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The action of berry phenolics against human intestinal pathogens

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Abstract. Phenolic compounds present in berries selectively inhibit the growth of human gastrointestinal pathogens. Especially cranberry, cloudberry, raspberry, strawberry and bilberry possess clear antimicrobial effects against e.g. salmonella and staphylococcus. Complex phenolic polymers, such as ellagitannins, are strong antibacterial agents present in cloudberry, raspberry and strawberry. Berry phenolics seem to affect the growth of different bacterial species with different mechanisms. Adherence of bacteria to epithelial surfaces is a prerequisite for colonization and infection of many pathogens. Antimicrobial activity of berries may also be related to antiadherence activity of the berries. Utilization of enzymes in berry processing increases the amount of phenolics and antimicrobial activity of the berry products. Antimicrobial berry compounds are likely to have many important applications in the future as natural antimicrobial agents for food industry as well as for medicine.

1. Introduction

Berries have a long tradition in folk medicine in many Nordic countries. Bilberry, for example, has through the ages been used as a medicinal herb for treatment of diarrhea and to improve night vision, and cranberry for urinary infections [40]. Today berries are important part of the daily diet for many people in the northern region, where almost 40 edible berries are grown. The most well-known and also commercially most important wild berries are lingonberry and bilberry, the annual crop of which has been estimated to be as large as 500 million kg and 250 million kg, respectively, in Finland only [45]. Thus wild berries in the Nordic region form an enormous natural source, only a fractional part of which has been advantaged so far. In North America cranberry along with blueberry are two important native fruits which are commercially grown. Around 500 million tons of cranberries are produced every year. Only 5% of the annual crop is harvested for fresh fruit and most of it is used for processing. Cranberries are used as ingredients in over 700 products. Cranberry juice is one of the important products produced by food processing industry [44].

Berries are rich in various phenolic compounds possessing interesting biological activities [20,21]. Antimicrobial activity of the berry compounds have attracted interest, because recent studies suggest that they may affect the behaviour of human pathogenic bacteria [6,24,29,32–34,43] (Fig. 1). Some studies also indicate that bioactive berry compounds act as new type antimicrobials, which control a wide range of pathogens and may over come the issue of antibiotic resistance. Pathogenic bacteria or toxins produced by bacteria often enter human body via food or drink causing symptoms or illness with several mechanisms. *Campylobacter jejuni, Escherichia coli* and *Salmonella enterica* sv. Typhimurium have

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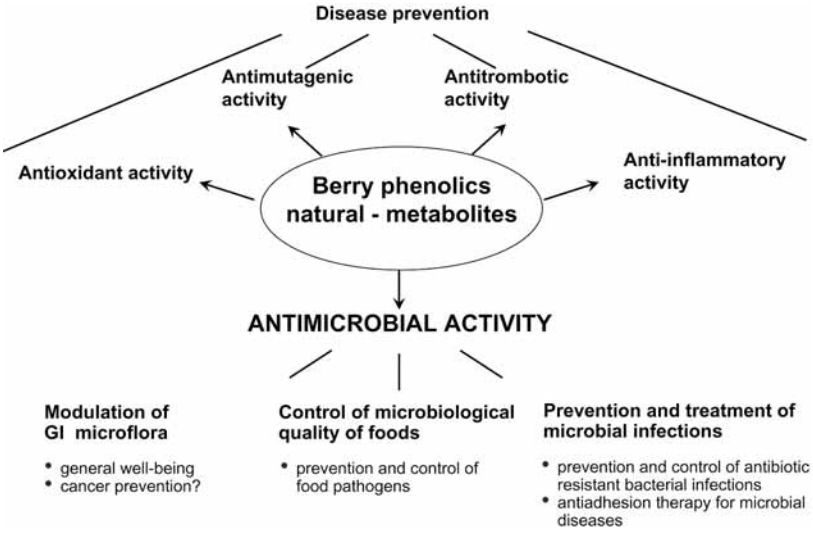

Fig. 1. Effects of antimicrobially active berry compounds against human pathogenic bacteria.

caused foodborne and waterborne outbreaks of GI tract infections in human, and *B. cereus*, *Clostridium perfringens* and *Staphylococcus aureus* are causative agents of food poisoning by producing toxin in food, followed by toxic symptoms in human. Opportunistic human pathogen *Candida albicans* is common organism in human normal flora, but also the most common pathogenic yeast causing infections both on the skin and in human body [39]. This review focuses on antibacterial properties of phenolic compounds present in berries and their effects on human microflora, especially gastrointestinal microflora.

2. Phenolic compounds in berries

Phenolic compounds are secondary metabolites ubiquitous in all higher plants. Many of them act in plant as defence compounds e.g. against plant pathogens, and they are often induced as a response to various stress conditions. Phenolics occur in plant tissues mainly as glycosides, or as complex polymerized molecules with high molecular weights. Flavonoids, phenolic acids, lignans, stilbenes and polymeric tannins are typical for berries (Fig. 2).

All berries contain flavonoids called flavonols. Biosynthesis of flavonols is stimulated by light, thus they are often accumulated in the outer surfaces of berry fruit, and peels are rich sources of them. Highest flavonol contents are found for example in cranberry, whortleberry, lingonberry and blackcurrant (50–200 mg kg^{-1} fresh weight) [20]. Anthocyanins (anthocyanidin glycosides) are the predominating group of flavonoids present in berries. They appeared as colored substances, responsible for the characteristic deep red or blue color of many berries. Their contents are highest in black currant and bilberry (up to 2000–5000 mg kg^{-1} fresh weight) [26]. Phenolic acids are also common in all berries. They are either derivatives of hydroxycinnamic acid or hydroxybenzoic acid. Chlorogenic acid, which is an ester between caffeic acid and quinic acid, is frequently found in many berries.

Flavonoids and phenolic acids form the building blocks for polymeric tannins, which can be classified into hydrolysable and condenced tannins. Hydrolysable tannins are glucose esters of gallic and ellagic acids and condenced tannins have a flavonoid core as a basic structure. Berries, especially of family *Rosaceae*, genus *Rubus* (red raspberry, arctic bramble and cloudberry) are rich in hydrolysable tannins

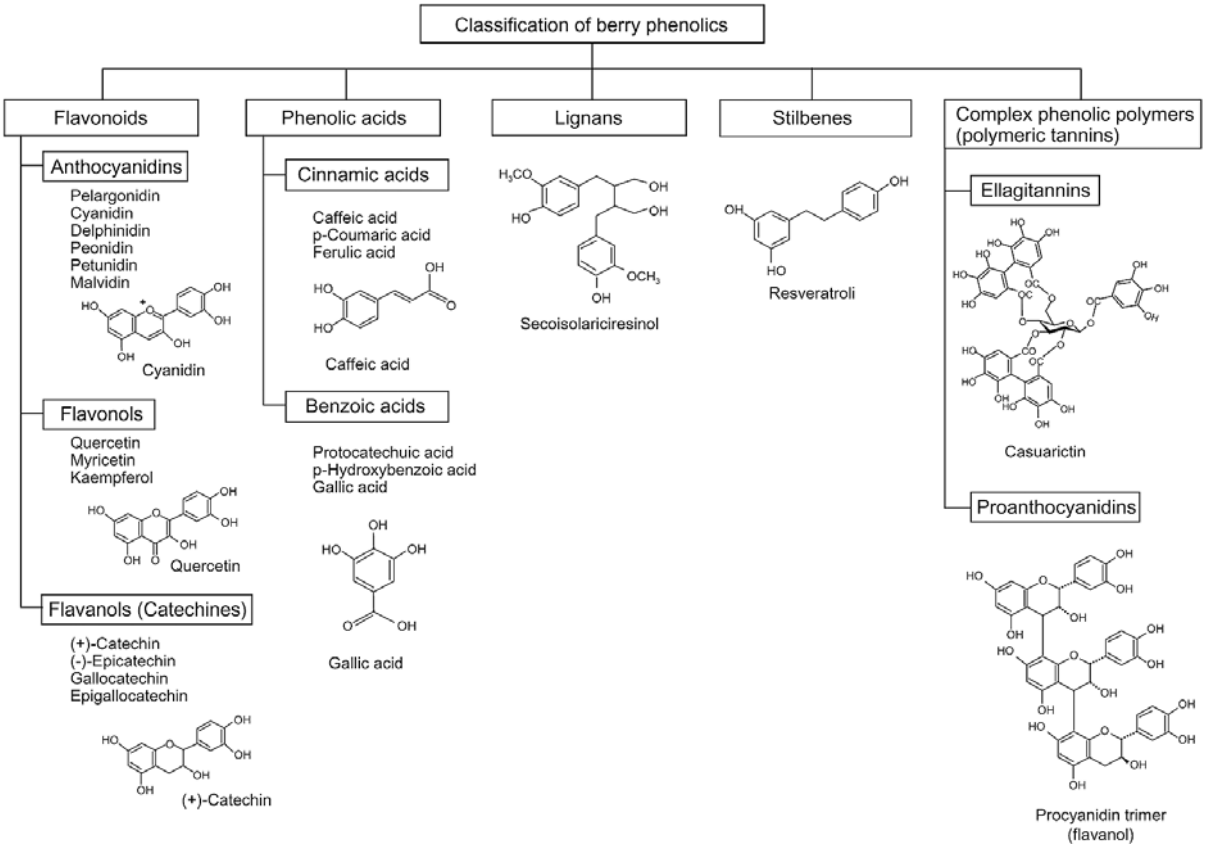

Fig. 2. Phenolic compounds present in berries.

called ellagitannins. Ellagitannins are not found in any other common foods in the Nordic diet, so these berries remain the most important sources of them. Some berries, such as cranberry, also contain condenced tannins, called proanthocyanidins, which are formed of catechin units [8]. Also stilbenes are found in some berries. Lingonberry, for example, contains as much resveratrol as grapes (6 mg kg^{-1} fresh weight) [37]. Lingonberry, strawberry and cranberry are examples of berries rich in diphenolic compounds called lignans, which belong to phytoestrogens (10–15 mg kg^{-1} dry weight) [25].

3. Antibacterial activity of berry phenolics

The antimicrobial activities of the pure phenolic compounds are widely studied. However, there is very little information about antimicrobial activity of the berries, which contain a very complex mixture of phenolic compounds, specific for each berry species. We have extensively studied the antimicrobial effects of Nordic berries, focusing to the effects of berry phenolics on human gastrointestinal bacteria. Antibacterial activity of 17 pure phenolic compounds representing flavonoids and phenolic acids, and 12 phenolic extracts from common Finnish berries against selected Gram-positive and Gram-negative bacterial species, including probiotic bacteria and a variety of intestinal pathogens and food poisoning bacteria, have been determined [29,32,33].

According to our results, the degree of hydroxylation might affect the antibacterial activity of pure phenolic compounds. The increasing number of hydroxyl groups in the B ring in flavonols and flavones correlates with increasing antimicrobial activity against lactic acid bacteria. The phenolic acids are active only against Gram-negative bacteria. No other structure/activity relationship was found. These variations in sensitivities against pure phenolic compounds may reflect differences in cell surface structures between Gram-positive and Gram-negative bacteria [33]. Antimicrobial activity of phenolic compounds depends on pH of the growth media in a complex way, which vary in different bacterial species and different phenolic compounds [12,32,46]. Thus, when evaluating the antimicrobial efficacy of phenolic compounds either in food matrices or in human body, pH is a very important parameter to be considered.

Phenolic berry extracts inhibited the growth of *Salmonella, Escherichia, Staphylococcus, Helicobacter, Bacillus, Clostridium* and *Campylobacter* species but not *Lactobacillus* and *Listeria* species. *Salmonella, Staphylococcus, Helicobacter* and *Bacillus* strains were the most sensitive bacteria for the berry extracts. The phenolic extract of cloudberry possessed the strongest antimicrobial activity, followed by raspberry and strawberry. The weakest antimicrobial effects were measured with chokeberry, rowanberry, crowberry and buckthorn berry. Cranberry extract was effective against *Bacillus cereus* and *Clostridium perfringens*. The sensitivity to cloudberry and bilberry phenolics of three different *Staphylococcus aureus* strains were similar showing bacteriocidic effect with cloudberry and bacteriostatic effect with bilberry [29,32,33]. Rauha et al. [34] have also studied antimicrobial effects of some Finnish berry extracts against food poisoning bacteria. They found that the widest bacteriocidal activity was also expressed by berries belonging to the genus *Rubus* (cloudberry and raspberry) which are rich in ellagitannins. Cavanagh et al. [6] confirmed that several fresh berries (liquefied with blender), including raspberry and blackcurrant, inhibited the growth of wide range of human pathogenic bacteria, both Gram-negative and Gram-positive. In their experiments mulberries and boysenberries did not inhibit any bacteria.

In our study [32], isolated ellagitannin fractions from cloudberry and raspberry were highly efficient against *S. aureus*. The antimicrobial properties of tannins present in many plant foods have been well documented [8]. Chung et al. [7] demonstrated that tannic acid but not gallic acid was inhibitory to a variety of food born bacteria, such as *Escherichia coli, Salmonella* ser. Enteritidis, S. ser. Paratyphi and *Staphylococcus aureus*. They found that inhibitory effects were associated with the ester linkage between gallic acid and polyols. In our experiments *Candida albicans* and *Campylobacter jejuni* were sensitive only to cloudberry, raspberry and strawberry extracts of all berries, suggesting that ellagitannins are the main antimicrobial compounds against these microbes [29]. Meanwhile growth inhibition of salmonella was only partly caused by the berry phenolics and ellagitannins, and most of the inhibition seemed to originate from other compounds, such as organic acids [31].

4. Effects of processing and storage on antimicrobial activity

There are very few reports on the effects of frozen storage on berry phenolics and their composition [13, 21,22]. In our recent study the stability of phenolic compounds and their antimicrobial activity against *Escherichia coli* and non-virulent *Salmonella enterica* sv. Typhimurium was studied in berries stored frozen up to one year [29]. The common trend was the decrease of phenolics in berry extracts (e.g. ellagitannins, hydroxycinnamic acids, anthocyanins, and flavonols) during the test period of 12 months, but their antimicrobial activity was not influenced accordingly. In general, antimicrobial activity of berry extracts against type strain of *Escherichia coli* and rough mutant strain of *Salmonella enterica* sv. Typhimurium was constant or increased in berries stored frozen for several months to a year. Especially cloudberry showed constantly strong antimicrobial activity during the storage. Interestingly,

clear increase in antimicrobial effect was observed with black currant against *S*. Typhimurium after nine month storage. This results suggest that structural changes occur in phenolics during long-term storage, and this may effect their antimicrobial activity. Probiotic *Lactobacillus rhamnosus* strains were not affected by berry extracts during the experiment. This data is important for food industry, since they almost entirely use frozen berries due to the short harvesting season.

Berry juices are the main berry products. Raspberry and blackcurrant juices display good antibacterial activity against various bacteria, such as *Enterococcus, Escherichia, Mycobacterium, Salmonella* and *Staphylococcus* species, whereas *Mycobacteria phlei* appeared to be susceptile to all the products [6]. Transportation and storage of berry juice concentrates at low temperatures prior to final packaging is a common practice in the juice industry and introduces a potential risk for postconcentration contamination with pathogenic bacteria. However, Nogueira and co-workers [28] have evaluated the likehood of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* to survive in cranberry juice concentrates at or above temperatures used for transportation or storage. Cranberry juice possessed intrinsic antimicrobial activity that will eliminate these bacterial pathogens in the events of postconcentration recontamination. At least 5-log reduction of all these bacteria was demonstrated.

We have found in our on-going study that enzymatic treatment of bilberry and cloudberry mash before juice pressing increases the amount of total phenolics and antimicrobial activity of the berry juices against *Salmonella* and *Staphylococcus* bacteria. Enzyme treatment released cell wall bound phenolics to berry juices resulting in increased antimicrobial activity. Most probably also structural changes occured in phenolic compounds, which might have effects on their antimicrobial activity. Vattem et al. [42,43] and Vattem and Shetty [44] have recently shown that solid-state bioprocessing of cranberry pomace, using food grade fungus *Rhizopus oligosporus* or *Lentinus edode*, resulted in enrichment of total soluble phenolics and of ellagic acid. They also found that bioprocessing improved the antimicrobial activities of the extracts against important food borne pathogens *Listeria monocytogenes, Vibrio parahaemolyticus* and *Escherichia coli* O157:H7. They hypothesized that the profile of phytochemicals that are present in natural sources confer a broad spectrum antimicrobial capacity and potentially limit the development of antimicrobial resistance because of their possible differences in modes of action. Thus enzyme-aided extraction or bioprocessing of berries may offer an innovative approach to produce broad spectrum antimicrobials against important pathogens.

5. Mechanisms of antibacterial activity of berries

The outer membrane (OM) of Gram-negative bacteria acts as a permeability barrier and is responsible for the intrinsic resistance of these micro-organisms to antimicrobial compounds [9,27]. The effect is mainly due to the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, resulting in many Gram-negative bacteria in an inherent resistance to hydrophobic antibiotics [16,27]. Besides LPS molecules various multidrug efflux pumps also contribute to the resistance of the cells [27].

Although the OM of Gram-negative bacteria protects the cells from many external agents, it is possible to specifically weaken it by various agents that disintegrate its LPS layer. Such agents are collectively termed as permeabilizers [41]. Certain small terpenoid and phenolic compounds found in herbal plants (e.g. essential oil components) have been reported to disintegrate the OM, releasing LPS and increasing the permeability of the cytoplasmic ATP [4,16]. Relatively few data, however, is available concerning the antimicrobial mechanisms from berry-derived purified compounds, although bacteriostatic and antimicrobial activity of berry samples has been reported [31,32,34]. We recently

reported that phenolic extracts of cloudberry and raspberry disintegrated OM of *Salmonella enterica* sv. Typhimurium and Infantis strains as indicated an increase in the uptake of an hydrophobic fluorochrome (NPN) and liberation of [^{14}C]Gal-LPS [29]. For S. Typhimurium and S. Infantis $MgCl_2$ addition abolished majority of the OM-disintegrating activity of raspberry and cloudberry phenolic extracts, suggesting that part of the activity occurs by chelation of divalent cations from the OM.

Nohynek et al. [29] reported that phenolic extracts of black currant, cranberry and bilberry did not increase the NPN uptake of tested *Salmonella* strains. However, in the LPS release assay phenolic extract of black currant, cranberry and bilberry efficiently released LPS from the *S*. Typhimurium and *S*. Infantis. The difference in the efficacy was probably caused by the organic acids present in the samples since in the LPS release assay the phenolic extracts resulted in lower pH (pH 3.4–4.2) than what was found in the NPN uptake assay (pH 5.5–5.8). Efficacy of the samples in LPS release assay can partially be due to the weak organic acids present which are known to be effective in undissociated state. Weak organic acids, such as citric and lactic acid, permeabilize the OM of Gram-negative bacteria [2,17]. Since phenolic extracts of cloudberry and raspberry in the NPN assay destabilized the OM also at pH 5, these preparations are likely to contain other active compounds besides weak organic acids.

Vattem and coworkers [42,43] proposed that antimicrobial activity of cranberry pomace was mainly caused by gallic acid. They suggested that partial hydrophobicity of the gallic acid would allow it to act efficiently on bacterial membranes destabilizing them. We observed that gallic acid effectively permeabilized the tested Salmonella strains destabilizing the OM by chelating divalent cations [29].

Ellagitannin and anthocyanin fractions of raspberry and cloudberry do not permeabilize or disintegrate the OM of the tested *Salmonella* strains [29]. Moreover, cloudberry ellagitannin fractions have been reported to have minor growth-inhibiting activity against *Salmonella* [32]. Hydroxycinnamic acids, due to their propenoid side chain, are less polar than corresponding hydrozybenzoic acids, and this property has been reported to facilitate the transport of these molecules across the cell membrane [5]. Several mechanisms are proposed to explain the antimicrobial activity of tannin include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation or iron deprivation [38]. Kolodzeij et al. [23] evaluated the antimicrobial activity of a total of 27 tannins and related compounds and observed weak to moderate antibacterial activities. Antibacterial action of several tannins on plasma coagulation by *S. aureus* have been examined and Akiyama et al. [1] suggested that mechanism of antimicrobial action of tannin was inhibition of fibrin formation by *S. aureus*.

6. Bacterial antiadhesion activity of berries

Adhesion of bacteria is a vital prerequisite for successful microbial colonization and infection [10, 11,14]. GI tract infections caused by salmonella are initiated by attachment of pathogenic bacterial cells to human intestinal mucus [11]. In our experiments [29] fluorescence viability staining of liquid cultures of salmonella showed viable cells not detectable by plate count adhering to phenolic berry extract. Sonication of the culture released part of the cells followed by 100 times higher numbers of culturable cells in plate count. Therefore, the antimicrobial effect observed by decreasing plate count numbers of *Salmonella enterica* sv. Typhimurium resulted partly from immobilization of viable bacterial cells by the cloudberry, strawberry and bilberry extract. Inactivation and even short-term immobilization of viable salmonella cells by berry extracts could be exploited to prevent their adhesion in human gut. In our experiments lactic acid bacteria did not adhere to berry extracts. We also showed, how *S. aureus* cells adhered to berry extracts, but were dead on the basis of their fluorescence and

plate count [29]. This capability of phenolic strawberry and cloudberry extracts to immobilize and kill staphylococcal cells could be used in further studies and development of pharmaceuticals and treatments against staphylococcal infections.

Bacteriostatic effects of berries due to antiadhesion have been demonstrated with cranberry, but it is very propable that also other *Vaccinium* berries (lingonberry, blueberry and bilberry) possess similar type activities. The antibacterial properties of cranberry juice have been associated with inhibition of *E. coli* adherence to uroepithelium and multiplying by cranberry juice [30,36]. Recent research has demonstrated that proanthocyanidins (condensed tannins) with unique molecular structures isolated from cranberry fruit exhibit potent bacterial antiadhesion activity [18]. Reed et al. [35] have compared differences in structure and bacterial antiadhesion activity of cranberry proanthocyanidins to proanthocyanidins from other foods. They found that only cranberry juice elicited bacterial antiadhesion activity in human urine after comsumption. Cranberry proanthocyanidins are also compositionally different from proanthocyanidins from other foods, characterized by a series of oligomers based on repeating unit structure of catechin with one or more A-type douple linkages. The A-type linkages in cranberry proanthocyanidins are associated with antiadhesion activity [35]. Mice feeding experiments with cranberry proanthocyanidins suggest that a bioactive proanthocyanidin metabolite is present in urine, or properties of the urine are altered by the proanthocyanidins in such a way that adhesion is inhibited [19]. Zafriri et al. [47] have given an interesting hypothesis that cranberry compounds could also be active in the colon. The metabolites of proanthocyanidins (e.g. released oligomers) could act on the colonic bacterial receptors making bacteria not capable to bind any more to the uroepithelium and proliferate [15].

High-molecular-weight constituent of cranberry juice have recently shown to inhibit also adhesion of *Helicobacter pylori* to immobilized human mucus, erythrocytes and cultured gastric epithelial cells [3].

Before these findings related to antiadhesion activities of berry phenolics on harmful bacteria in gastrointestinal tract may lead to new therapeutic strategies, it must be verified that influence on colonization is selective to pathogenic strains, and colonization of beneficial bacteria is not inhibited. In our laboratory such experiments with Caco-2 cell model using various purified phenolic fractions of berries are just in process.

7. Conclusions and future outlook

In recent years knowledge about bioactive berry compounds, especially the phenolic compounds, has increased a lot. Several studies show that berry compounds inhibit the growth of human pathogenic bacteria, such as salmonella, staphylococcus, helicobacter and *E. coli* O157:H7. Potential utilization areas of antimicrobial berry compounds in food industry or in medicine are the following:

- functional foods or beverages targeted for gut well-being and balanced microflora;
- natural food preservatives for foods which are easily contaminated by bacteria;
- packages, dressings and marinades for chicken, meat and seafood;
- products to balance gut microflora and to prevent diarrhoea;
- products to prevent gastrointestinal tract and urinary tract infections;
- products to prevent and treat antibiotic resistant bacterial infections.

Recent findings reinforce the suggestions that berry derived antimicrobials might act on a broad spectrum of bacteria and could be included as an effective addition to traditional antimicrobial compounds and treatments. However, further studies in conditions mimicking food matrix or physiological conditions in humans and clinical trials are needed to verify the mechanisms and antimicrobial activity of the compounds.

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