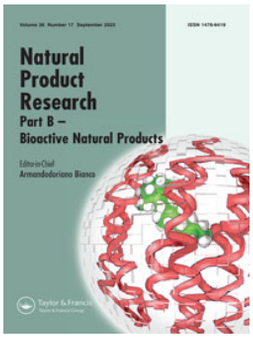

Natural Product Research

Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: Akash Maurya, Susheel Kumar, Bijendra Kumar Singh, Anand Kumar Chaudhari, Abhishek Kumar Dwivedy, Bhanu Prakash & Nawal Kishore Dubey (2022) Mechanistic investigations on antifungal and antiaflatoxigenic activities of chemically characterised *Carum carvi* L. essential oil against fungal infestation and aflatoxin contamination of herbal raw materials, Natural Product Research, 36:17, 4569-4574, DOI: 10.1080/14786419.2021.1994566

To link to this article: https://doi.org/10.1080/14786419.2021.1994566

Published online: 21 Oct 2021.

NATURAL PRODUCT RESEARCH
2022, VOL. 36, NO. 17, 4569–4574
https://doi.org/10.1080/14786419.2021.1994566

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SHORT COMMUNICATION

Mechanistic investigations on antifungal and antiaflatoxigenic activities of chemically characterised *Carum carvi* L. essential oil against fungal infestation and aflatoxin contamination of herbal raw materials

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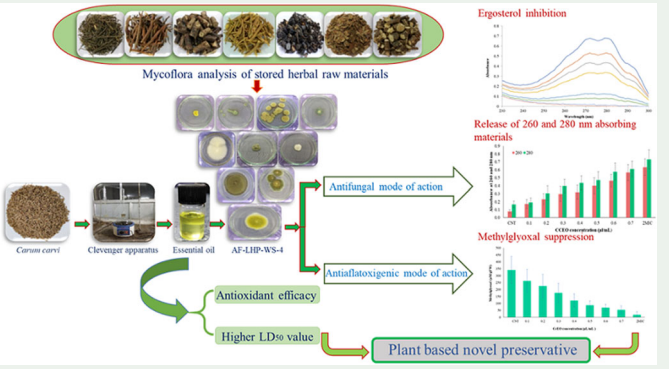
ABSTRACT

This study aimed to investigate the efficiency of chemically characterised *Carum carvi* essential oil (CcEO) against aflatoxin B_1 (AFB_1) producing strain of *Aspergillus flavus* (AF-LHP-WS-4) causing deterioration of herbal raw materials (HRM). GC-MS analysis of the EO revealed the presence of carvone (69.85%) as a dominant component. CcEO caused complete suppression of *A. flavus* growth and AFB_1 secretion at 0.7 and 0.6 µL/mL, respectively. The investigation on antifungal mode of action showed that CcEO inhibited fungal growth via abrogating ergosterol biosynthesis and triggered efflux of vital cellular ions. The inhibition of AFB_1 biosynthesis was attributed to the inhibition of cellular methylglyoxal (MG) biosynthesis. In addition, CcEO showed remarkable antioxidant activity (IC_{50} = 10.564 µL/mL) against DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals. Based on overall results, it can be concluded that the CcEO may be recommended as potential antifungal agent for protection of HRM from fungal infestation and AFB_1 contamination.



ARTICLE HISTORY
Received 23 July 2021
Accepted 7 October 2021

KEYWORDS
Carum carvi essential oil; aflatoxin B_1; methylglyoxal; herbal raw materials; safety profile

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2021.1994566.

1. Introduction

Herbal raw materials (HRM) are extremely popular worldwide due to the presence of a large number of bioactive components with proved therapeutic potential (Caldeirão et al. 2021). However, natural mycobiota, especially mycotoxigenic fungi can contaminate the HRM and degrade their bioactive components, which may cause serious health hazards to consumers. Mycotoxins are low molecular weight secondary metabolites of fungi mostly produced by the genera *Aspergillus*, *Fusarium*, and *Penicillium* (Chaudhari et al. 2019). Among different aflatoxins, aflatoxin B_1 (AFB_1) is the most toxic and hence classified as class I human carcinogen (Maurya et al. 2021). Presently, different chemical fungicides have been applied to inhibit the fungal infestation and aflatoxin secretion in HRM, however, their overuse many times may lead to residual toxicity, emergence of fungal resistance, and environmental pollution (Tian et al. 2012). In this context, the use of plant derived products (Frezza et al. 2019), especially essential oils (EOs) are attracting enormous attention to control fungal and aflatoxin B_1 contamination in stored HRM.

Carum carvi L., popularly known as 'caraway', is a biennial plant belonging to the family Apiaceae and is indigenous to central Asia and northern and central Europe. The caraway EO, which is isolated from the seeds have been used since long time and prescribed as antispasmodic, antidiarrheal, antiflatulence, antidysmenorrhea, and appetizer (Homayounpour et al. 2021). This research aimed to evaluate the presence of fungal and AFB_1 contamination in stored HRM sold in different parts of India. The EO was characterised and evaluated for its efficacy against the AFB_1 secreting strain of *A. flavus* (AF-LHP-WS-4) isolated during mycobiota investigation of the stored HRM. Furthermore, the antifungal and anti-aflatoxigenic mode of action of EO was unraveled. Moreover, the antioxidant activity and safety profile assessment of *Carum carvi* essential oil (CcEO) on mice model was determined.

2. Results and discussion

All the tables and figures are provided as supplementary material.

2.1. Mycobiota investigation and selection of most aflatoxigenic strain

In the present investigation, the moisture content of the selected HRM varied from 10.14%–11.26% (Table S1). This range of moisture content accelerates the growth of molds and accumulation of their associated toxins in stored HRM (Das et al. 2020). The pH of HRM ranged between 2.94 and 5.96 (Table S1), which favors deterioration of stored HRM with the help of hydrolytic enzymes which are released by stored mold species (Guatam et al. 2009). During mycobiota investigation, a total of 698 fungal isolates belonging to 4 different genera and 10 species were isolated. Among different fungal isolates, *A. flavus* was found to be the dominant fungus with highest per cent relative density (33.66%). During toxigenicity assay, out of 49 different *A. flavus* strains screened, 47 were found aflatoxigenic and the strain AF-LHP-WS-4 isolated from the roots of *Withania somnifera* was reported to produce highest AFB_1 (5939.44 μg/L) in

the medium (Table S2), and hence selected as test fungus for the detailed investigation.

2.2. Extraction and chemical characterization of CcEO

The CcEO yield through hydro-distillation was found to be 12.7 mL/kg of seeds of *C. carvi*. The chemical characterization of CcEO determined through GC-MS analysis identified 17 different components representing 94.94% of the total oil (Table S3). However, carvone (69.85%) was found as major component followed by limonene (13.62%), β-linalool (4.26%), and *p*-cymene (3.12%), which all together comprised 90.85% of total oil. The presence of carvone as the major component in CcEO has also been verified by Yakoubi et al. (2020), however percentage of other organic components varies due to phenotypic and genotypic nature of plants. The antimicrobial activity of CcEO may also depend on mixture of major and minor organic components and their synergistic activity.

2.3. Antifungal, anti-aflatoxigenic and fungitoxic activity of CcEO against AF-LHP-WS-4

During investigation of efficacy against test fungus, CcEO caused complete inhibition of AF-LHP-WS-4 mycelial growth and AFB_1 secretion at 0.7 and 0.6 μL/mL concentrations, which was reported as its MIC and MAIC, respectively (Table S3). The antifungal and anti-aflatoxigenic activity reported here was found far efficacious than some of the previously explored EOs such as *Myristica fragrans* (with MIC and MAIC = 2.75, and 1.75 mg/mL, respectively) and *Pimenta dioica* (MIC and MAIC = 2.5 and 1.5 μL/mL, respectively) (Das et al. 2020; Chaudhari et al. 2020a). In addition, CcEO at MIC concentration absolutely suppressed the visible growth of other tested moulds viz., *A. flavus*, *A. sydowii*, *A. luchuensis*, *A. fumigatus*, *A. niger*, *A. candidus*, *A. wentii*, *C. epiphyllum*, and *F. oxysporum*, except *P. substellium* (85.641%) (Figure S1). These results confirmed that CcEO possesses extensive fungitoxic spectrum, and therefore could be recommended as potential fungistatic and fungicidal agent for protection of stored HRM from a range of storage fungi.

2.4. Antifungal and anti-aflatoxigenic mode of action of CcEO against AF-LHP-WS-4

The detailed antifungal mode of action of CcEO against AF-LHP-WS-4 cells was determined by measurement of intracellular ergosterol content, release of vital cellular ions, and 260 and 280 nm absorbing materials, while anti-aflatoxigenic mode of action was determined by measuring the amount of cellular methylglyoxal (MG) in treated *A. flavus* cells. A substantial reduction of ergosterol content with value 16.68%, 29.32%, 36.53%, 62.97%, 78.80%, 100% was noticed at the treatment concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 μL/mL, respectively (Table S5 and Figure S2). Ergosterol is the prime sterol present in fungal plasma membrane responsible for retaining the integrity and functionality of the cells. A dose dependent reduction of ergosterol contents may

lead to loss of permeability of the cells resulting in the enhanced leakage of important cellular ions (Tian et al. 2012). The excessive leakage of cellular contents outside the cells may cause loss of function of the cells, thereby resulting in programmed cell death. To support this result, intracellular ions and 260 and 280 nm absorbing materials from treated AF-LHP-WS-4 cells were measured. A dose dependent rise in the leakage of intracellular ions (viz., K^+, Ca^{2+}, and Mg^{2+}) and 260 and 280 nm absorbing materials with increasing concentrations of CcEO from $^1/_2$ MIC to 2 MIC was observed (Figure S3), confirming the above finding reporting loss of plasma membrane integrity.

In addition, the CcEO caused dose dependent inhibition of cellular MG levels (Figure S4). MG has been reported to be an important metabolite responsible for enhancing the production of AFB_1 content in *A. flavus* cells, possibly via upregulating the expression of AFB_1 biosynthesis genes (Chaudhari et al. 2020b). Thus, the reduction of MG content in *A. flavus* treated cells may be related to the reduction of aflatoxin B_1 production. This result revealed a novel anti-aflatoxigenic mode of action of CcEO, which can be used as novel approach for the development of AFB_1 resistance herbal plants through green transgenic approach. This is the first time report on anti-aflatoxigenic mechanism of action of CcEO through reduction of intracellular MG content.

2.5. Determination of radical scavenging activity of CcEO through DPPH assay

Results revealed that CcEO caused dose dependent scavenging of DPPH radicals with IC_{50} value 10.564 μL/mL as determined through DPPH assay (Figure S5). The free radical scavenging activity presented by CcEO was found better than some of the formerly investigated EOs viz., *Gaultheria fragrantissima* (13.09 μL/mL) and *Zingiber officinale* (14.44 μL/mL) (Ramsdam et al. 2021). Based on remarkable antioxidant activity, CcEO may be suggested as a suitable substitute to the synthetic antioxidants viz., BHA, BHT, TBHQ, and PG to improve the shelf-life of stored HRM.

2.6. Determination of safety profile of CcEO

Before suggesting any preservatives for food application, it is important to demonstrate their safety profile on model animals like rat and mice. The experiment regarding determination of safety profile on mice model revealed that CcEO exhibited high safety profile with LD_{50} equivalent to 9074.149 μL/Kg. This value was found much greater in comparison to commonly used botanical preservatives viz., azadirachtin (~5000 μl/kg) and pyrethrum (350–500 μl/kg) (Coats 1994), and hence, may be considered as much safer for consumption as well as preservation purpose.

3. Experimental

Experimental section is provided as supplementary material.

4. Conclusion

On the basis of remarkable antifungal, anti-aflatoxigenic, and free radical scavenging activity with high value of mammalian toxicity, *Carum carvi* EO may be proposed as a potential shelf-life enhancer of stored HRM. The reduction of MG suggests novel mechanism of action for the inhibition of aflatoxin biosynthesis, which can be used for developing AFB_1 resistant herbal plants via green transgenic approach.

Acknowledgments

Authors are thankful to the Head, Centre of Advance Study (CAS), in Botany, DST-PURSE, DST-FIST, ISLS and Institute of Eminence (IoE) Cell, Banaras Hindu University for providing laboratory facilities.

Disclosure statement

The authors declare that they have no potential conflicts of interest.

Funding

Akash Maurya and Susheel Kumar are thankful to the National Medicinal Plant Board (NMPB) [Project code: P 21/14], Ministry of Ayush, Government of India, New Delhi, India for financial assistance.

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