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Antimicrobial properties of the medicinal plant *Cardiospermum halicacabum L*: new evidence and future perspectives

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Abstract. – The emergence and rapid spread of multidrug-resistance in human pathogenic microorganisms urgently require the development of novel therapeutic strategies for the treatment of infectious diseases. From this perspective, the antimicrobial properties of the natural plant-derived products may represent an important alternative therapeutic option to synthetic drugs. Among medicinal plants, the *Cardiospermum halicacabum L. (C. halicacabum)*, belonging to *Sapindaceae* family, could be a very promising candidate for its antimicrobial activity against a wide range of microorganisms, including both Gram positive and Gram negative bacteria, as well as fungal pathogens. Although the antimicrobial properties of *C. halicacabum* have been intensively studied, the mechanism/s by which it exerts the inhibitory activity towards the pathogenic microbes have not yet been completely understood. This review focuses on the main antimicrobial activities displayed *in vitro* by the plant extract, with particular attention on our recent advances. We demonstrated that *C. halicacabum* is able to exert *in vitro* a dose-dependent fungistatic effect against *Trychophyton rubrum (T. rubrum)* through molecular interaction with the fungal heat shock protein (Hsp)-90 chaperone. These findings are supported by a growing body of research indicating the crucial role played by the Hsp90 in the virulence of the pathogenic microorganisms, including fungal pathogens. The possible future use of *C. halicacabum* for treating a wide range of infectious diseases is also discussed.

Key Words:
C. halicacabum, Antimicrobial, *T. rubrum*, Hsp90, Molecular modeling, Antifungal therapeutic strategies.

Introduction

The increasing resistance of the microorganisms towards conventional antimicrobial agents has led to serious health problems in recent years and even more it is be expected in the future. This global phenomenon encourages the development of new agents which can effectively inhibit microbial growth. An alternative and very promising approach to overcome this issue might be the use of natural antimicrobial products. The Middle East represents an important source of medicinal plants, which have been used for treating diseases for thousands of years in traditional medicine. The bioactive aromatic products extracted from some of these plants have been shown to possess potential antimicrobial properties[1]. Nowadays people in developing countries prefer plant-derived natural products limiting their exposure to the side effects and toxicity of synthetic drugs. Among medicinal plants, *Cardiospermum halicacabum L.* (*C. halicacabum*) is an herbaceous climber belonging to the Sapindaceae family, naturally distributed in tropical and subtropical

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regions of the world. It grows in the plains of Africa, America, Bangladesh, India, Malacca, and Pakistan. Its roots, leaves, and seeds have been employed as herbal medication[2]. The phytochemical characterization of the constituents of the plant extracts revealed the presence of β-arachidic acid, apigenin, apigenin-7-*O*-glucuronide, and chrysoeriol-7-*O*-glucuronide[3,4]. Various fatty acids and volatile esters were also isolated from seed oil[5,6]. Furthermore, other secondary metabolites have been found in small quantities in the extracts including alkaloids, carbohydrates, proteins, saponins, lignin, steroids, flavonoids, terpenoids, and cardiac glycosides[7,8]. The plant-based herbal products are commercially available as a gel, cream, shampoo, spray, medical drops, and pills. They are effective especially in the treatment of dry itchy skin and scalp and for treating chronic skin inflammatory disorders, including psoriasis[4,9]. The plant has been used in Ayurveda and folk medicine for a long time in the treatment of rheumatism, lumbago, cough, and hyperthermia[10]. Furthermore, it has been employed to treat nervous diseases like epilepsy and anxiety disorders, as a demulcent in orchitis, and in dropsy[11-13]. *C. halicacabum* also acts as a diaphoretic, diuretic, laxative, and anti-diarrheal[6,14]. Additionally, it exhibits antioxidant and anti-inflammatory activities *via* suppression of TNF-α production, vasodepressant, and even anticancer effect *in vitro*[15-18]. Remarkable antipyretic and antiulcer activities of *C. halicacabum*, as well as an antihyperglycemic effect against streptozotocin-induced diabetes, have also been shown in experimental animal models[19-21]. Its aqueous leaf extract increases fertility in male rats and exhibits a significant hepatoprotective effect[22]. The herb has also been shown to have strong protective activity against diverse infectious diseases for its antibacterial, antifungal, and antiparasitic activity[3,23]. Although the antimicrobial properties of the plant have extensively been studied, the precise mechanisms underlying its inhibitory effect against microbial pathogens have not yet been completely clarified. This review summarizes available scientific data reported by the most recent studies describing the antimicrobial activity of *C. halicacabum.* Furthermore, the review highlights our recent results showing that *C. halicacabum* exerted an inhibitory effect against the dermatophytic fungus *Trychophyton rubrum.* In addition, by molecular docking simulations, we showed that the fungistatic effect of *C. halicacabum* may be related to the molecular interaction of its bioactive compounds with the fungal Hsp90, compromising the biological functions of the chaperone[24]. These findings are supported by recent reports demonstrating the pivotal role played by the Hsp90 in the virulence of the pathogenic microorganisms like bacteria, fungi, and human protozoan. Based on our results, herein we firstly propose that the chaperone Hsp90 in pathogens could be a potential therapeutic target for *C. halicacabum*, defining a possible mechanism of action underlying its antimicrobial properties. Finally, the future therapeutic utility of this herb in the treatment of infectious diseases is also discussed.

Antimicrobial Properties of *Cardiospermum Halicacabum*

Antibacterial Activity

It has been reported that *Cardiospermum halicacabum* exerts an important antimicrobial activity against numerous bacteria responsible for the most common human infectious diseases. Depending on the different solvents or extraction techniques, a different degree of inhibition can be detected. Both the aqueous and alcoholic extracts exhibit a marked activity against Gram positive and Gram-negative bacterial strains, although some reports[7] suggest that the aqueous extract displays more effective than the alcoholic one. In detail, among Gram positive species, *Bacillus subtilis* and *Bacillus cereus* appeared to be more susceptible than *Staphylococcus aureus* to the aqueous leaf extract, whereas among Gram negative bacteria, *Escherichia coli* was more sensitive to aqueous than the alcoholic extract[7]. On the other hand, the ethanolic fraction was found to be more effective than the aqueous extract against Gram positive bacterial strains, especially *Staphylococcus epidermidis, Streptococcus faecalis, Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus*[23,25-27]. The ethanolic extract also displayed remarkable inhibitory activity against various Gram-negative bacteria, especially *Escherichia coli*[23] and *Citrobacter freundii*[26]. By contrast, it exerted only moderate inhibition of the multidrug resistant *Shigella spp., Salmonella typhi, Klebsiella pneumoniae,* and *Enterobacter*[25] while exerted minimal or no activity against *Pseudomonas spp.*[23,25]. The last one was found to be more susceptible to benzene followed by acetone, ethanol, and aqueous extracts[23,25-27]. A considerable

activity against *Streptococcus faecalis, Bacillus cereus, Bacillus steadher,* and some Gram-negative species, including *Salmonella typhi, Shigella boydii, Klebsiella pneumoniae*, and *Enterobacter,* was also obtained with the oil fraction[25]. Furthermore, the butanol extraction showed a strong inhibitory effect against *Micrococcus luteus* and a modest activity against *Staphylococcus epidermidis* and *Streptococcus faecalis*[25]. The ethyl acetate fraction was found to exert a remarkable antibacterial activity against *Streptococcus faecalis, Staphylococcus epidermidis, Micrococcus luteus*, and *Klebsiella pneumoniae.* The same fraction also displayed a significant inhibition against *Shigella boydii, Salmonella typhi* and *paratyphi B*, and *Enterobacter*[25]. Finally, the chloroform was seen to be more effective against *Staphylococcus aureus* than alcohol and ether extractions but showed no inhibition against *Escherichia coli*[26]. Altogether these data suggest that the natural extract of this plant is able to exert antibacterial activity against a broad spectrum of bacterial pathogens. The type of the extracting solvent can significantly influence its antimicrobial properties, probably by affecting the content of the bioactive compounds present in the plant.

Antifungal Activity

C. halicacabum displays *in vitro* strong antifungal properties against the most common pathogenic fungi responsible for opportunistic infections in immunocompromised individuals. As reported by Shareef et al[25] the ethanol extract showed a consistent inhibitory effect against the opportunistic fungal species S*accharomyces cerevisiae* and *Aspergillus niger.* However, the ethanol, aqueous, and oil extracts displayed only a moderate inhibition against the opportunistic yeast *Candida albicans*[25]. On the other hand, the ethanol extract exhibited an important antifungal activity against *Candida albicans* ATCC 227[23]. Additionally, we previously demonstrated that the methanol extract exerts a clear-cut and dose-dependent inhibitory effect against the anthropophilic dermatophyte species *Trichophyton rubrum*[24]. Furthermore, seed oil has been shown to be active against zoophilic pathogenic dermatophytes *Micosporillum gypsiccus* and *Trychophyton mentagrophytes*[25]. By contrast, ethanol, aqueous, and oil extracts did not influence the *in vitro* growth of dermatophytes *Trichophyton longifusis*, *Trichophyton tonsurans*, and *Microsporum canis*[25].

Antiparasitic Activity

Among medicinal plants, *C. halicacabum* holds promising antiparasitic properties. The leaves ethyl acetate extract of the plant was found to have moderate antiplasmodial activity against a Chloroquine (CQ)-resistant strain of *Plasmodium falciparum*, whereas the dichloromethane methanol (1:1) whole balloon plant extract showed good *in vitro* antiplasmodial activity[28,29]. Furthermore, its shoot water extract displayed a weak antiplasmodial activity *in vitro*, while there was no evidence of its protective effect in a murine model of malaria[30]. The *in vitro* antifilarial activity of ethanol and aqueous extracts of *C. halicacabum* against *Brugia pahangi* was also investigated[31]. The aqueous extracts showed a mild reduction of the motility of adult worms in a concentration and time dependent manner, and significantly reduced the pattern of microfilarial release from female worms. Furthermore, the ethanol extract, at the highest concentrations used, rapidly inhibited both the motility of adult worms and the release of microfilariae from females.

Hsp90 Chaperone as a Potential Molecular Target of Cardiospermum Halicacabum in Pathogens

While the antimicrobial activity of *C. halicacabum* has been well established, there is no evidence regarding the cellular/molecular mechanisms underlying its inhibitory activity against pathogenic microorganisms. By using the molecular docking analysis we recently proposed, for the first time, that Hsp90 chaperone could be involved in the fungistatic activity of *C. halicacabum* against the fungus *Trychophyton rubrum*[24]. Targeting the Hsp90 chaperone, *C. halicacabum* could interfere with the biological functions of the protein, similarly to the Hsp90 inhibitor geldanamycin, a benzoquinone ansamycin antibiotic, which targets the ADP/ATP binding pocket in the Hsp90 chaperone[32,33].

Hsp90, a highly conserved and ubiquitous ATP-dependent molecular chaperone, has been identified from bacteria to mammals. In eukaryotes, Hsp90 is essential for cell viability under all tested growth conditions[34]. Its activity requires collaboration with several Hsp90 co-chaperones. Together with Hsp70 chaperone, it plays a crucial role in the activation and stabilization of a variety of different client proteins, including many kinases involved in cell-signalling pathways. As for other classes of molecular chaperones, Hsp90 has a critical ATPase activity. Consistently, the

structural changes induced by the ATP binding in Hsp90 play a key role in co-chaperone and chaperone client protein interactions. Hsp90 is a conformationally dynamic dimeric protein (Figure 1). The N-terminal domain (NTD) contains a unique ATP-binding pocket and co-chaperone interacting motifs[35,36]. The ATP binding to the N-terminal domain and its subsequent hydrolysis by Hsp90 triggers a conformational cycle that is crucial for the chaperone activity[37]. The heat shock proteins have been extensively studied in cancer because they are important regulators of cellular proliferation and differentiation. Moreover, they are strongly implicated in the molecular orchestration, which leads to the initiation and/or progression of cancer[38]. This fact conditioned an increasing development of several Hsp90 and other Hsps inhibitors that have shown promising results, both in pre-clinical and clinical studies, in the treatment of tumor diseases[39,40]. The application of some Hsp90 inhibitors as anticancer agents has raised considerable interest in this protein also as a target for new antifungal therapies. Recently, many investigations suggest the crucial role played by Hsp90 in the microbial virulence and pathogenicity[41-43]. Studies on the interrelationship among antifungal drug resistance, survival under stressful conditions, and the signalling pathways involved in these processes in fungi, highlighted Hsp90 as a novel potential molecular target for antifungal therapy. Its involvement in the pathogenicity and resistance to azole and echinocandin antifungal drugs of the most clinically important fungi, i.e., *Aspergillus fumigatus*, *Candida albicans*, *Cryptoccoccus neoformans*, and *Trychophyton rubrum*, is well established[41,42,44,45]. In particular, the genetic or pharmacological inhibition of Hsp90 by geldanamycin blocks the emergence of azole resistance in both fungal yeasts *Saccharomyces cerevisiae* and *Candida albicans*[44]. Furthemore, Hsp90 governs the phenotypic switching of *Candida albicans*

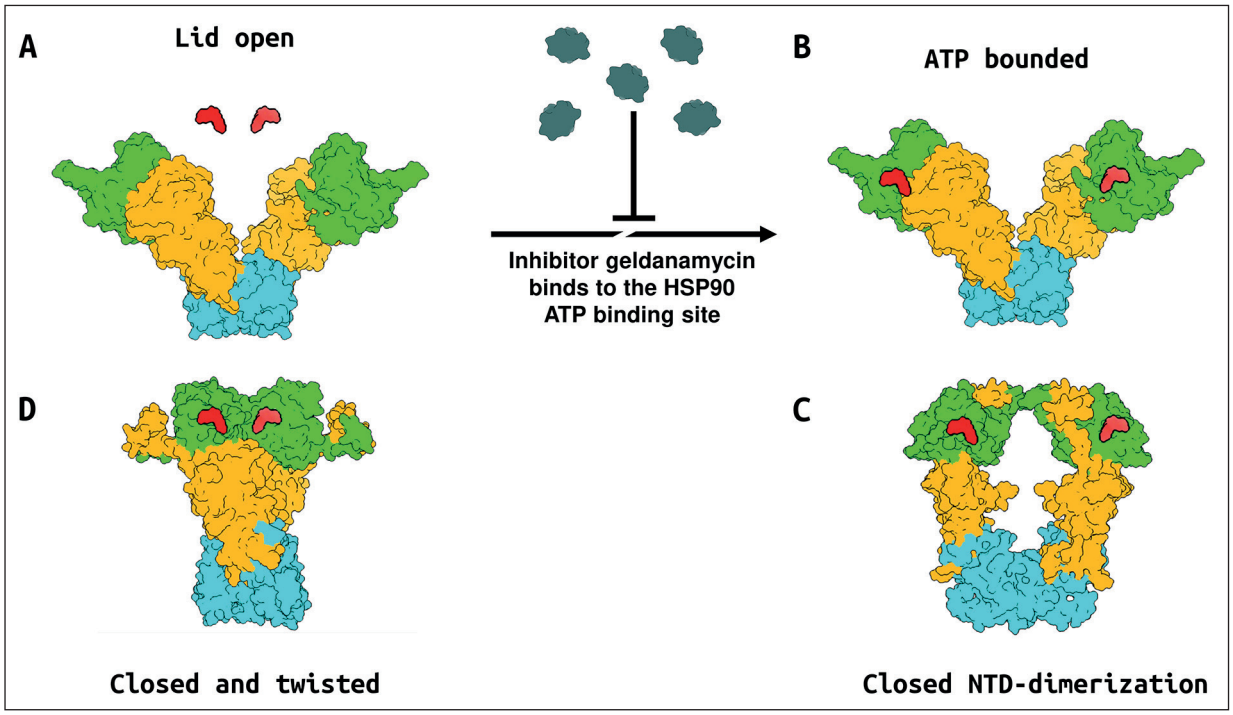

Figure 1. ATP-coupled functioning cycle of Hsp90. ***A,*** In the resting state Hsp90, through the dimerization of its C-terminal domain (cyan), forms a homodimer with an open V-shaped conformation. ***B,*** Upon ATP (red) binding to the N-terminal ATPase domain, Hsp90 undergoes significant conformational changes and through an intermediate-state the N-terminal lids close and subsequently ATP is incorporated in the nucleotide-binding pocket. ***C,*** This results in a dimerization of the N-terminal domains (green) of each homodimer followed by closure of Hsp90 ***(D)*** and recruitment of the M domain (orange) for ATP hydrolysis. Finally, ATP is hydrolyzed and Hsp90 reverts to the open conformation where the N-terminal domains dissociate, allowing repetition of the cycle. Hsp90 inhibitors like geldanamycin (grey), radicicol, and purine derivatives bind to the N-terminal domain competing with ATP for binding. This picture has been produced using the program Chimera[35] using X-ray structures (PDB IDs: 2CG9, 2IOQ) deposited in the Protein Data Bank[36].

from yeast to hyphal growth. The morphogenetic plasticity of the fungus is implicated in its virulence, since mutants defective in the yeast to filament transition are attenuated in terms of virulence[46,47].

These reports prompted us, in a previous work, to speculate about the potential interaction of *C. halicacabum* with the Hsp90 of *Trychophyton rubrum*, the main fungus involved in superficial mycoses. Using molecular docking simulations, we analyzed the interaction of 13 compounds of *C. halicacabum* (Table I) with the ATP-binding site of the fungal Hsp90[24]. Intriguingly, among the 13 plant compounds tested, the flavonoids luteolin-7-*O*-glucoside and rutin were identified as the most important potential Hsp90 inhibitors, although all tested compounds showed an affinity for the fungal Hsp90 ATP-binding pocket. These results were supported by *in vitro* agar diffusion tests showing a direct remarkable antifungal activity of the single flavonoid components on *Trychophyton rubrum*. A considerable antifungal activity of luteolin-7-*O*-glucoside against other fungi like *Aspergillus fumigatus*, *Aspergillus niger*, and *Alternaria alternata,* has already been described by Chiruvella et al[48]. Furthermore, herein we demonstrate that among the numerous components of *C. halicacabum*, also the all-trans-retinoic acid (ATRA), an isomer of the retinoic acid representing the oxidized form of vitamin A, is capable of exerting a clear dose-dependent fungistatic activity *in vitro* against *Trychophyton rubrum* (Figure 2). These results are in line with our previous analyses showing that ATRA inhibited *in vitro* the germination of *Candida albicans* and *Aspergillus fumigatus* in a dose-dependent manner[49]. Of note, when the compounds luteolin-7-*O*-glucoside, rutin, or ATRA were individually tested against the *Trychophyton rubrum* growth, their antifungal action resulted in

Table I. *Cardiospermum halicacabum* compounds are potential molecular interactors of *Trychophyton rubrum* Hsp90. In a previous work the docking energies of all listed *C. halicacabum* compounds have been obtained through a molecular docking simulation, using a structural model of *Trichophyton rubrum* Hsp90 as a receptor. The results indicated that all 13 bioactive compounds tested were potential Hsp90 interactors, although the 2 flavonoids, rutin, and luteolin-7-O-glucoside, showed the highest affinity for the ATP site-binding of fungal Hsp90[24].

Compound name
Rutin
Luteolin-7-O-glucoside
Kaempferol
Apigenin
Cholecalciferol
All-trans-retinoic acid (ATRA)
Quercetin
Chrysoeriol
Calycosin-7-O-beta-D-glucopyranoside
1-Hentriacontanol
Pentadecanoic acid
3,4-Dihydroxybenzoic acid
3,4-Dihydroxybenzaldehyde

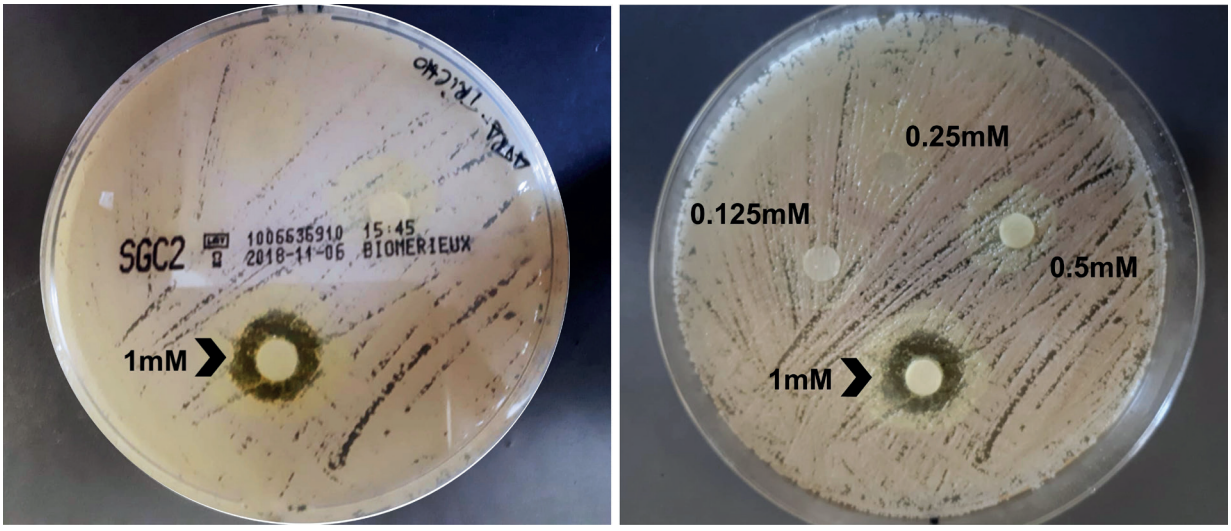

Figure 2. Disc diffusion assay of antifungal activity of ATRA against *Trichophyton rubrum*. Fungal cultures were incubated at 30°C with all trans retinoic acid ATRA at 1, 0.5, 0.25, and 0.125 mM/disc. After 24 hours of incubation, a clear-cut zone of inhibition was observed in the area treated with 1 mM of ATRA. One of the 3 representative experiments is shown.

being lower than that obtained with the total plant extract. Therefore, the antimicrobial effect of *C. halicacabum* could be the result of a synergistic interaction between the individual bioactive compounds, which altogether can work in a concerted way, enhancing the antimicrobial properties of the whole plant extract.

In addition to fungi, the role of the heat shock proteins as virulence factors in human protozoan parasites and bacteria has been speculated. Particularly, Hsp90 is involved in critical cellular processes such as stage development in important human pathogens like *Leishmania donovani*[50], *Trypanosoma cruzi*[51], *Toxoplasma gondii*[52,53], and *Plasmodium falciparum*[54]. In bacteria, similarly with their eukaryotic counterparts, Hsp90 is involved in protein folding and it has also been implicated in the virulence of these microorganisms[55]. It has been reported that the antibacterial activity of the selected tropical plant extracts may be attributed to their high content of phenols and flavonoids[56,57]. In particular, flavonoids have multiple bacterial cell targets. One of their mechanisms of action consists in their ability to interact with bacterial proteins either by non-specific forces or by covalent bond formation, inactivating microbial adhesins, enzymes, and cell envelope transport proteins[23,58,59]. Lipophilic flavonoids may also disrupt microbial membranes reducing the fluidity of outer and inner layers[60]. Based on our results, it may be assumed that the antibacterial activity of the *C. halicacabum* total extract could be due, not only to the ability of flavonoids to form complexes with extracellular soluble proteins and cell wall, but also to its ability to interfere with the Hsp90 function by competing with ATP. Therefore, the Hsp90 might be a novel and very promising therapeutic target of *C. halicacabum* against clinically important pathogens.

Conclusions

Our results suggest that the crude plant extract of *C. halicacabum* may be an important natural source of biologically active compounds able to exert an efficient antimicrobial activity against a wide range of microorganisms. Particularly, the antibacterial property of the plant involves a broad spectrum of Gram positive and Gram negative microbes responsible for hospital-acquired/associated infections. Among Gram positive bacteria, *C. halicacabum* exerts an inhibitory action on *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*, which are the major agents implicated in nosocomial infections. Over the past two decades mainly multi-drug resistant bacterial strains such as methicillin (MRSA) or vancomycin (VRSA) resistant staphylococci or vancomycin resistant enterococci (VRE), represent a global concern[61-63]. So, there has been an increasing need for the discovery and development of new antimicrobial agents. Natural products, from this perspective, could represent an alternative therapeutic strategy, overcoming the drug resistance in pathogens. Furthermore, *C. halicacabum* is also effective against the Gram negative *Escherichia coli, Klebsiella pneumoniae*, *Citrobacter*, *Enterobacter,* and *Pseudomonas spp.* These bacteria are considered the emerging nosocomial pathogens responsible for different types of infections, ranging from uncomplicated urinary tract infections to life-threatening sepsis in health care settings. The latter is to date still uncontrollable, especially those caused by extended spectrum beta-lactamase (ESBL)-producing bacteria. In fact, these strains are resistant to the most beta-lactam antibiotics and often also exhibit co-resistance to many other classes of antimicrobial drugs, such as aminoglycosides and quinolones, resulting in a marked limitation of the therapeutic options[64]. In addition to its antibacterial action, an important fungistatic activity of *C. halicacabum* has been shown against *Candida albicans* and *Aspergillus fumigatus*, the two most common opportunistic fungi which can be harmful to health. Indeed, they are responsible for systemic and fatal infections in immunocompromised individuals, especially those undergoing solid organ or bone marrow transplantation, with diabetes mellitus, haematological malignancy, human immunodeficiency disorder, or long-term corticosteroid therapy recipient. The plant also exhibits antifungal activity against *Trychophyton spp.*, a group of pathogenic fungi that are involved in superficial mycoses but can also cause deep and invasive infections, especially in severely immunocompromised patients[65]. Despite the increase in the spectrum of available antifungal drugs, the choice of suitable therapies for treating systemic or superficial mycoses remains relatively limited. This is due to multiple factors, such as the emergence of drug-resistant fungal strains and the heavy side effects and toxicity associated with current antifungal agents. Over-

all, these investigations make *C. halicacabum* a very promising candidate for novel antimicrobial therapeutic strategies, overcoming the major problems related to the drug resistance in microbes and to the side effects of conventional antimicrobial therapies. Furthermore, the Hsp90 chaperone of microbial pathogens could be a possible therapeutic target for *C. halicacabum*. The potential use of chaperones as molecular drug targets could be considered unattractive because of the similarity of the molecular structure between human and microbial chaperones. However, they exhibit different dependencies on chaperone-dependent pathways and could therefore display differences in the sensitivity of their inhibition[43]. In conclusion, Hsp90 could not be the only possible target for *C. halicacabum,* since multiple plant bioactive compounds may interact with different molecular targets both in a single or multiple intracellular pathways. Additional studies are required to fully define the precise mechanisms underlying the antimicrobial effect of the plant. Furthermore, despite the great body of evidence regarding the antimicrobial activity of *C. halicacabum,* most of the studies reported in the literature have been conducted *in vitro*. The traditional practitioners in India currently prescribe the leaves of the plant to the patients, in consideration of their many beneficial uses without any toxic effect[16]. Therefore, further investigations are required, in *in vivo* experimental models, to assess the efficacy and safety of this plant, for its potential future research translation into clinical practice.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

All authors contributed toward drafting and critically revising the manuscript, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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