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Phytochemical Assay, Cytotoxicity and Antibacterial Activity of Selected Indian Medicinal Plants for Bioprinting Applications

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Over the decade, finding an appropriate biocompatible material has found to be one of the most challenging tasks in translational bioprinting. Developing a biomaterial from herbal plants would support the cell growth and improve the biocompatibility. Indian medicinal plants have potential antimicrobial activity and provide an alternative therapeutic source for treating uropathogens. Hence, the present study evaluated the phytochemical analysis, cytotoxic effects and antibacterial activity of selected Indian medicinal plants against uropathogenic *Klebsiella pneumoniae.* Twenty-four selected Indian plants were used in this study. Biochemical tests and GC-MS methods were used to analyse the phytochemical components of plant extracts. Cytotoxic potential of plant extracts was assessed using human dermal fibroblast cells. Antimicrobial susceptibility test for plant extracts was performed by agar well diffusion method and minimum inhibitory concentration assay. Among the medicinal plants, eight plants showed antibacterial activity against the *K. pneumoniae.* In our study wide variety of phytochemicals were identified in the plant extracts. The MTT assay yielded IC_{50} <250µg for the all the extracts.

Introduction

The use of medicinal plants in the drug preparation to cure illnesses is one of the oldest methods of medical practise. Medicinal plants have made resurgence and they are used to treat wide range of diseases across the world. Biomimetic compounds derived from plants, in particular, are an emerging field of research in medical and dental implants. Over the decade, finding an appropriate biocompatible material has found to be one of the most challenging tasks in translational bioprinting. Developing a biomaterial from herbal plants would confer less immunogenic response, facilitate cell growth and antimicrobial properties. Antimicrobial resistance among the *K. pneumoniae* is alarming and it is important to identify solutions to combat these challenges. Perhaps, natural products can be used as an alternative therapy to fight against the drug resistant *K. pneumoniae* [1]. Long term exposures of antibiotics lead to disruption of normal flora and increased chance of transferring the resistant genes to non-pathogenic organism [2]. Thus, the use of plant based natural products is an attractive field of medicine, since the usage is not only restricted to treating the resistant bacteria, it can be applied in other treatment approaches such as immunomodulatory therapy. Plant based products have been traditionally utilised for centuries and there is ample evidence about the therapeutic potential of plants from ancient epoch till now. Plants produce a large variety of compounds belonging to distinct classes which are responsible for antimicrobial activity [3]. Plants acts as traditional healers which has almost limitless ability to manage and cure common ailments [4]. Plants holds enormous phytochemicals with immense curing properties which aren't explored yet. Hence, in the present study Indian medicinal plants which were traditionally used as food additives were evaluated for phytochemical components, cytotoxicity and antimicrobial activity. Further, blending them with biopolymers like polycaprolactone (PCL), poly lactic acid (PLA) would enable us in the future development of biomaterials using bioprinting technology.

Materials and Methods

The medicinal plants selected for the study were purchased freshly

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Table 1: List of Indian medicinal plants used in this study

Scientific Name	Common Name	Plant Part
Carica papaya	Papaya	Leaf
Vitex negundo linn	Notchi	Leaf
Aegle marmelos (l.) Corr. Serr	Vilvum	Leaf
Elettaria cardamomum	Cardamom	Seeds
Curcuma longa	Turmeric	Dried rhizome
Allium cepa	Onion	Bulb
Allium sativum	Garlic	Bulb
Piper nigrum	Black pepper	Fruit
Illicium verum	Star anise	Seeds
Syzygium aromaticum	Clove	fruit bud
Foeniculum vulgare	Fennel	Seeds
Nigella sativa	Black cumin	Seeds
Cinnamomum tamala	Indian bay leaves	Leaf
Cinnamomum cassia	Cinnamon	Bark
Piper betle	Betel leaf	Leaf
Mentha piperita	Mint	Leaf
Emblica officinalis	Amla/gooseberry	Fruit
Citrus limon	Lemon	Fruit
Aloe barbadensis	Aloe vera	Bark
Ocimum sanctum	Tulsi	Leaf
Murraya koenigii	Curry	Leaf
Azadirachta indica	Neem	Leaf
Coriandrum sativum	Coriander	Leaf
Zingiber officinale	Ginger	Rhizome

in local market (table 1). Crude/fresh extract preparation of selected Indian medicinal plants: Fresh extract of sliced lemon, deskinned ginger, deskinned garlic and deskinned onion was obtained by mashing in sterile mortar and pestle and by filtering through sterile muslin cloth [5,6]. Methanolic and ethanolic extract of selected Indian medicinal plant: plant materials like leaves, seeds, shallots, roots, fruit, flower buds in respect to specific medicinal plant used in the study (table 1) were collected and washed with sterile water and allowed to dry completely for 10-20 days. The dried plant materials were then grinded into fine/coarse powder using an electrical grinder.

Each plant powder was labelled and divided into 2 parts and one part was dissolved in methanol and other one in ethanol in the ratio of 1:10 and then subjected to Soxhlet extraction procedure [5,6]. The product obtained after Soxhlet extraction procedure was subjected to vacuum drying and the final extract powder obtained was mixed to a suitable vehicle i.e., dimethyl sulfoxide (DMSO) and then subjected to antimicrobial susceptibility test by agar well diffusion method.

Antibacterial activity of plant extracts

Agar well diffusion method was used for screening in-vitro antimicrobial activity of the plant extracts against control strain of *K. pneumoniae* [7]. Stock concentration of 1000 mg/ml prepared by dissolving 1g of the crude extract into 1ml of (0.1%) DMSO was used as test concentration. Bacterial suspension was swab cultured on Muller Hinton agar (MHA) plate and wells were cut using well cutter in each plate. 20µl of fresh methanol and ethanol extract (1000mg/ml) were dispensed in each well and plates were kept in refrigerator for 10 minutes to allow plant extracts to diffuse into the medium and then incubated at 37°C for 24 hours. After incubation, zone of inhibition (diameter of the inhibition zone) was measured to determine the antimicrobial activity. DMSO and cotrimoxazole (HI media, India) were used as controls in the study.

Minimum inhibitory concentration of plant extracts

K. pnuemoniae ATCC 700603 strain was used as a control in the study. The test extracts with good antibacterial activity were further subjected to MIC assay using broth microdilution method to evaluate the minimum inhibitory concentration that completely inhibit the visible bacterial growth [8]. Based on the CLSI guidelines, the concentrations of plant extracts used for MICs were ranging from 0.125 µg -32µg. 100µl of inoculums with two-fold dilution of plant extracts for each strain were dispensed in microtiter plate with 96 flat bottom wells. Plates were then incubated at 37°C for 24 hrs and the results were interpreted according to CLSI (2018) guidelines. Sterile MHB and bacterial inoculums in MHB without any extracts were used as sterility and negative control, respectively.

Qualitative phytochemical analysis

Phytochemical analysis of the plant extracts for key phytoconstituents was performed for biologically active compounds such as proteins, glycosides, phenols, alkaloids, tannins, flavonoids, saponins, terpenoids, quinones, glycosides and steroids were tested in the plant extracts [9-11].

Table 2: Minimum inhibitory concentration assay, cytotoxicity assay and phytochemical analysis of plant extracts

Scientific name	Part of plant used	Minimum inhibitory concentration (µg/ml) against ATCC *K. pneumoniae* 700603 strain		Cytotoxic activity IC50 (µg/ml)		Phytochemical Analysis																			
						Tannins		Saponins		Flavonoids		Steroids		Glycosides		Alkaloids		Protein		Quinones		Terpenoids		Phenol	
		E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M
Carica papaya	leaf	16 µg	16 µg	35.52	26.52	+	+	-	-	+	+	+	-	-	-	+	+	-	-	-	-	+	-	-	+
Vitex negundo Linn	leaf	16 µg	8 µg	48.56	36.65	+	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Curcuma longa	rhizome	32µg	32 µg	59.32	52.63	-	-	-	-	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	-
Allium sativum	blub	16 µg	32 µg	13.84	15.24	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Citrus limon L	fruit	16 µg	8 µg	69.63	21.58	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Ocimum sanctum	leaf	16 µg	8 µg	60.52	20.37	-	+	-	-	-	+	+	-	-	-	+	+	-	+	+	-	+	-	-	-
Azadirachta indica	leaf	16 µg	16 µg	32.52	13.92	-	+	-	-	-	+	-	+	-	+	+	+	-	-	+	-	+	+	-	+
Zingiber officinale	rhizome	16 µg	16 µg	11.93	32.52	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	-	-	-

* E - Ethanol extract; *M - Methanol extract; *+ Positive; *- Negative

MTT Assay

The cytotoxicity of the methanolic and ethanolic extracts of plants, using MTT assay, was evaluated on Human dermal Fibroblast Cells (HDF) in 96 well microplate [12]. Varying concentrations (0.7185-250µg/ml) of plant extracts were incubated along with the HDF maintained in *Dulbecco's modified eagle's medium* (DMEM) with 10 % foetal bovine serum. Following incubation, media was removed and 5mg/ml MTT dissolved in phosphate-buffered saline (PBS) and added in each well. The plate was incubated at 37°C for 3.5 hours in the presence of 5% CO_2. After incubation, media was removed and 150 µl solubilising solution (DMSO) was added into each well. Optical density was determined at a wavelength of 590 nm using microplate reader. IC 50 (Inhibitory 50 concentration) was used to express the cytotoxic effects of plant extracts.

Characterization of medicinal plants by Gas Chromatography and Mass Spectrophotometry (GC-MS)

Plant extracts with antibacterial activity were subjected to GC-MS using Perkin Elmer Claurus 500 system (Perkin Elmer, USA), which was packed with an Elite-1 (composed of 100% dimethyl poly siloxane) fused silica capillary column (30 m x 0.25 mm IDx1µMdf, gas chromatograph interfaced to a mass spectrometer). Pure helium gas was used as the carrier at constant flow rate of 1 ml/min and 2µl of a sample was injected into the system (split ratio of 10:1). GC MS spectral detection was performed using electron ionization energy method with an ionising energy of 70 eV with a scan period of 0.5 seconds and fragments ranging from 45 to 450 Da. The injector and ion-source temperature maintained at 250°C and 280°C, respectively. The oven temperature was programmed initially at 110°C for 2 min, raised at 10°C/min to 200°C and the final temperature increased at 5°C/min to 280°C. The components were identified by comparing them to mass spectral fragmentation patterns in the MS library. The Phyto-components in plant extracts were identified mass spectral patterns, retention time, peak area and peak height to those in MS library (National Institute of Standards and Technology (NIST) library's spectrum database of genuine compounds, USA) [13].

Results

Among the fresh extracts, garlic bulb (*Allium sativum*) showed good antibacterial activity against standard strain of *K. pnuemoniae* (table 2). Methanolic and ethanolic extracts of papaya leaves (*Carica papaya*), notchi leaves (*Vitex negundo Linn*), turmeric (*Curcuma longa*), garlic bulb (*Allium sativum*), lemon Fruit (*Citrus limon*), tulsi leaves (*Ocimum sanctum*), neem leaves (*Azadirachta indica*) and ginger rhizome (*Zingiber officinale*) demonstrated good and consistent antimicrobial activity with their MIC values ranging between ≥ 8-32µg/ml (figure 1, table 2). The highest antibacterial activity was found in methanolic extracts of papaya leaves (*Carica papaya*) and turmeric (*Curcuma longa*), as well as ethanolic extracts of tulsi leaves (*Ocimum sanctum*) and ginger rhizome (*Zingiber officinale*) (table 2). Plants extracts showed antibacterial activity against uropathogenic *K. pneumoniae*.

Qualitative screening of phytochemicals present in methanolic and ethanolic extracts of plants was shown in table 2. Alkaloids were found in all the 8 plant extracts and none of the plant extracts were positive for saponins. Plant extracts cytotoxic assay demonstrated significant IC_{50} values and inhibitory concentration, documented in table 2. In our study, all the plant extracts had a significant antiproliferative effect in HDF cells, with 12.5 % of the extracts having an IC_{50} of greater than 60% (ethanolic extract of *Citrus limon L* and *Ocimum sanctum*), 12.5 % of the extracts having an IC_{50} of greater than 50% (methanolic and ethanolic extract of *Curcuma longa*), 6.25 % of the extracts having an IC_{50} of greater than 40% (ethanolic extract of *Vitex negundo Linn*), 25% of the extracts having IC_{50} above 30% (methanolic extract of *Vitex negundo Linn*, *Allium sativum* and ethanolic extract of *Carica papaya*) and the remaining were below 30% (methanolic extract of *Carica papaya*, *Ocimum sanctum*, *Citrus limon L*, *Azadirachta indica*, *Allium sativum* and ethanolic extract of *Azadirachta indica* and *Allium sativum*) (table 2). GC-MS spectrum of ethanolic and methanolic extracts of the plant for various retention times were listed in table 3. Each ethanolic and methanolic plant extracts predicted 10 to 20 chemicals, which were compared with GC-MS library. The most prevailing compounds found among the eight plant extracts were tumerone, tetradecane, sulphurous acid, silane, piperine, phytol, phenol, 2-methoxy, phenol, 2,4-

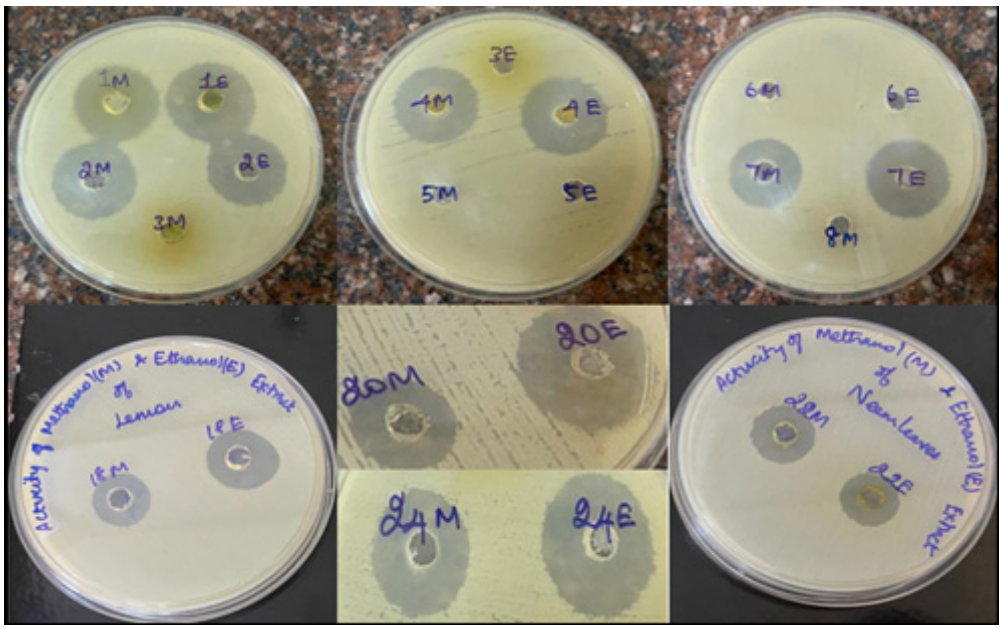

Figure 1: Antibacterial activity of Indian medicinal plant extracts

Table 3: Gas Chromatography Mass Spectrometric analysis of plant extracts

Plant name	Extract type	Retention Time (min)	Name of the compound With highest peak	Area %
PAPAYA	Ethanol	18.674	2H,8H-Benzo[1,2-b:5,4-b'] dipyran	36.06
	Methanol	13.097	Butylated Hydroxytoluene	20.54
NOCHI	Ethanol	27.129	Eicosane	12.07
	Methanol	13.086	Butylated Hydroxytoluene	35.02
TURMERIC	Ethanol	15.019	Ar-tumerone	26.61
	Methanol	15.074	Tumerone	23.12
GARLIC	Ethanol	25.928	Piperine	43.36
	Methanol	12.452	Oxalic acid, monoamide, n-propyl	66.04
LEMON	Ethanol	13.108	Phenol, 2,4-bis(1,1-dimethylethyl)	20.55
	Methanol	13.086	Butylated Hydroxytoluene	64.36
TULSI	Ethanol	11.764	Benzene, 1,2-dimethoxy	55.49
	Methanol	13.086	Butylated Hydroxytoluene	26.09
NEEM	Ethanol	19.485	Phytol	19.75
	Methanol	13.086	Butylated Hydroxytoluene	29.94
GINGER	Ethanol	14.830	Butan-2-one, 4-(3-hydroxy-2-meth...	31.37
	Methanol	13.097	Butylated Hydroxytoluene	29.82

bis(1,1-dimethylethyl), octadecanoic acid methyl ester, octacaine, octadecane, naphthalene, methyl ricinolate, hexadecenoic acid, ethyl ester, hexadecane, furane-2-carbohydrazide, eicosane, dodecane, diethyl phthalate, disulphide, di-tert-dodecyl, decane, cyclopropyl phenyl carbinol, cyclohexane, caryophyllene oxide, caryophyllene, butylated hydroxytoluene, butane dioic acid, bi-cycloheptane, benzene propanoic acid, benzene, alpha-caryophyllene, ar-tumerone, 9-octadecene, 9,12,15-octadecatrienoicacid, 2-methoxy-4-vinylphenol, 17-pentatriacontene. The most common constituents found in all the plant extract were represented in table 3.

Discussion

In the present study, plants such as *Carica papaya, Vitex negundo Linn, Curcuma longa, Allium sativum, Citrus limon, Ocimum sanctum, Azadirachta indica*, and *Zingiber officinale* demonstrated antibacterial activity against *K. pneumoniae.* Fresh crude extracts of *Allium sativum* and *Citrus limon* showed significant antibacterial activity, which was similar to the previous reports [14,15]. Sharma *et al.* reported antibacterial activity of ethanolic extract of *Z. Officinale, A. indica* and *Ocimum sanctum* against the UTI causing micro-organisms [16]. Ethanol extracts of *Ocimum sanctum* demonstrated potential antibacterial activity which was similar to the previous studies [17]. Methanol extracts of *Carica papaya* exhibited highest antibacterial activity among the plant extracts. Adetunde *et al.,* reported similar results for the methanolic extracts of *Carica papaya against* the *K. pneumoniae* [18]. In our study, ethanolic and methanolic extracts of *Curcuma longa* showed highest antibacterial activity against *K. pneumoniae* [19,20]. The pharmacological efficacy of a plant is determined by the presence of abundant secondary metabolites and a specific molecule may have significant therapeutic potential. In the current study, phytochemical analysis of all the plant extracts were confirmed the presence of metabolites. According to Sodipo *et al.,* most phytochemicals work as natural antibiotics that aid in the body's defence against infections and microbial invasion [21]. Alkaloids, for example, are chemical compounds primarily composed of basic nitrogen atoms that occur naturally in plants. In our study, alkaloids were detected in all plant extracts, while saponins was not detected in any plant extracts. In comparison to other plant extracts, *Azadirachta indica, Curcuma longa* and *Carica papaya* were high in phytochemicals. The study findings are consistent with other studies [22]. Identifying these bioactive compounds would support the development of new drugs for many diseases. Tannins, a major active ingredient in plant-based therapies with antiviral and antibacterial characteristics [23]. Tannins are found in *Carica papaya* and *Azadirachta indica* in our study. Flavonoids found in *Carica papaya*, *Curcuma longa* and *Azadirachta indica* have been shown to have antibacterial properties [24]. Butylated Hydroxytoluene, a common phytoconstituent found in all plant extracts, was identified in this study. Antimicrobial properties of butylated hydroxytoluene have been documented in previous studies [25]. Similarly, antibacterial activity has been observed for tumerone, Ar-tumerone, piperine, phytol and eicosane [26-29]. Hence, it's possible that study plant extracts have antibacterial property linked to the presence of these bioactive compounds. Plant extracts with IC_{50} < 100 µg/ml is generally considered to be therapeutically important [30]. In our study, majority of plant extracts demonstrated cytotoxicity below 50 ug/ml. As a result, the study plant extract found to be potential candidates for antimicrobial research. The present study aids in biomolecule identification and further structural elucidation will aid in bioprinting of biomaterials and drug development.

Conclusion

The majority of Indian medicinal plants are widely used in traditional medicine, but their use in treating various ailments is limited. Medicinal plants will play a significant role in the preparation of biomaterials for implantable medical devices and drug delivery system. Hence, the current study demonstrated the therapeutic

potential of a few Indian medicinal plants. The medicinal plants used in the study were rich in phytochemical compounds, which might be used as a substitute or source for antibacterial medications. In the future, more research into the bioactive chemicals found in plant extracts will be conducted to confirm their potential benefits.

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