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Towards sustainable antimicrobials from plants: Some ways to abridge current methodological approaches

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ABSTRACT

Plants are potential sources for new antimicrobials that might be helpful in combating antimicrobial resistance. Screening of plant materials for antimicrobial activities is an essential step towards discovery of new alternatives to current antibiotics. This article presents strategies allowing to simplify some methodological approaches used in initial testing of plant materials for their antimicrobial properties. Particular attention is given to sensitivity testing without extraction based on utilisation of agarose hydrogel tablets and dry powders. Other aspects relate to testing fractionated extracts and rapid determination of inhibition modes directly from zones of inhibition in diffusion assays. Using these approaches could facilitate standardised preliminary screening for new antimicrobials to make this process more productive.

1. The need for new antimicrobials

Antimicrobials are substances used to prevent replication of microorganisms in order to control the spread of pathogens in the environment, and to treat infections. Antibiotics are specifically important antimicrobials for treating human infections. However, their extensive use has led to an increasing global problem of antibiotic resistance [1], with the prediction of 10 million deaths by 2050, if no action is taken [2]. Most antibiotics become ineffective due to the evolution of resistance or tolerance by gene acquisition. The release of antibiotics into the environment (as poorly metabolised waste) selects for antibiotic resistant bacteria that represent an important reservoir of antimicrobial resistance [1,3], with the possibility of further horizontal transfer of genetic determinants of resistance to various microorganisms [4]. Therefore, the development of alternatives or adjuncts to antibiotics is an urgent need [5]. However, this is not an easy task as identification and development of alternative agents requires sustainability at all stages in the pathway from discovery to clinical use [1]. Well-justified and effective resources as well as simple and cheap (but productive) approaches are required. Alternative antimicrobials from diverse natural and synthetic sources should be considered to maximise the chances of finding novel, effective and safe antimicrobials for future use [5].

2. Plants as potential sources of alternatives to antibiotics

Plants have been considered as attractive sources of antimicrobial substances for a variety of different reasons. The number of plant species with significant antimicrobial activity is huge, with many products characterised [6–8]. Many plants with antimicrobial activities are edible and, therefore, not toxic for humans [9]. Plants represent an easily available localised reservoir separated from the bulk environment (e.g. soil and water with their specific biosphere). The attraction to plants is also explained by the long history of traditional medicine that still remains a major modality for treating different diseases (including infections) in many countries [6]. An inspirational example of the power of folk medicine was the Nobel Prize that Youyou Tu received in 2015 for purification of an effective drug artemisinin from *Artemisia annua* that was historically used for the treatment of malaria in China. Purified artemisinin is much more effective than traditional preparations, and its utilisation has saved millions of lives [10,11]. This success story illustrates that the identification and purification of active ingredients and testing their efficacy should be a prerequisite stage in the search for new and effective plant antimicrobials. Determination of the minimum inhibitory concentration (MIC) [12] of high purity drug substance should be an essential step in selecting suitable drug candidates for future development. At the primary characterisation stage, using crude plant extracts may prove misleading as effective natural antimicrobials may be present in only small amounts with limited antimicrobial effect. Using such extracts directly would not adequately represent their full potential for curing infections.

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3. Essential steps from primary screening to purification of new antimicrobials

Generalised schemes and strategies for drug discovery and development pathway from natural products are outlined in a recent comprehensive review [13]. The principles highlighted in this work are highly relevant for the discussion of some specific aspects of discovering new antimicrobials from plants. In particular, the simplification of some approaches and methodologies is discussed below, with the aim of making them more efficient and sustainable.

3.1. Selection of plant material

Choosing plant material, in principle, could be random or based on any preliminary information from the previous research or practice [6]. At this stage, preferences could be given to edible and medicinal plants. Unique experience and knowledge from using medicinal plants could facilitate identifying new biocompatible antimicrobials. This knowledge might be also useful for designing sustainable methodologies for purification and exploitation of novel antimicrobials [8]. Edible plants in particular are attractive due to their safety profile and high contents of polyphenols, which are potent antimicrobials used in food preservation [9]. It is debatable if poisonous plants should be excluded from consideration or not, because the status and nature of their toxicity were rarely evaluated [8] but it is possible that valuable antimicrobials could be separated from poisonous substances. Plant waste from the environment (e.g., fallen senescent leaves and trees) or from the food industry (e.g., non-edible plant parts) could be a valuable source of antimicrobials [14]. Use of such resources can be considered from the angle of effective, sustainable waste management technology. In any plant product evaluation, robust determination of antimicrobial properties of materials should take place.

3.2. Screening for antimicrobial properties

During the evaluation it is essential to initially demonstrate obvious antimicrobial activities of chosen plant materials against a range of bacterial and/or fungal species (especially pathogenic strains). In particular, it is important to include into testing microorganisms from identified “threat lists” highlighting drug-resistant bacteria [2] and pathogenic fungi [15,16] that pose the highest hazard to health and require new drugs to be treated successfully in future.

The most popular assays for detecting antimicrobial properties include disc- and well-diffusion methods, as well as broth microdilution techniques [17,18]. In the diffusion assays, inhibitory effects are visually detectable as circular zones of inhibition (ZOIs) of microorganisms around paper discs or wells containing extracts. Broth microdilution methods rely on measuring reduced density of viable microbial cells as a result of their growth inhibition in a liquid medium. The main limitations and challenges in these methodologies are associated with the lack of standardization and, therefore, reproducibility of results in independent experiments, due to countless protocols at different stages of screening [8]. In addition, all these methods involve preparation of crude extracts in different solvents [6], which contributes to further variations of results.

3.3. Solvents

The choice of solvents for extraction is key when examining the activity of plant extracts. Antimicrobial properties of particular materials often depend on compounds used for extraction. The most prevalent solvents for extraction include water, ethanol, methanol, chloroform, ether and acetone [6] Not every solvent allows preparation of extracts with detectable antimicrobial activities. For example, some water-based extracts do not manifest antimicrobial activities but ethanol-based extracts from the same plant material produce pronounced ZOIs in disc diffusion assays (personal observations). The easiest explanation here would be differential solubility of plant compounds in different solvents. However, actual reasons for such results are not always clear, because after drying out ethanol from discs loaded with ethanolic extracts, ZOIs are generated by diffusion of antimicrobial substances from the discs into water-based media. Similarly, ZOIs from all extracts prepared on any other organic solvents are caused by diffusion of antimicrobial compounds in water-based media. Ideal solvents should not be toxic, to prevent possible interference with bioassays [18]. In most cases, the use of the two most popular solvents (water and ethanol) is sufficient to detect antimicrobial properties of a particular plant material [6]. Both solvents are sustainable as they are environmentally friendly, non-toxic for humans and easily degradable [19].

3.4. Is quantitation needed?

The methods for sensitivity testing mentioned above are quantitative, based on measuring size of ZOIs and MIC. Quantitation of ZOIs is useful for comparing antimicrobial strength of pure antimicrobials, defined mixtures or different crude extracts if the latter are used directly for curing infections (e.g., in topical applications). However, if the ultimate aim is purification of antimicrobial products, quantitation of ZOIs and determination of MIC values in crude extracts are not important at the stage of initial screening - because MIC of a crude extract does not reflect actual MIC of a pure antimicrobial present in the extract. Therefore, drying plant materials for extraction could be omitted, and using juices or crude extracts from fresh materials would be sufficient for initial confirmation of antimicrobial activity.

3.5. Is extraction always necessary?

Undoubtedly, the stage of extraction is essential for characterisation of plant compounds and their purification. The extraction is time consuming, costly, and laborious process requiring solvents, other consumables, and equipment. However, in initial screening of plant materials for the presence of antimicrobials extraction is not always necessary. Fig. 1 shows an example of initial verification of antibacterial activity in anthers of tulips by placing them directly onto bacterial lawns on agar [20]. Although this approach is not accurately quantitative, its value for preliminary screening is obvious. A potentially quantitative approach for sensitivity testing without extraction was used in assessing antimicrobial characteristics of wood materials [21], where solid discs of wood were applied directly to microbes swabbed onto agar, and ZOIs were measured (Fig. 2). Antimicrobial activities of different wood samples were studied by loading sawdust into wells in agar, as depicted in Fig. 3 [21]. Presumably sizes of ZOIs in such assays depend on size of wood particles and their physical contacts with agar surface. In case of gaps between large particles (especially if they are irregular in shape), the contacts are limited, and this in turn compromises the detection of

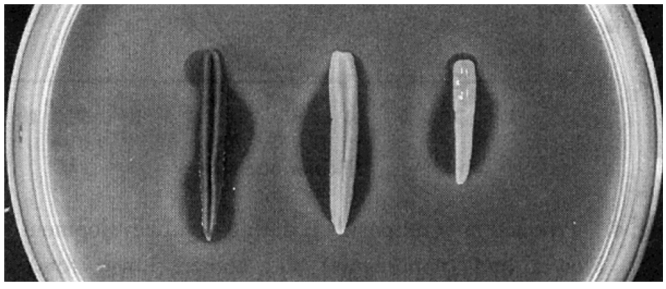

Fig. 1. Antibacterial activity of anthers from different tulips.
Varieties of the tulips include: Halcro (left), Dordogne (middle), and Mirella (right). The bacterial species is not specified. Translucent ZOIs with irregular shape are visible around the anthers. (With the journal’s permission adapted from: [20]).

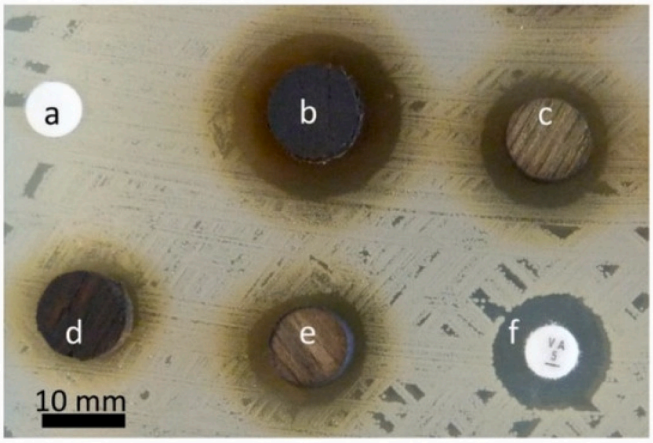

Fig. 2. Testing inhibition of *Staphylococcus aureus.*
Paper discs (6 mm) and wood discs (10 × 3 mm) were applied on a Mueller–Hinton agar plate inoculated with bacteria: (a) negative control (a blank filter paper disc); (b) oak wood transversal cut; (c–e) oak wood longitudinal cut, (f) positive control (a disc with antibiotic vancomycin). (With the journal's permission adapted from: [21]).

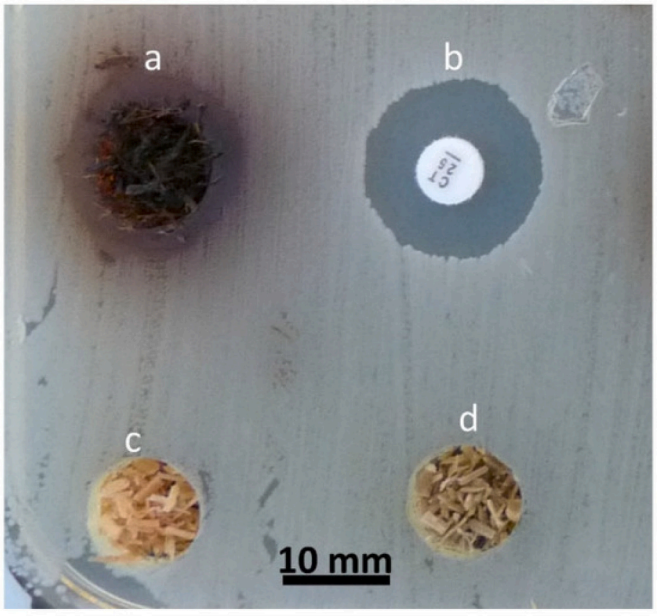

Fig. 3. Testing inhibition of *Acinetobacter baumannii.*
In well-diffusion assays using a Mueller–Hinton agar plate: (a) ZOI around oak wood; (b) positive control (ntibiotic disc with colistin); (c) poplar sawdust with no activity; (d) ash sawdust with no antimicrobial activity. (With the journal's permission adapted from: [21]).

inhibitory effects (and therefore size of ZOIs). To circumvent this, the contact between particles could be increased which will increase diffusion. Therefore, making standardised fine powders with increased surface area is a solution suggested before [18].

We have explored the use of fine dry powders of plant materials embedded into hydrogels in the form of tablets. Application of the tablets directly to agar swabbed with microbial suspensions appears to work well. An example of such assays mimicking disc-diffusion method is shown in Fig. 4. This approach does not require preparation of extracts; it is quick, reproducible, and quantitative.

Using dry powders was quite effective for primary testing of their antimicrobial potential in the author's laboratory. Fig. 5 shows ZOIs generated by powders from a few dry plant materials that were ground (using a coffee grinder) and applied directly to bacteria swabbed onto Mueller-Hinton agar plates. This is the simplest possible approach to sensitivity testing without making extracts. The formation of ZOIs in these assays resulted from the soaking of dry powders in water used to prepare agar plates, local extraction of water-soluble compounds from the powders, and diffusion of these compounds into the agar. Presumably, the particle size in the powders (and therefore total surface area) could affect the size of ZOIs. This trend is observable in Fig. 5; however, the extent of the effects is not massive. Beyond the simplicity and time efficiency this method is economical and environmentally friendly as it does not involve any chemicals routinely used for extractions. Obviously, those materials that produce ZOIs in the form of dry powders should be active in the form of at least water extracts (in disc- and well-diffusion assays). It is still not clear whether this method allows detection of antimicrobial activities of materials for which water extracts do not display microbial inhibition. Nevertheless, the method is very attractive because it allows rapid testing of a wide range of dry powders from different sources in nature.

It is generally accepted that an important characteristic of antimicrobials is the general mode of inhibition (MoI). It could be microbiostatic (not associated with microbial death) or microbiocidal (attributed to killing microbes) [22]. Despite some relativity and peculiarities of these definitions, researchers intuitively tend to think that an effective antimicrobial should preferably kill pathogens rather than to just inhibit them. Nevertheless, a recent systematic analysis showed that overall there is no superiority of bactericidal drugs over bacteriostatic ones in clinical trials [23]. Methods for MoI are based on determining viability of microorganisms exposed to antimicrobials [18,22,24], and they are of different complexity. We proposed a very simple MoI assay based on rubbing agar in central areas of ZOIs (close to discs) with sterile toothpicks or forceps, and streaking onto sectors of a nutrient agar plate. Re-growth of a tested microorganism indicates microbiostatic MoI, and the absence of growth could be interpreted as microbiocidal activity [25]. The advantage of this approach is the possibility to determine general MoI directly from plates with disc-diffusion assays, with the prerequisite that ZOIs are free of contaminants. If contaminants on ZOIs are not excluded, at least the results in favour of microbiocidal inhibition (the absence of growth) are still reliable. In principle, this simple approach could be adapted to well-diffusion techniques, therefore expanding the potential of both diffusion methods, which do not allow discrimination between microbiostatic and microbiocidal MoI [18].

3.6. Fractionation

For discovery of new antimicrobials, initial screening of materials for obvious antimicrobial properties is the first key step. However, antimicrobial activities of crude extracts could result from more than one compound, most likely from a mixture of substances and their interactions [6,8,18]. The same is true for antimicrobial activities of bulk plant materials (including dry powders) without extraction. Fractionation and testing antimicrobial properties of fractions are essential for purification of individual antimicrobials [13]. Fractionation requires preparation of crude extracts from fresh or dried plant tissues. Extraction in water could be justified for a few reasons. Water soluble drugs would be practically preferable for oral and parenteral administration. Water is the cheapest and most sustainable solvent. Homogenisation of fresh plant materials (e.g. leaves and berries) would be an easy straightforward choice for initial sensitivity testing in diffusion assays, primary fractionation and testing antimicrobial activities of fractions in broth dilutions assays.

Gel-filtration chromatography [26] is a good option for initial fractionation of crude extracts because it provides yields of fractions sufficient for antimicrobial activity testing and for further characterisation of active fractions towards purification of individual compounds. Although gel-filtration fractions are quite diluted in concentrations of

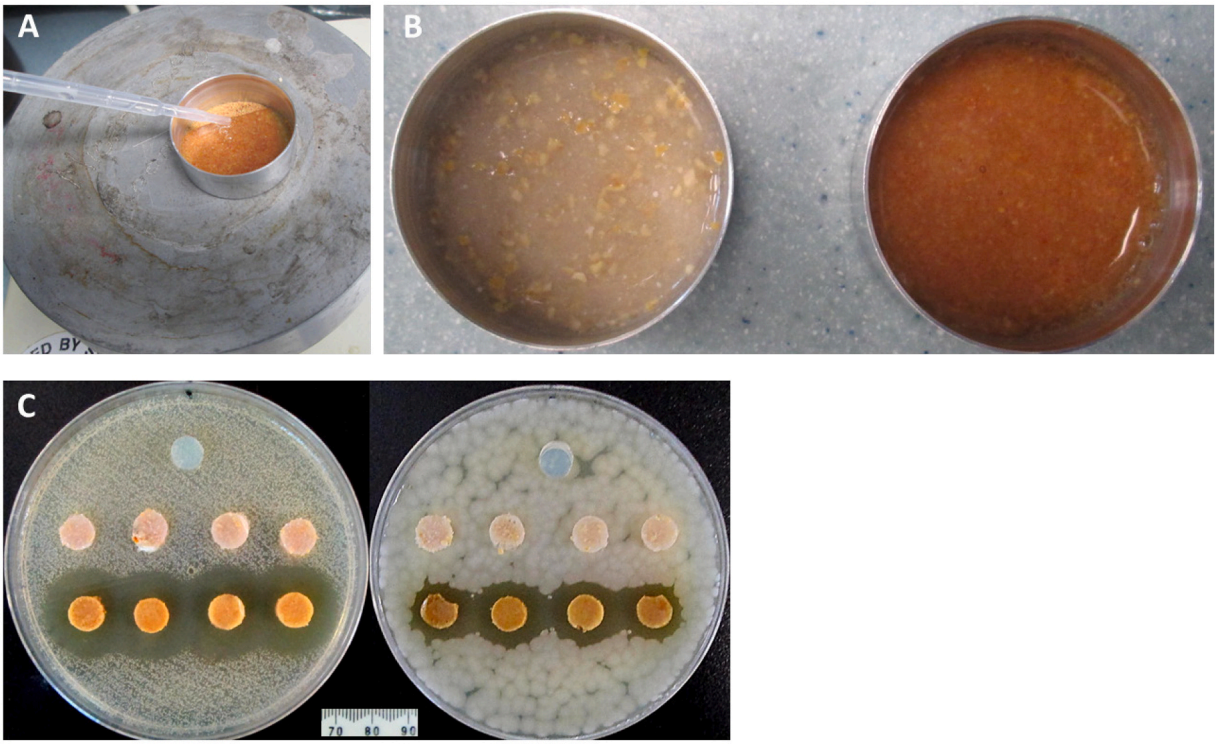

Fig. 4. Using agarose tablets containing pomegranate dry powders for quick testing their antimicrobial properties.
(A) Embedding pomegranate powder (10% in total, *w*/w) into 3% agarose by mixing the components on a hot plate (at temperature of the mix ~75 °C inside of the aluminium tin). Diameter of the tin is 3 cm. (B) Solidified agarose hydrogels containing ground material from seeds (left) and peels (right). Tablets from them (9 mm in diameter) were cut out by using cork borers. (C) ZOIs (bottom rows) after application of tablets with pomegranate peel powder to Mueller-Hinton agar plates swabbed with suspensions of *Staphylococcus aureus* (on the left) and *Bacillus cereus* (on the right), after exposure of the plates to 37 °C overnight. Tablets containing seed powder (upper rows) and controls (single blank agarose tablets at the top) did not manifest inhibition of the bacteria.

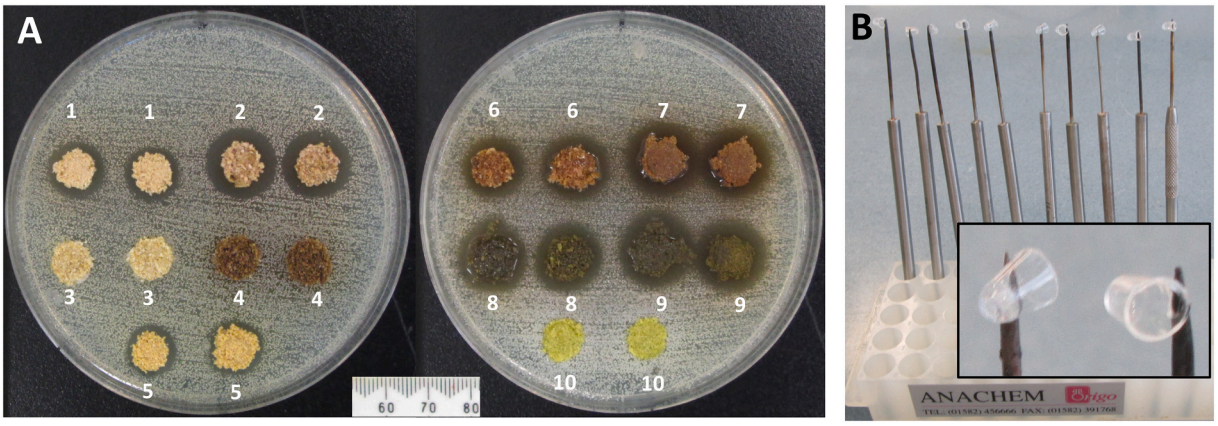

Fig. 5. Testing antimicrobial properties of plant dry powders via direct application of them to bacteria on agar plates.
The same volumes of dry powders were loaded on MRSA bacteria swabbed onto MH agar (A) by using mini scoops (B) made via touching cut bottoms of 0.5 ml Eppendorf tubes with hot (flamed) dissection needles. Alternatively, mini scoops could be made in the same way by using caps cut from Eppendorf tubes (0.5 ml or 1.5 ml). In this experiment, ten different samples (1–10) were used in duplicates: 1 – pink rose petals; 2 – scarlet rose petals; 3 – roots of horseradish; 4 – fig leaves; 5 – senescent leaves of ginkgo; 6 – red rose petals grinded in 10 pulses (2 s each); 7 – (red rose petals grinded in 50 pulses; 8 – red rose leaves grinded in 10 pulses; 9 – red rose leaves grinded in 50 pulses; 10 – inflorescences of parsley. Plates were photographed after incubation at 37 °C during 20 h. ZOIs were observed for seven powders. Three powders did not manifest inhibitory effects.

antimicrobial substances (in comparison to crude extracts) their inhibition of microorganisms may still be detectable in disc- and well-diffusion assays [20] or in automated assays [13]. If detection is not achievable via diffusion-based assays, broth microdilution could be used as a more sensitive method instead, in particular when low concentrations of inoculum of tested microorganisms are used. By using this method, we successfully detected inhibitory capacities of fractions from water extracts of *Viburnum opulus* berries followed by determination of MIC to identify the most active fractions [27]; Fig. 6). Despite fractions were diluted 50 times (due to elution from the column), antibacterial activities against *Pseudomonas aeruginosa* were easily detectable in broth dilution assays at low bacterial load.

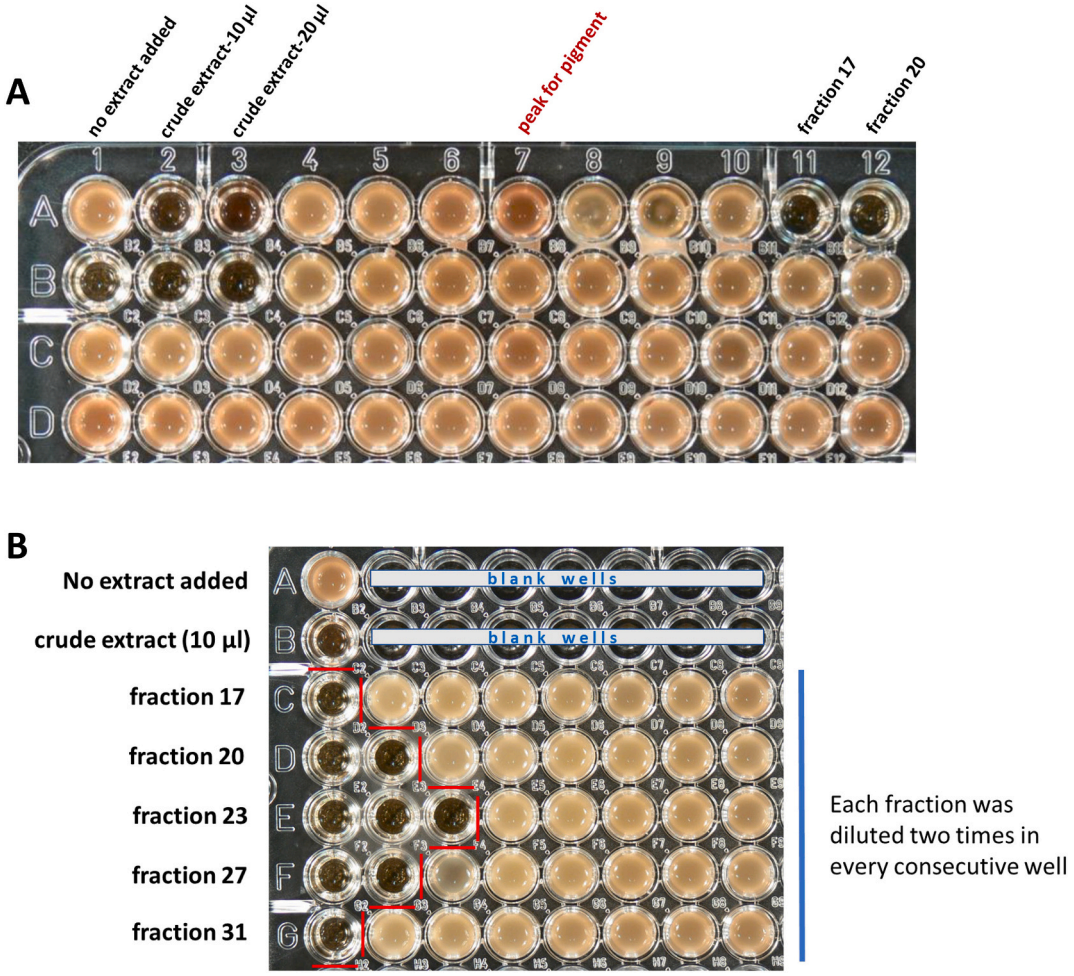

Fig. 6. Identification of active fractions from *Viburnum opulus* extract possessing antimicrobial activities against *Pseudomonas aeruginosa,* and quantification of their inhibitory potential.
(A) Detection of antibacterial fractions in 96-well plate broth dilution assay. Active fractions correspond to wells with no growth (no turbidity). (B) Determination of relative MIC for fractions manifesting antimicrobial activities. The pattern of inhibition of *P. aeruginosa* with diluted fractions reflects peak-like distribution of fractions with different concentration of active substances after chromatography. Images are taken from [27].

3.7. Further work with active fractions

Biologically active fractions identified by resolving peaks of inhibitory activity need to be characterised further for chemical composition and purification of individual substances possessing desirable antimicrobial activities. One of the important criteria for selecting potentially valuable antimicrobials is their low MIC (in pure form), comparable with that of antibiotics.

Ensuring that substances of interest do not manifest mutagenic effects is another important criterium. Preliminary testing of mutagenicity could be easily done using Ames test [28,29]. In this prokaryotic system mutation rates are calculated based on the increased proportion of reverse mutations of prototrophy in *Salmonella* cells treated with substances possessing mutagenic properties. Popular eukaryotic systems for scoring mutation rates in budding yeast operate on varieties of approaches including reversion of auxotrophic point mutations [28], mutations of resistance [30], or high throughput sequencing [31].

Toxicity for host cells is another issue in developing new drugs [32,33]. It has to be evaluated before recommending antimicrobials for trials.

In summary, most effective candidates for new alternatives to antibiotics should possess low MIC values, have no mutagenic effects and low toxicity. Such substances could then be selected for pre-clinical development.

4. Conclusive remarks

Discovery of new antimicrobials is an important step in combatting antibiotic resistance. Exploring plant resources, in particular those used in traditional medicines and edible products, is an attractive option due to ready availability, information on past use, and reported historical success of treatments. They will already have a profile of economic benefits, sound environmental safety, and overall sustainability.

A key prerequisite for succeeding in the discovery of alternatives to current antibiotics is optimising and simplifying multi-factorial research in this area – from justifying resources to implementation of pure antimicrobial products. Abridging any methodological and strategic aspects of typical pathways in such research would promote time-efficient and sustainable research and development.

Initial screening of plant materials could be made easier, cheaper, and more productive. The extraction stage could be omitted in many scenarios of preliminary testing of antimicrobial activities, with the introduction of dry powders or their hydrogels that can be directly applied to tested microorganisms. MoI could then be determined straightforward from ZOIs.

Fractionation, testing antimicrobial activities of fractions, their chemical characterisation, and further purification of active antimicrobial substances would facilitate the entire process of identifying new antimicrobials. Utilisation of sustainable, ecologically friendly

procedures should be considered as a significant criterium at this stage. Obtaining chemically pure antimicrobials with low MIC values and favourable in vitro safety profiles will help to maximise the potential of antimicrobial drug discovery from plants, an abundant and extremely diverse resource.

CRediT authorship contribution statement

Mikhajlo K. Zubko: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

I have analysed data from published literature and some my unpublished experimental data

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