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ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF BIO-OIL FROM *PONGAMIA GLABRA*, *MESUA FERREA* AND *PARACHLORELLA* SPP DEOILED CAKE

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ABSTRACT

The present study deals with the assessment of antimicrobial activity (antibacterial, antifungal and antiyeast) of bio-oil from *Pongamia glabra*, *Mesua ferrea* and *Parachlorella* spp deoiled cake. The bio-oil from the respective deoiled cakes were obtained via the process of pyrolysis (ambient to 500°C at a heating rate of 40°C/min) in a vertical tubular fixed bed reactor in nitrogen atmosphere. Bio-oil from *Mesua ferrea* deoiled cake (BMFDC) and *Pongamia glabra* deoiled cake (BPGDC) recorded the most effective Zone of Inhibition (ZOI) against *S. aureus* viz., 28 and 29 mm respectively. BMFDC and BPGDC were more effective in terms of antimicrobial efficacy in contrast to bio-oil from *Parachlorella* deoiled cake (BPCDC). The antimicrobial activity of the bio-oil samples may be due to the presence of phenolic and carboxyl groups (detectable by FTIR spectroscopy). The results of this study indicate the presence of bio-active agents in bio-oils which may lead to development of new pharmaceuticals.

KEYWORDS: Antimicrobial, De-oiled cake, Bio-oil, Microalgae

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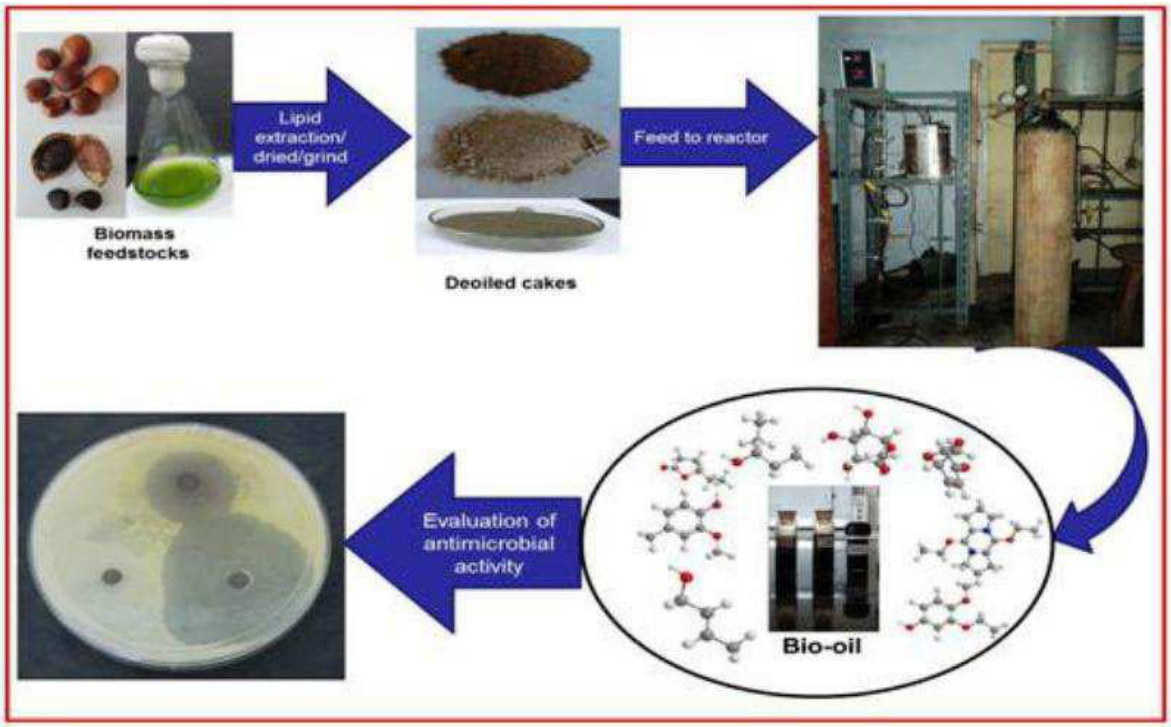
GRAPHICAL ABSTRACT



INTRODUCTION

Antibiotics have always been regarded as one of the greatest discoveries of the preceding century. Once after their discovery a popular belief prevailed in the medical fraternity that they might lead to the eventual eradication of infectious diseases [1]. But however a decisive blow to this belief came in the form of a future medical disaster viz., the troublesome trend of antibiotic resistance. The global emergence of bacterial strains exhibiting resistance to multiple antibiotics (multi drug resistant) is progressively restraining the efficiency of existent drugs with resultant failure of available treatment regimes. Certain gram positive bacteria (methicillin-resistant *Staphylococcus aureus, vancomycin-resistant enterococci,* and *quinolone-resistant Streptococcus pneumonia*) in this regard have achieved the status of "superbugs" in that a very few or no antibiotics are available for therapy against these pathogens[2]. These developments have resulted in disastrous consequences like increased morbidity and mortality of patients and need for more expensive treatment regimes. This worrisome inclination towards antibiotic resistance has necessitated the need for search operations for the quest of new antimicrobial compounds. In this regard plant resources are possibly the best available repertoire for bioactive compounds that might result in the development of new pharmaceutical agents. Owing to the cost effectiveness, safety, increasing failure of chemotherapy and antibiotic resistance, search for plant resources has considerably increased for their potential antimicrobial activity[3]. The most significant bioactive compounds reported from plants are alkaloids, tannins, flavonoids, and phenolic compounds[4]. Alkaloids possesses antimicrobial, anticancer, antimalarial and cytotoxic properties while flavonoids have high antibacterial activity and are more efficient in treatment of inflammation, allergy, cancer, viral infection and hypertension[5]. Tannin has high activities against bacterial and viral infections and also acts as strong antioxidant[6].The chemical compounds responsible for the antibacterial activity in algae have been variously identified as organic and fatty acids, terpenes, carbonyls, bromophenols, halogenated aliphatic and

sulfur-containing heterocyclic compounds, isoprenylated and brominated hydroquinones, as well as phlorotannins[7; 8]. The study of natural products from the plant kingdom has long been the source of pharmaceuticals, and as such it makes sense that bio-oil (thermochemically converted biomass) may possess new functional moieties (via making and breaking of bonds resultant from breakdown of larger biopolymers into smaller molecules) with prospective pharmaceutical value. The energy intensive future research scenario has high lightened the importance of biomass conversion as a promising route for biofuel production. In wake of recent advancements in bioenergy research bio-oils have already gathered the attention of the scientific community in that they offer prospective applicability as chemical feedstock apart from being an increasingly attractive fuel option. There is paucity of evidence regarding possession of antimicrobial activity by bio-oil. Only a few reports pertaining to antifungal activity of bio-oil has been reported till date[9,10]. As such the present study was carried out to investigate the utility of bio-oil, produced via pyrolysis process (from *Mesua ferrea, Pongamia glabra* and *Parachlorella* deoiled cake), as an antimicrobial agent against gram positive and gram negative bacteria [*Staphylococcus aureus* (MTCC96) and *Escherichia coli* (MTCC723]. An attempt was also made to assess the antimicrobial activity against eukaryotic systems [*Candida albicans* (ATCC 183) and *Saccharomyces cerevisiae* (ATCC 4126)], the results of which are being reported in the present communication. This is also the first report of assessing the antimicrobial activity of bio-oil derived from deoiled cakes of terrestrial energy crops (*Mesua ferrea* and *Pongamia glabra*) and microalgae (*Parachlorella spp)*.

MATERIALS AND METHODS

SAMPLE PREPARATION

The sample (bio-oil) used for this study was prepared by pyrolysis of *Pongamia glabra*, *Mesua ferrea* and *Parachlorella* de-oiled cake in a lab scale fixed bed pyrolysis reactor (ambient to 500°C at a heating rate of 40°C/min in nitrogen atmosphere).

Preparation of cultures

Fresh bacterial cultures (*E. coli* MTCC723 and *Staphylococcus aureus* MTCC96) were prepared by adding a loopful of stock culture to sterilized nutrient broth. The media was incubated at 37°C for 24hr and used for accessing antibacterial activity. Fresh cultures of *Candida albicans* ATCC 183 and *Sachharomyces cerevisiae* ATCC 4126 were prepared by adding a loopful of stock culture to Potato Dextrose Broth (PDB) and YPD media respectively. The former culture was incubated at 37°C (24hr) and the later at 28°C (48hr) respectively, for evaluating antiyeast and antifungal activity.

AGAR WELL DIFFUSION METHOD

The antimicrobial assay was done by using the agar well diffusion method. The assay was carried out to find out if the respective bio-oils had any antimicrobial activity. Microbial cultures were adjusted to 0.5 McFarland standards before the tests. The media used for antibacterial assay was Muller Hinton Agar, whereas PDB and YPD media were used to access the antifungal and antiyeast activity. The standard solutions of the samples (BMFDC and BPGDC) were 500µg/ml DMSO, whereas 200 µg/ml DMSO for BPCDC. The sterile liquid culture media (25mL) was poured into petriplates and after solidification were inoculated by spread plate method with an inoculum corresponding to 0.5 McFarland standards. Three (3) wells of 5mm diameter were punched into the agar with the help of a sterilized well puncturer (5mm diameter) and 50µl of the sample was added to the sample well. Chloramphenicol (10 mg/ml stock) was used as the positive control for antibacterial assay, whereas Indofil M-45 a commercial fungicide (50mg/ml stock) was used as the positive control for antifungal and antiyeast assay. 1% DMSO was used as the negative control for all the tests. The plates were then sealed with paraffin and kept for incubation at 37°C for 24h (for *S. aureus*, *E. coli* and *S. cerevisiae*) and 28°C for 48hrs (for *C. albicans*). Antimicrobial activity was accessed

by measuring the ZOI diameter using a zone scale (Antibiotic Zone Scale, HIMEDIA).

FTIR ANALYSIS

The FTIR spectrum of the samples (Bio-oil of *Mesua ferrea*, *Pongamia glabra* and *Parachlorella* deoiled cake) was recorded in a PERKIN ELMER Spectrum 100 spectrometer at room temperature (28±2˚C). A region in the spectral range of 4000–400 cm^{-1} was used for scanning.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC was determined following the protocol of Wang et al., 2010 with slight modification. The MIC activity was determined using a 96-well microtitre plate. The stock solutions for the sample oil were (200µl/ml DMSO) for BMFDC and BPGDC, whereas (100µl/ml DMSO) for BPCDC. 10ml culture of *E. coli*, *S. aureus* were prepared in LB broth, whereas the same amount of culture of *C. albicans* and *S. cerevisiae* were prepared in PDB and YPD broth respectively. Saturated cultures of all the four strains were then diluted to form an approximately 1×10^6 colony forming units (CFU)/ml. In the 1st well 200 µl of stock solution was added and serially diluted (8 fold) by adding the respective culture media. Following this 100 µl of culture was added to the respective wells. Kanamycin (50mg/ml) was used as the positive control for bacteria, whereas Indofil (50mg/ml, commercial fungicide) as the positive control for *C. albicans* and *S. cerevisiae*. DMSO (1%) was used as negative control for all the test samples. The plates were covered and incubated overnight at 37 °C (for *S. aureus* and *E. coli*), and 28°C for 48 hrs (for *C. albicans* and *S. cerevisiae*). At the end of the incubation period, 40µL of MTT solution (0.2 mg/mL) was added into each well and then further incubated at 37°C for 45 minutes. The culture absorbance was recorded at 570 nm.

DISCUSSION

Pyrolysis, the thermochemical conversion of biomass in oxygen starved atmosphere has the potential to convert waste or remnants into bio-oil for upgrading into fuels and other value added products [11]. With a view to sincerely realize the value addition of bio-oils, the assessment of antimicrobial activity of bio-oil from deoiled cakes of terrestrial energy crops and microalgae is taken into consideration in the present investigation. The bioassay results for antimicrobial activity of the respective bio-oil samples are shown in Table 1.

Table 1
Bioassay results for antimicrobial activity of BMFDC, BPGDC and BPCDC

Species	Bio-oil	ZOI
S. cervisiae	BMFDC	20mm±0.32
S. cervisiae	BPGDC	20mm±0.52
S. cervisiae	BPCDC	No zone
C. albicans	BMFDC	20mm±0.36
C. albicans	BPGDC	22mm±0.45
C. albicans	BPCDC	No zone
S. aureus	BMFDC	29mm±0.43
S. aureus	BPGDC	28mm±0.35
S. aureus	BPCDC	12mm±0.3
E. coli	BMFDC	15mm±0.4
E. coli	BPGDC	22mm±0.3
E. coli	BPCDC	12mm±0.23

Figure 1
Antimicrobial activity histogram for bio-oil samples.

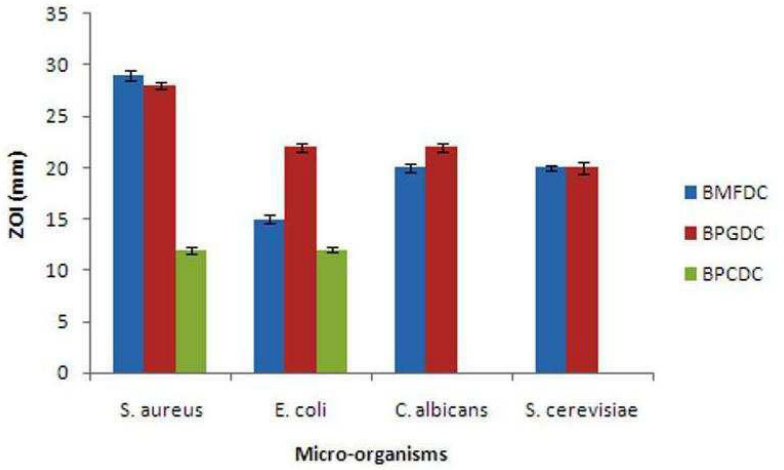


Fig 2, 3, and 4 shows the ZOI produced by the bio-oil samples viz. BMFDC, BPGDC and BPCDC on the test micro-organisms viz. *S. cerevisiae*, *S. aureus*, *C. albicans* and *E. coli*. The ZOI of the sample extract was compared with the standard antibiotic chloramphenicol for antibacterial and Indofil M-45 (commercial antifungal) for antifungal and antiyeast assay. All the tested samples showed varying degrees of antimicrobial activity against the tested micro-organisms. From Table 1and Fig 3 it can be concluded that, BMFDC showed maximum inhibition zone against *S. aureus* (29 mm) and moderate against *E. coli* (15 mm). Similarly, BPGDC exhibited highest antibacterial activity against *S. aureus* (28 mm) and comparatively lesser activity against *E. coli* (22 mm). BMFDC was found to be more effective against gram positive bacteria whereas, BPGDC against gram negative bacteria. BPCDC showed a moderate inhibition zone (12 mm) against both the tested bacterial strains.

Figure 2
Inhibition zones of BMFDC against E. coli, S. aureus, S. cerevisiae and C. albicans

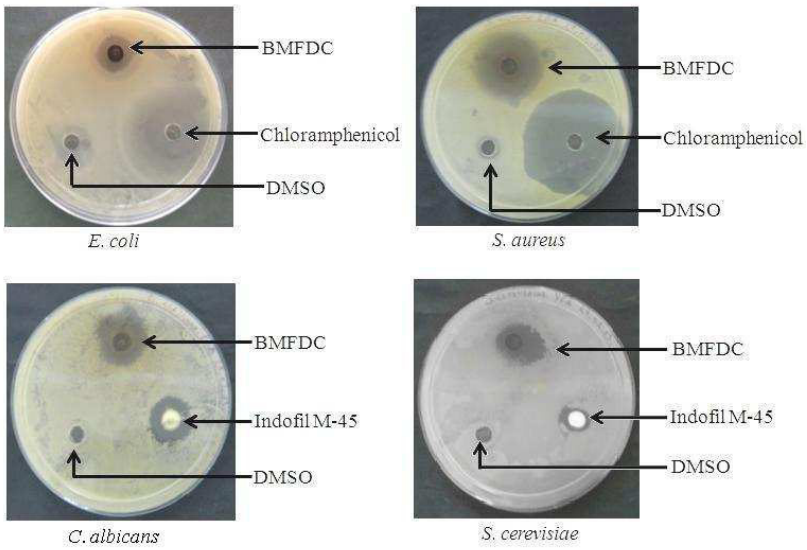


Figure 3
Inhibition zones of BPGDC against E. coli, S. aureus, S. cerevisiae and C. albicans

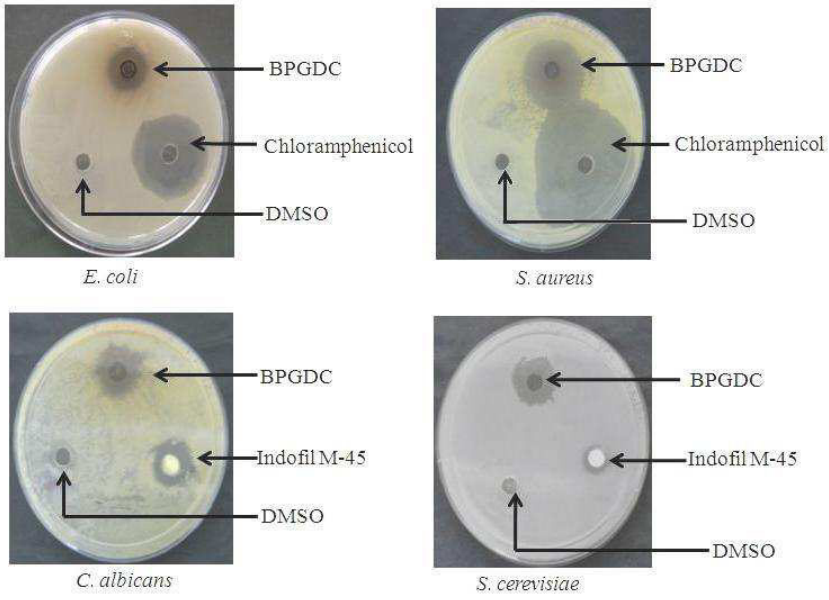
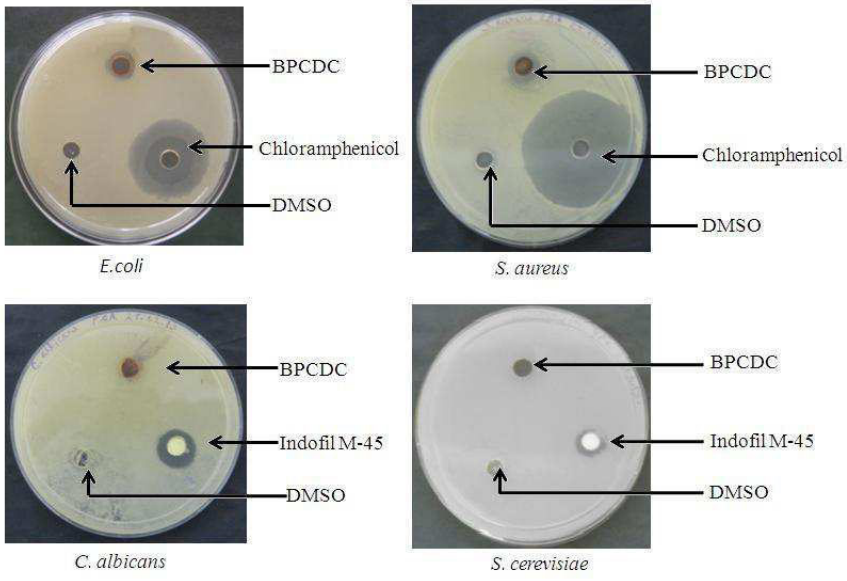


Figure 4
Inhibition zones of BPCDC against E. coli, S. aureus. No ZOI was observed against S. cerevisiae and C. albicans

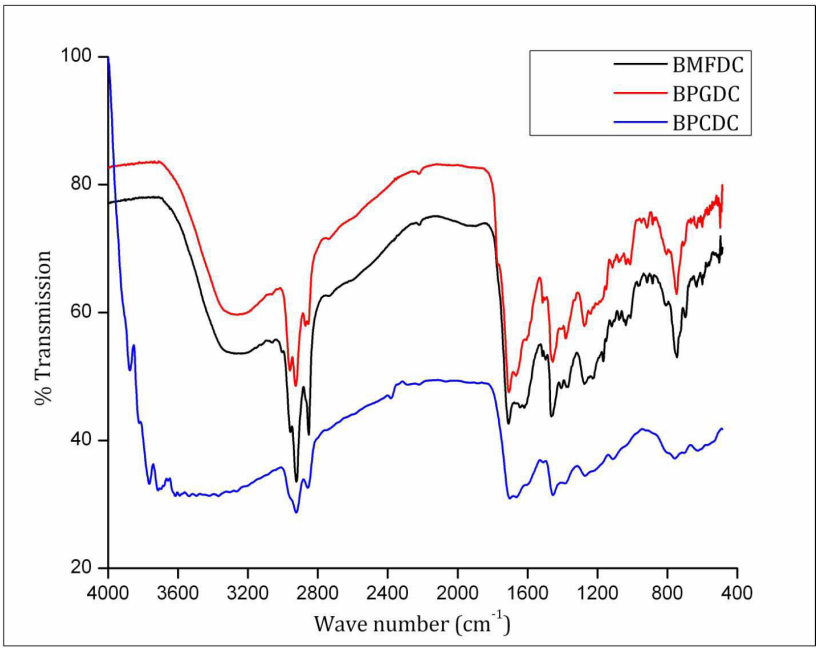


Antifungal activity against human pathogens is highly sought after in pharmaceutical and pharmacological research. As such, antifungal assay was performed to evaluate the activity of the bio-oil samples against *C. albicans*. BMFDC showed a 20 mm ZOI against *C. albicans* whereas BPGDC showed a comparatively higher 22 mm zone. Both BMFDC and BPGDC showed a 20 mm ZOI against *S. cerevisiae*. It is noteworthy to mention here that no sign of antifungal and antiyeast activity was shown by BPCDC.

Minimum inhibition concentration of all three bio-oil samples viz., BMFDC, BPGDC and

BPCDC at different concentrations (100µg, 50µg, 25µg, 12.5µg, 6.25µg, 3.125µg, 1.56µg and 0.78µg/ml) was determined against two prokaryotic and two eukaryotic systems. After 24 hours / and 48 hours of incubation the cultures were subjected to MTT assay. Kanamycin (50mg/ml) and Indofil (50mg/ml) were used as positive control and cells mixed with DMSO (1%) as a negative control. A concentration-dependent increase in inhibition of prokaryotic and eukaryotic cells was observed for all the three samples. MIC for BMFDC was minimum and it inhibits *E. coli* and *S. aureaus* at concentrations of 1.56 and 3.12 µg/ml. On the contrary, higher concentration was required against eukaryotes viz., *C. albicans* (3.12 µg/ml) and *S. cerevisiae* (6.25µg/ml). Antagonism of BPGDC against all the tested strains except *S. cerevisiae* was same. A concentration of 6.25 µg/ml was required to inhibit the growth of *E. coli, S. aureus,* and *S. cerevisiae*, whereas for *C. albicans* a higher concentration of 12.5µg/ml was required. BPCDC was found to be effective only against prokaryotes, *E.coli* (25µg/ml) and *S. aureaus* (12.5µg/ml). Chemically, bio-oil is a complex mixture of water,guaiacols,syringols,furancarboxyaldehydes, pyrones, isoeugenol, vanillins, catechols, acetic acid, formic acid and other carboxylic acids[12]. Bio-oils also contain other major group of compounds including hydroxyaldehydes, hydroxyketones, sugars, carboxylic acid and phenols[13]. Fig 5 presents the FTIR spectra for the three bio-oils.

Figure 5
FTIR spectra of BMFDC, BPGDC and BPCDC



The FTIR spectra for all the three bio-oils are almost similar due to the presence of similar functional groups. The major spectral assignments are C-H stretch (2928 cm^{-1}), -CH_2- (bend) [1465 cm^{-1}], OH (H bonded) [3400-3200 cm^{-1}], C=O (1700-1725 cm^{-1}) and CH_2 (rocking) [743 cm^{-1}]. The FTIR spectra of the bio-oil samples show the presence of hydroxyl group which is common in all phenolic compounds. The C=O (attributed to the presence of carboxylic acids) was another important functional component observed in the spectra of the bio-oils. Phenolic compounds have been reported to be associated with antifungal activity[14], whereas carboxylic acids with many antibacterial and antimicrobial activities[15]. Since, bio-oils are obtained from plant and algal feedstocks it is arguable to expect a diversity of compounds (obtainable by breakdown of larger polymers

to simple compounds) in bio-oils with specific as well as broad-spectrum antimicrobial activity. The exploitation of bio-oil for the discovery of pharmaceutically important products is a futuristic top notch research area with tremendous biological prospects. In wake of recent hike in microbial resistance (especially bacteria) to bactericides and antibiotics[16], bio-oils may well be viewed as a resource material for pharmaceutical studies. Ongoing research is currently focused on the isolation, identification and characterization of compounds responsible for bioactivity of bio-oils with high antimicrobial activity.

CONCLUSION

Bio-oils, if properly investigated may offer an unexplored repertoire for chemicals with huge potential to provide significant therapeutic benefits for the human civilization. From the above study, it can be concluded that BMFDC was found to be most effective against the growth of *S. aureus*, whereas BPGDC against *E. coli* and *C. albicans*. BPGDC was found to be moderately effective against prokaryotic system whereas, completely ineffective against eukaryotic systems. Bio-oil produced from the deoiled cake of terrestrial energy crops (*Mesua ferrea* and *Pongamia glabra*) was found to be more effective than bio-oil from microalgal (*Parachlorella spp*) deoiled cake in terms of ZOI. Antimicrobial activity exhibited by two of the bio-oil samples viz., BMFDC and BPGDC used in this study indicates that they possess bioactive natural products (may lead to development of new pharmaceuticals),and henceforth warrants further research investigations (greater research capacities would enable a better comprehensive understanding of drug discovery from fast pyrolysis technology). The study also suggests that bio-oil from different plant and algal feedstocks which remain grossly unexplored should be evaluated for antimicrobial activity against multidrug resistant bacteria (MDR) and phytopathogens. There is every possibility that Mother Nature endowed with enormous plant and algal diversity may literally unfold the elixir of life against the worrisome trend of drug resistance.

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