Indian nutraceutical plant leaves as a potential source of natural antimicrobial agents

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The quest for plants with medicinal properties continues to receive attention as they are known for a range of biological activities which range from antibiotics to antitumor. Medicinal plants represent one of the most important fields of traditional medicine all over the world. In light of the recent emergence of bacteria which are resistant to multiple antimicrobial drugs posing a challenge for the treatment of infections, the need to discover new antimicrobial substances for use in combating such microorganisms become pertinent. Bioactive compounds are normally accumulated as secondary metabolites in all plant cells but their concentration varies according to the plant parts. Leaf is one of the highest accumulatory plant part of such compounds and generally it is preferred for therapeutic purpose. Some of the active compounds that inhibit the disease causing microbial growth act either singly or in combination. They can inhibit the growth of the microbes by many mechanisms of actions. It is believed that crude extracts from plants are more effective than isolated compounds due to their synergistic effect. Here we report the antimicrobial activity of five different solvent extracts of four Indian nutraceutical plants viz. *Manilkara zapota* (L.) var. Royen., *Psidium guajava* L., *Punica granatum* L. and *Syzygium cumini* L. leaves against 14 pathogenic microorganisms. The antimicrobial activity was evaluated by agar well diffusion method. *P. guajava* leaves showed best and promising antimicrobial activity, indicating the possibilities of its potential use in the formula of natural remedies for the treatment of infections.

**Keywords** antimicrobial activity; solvent extracts; *Manilkara zapota*; *Psidium guajava*; *Punica granatum*; *Syzygium cumini*

1. Introduction

Infectious diseases account for a high proportion of health problems and burden in India. Large amounts of antibiotics are consumed in fighting infections, some of them saving lives, but every use adds antibiotic resistance in bacteria. Antimicrobial resistance has in fact turned into a global health issue, which hampers the control of infectious diseases; multi-drug resistant bacteria are thus posing a problem in treatment regimens. Multi-drug resistant organisms are also an epidemiological concern as they may spread locally, regionally or globally through individual contacts, poor sanitation, travel or the food chain. During the present millennium, overcoming resistance to antibiotics is one of the major issues facing WHO [1]. Hence, WHO has declared antimicrobial resistance as the theme for the World Health Day, 2011 [2]. Antimicrobials have been used successfully for over six decades to treat infectious diseases; but antimicrobial resistance threatens the continued effectiveness of antimicrobials and simultaneously the downward trend in the development of new antibiotics has serious implications [3].

Resistant bacteria dramatically reduce the possibilities of treating infectious diseases effectively and increase the risk of complications and fatal outcomes for patients with severe infections. The increasing prevalence of multi - drug resistant bacteria and fungi, the recent appearance of strains with reduced susceptibility to antibiotics, the side effects associated with antibiotics, the high costs of antimicrobial drugs and the re-emergence of diseases are the key factors that obstruct effective management of bacterial infections in many developing countries. Unfortunately, the future does not look bright in the war against infectious diseases, as multi drug resistant strains of microbes continue to increase at an alarming rate. In fact, multi drug resistant strains are adapting faster than the introduction of new, more potent antibiotics. Consequently, this has raised the specter of untreatable microbial infections and adds urgency to the search for new infection-fighting strategies. This necessitates greater efforts to discover new potent antibiotics. A potential post-antibiotic era is threatening present and future medical advances.

The development of antibiotic resistance can be natural or acquired. Bacteria usually become resistant to antibiotics by one of the following mechanisms (Fig. 1). Natural resistance is achieved by spontaneous gene mutation and acquired resistance is through the transfer of DNA fragments like transposons from one bacterium to another. Bacteria gain antibiotic resistance due to any of the four reasons namely: (I) by alteration of drug binding or target sites; by alteration of membrane permeability; (II) direct destruction or in ability of the drug to enter in the cell; (III) by developing the ability to produce multi resistance pumps, due to this pump drug drained out of the cells before drug can damage or kill the cell or (IV) replacement of a sensitive pathway and acquisition of a new enzyme to replace a sensitive one [4].

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In light of the recent emergence of bacteria which are resistant to multiple antimicrobial drugs causing a challenge for the treatment of infections, the need to discover new antimicrobial substances for use in combating such microorganisms becomes pertinent. Thus, there is a constant and urgent need to develop new antimicrobial drugs for the treatment of infectious diseases from medicinal plants. Naturally occurring compounds have been and continue to be, an important source of new leads for many pharmaceuticals and agrochemicals. In this context, there is a growing popularity in nutraceutical products or functional foods.

1.1 Medicinal plants as antimicrobials

India is a varietal emporium of medicinal plants and is one of the richest countries in the world with regard to genetic resources for medicinal plants. India is rich in medicinal plant diversity; all known types of agroclimatic, ecologic and edaphic conditions are found within India. India is rich in all the three levels of biodiversity viz. species diversity, genetic diversity and habitat diversity. The increased interest in medicinal plant cures is because, primarily plants as medicines are safe, less rigorous and more affordable than synthetic alternatives. In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines [5]. Many plants contain non-toxic glycosides that can get hydrolyzed to release phenolics that are toxic to microbial pathogens [6]. Phytochemicals or the bioactive compounds in medicinal plants could be used as a novel source in infectious disease management as an alternative to synthetic drugs. Phytochemicals can be derived from any part of the plant like bark, leaves, roots, fruits, seeds, fruit rind, flowers, stem, whole plant, etc. and antimicrobial activity of different parts of the plant is reported in literature [7]. However, leaf is generally the preferred part for therapeutic purpose [8]. This may be because leaves contain more number of secondary metabolites which may be responsible for its antimicrobial activity. They can inhibit the growth of microbes in many ways such as by inhibiting protein synthesis, interfering with nucleic acid synthesis, breaking the peptide bonds, acting as chelating agents, inhibiting metabolic pathway, interfering with cell wall synthesis or by preventing utilization of available nutrients by the microorganisms. Some compounds also cause lyses of microbial cells.

1.2 Nutraceuticals as a novel source of antimicrobials

The traditional and herbal medicines are known as essential resources of nutraceuticals which could provide a variety of beneficial effects on human health. Nutraceuticals or functional foods are natural bioactive, chemical compounds that have health promoting, disease preventing or medicinal properties. The secondary metabolites present in them give them a specific medical benefit other than a purely nutritional. Nutraceuticals have thus dual role to play: as food and therapeutic agent i.e. aids in prevention and/or treatment of disease and/or disorder. The other benefit is, being natural, they have no side effects as other therapeutic agents. Nutraceuticals may range from single isolated nutrients, dietary supplements, or secondary metabolites to genetically engineered designer foods [9]. There are many reports of nutraceutical plant leaves that are effective as antimicrobials (Table 1).
<table>
<thead>
<tr>
<th>Plant name</th>
<th>Extracts</th>
<th>Microorganisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anacardium occidentale Linn.</td>
<td>Petroleum ether, 70% ethanol</td>
<td>Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans, Aspergillus niger</td>
<td>[23]</td>
</tr>
<tr>
<td>Carissa edulis Vahl</td>
<td>Water</td>
<td>Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis</td>
<td>[24]</td>
</tr>
<tr>
<td>Ficus exasperata Vahl</td>
<td>Ethanol</td>
<td>Escherichia coli, Staphylococcus albus</td>
<td>[26]</td>
</tr>
<tr>
<td>Rumex dentatus L. (Polygonaceae)</td>
<td>Hexane, methanol</td>
<td>Staphylococcus aureus, S. Setubal, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Pseudomonas picketti, Bordetella bronchiseptica</td>
<td>[30]</td>
</tr>
<tr>
<td>Rumex ecklonianus Meissner (Polygonaceae)</td>
<td>Acetone, methanol, water</td>
<td>Bacillus cereus, Staphylococcus epidermidis, S. aureus, Micrococcus kristinae, Streptococcus pyogenes, Escherichia coli, Salmonella pooni, Serratia marcescens, Pseudomonas aeruginosa, Klebsiella pneumoniae</td>
<td>[31]</td>
</tr>
<tr>
<td>Thymus vulgaris L. (Lamiaceae)</td>
<td>Water, ethanol, methanol</td>
<td>Pseudomonas aeruginosa</td>
<td>[33]</td>
</tr>
<tr>
<td>Ziziphus mauritiana Lam. (Rhamnaceae)</td>
<td>Aqueous, methanol, saponins</td>
<td>Staphylococcus aureus, Pseudomonas aeruginosa, S. facalis, Klebsiella pneumoniae, Escherichia coli, Enterobacter facalis, Enterobacter faecium, Proteus mirabilis</td>
<td>[34]</td>
</tr>
</tbody>
</table>
2. Objective

The purpose of this work was to investigate the potential of four Indian nutraceutical plant leaves as natural antimicrobial agents. Therefore leaves of four plants viz. Manilkara zapota L. var. Royen., Psidium guajava L., Punica granatum L. and Syzygium cumini L. were selected and their antimicrobial potential was explored against some common pathogenic bacteria and fungi.

3. Experimental methodology

3.1 Chemicals

Petroleum ether, toluene, ethyl acetate, acetone, dimethyl sulphoxide (DMSO), Nutrient broth, Sabouraud dextrose broth, Muller Hinton agar No. 2 and Sabouraud dextrose agar, agar powder were obtained from Hi-media or Merck. All reagents used were of analytical grade.

3.2 Collection of plant materials and preparation of plant extracts

Fresh leaves of four nutraceutical plants were collected in September, 2008, from Jam-jodhpur, Gujarat, India. The ethnobotanical description of the four plants is given in table 2 [10]. The leaves were separated, washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in airtight bottles.

The dried powder of the leaves was extracted sequentially [11] by cold percolation method [12], using different solvents depending upon their polarities like petroleum ether, toluene, ethyl acetate, acetone and water (Flow chart 1).

The extracts were concentrated under reduced pressure, using rotary vacuum evaporator (Equitron, India) to dryness. The dried crude concentrated extracts were stored at 4 °C in a refrigerator in air tight bottles.

Flow Chart. 1 Systematic representation of different solvent extracts of leaves by sequential extraction.
Table 2 Ethnobotanical description of four studied nutraceutical plants.

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant name (Voucher specimen No.)</th>
<th>Vernacular name</th>
<th>Family</th>
<th>Therapeutic uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Manilkara zapota</em> (L.) var. Royen. (PSN429)</td>
<td>Chiku</td>
<td>Sapotaceae</td>
<td>The seeds are aperients, diuretic, tonic and febrifuge. Bark is antibiotic, astringent and febrifuge. Fruits are edible, sweet with rich fine flavour. Chicle from bark is used in dental surgery. Bark is used as tonic and the decoction is given in diarrhoea and peludism.</td>
</tr>
<tr>
<td>2</td>
<td><em>Psidium guajava</em> L. (SU/BIO/510/Thakrar)</td>
<td>Jamphal</td>
<td>Myrtaceae</td>
<td>The roots are astringent, haemostatic, constipating, antiemetic. Leaves are astringent, anodyne, febrifuge, antispasmodic, tonic. Flowers are cooling, laxative, tonic. Fruits are sweet, astringent, sour, cooling, aphrodisiac, laxative, tonic. Roots are used in haemorrhages, diarrhoea, dysentery, ulcers, gingivitis, proctoptisis, vomiting. Leaves are used in wounds, ulcers, cholera, diarrhoea, vomiting, nephritis, cachexia, vata, epilepsy, odontalgia, gum boils. Flowers are used in bronchitis, ophthalmodynia, colic, ulemorrhagia. Fruits are used in pitta, dipsia, burning sensation, colic, ulemorrhagia, diarrhoea, dysentery, debility.</td>
</tr>
<tr>
<td>3</td>
<td><em>Punica granatum</em> L. (PSN311)</td>
<td>Dadum</td>
<td>Punicaceae</td>
<td>The root and stem bark are astringent, cooling and anthelmintic. Flowers are stypic. Fruits are sweet, sour, astringent, cooling, tonic, aphrodisiac, laxative, diuretic. Seeds are astringent, stomachic, diuretic, cardiotoxic. Root and stem bark is used in tapeworm infection, vomiting. Flowers are used in vomiting, pitta, ophthalmodynia, ulcers, pharyngodynia, hydrocele. Fruits are used in anaemia, hyperdipsia, pharyngodynia, ophthalmodynia, pectoral diseases, splenopathy, bronchitis, otalgia. Fruit rind is used in dysentery, diarrhoea and gastralgia. Seeds are used in vomiting, ophthalmodynia, pitta, scabies, hepatopathy, splenopathy.</td>
</tr>
<tr>
<td>4</td>
<td><em>Syzygium cumini</em> L. (PSN295)</td>
<td>Jambu</td>
<td>Myrtaceae</td>
<td>The bark is astringent, sweet, sour, acrid, refrigerant, carminative, diuretic, digestive, anthelmintic, febrifuge, constipating, stomachic and antibacterial. The fruits and seeds are sweet, acrid, sour, tonic and cooling. The bark is useful in diabetes, leucorrhoea, stomachaigia, fever, gastropathy, strangury and dermatopathy. The tender leaves are used for vomiting. The leaves are used for strengthening the teeth and gums. The fruits and seeds are used in diabetes, diarrhoea, pharyngitis, splenopathy, urethrorrhoea and ring worm.</td>
</tr>
</tbody>
</table>
3.3 Microbial strains and culture condition

The bacterial and fungal strains used to assess the antimicrobial properties included five Gram positive bacteria [Bacillus megaterium ATCC9885 (BM), Bacillus subtilis ATCC6633 (BS), Corynebacterium rubrum ATCC14898 (CR), Staphylococcus aureus ATCC25923 (SA), Staphylococcus epidermidis ATCC12228 (SE)], five Gram negative bacteria [Citrobacter freundii ATCC10787 (CF), Enterobacter aerogenes ATCC13048 (EA), Klebsiella pneumoniae NCIM2719 (KP), Proteus mirabilis NCIM2241 (PM), Salmonella typhimurium ATCC23564 (ST)] and four fungi [Candida albicans ATCC2091 (CA), Candida epicola NCIM3367 (CE), Candida glabrata NCIM3448 (CG), Candida neoforms NCIM3542 (CN)]. The investigated bacterial and fungal strains were obtained from National Chemical Laboratory (NCL), Pune, India. The organisms were maintained on nutrient agar and MGYP medium (Hi-media, India) bacteria and fungi respectively, at 4 °C and sub-cultured before use. The microorganisms studied are clinically important ones causing several infections, food born diseases, spoilages, skin infections and it is essential to overcome them through some active therapeutic agents.

3.4 Antimicrobial assay

In vitro antimicrobial activity of the different solvent extracts of the four screened plants was studied against 14 pathogenic microbial strains by the agar well diffusion method [13, 14]. Muller Hinton No. 2 / Sabouraud dextrose agar (Hi-media) was used for antibacterial and antifungal susceptibility test respectively. The extracts were prepared in 100% DMSO at a concentration of 20 mg/ml. The microbial activity was evaluated at a concentration 2.0 mg/well. The Muller Hinton agar / Sabouraud dextrose agar was melted and cooled to 48-50 °C and a standardized inoculum (1.5 × 100% DMSO at a concentration of 20 mg/ml. The microbial activity was evaluated at a concentration 2.0 mg/well. The Muller Hinton agar / Sabouraud dextrose agar was melted and cooled to 48-50 °C and a standardized inoculum (1.5 × 10³ CFU/ml, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile Petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound (100 µl) was introduced in the well (8.5 mm). The plates were incubated over night at 37 °C and 28 °C for 24 h and 48 h for bacteria and fungi respectively. The antimicrobial spectrum of the extract was determined in terms of zone sizes around each well. DMSO was used as negative control. The control zones were subtracted from the test zones and the resulting zone diameter is shown in Fig. 2. The experiment was performed three times to minimize the error.

3.5 Data analysis

The results are expressed as the mean value ± Standard Error of Mean (S.E.M.).

4. Results and discussion

In the present work, the four nutraceutical plant extracts showed considerable antibacterial activity; the various solvent extracts showed 71% activity against Gram positive bacteria and 79% activity against Gram negative bacteria. Generally, it is believed that Gram negative bacteria are more resistant to plant based antimicrobials than Gram positive bacteria [15-17]. This is because the Gram negative bacteria have an effective permeability barrier, comprised of outer membrane, which restricts the penetration of antimicrobial compounds, which extrude plant extracts across this barrier. The single membrane of Gram positive bacteria is considerably more accessible to permeation by plant extracts in region where these bacteria provide limited protection [7, 12, 18]. But contradicting this general belief, in the present work, Gram negative bacteria were more susceptible than Gram positive bacteria. All the five different solvent extracts of P. guajava showed activity against all the ten tested bacteria, while all the five different solvent extracts of P. granatum showed activity against all the five tested Gram negative bacteria (Fig. 2). The most resistant bacteria was S. aureus. The antifungal activity was comparatively less than that of antibacterial activity. It was only 61%. It appears that fungi were more resistant than bacteria. Resistance of fungi to plant extracts can be explained by chitinous structure of the cell wall, which does not allow easy penetration of bioactive substances [19]. There are many reports in literature where plant extracts showed good antibacterial activity but poor antifungal activity [20-22]. All plants extracts showed better antibacterial activity than antifungal activity. P. guajava showed best antimicrobial activity. Overall, a broad spectrum antimicrobial activity was exhibited by four nutraceutical plants. Therefore, it can be stated that all the four nutraceutical plant leaf extracts can be a good source of natural antimicrobics.
Fig. 2 Antimicrobial activity of five different solvent extracts of leaves of *Manilkara zapota*, *Psidium guajava*, *Punica granatum* and *Syzygium cumini*. 
5. Concluding remarks

The broad spectrum activity exhibited by four nutraceutical plant extracts, establishes the scientific basis of their use as ethnomedicine and use their potential for development of novel antimicrobial agents effective for treatment of microbial infectious diseases. Its usefulness in the formulation of antiseptics and disinfectants is also recommended if the active principles can be isolated and purified. On the basis of the present findings, we conclude that amongst four nutraceutical plants, *P. guajava* showed best antimicrobial activity. It might turn out to be a good candidate in the search for effective and efficient antimicrobial agents.

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