents the mean of three replicates.
  c Values within a column followed by the same letter(s) are not significantly different according to Duncan’s multiple range test (*P* = 0.01).

**Table 5.** Mean tomato fruit yields from field-grown plants treated with fungicide, or aqueous of ethanol extracts from three medicinal plants.*

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Aqueous extracts</th>
<th>Ethanol extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (g)</td>
<td>Increase (%)</td>
</tr>
<tr>
<td>Inoculated and not treated</td>
<td>258.6a e</td>
<td>0.0 e</td>
</tr>
<tr>
<td>Mancozeb (0.2%)</td>
<td>493.5 a</td>
<td>90.9 a</td>
</tr>
<tr>
<td><em>Calotropis procera</em></td>
<td>481.4 b</td>
<td>86.2 b</td>
</tr>
<tr>
<td><em>Acacia nilotica</em></td>
<td>431.6 c</td>
<td>66.7 c</td>
</tr>
<tr>
<td><em>Achillea fragrantissima</em></td>
<td>373.7 d</td>
<td>44.5 d</td>
</tr>
</tbody>
</table>

* Each value represents the mean of three replicates.
  b Values within a column followed by the same letter(s) are not significantly different according to Duncan’s multiple range test (*P* = 0.01).
The efficacy of medicinal plant extracts may induce a systemic resistance in host plants against pathogens (Lubaina and Murugan, 2013). The extracts may also delay development of infections at early growth stages by inhibiting the mycelial growth of pathogens (Krebs and Forrer, 2001), or the toxic effect of active principles may act directly on the pathogens (Amadioha, 2000).

The extract from C. procera was the most effective against fungal growth compared with the other plant extracts. The results confirmed the presence of phytochemicals such as flavonoids, alkaloids and cardiac glycosides in high amounts in C. procera as compared with the other two plant species. The aggressive antifungal potential of C. procera may be attributed to these phytochemicals. Our results are in accordance with those of Mainasara et al. (2012), Tiwari and Singh (2014) and Ajiboso et al. (2015). Latex produced by C. procera and the members of the Asclepiadaceae contains many bioactive components which can be used for their antimicrobial activity (Nenaah, 2013; Ujwala and Karpagan, 2013).

Many studies have shown effects of active compounds in plant extracts which act as natural fungicides. However, few studies have referred to the action mechanism of these compounds on the microbial cells. For instance, these compounds may increase fungal cell plasma membrane permeability (Imelouane et al., 2009). In addition, these compounds at low concentrations lead to lipid peroxidation in fungi and increase ergosterol biosynthesis (Lucini et al., 2006). Moreover, the toxic effects of phenolic compounds possibly result from denature of fungal proteins (Cowan, 1999). These compounds can also inhibit the protective enzyme activity and subsequently inhibit one or more biochemical pathways (Xing et al., 2012).

In conclusion, this study has revealed the potential of aqueous and ethanol extracts of A. nilotica, A. fragrantissima and C. procera as alternatives to synthetic chemicals for control of early blight of tomato. Since these plants are wild and available, they could be an economic and eco-friendly disease management. With ease of extraction methods, these plant extracts have potential to be harnessed for control this important disease.

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Literature cited


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