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Antifungal effect of wood vinegar from selected feedstocks on *Ascochyta rabiei in vitro*

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A study to evaluate the antifungal activity of wood vinegar (pyrogenous acid) from maize cobs, acacia twigs, bean straw and an invasive tree species *Prosopis juliflora* against *Ascochyta rabiei* was conducted *in vitro* and at Egerton University Njoro, Kenya. The physicochemical characteristics of the different wood vinegar were also determined. Antifungal effects of wood vinegar were evaluated at different concentrations (0.5, 1, 1.5, 2, 2.5 and 3% v/v) using a Petri dish bioassay arranged in a completely randomized design. A fungicide (Metalaxy-M-40 g/kg) and water were used as positive and negative controls, respectively. All the wood vinegars had a smoky odor with brown/yellow coloration and an average density of 1.06 g/cm^3. The wood vinegar from maize cobs showed a near acidic pH of 3.90 while bean straws showed a near neutral pH of 7.16. Prosopis and acacia showed moderate acidity of 5.10 and 5.43, respectively. The highest concentration of phenols was recorded in wood vinegar from maize cobs (4.56 mg/ml) followed by acacia (3.52 mg/ml). The results from the antifungal assay showed wood vinegar treatment significantly ($P \leq 0.001$) reduced *A. rabiei* mycelia growth at all tested concentrations when compared to the untreated control. The minimum inhibition concentration was 0.5% v/v for all the tested wood vinegars. The percent mycelia growth inhibition generally increased with increasing concentration except for maize cob which showed 99.33% and complete inhibition at 0.5 and 1.5 % v/v concentrations, respectively. Complete inhibition of the pathogen's growth (100%) for all the wood vinegars tested was achieved at 2.5% v/v concentration. Plant-based wood vinegar has antifungal activity against *A. rabiei*.

Key words: Antifungal, *Ascochyta rabiei,* polyphenols, pyrogenous acid, wood vinegar.

INTRODUCTION

Chickpea (*Cicer arietinum* L.), is a drought-tolerant and nutritious legume with great potential to combat malnourishment in human populations (Fikre et al., 2020). Global production of chickpeas is, however, limited by several fungal diseases key among them is Ascochyta blight (AB) caused by the *Ascochyta rabiei* pathogen (Tadesse et al., 2017). This is a well-pronounced world pathogen that can cause yield losses of up to 100% under favorable weather and susceptible cultivars (Singh et al., 2022). Ascochyta blight disease symptoms occur on aerial parts of the plant including leaves, stems, pods and flowers. Infected seeds are

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shrunken and shrivelled and in severe cases, lesions having dark pycnidia are present (Zhang et al., 2019).

Limited commercial chickpea cultivars with sufficient resistance have led to over reliance on foliar applied fungicides for the management of this disease (Fanning et al., 2022). There is an increasing demand for natural pesticides that are efficient and safe to humans and the environment. Biopesticides are potential alternatives and are gaining great research attention (Khursheed et al., 2022). Plant-derived biopesticides are reported to be efficacious against several plant pathogens without imposing ill side effects (Shimira et al., 2021; Souto et al. 2021). The antifungal effect of various plant derived products has been demonstrated both *in-vitro* and *in-vivo* (Kundu et al., 2021; Kursa et al., 2022).

Wood vinegar (pyrogenous acid) is a highly condensed aqueous crude liquid produced through fast or slow pyrolysis of plant biomass materials in an oxygen-limited environment (Sangsuk et al., 2018; Wang et al., 2018). Several studies have reported antifungal, antibacterial, insecticidal, larvicidal and herbicidal properties of wood vinegar (Arsyad et al., 2020; Lee et al., 2022; Mhamd, 2023). Theapparat et al. (2015) reported that wood vinegar produced from *Dendrocalamus* asper (bamboo), *Eucalyptus camaldulensis*, *Azadirachta indica,* and *Leucaena leucocephala* exhibited antifungal properties against brown rot and sapstain fungi. Oramahi et al. (2018) also reported fungicidal effect of wood vinegar from oil palm trunk against a white-rot fungus, *Trametes versicolor*, and a brown-rot fungus, *Fomitopsis palustris*

The physico-chemistry and biological activity of wood vinegar are affected by many factors such as chemical composition of biomass (Zhao et al., 2014), pyrolysis system and refining method. It is on this premise that the current study was undertaken to determine the physico-chemical characteristics and the antifungal effects of wood vinegar from an invasive plant, *Prosopis juliflora*, acacia twigs, maize cobs and bean straws, on Ascochyta blight in-vitro. The study hypothesized differences in the efficacy of the wood vinegar from the different feedstocks.

MATERIALS AND METHODS

Description of the botanical plants

Maize cob waste (MCW) is the leftover material from maize after the kernels have been removed. It is the most produced crop in the world and a sort of agricultural waste that is widely available worldwide. MCW is mostly made up of lignin, cellulose and hemicellulose integrated into a complex matrix. Bean straws are dry stalks left over after the grains have been taken from plants and are a byproduct of agriculture. *Prosopis juliflora* is a type of mesquite shrub or small tree in the Fabaceae family. It is indigenous to the Caribbean, South America and Mexico. In Africa, Asia, Australia and other places, it has established itself as an invasive weed. The tree does not reproduce vegetatively; it reproduces only through seeds. Within the Fabaceae pea family's Mimosoideae subfamily, the huge genus *Acacia* comprises shrubs and trees. In order to adapt to hot weather and dehydration, species in the genus possess vertically oriented phyllodes, which are green, expanded leaf petioles that resemble leaf blades

Preparation of wood vinegar

Wood vinegar was produced from maize cobs, acacia, bean straws and *P. juliflora* locally known as Mathenge in Kenya. Maize cobs, bean straw and *P. juliflora* were provided by the Perkerra National Irrigation Authority (NIA) at Marigat, Baringo County. Acacia trimmings were collected around Egerton University Njoro campus. All the materials were sun-dried to a moisture content of 12%. Wood vinegar from the different feedstocks was separately collected as condensed smoke produced through slow pyrolysis at 350 to 400°C (Doti et al., 2023) using a small vertical drum kiln developed by the Bioenergy Kenya Climate Smart Agriculture Project in the Department of Agricultural Engineering, Egerton University. The smoke condensate was allowed to settle at room temperature for 90 days, separating into layers of tar (at bottom), wood vinegar and water according to Zhai et al. (2015).

Physico-chemical properties of wood vinegar from the selected feedstocks

The odor of the wood vinegar was determined by raising the hand above the container and wafting the air towards the nose. Color was determined by visual observation of the liquid and comparisons made against a color chart. The pH values of wood vinegar were measured using a pH meter model (PHS-3CHS). The density was calculated using the formula (Equation 1):

$$\text{D}ensity = \frac{\text{mass (kg)}}{\text{volume}} \quad (1)$$

Preparation of standard garlic acid and Folin–Ciocalteu reagent

The garlic acid standards were prepared according to Shirazi et al. (2014) with some modifications. One gram (1 g) of garlic acid was weighed and dissolved in 100 ml of methanol resulting in a 1% solution of the garlic acid (10 mg/ml). Folin-Ciocalteu suspension was prepared by dissolving 10 g of sodium tungstate and 2.5 g of sodium molybdate in 70 ml of distilled water. This was followed by adding 5 ml of 85% phosphoric acid and 10 ml of concentrated hydrochloric acid. The resulting solution was refluxed for 10 h before adding 15 g of lithium sulfate followed by 5 ml of distilled water and one drop of bromine water. The solution was refluxed again for 15 min and cooled to room temperature before use.

Determination of the phenol concentration

The phenol concentration in wood vinegar was determined using Folin-Ciocalteu method according to Yang et al. (2016) with some modifications. An aliquot of wood vinegar (1 mL) was added to 25% Folin-Ciocalteu suspension followed by 2 ml of 2% Na_2CO_3.The procedure was repeated with standard garlic solution whereby 1 mL aliquots (0.2, 0.4, 0.6 and 0.8 mg/ml) were used. The solutions were thoroughly shaken and allowed to incubate for 30 min at room temperature. Methanol was used as a blank solution while distilled water was used to zero the spectrophotometer. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer. The measurement for each sample was done in triplicates. A standard garlic acid curve was drawn from the different dilutions. The regression equation derived from the standard garlic acid curve was

used to calculate the phenol concentrations of the selected wood vinegar.

Ascochyta rabiei isolation and culture preparation

Chickpea plants exhibiting Ascochyta blight disease symptoms were collected from the field. Small pieces of diseased parts were cut off the plants using a sterile scalpel. The pieces were sterilized by dipping them in 2% sodium hypochlorite for 3 min, washing in 70% ethanol for 30 s and rinsing them five times in sterile distilled water. Potato Dextrose Agar (PDA) was prepared and added with doxycycline 300 g/L to prevent the growth of opportunistic pathogens. The mixture was dispensed into sterile petri dishes and left to set. The diseased materials were aseptically transferred onto the PDA plates and incubated at 24°C for 14 days. The plates were observed daily for fungal growth. Thereafter, the plates were flooded with sterile distilled and *A. rabiei* spores were scrapped and harvested using a sterile spatula. The spore suspension was filtered through several layers of a Whatman paper to remove mycelia fragments. The pure culture of the resulting fungal growth was stained with lactophenol cotton blue solution and placed under a compound microscope magnification (×100) for identification based on morphological characteristics of *A. rabiei.*

Antifungal assay

Fungal growth inhibition was conducted according to Oramahi et al. (2018) using a completely randomized design (CRD) with three replicates. The different wood vinegar (maize cobs, acacia, bean straws and Prosopis) were tested at 0.5, 1.0, 1.5, 2, 2.5 and 3% v/v concentrations. The fungicide Metalaxy-M-40 g/kg and distilled water were used as positive and negative checks, respectively. PDA media was separately mixed with the varying concentrations of the different wood vinegars except maize cobs. The mixture was autoclaved for 15 min at 121°C and then poured into 90 mm diameter Petri dishes. In the case of the wood vinegar from maize cobs, the PDA was sterilized separately for 15 min at 121°C and mixed when the media was about to gel. After cooling, 5 mm plug mycelia from previously prepared pure culture was cut using a cock borer and centrally inoculated into the treatment plates. The plates were incubated at 25°C and monitored daily for the radial growth of fungi until maximum growth was observed (mycelia reached the edge) in the control plates. The presence of growth inhibition zones, an indication of antifungal activity, was observed. The minimum inhibitory concentration (MIC) for the different wood vinegars was also determined.

Fungal growth inhibition

The radial growth (diameter) of the colonies from each Petri dish was measured and the percentage of fungal growth inhibition was calculated using Equation 2:

$$I = \frac{C-T}{C} \times 100 \quad (2)$$

where I = inhibition, as a percentage; C = colony diameter of mycelium from control Petri dishes (mm); and T = colony diameter of mycelium from the Petri dishes containing the wood vinegar.

Data analysis

All the data was analysed using SAS version 9.4 following *PROC GLM* procedures. Means were separated using Fischer's least significance difference at $\alpha = 0.05$.

RESULTS

Physicochemical properties of the selected wood vinegar

All the wood vinegars tested had a smoky odor with brown/yellow coloration. The wood vinegar from maize cobs showed a near acidic pH of 3.90 while bean straws had a near neutral pH of 7.16. Prosopis and acacia had a moderate acidity of 5.10 and 5.43, respectively. The average density of all the tested wood vinegar sources was 1.07 g/cm^3 (Table 1).

Phenol concentration

The results showed significant differences in the concentration of phenols in the wood vinegars tested. The highest concentration of phenols 4.56 mg/ml was recorded in the vinegar from maize cobs, followed by acacia (3.52) which was not significantly different from that from bean straw and Prosopis (Figure 1).

Morphological identification of *A. rabiei*

Morphological characteristics consistent with *A. rabiei* as described by Baite et al. (2016) and Crociara et al. (2022) were observed. These included isolated fungal colonies exhibiting circular dark brown shapes with spherical pycnidium indicating an opening/ostiole at the center. The conidiophores were light brown in color and submerged in PDA.

Antifungal effects of wood vinegar at different concentrations

Wood vinegar from all the feedstocks differed significantly and they exhibited a decline in the growth of the fungus with wood vinegar from cobs and acacia showing maximum results on fungal growth inhibition with means of 99.82 and 86.65% respectively (Table 2). The results further showed that wood vinegar treatment, significantly ($P \leq 0.001$) reduced *A. rabiei* mycelia growth at all tested concentrations compared to the untreated control (Table 3). The minimum inhibition concentration was 0.5% for all the tested wood vinegar. The percentage of mycelia growth inhibition generally increased with increasing concentration with maize cob showing complete inhibition even at low concentrations. Complete inhibition of the pathogen's growth (100%) was achieved at a concentration of 2.5% v/v for all the wood vinegars. Antifungal activity of the different wood vinegars was indicated by the presence of clear growth inhibition zones in the inoculated plates. Plate 1 shows plates of radial fungal inhibition zones for the different vinegars at six concentrations with water and Metalaxyl-M 40 g/kg

Table 1. Absorbance, density and pH of wood vinegar from selected feedstock.

Wood vinegar	Absorbance (760 nm)	pH	Density/cm^3
Maize cobs	0.178	3.90	1.08
Acacia	0.126	5.43	1.06
Prosopis	0.113	5.10	1.06
Bean straw	0.112	7.16	1.06
Methanol	-0.05		

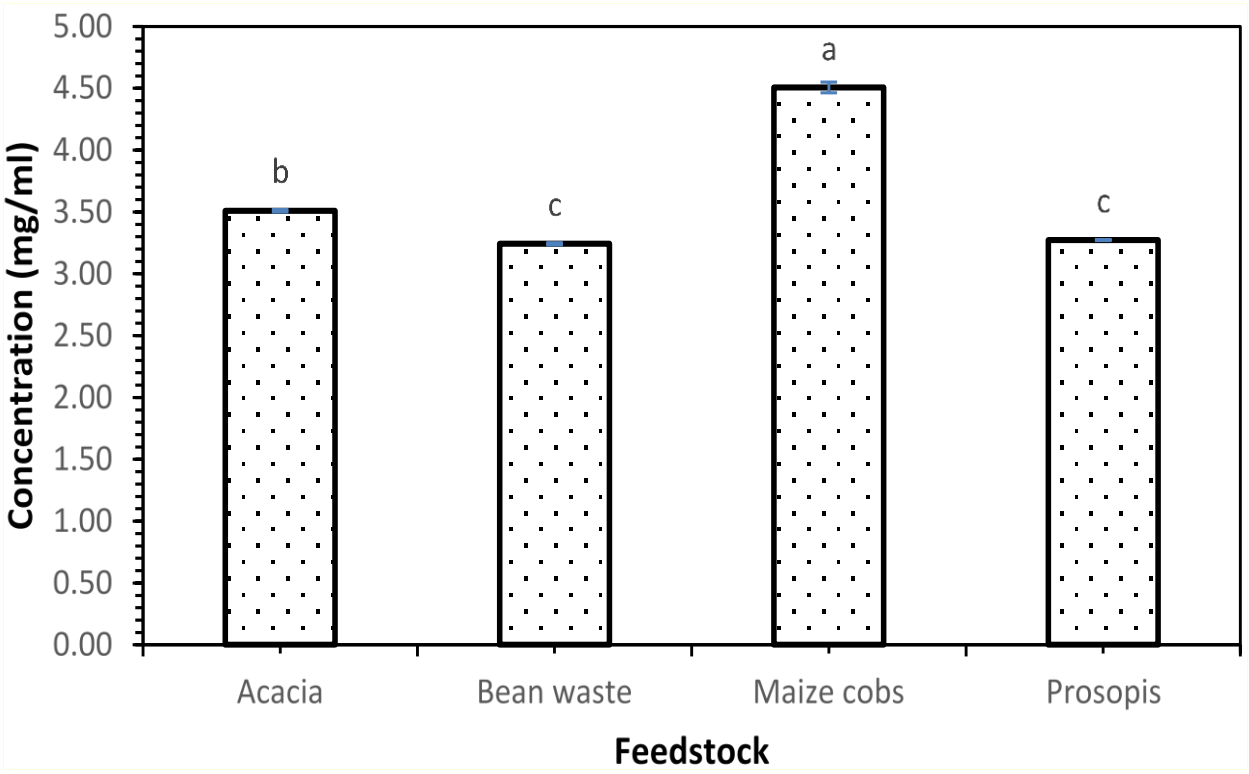

Figure 1. Phenol concentration of the different wood vinegar from selected feedstocks. Bars with similar letters are not significantly different at P≤0.05 using least significance difference (LSD).

Table 2. Performance of the fungicide and wood vinegar from selected feedstocks on growth inhibition of *A. rabiei in vitro.*

Feedstock	Growth
Acacia	86.65^{b}
Bean waste	67.97^{e}
Maize cobs	99.82^{a}
Metalaxyl-M	75.31^{c}
Prosopis juliflora	73.73^{d}
Negative control	0.00^{f}
Lsd	**0.58**

Means followed by the same letters along the column are not significantly different according to Fischer's least significance difference at *P≤0.05.*

fungicide with a standard application rate of 50 g/L being used as controls.

DISCUSSION

All the wood vinegar tested appeared to be of good quality in terms of physical properties; a smoky odor with brown/yellow coloration and an average specific density of 1.06 g.mL^{-1}. Theapparat et al. (2018) described the qualities of good wood vinegar as having a smoky odor, no visible suspended matter, transparent liquid with a brown/ yellow coloration, pH value of about 3 and a specific density of 1.010 to 1.050 g.mL^{-1}. The near ideal pH of 3.90 was only observed in wood vinegar from maize cobs followed by acacia with 5.10. The organic

Table 3. Mycelial growth inhibition of *A. rabiei* upon exposure on semi-volatile organic compounds of wood vinegar from acacia, bean waste, maize cobs, bean straws, fungicide and Prosopis at different concentrations (Values are the mean of three replicates ± SD)**.**

Feedstock	Concentration					
	0.5	1	1.5	2	2.5	3
Maize cobs	99.33±0.33	99.57±0.43	100±0.00	100±0.00	100±0.00	100±0.00
Acacia	50.81±0.22	70.41±0.30	98.67±0.67	100±0.00	100±0.00	100±0.00
Prosopis	24.08±0.61	38.81±1.86	89.00±0.76	100.00±0.00	100.00±0.00	100.00±0.00
Bean straws	11.31±0.39	46.99±0.03	65.11±0.39	84.41±1.34	100±0.00	100±0.00
Metalaxyl-M	24.08±0.61	38.81±1.86	89.00±0.00	100±0.76	100±0.00	100±0.00
Water	0.00±0.00					

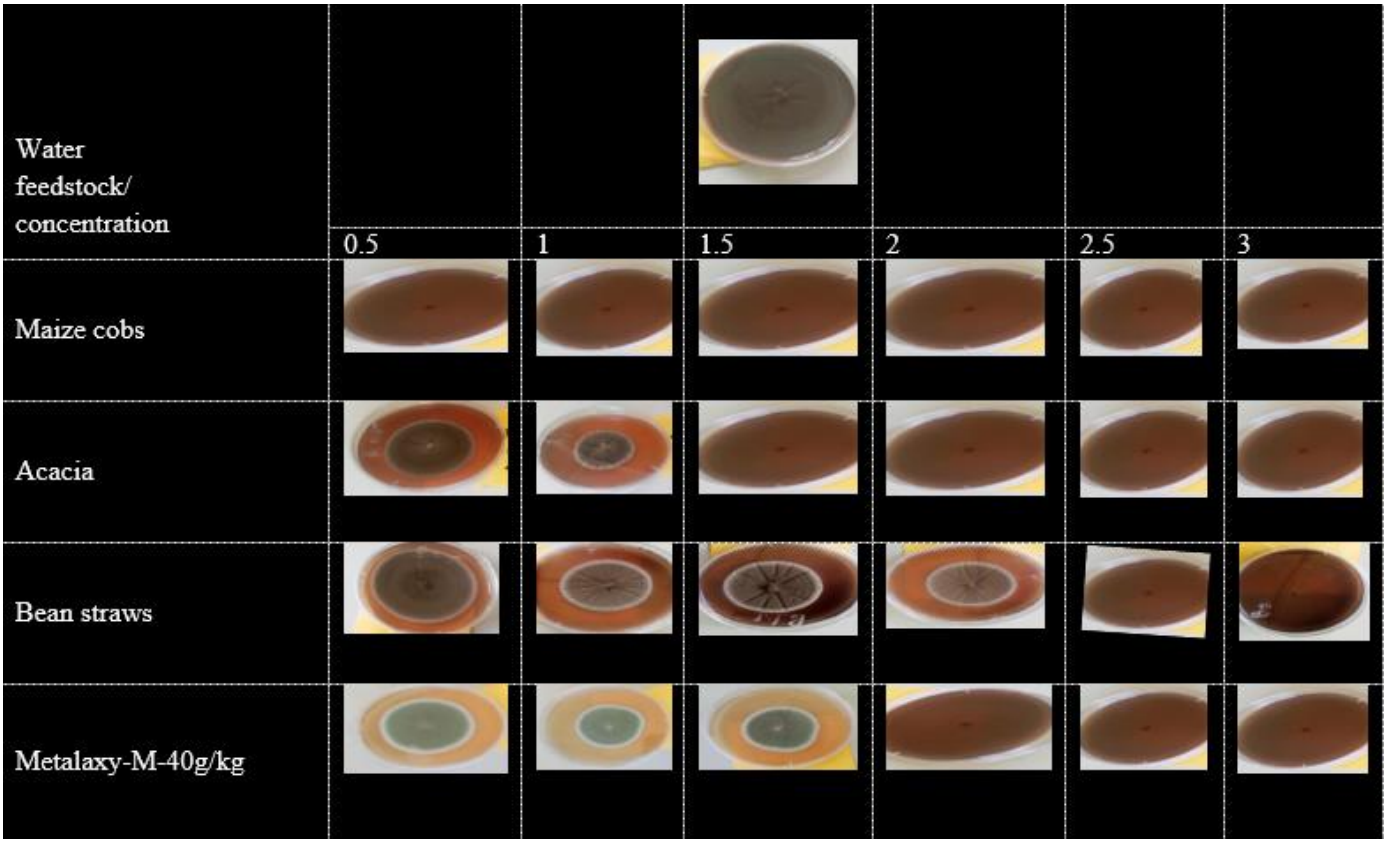

Plate 1. Radial fungal growth inhibition zones for *A. rabiei* following *in vitro* assay of wood vinegar and fungicide at different concentrations.

and inorganic components of the acid contribute to the yellow color as well as the smoky odor of the acid as further confirmed by Mathew and Zakaria (2015).

Results from the current study also showed the presence of varying concentrations of phenols in the wood vinegar tested. The highest concentration of phenols 4.56 mg/ml was obtained from maize cobs followed by acacia with 3.52 mg/ml. Previous studies have reported that the physical and chemical properties of wood vinegar are influenced by several factors key among them being the type of feedstock carbonized (Xue et al., 2022) Wood vinegar from biomass varies in their chemical structure due to differences in the approximate amount of lignin, cellulose and hemicellulose (Cheng et al., 2022). Differences in the proportion of these constituents not only depend on the plant species but are also influenced by other factors such as habitat, age and part of the plant among others (Grewal et al., 2018). Variations in the composition of feedstock used for the preparation of wood vinegar are therefore the most likely

reason for the different properties of the product. Most research has found wood vinegar possesses high contents of phenols. The phenolic compounds are biopolymers that form the major components of cell walls in woody plant bark. According to Chenari Bouket et al. (2023), wood vinegar contains phenolic compounds that are either syringol or catechol type. The results are further confirmed by Liu et al. (2021) who reported that wood vinegar contains around 70% of phenolic compounds. These components of wood vinegar have demonstrated excellent antifungal activity against different fungal pathogens (Alves et al., 2014; Simonetti et al., 2019; Silva et al., 2020; Cadenillas et al., 2022) which is consistent with the findings of the current study. It is therefore a prompt to carry out further research to test the components of wood vinegar individually and their specific antifungal activities. However, several authors have indicated that it is not possible to find out the mode of action of wood vinegar due to the diversity of volatile organic components. The plant-based bioactive compounds including alcohols, alkaloids, phenols, tannins and terpenes present in wood vinegar have been found to delay sporulation and mycelial development as well as inhibit germ tube elongation (Ngegba et al., 2022; Mansour et al., 2023). In the current study, wood vinegar from maize cobs caused complete inhibition of *A. rabiei* mycelial growth *in vitro* even at low concentrations of 0.5% V/V. Complete inhibition of fungal growth for acacia, bean straw and Prosopis was achieved at the concentration of 2.5% V/V *in-vitro*. The stronger antifungal activity of wood vinegar from maize cobs can be attributed to the higher phenol content and acetic acid indicated by the near-ideal pH of 3.90. High contents of organic acids and phenols may correlate with strong antimicrobial activity (Deng et al., 2023; Shiny et al., 2024). A similar study by Chenari Bouket et al. (2022) reported differences in fungal growth inhibition of Pythium mycelia by wood vinegar from Almond, pomate, pine, pomegranate and walnut.

Conclusion

This study demonstrated that the tested wood vinegars possess antifungal activity against *A. rabiei* pathogen well exemplified by their ability to completely inhibit mycelial growth at the concentration of 2.5% v/v. The higher potential of wood vinegar from maize which showed complete fungal growth inhibition at 0.5% v/v, is due to the higher proportion of phenols and organic acids indicated by the lower pH. Farmers can produce wood vinegar from biomass waste such as branches trimmed from trees and crop residues making it a clean energy material with low cost and high benefits. The low cost of production can also be attributed to savings from the use of synthetic pesticides. Further research is however recommended to establish specific compounds in wood vinegar that are responsible for fungal growth inhibition of *A. rabiei* and their stability under field conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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