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EXPLORATION OF BETEL LEAF WASTE FOR ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT
In an attempt to reuse agrowaste, betel leaf stalk extract was found as potent antimicrobial agent. The principal compound showed antibacterial activity against Gram positive and Gram negative bacteria. The MIC determined between 25μg/mL to 250μg/mL and was supported by time kill experiment. The potentiality of the active principal was measured against ciprofloxacin as a standard. The active compound tentatively identified as phenolic.

INTRODUCTION

India is one of the megadiversity hot spots with rich heritage of traditional knowledge of folk medicines. It encourages the scientists to explore further for the processing of bioactive compounds. Thus, there is growing interest in the development of bioprocess for the production and extraction of compounds from natural renewable sources for their potential applications in food, cosmetics and pharmaceuticals (Oreopoulou and Tzia, 2007). Use of phytochemicals as major bioactive compounds with multidimensional benefits are gaining momentum.

Efforts have been made by researchers to explore possibility of reusing plant wastes as the source of organics. The processing of agro product results the formation of waste materials in high amount and commonly are in the form of peels, seeds, and oilseed meals that pose a problem to the environment and transportation. In addition, the cost of drying, storage or transportation concern with financial limitation to waste utilization. Therefore, it needs to be managed by utilization or by transformation. To comply with, there is a growing interest in recycling of waste biomass of agro products that sometimes focusssed to judge as potential therapeutic agent.

Plant metabolites represent an important source of sugars, minerals, organic acids, dietary fibre, as well as bioacitive compounds like phenolics which are a much diversified group of secondary plant metabolites (Dewick, 2002, Spatafora and Tringali, 2012). Many of such compounds are having strong antioxidant properties as oxygen scavengers, peroxide decomposers, metal chelating agents and free radical inhibitors (van Acker *et al*., 1998; Nijveldt *et al*., 2001). Researchers are increasingly attracted for the development of phytomedicine and to look for new potential leads to combat against microbial pathogens (Braga *et al*., 2005). To comply with, scientists have studied the antimicrobial activity from different organic wastes eg, pomegranate peels (Oliveira *et al*., 2008; Mahmud *et al*., 2009), green walnut husks (Al-Zoreky, 2009), lemon peels (Katalinic *et al*., 2010), etc. To identify the therapeutic potential of such novel compounds are of much advantage as they are eco-friendly and cheap also used as alternative to synthetics (Martin *et al*., 2012). In this context, the exploration of different organic raw material would be of value addition to the waste.

The present investigation explores the possibility of using plant waste as a source of low-cost natural antimicrobials.

MATERIALS AND METHODS

Plant materials

Different types of agricultural wastes were collected from Bolpur, West Bengal within a radius of 50 km, West Bengal. After repeated washing the wastes were shade dried and crushed into fine powder. The powdered samples were sealed in polythene bag and were stored in dessicator at room temperature.

Preparation of extract

Extraction

The dried and powdered wastes (1kg) were extracted successively with 500mL of solvent non-polar to polar seperately by using soxhlet extractor (Lin *et al*., 1999). The extract was concentrated to dryness in rotary vacuum

evaporator below 50°C. Yield of the extract was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of the extract recovered}}{\text{Weight of the dry powder}} \times 100$$

The crude extracts were transferred to different glass vials and kept at 4°C for further use. For antimicrobial testing the different extracts were dissolved in DMSO (1%), a non toxic solvent.

Purification of the active compound

The desired sample after screening was subjected for purification using chromatographic procedures like, paper, column and thin layer chromatography (Fig. 1). The concentrate of solvent extracted sample was subjected to preparative paper chromatography (PPC) and run in varied solvent system. Paper chromatography was performed in triplicate using Whatman No.1 filter paper. The first set of paper was observed under UV (254 and 366nm) illumination and in Iodine vapour chamber. The R_f value of the bands were determined. The second set was taken for bioautography. The third set was used to collect the corresponding fraction showing antimicrobial activity maximally. Performing repeat PPC the active fraction was eluted from papers, pulled together and evaporated to make it concentrate for column chromatography.

The partially purified concentrate was loaded to column, packed with silica gel (60 -120 mesh). After saturation with desired solvent system the sample was loaded at the top of the column and eluted with same solvent system, at a flow rate of 1mL/min. The fractions were collected separately and assayed for antibacterial activity. The active fractions were pooled together, concentrated and considered as semipurified sample loaded to TLC plate. The concentrate was applied for TLC in duplicate plates. One plate was put into iodine chamber to identify the sample spot of active principal and the other to observe under UV light.

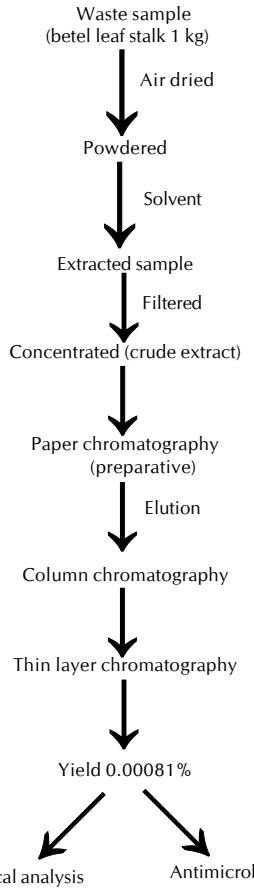

Figure 1: Schematic representation of extraction and purification of the active principal

Microorganisms used and inoculum preparation

The test bacteria were *Bacillus subtilis* (ATCC 6633), *Eschericia coli* (ATCC 25922), *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (MTCC 737). Stock cultures were maintained at 4°C on nutrient agar medium. Active cultures were prepared by inoculating fresh nutrient broth medium with a loopful of cells from the stock and incubated at 37°C for 24h, to get a desirable cell count (10^6 CFU/mL) for bioassay.

Antibacterial screening and MIC determination

Antibacterial activity of the purified sample was made against the test bacteria using agar diffusion. The agar plates were prepared by pouring 20mL of molten nutrient agar medium into sterile petri plates. The plates were allowed to solidify and 0.1 % cell suspension (10^6CFU/mL) of test organisms were spread uniformly and kept undisturbed for 15mins. Purified sample was used in wells or as paper discs. Whether in wells or in paper discs the plates were kept at low temperature in a refrigerator for 20mins and then incubated at 37°C for 24h. At the end of incubation, inhibition zones formed around the wells/discs were recorded. The sample with highest antibacterial activity was used to determine the MIC value.

Time kill kinetics of active principal

Each test organism was grown in presence of the purified active principal of the selected waste (betel leaf stalk, BLS) to understand the time-kill kinetics (Datta *et al*., 2011). Freshly grown test bacteria 100μL (10^6 CFU/mL) in nutrient broth was inoculated in tubes containing different concentrations (0, 25, 50, 100 and 200μg/mL) of the active principal and incubated at 37°C for 24h and optical density was recorded at 2h intervals up to 12h and the number of CFU was determined on nutrient agar plate using a serial dilution method after 24h. Ciprofloxacin (1mg/mL) was used as positive control.

Phytochemical screening

For detection of phenolic compound, the sample was loaded to TLC plate and run in the desired solvent system and was sprayed with Folin–Ciocalteu (FC) reagent. It produces a deep violet color with FC reagent, indicating the presence of phenolic (Nalina and Rahim, 2007).

Statistical analysis

Each test was performed in triplicate and data are represented as mean $\pm$ SD using Excel 2007 software.

RESULTS AND DISCUSSION

The dried waste samples (1kg each) were powdered and attempted to extract with polar to non-polar solvents. The extracted samples were filtered and concentrated in vacuum evaporator to dryness and stored at 4°C for further use. The

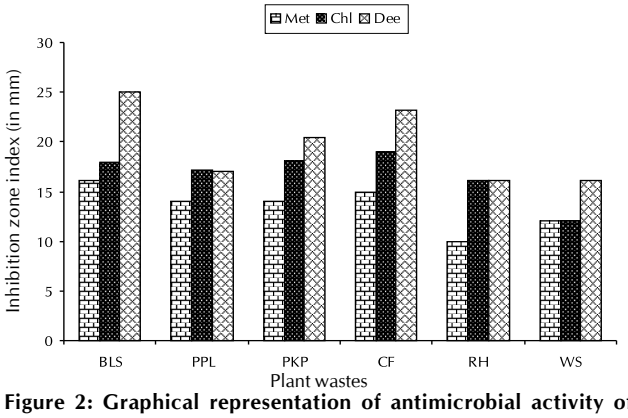

Figure 2: Graphical representation of antimicrobial activity of different wastes tested

BLS-Betel leaf stalk, PPL- Potato peel, PKP-Pumpkin peel, CF-Coconut fibre, RH-Rice husk, WS- wheat straw,Met-Methanol,Chl-Chloroform, Dee-Diethylether

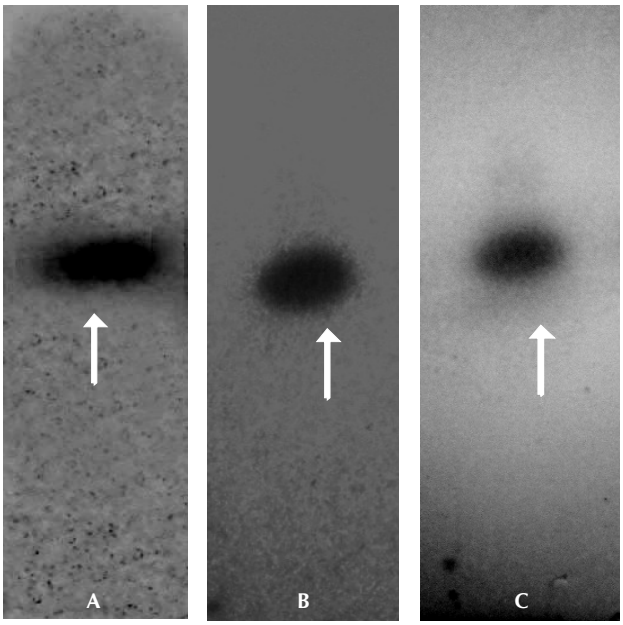

Figure 3: TLC plates observed under conditions- A: at 254 nm, B: at 366 nm, C: iodine vapour

concentrates were redissolved in DMSO (1%) and used for antibacterial testing. All the samples showed positive activity against the test bacteria both in agar cup and in disc assay, showing an inhibition zone diameter between 15-24mm. It appeared that the solvent used were with certain role in the process of extraction extracts with diethylether showed the highest inhibition followed by those extracted with chloroform and methanol. However among all the extracted samples of different solvent system, the BLS sample exhibited highest activity (Fig. 2). Studies showed that suitability of extraction of antibacterial metabolites depended on polarity of solvents but the activity varied with test organisms (Khan and Kumar, 2011).

Showing maximum activity and indicating highest extraction, BLS sample extract was attempted for purification near homogeneity. The crude sample was loaded to PPC for purification and run with standardized solvent system. A portion of the chromatogram was cut vertically to observe under UV illumination, thereafter, placed within iodine

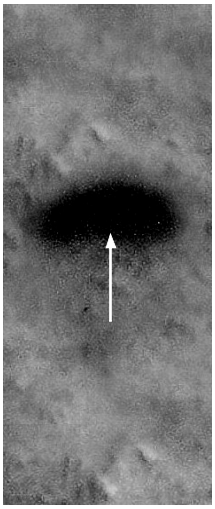

Figure 4: Deep colour spot indicates the presence of phenolic

chamber to understand the band location. The band was then cut horizontally from the main chromatogram and eluted with identified solvent and loaded to silica gel column for further purification. The eluted fractions were tested for antibacterial activity and the active fraction (F_3), after repeat collections, was pooled together for performing TLC to confirm its purity (Fig. 3). The pooled fraction (F_3) was concentrated and used as stock (1mg/mL). However, the active principal showing the antibacterial activity was identified and purified from leaf part of betel plant using chromatographic techniques (Josenbabu *et al.*, 2011).

For detecting the chemical nature, the active principal was reacted over a TLC plate with FC reagent and found positive to phenolic (Fig. 4). The antimicrobial activity of phenolics was detected from red wine (Shi and Schwendt, 1995), buckwheat

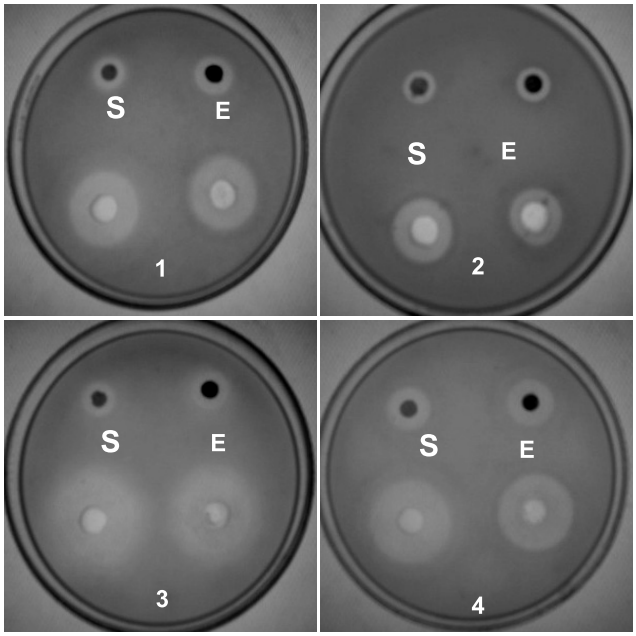

Figure 5: MIC determination using both well and paper disc method. S – Standard, E - Extract (100μg/mL)
(1) *B. subtilis* (2) *S. aureus* (3) *E. coli* (4) *P. aeruginosa*

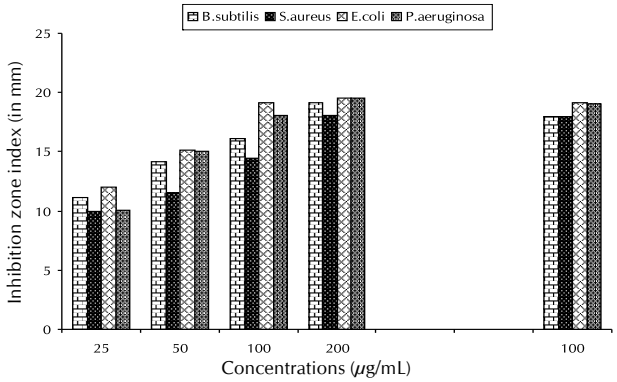

Figure 6: MIC against test bacteria

groats (Watanabe, 1998) also from *Tectona grandis*, *Shilajit* and *Valeriana* (Shalini and Srivastava, 2009).

To evaluate the MIC of the active principal against both Gram negative and Gram positive test bacteria in agar cup as well as by disc method were performed (Fig. 5). The inhibitory effect of active principal (25μg/mL) of BLS sample against test bacteria was observed between 11-18mm (inhibition zone in diameter). Other workers investigated antibacterial effect of BLS extract and found MIC between 50-400μg/mL (Jesonbabu *et al.*, 2011; Hemamalini *et al.*, 2012). While phenolics obtained from seeds of *Elettaria cardamomum* showed MIC at a concentration of 12mg/mL (Al-Maliki, 2011). However, the antibacterial activity of the purified compound was compared with a standard ciprofloxacin (1mg/mL) and was found quite comparable with the experimental sample (Fig. 6).

Further, experiment was conducted to understand the effect of the active principal on time dependent killing *i.e.,* time required for actual inhibition of the selected test bacteria. From the experimental results, it appeared that the growth decline sharply within 8h of treatment (25μg/mL), in all the sets having different test bacterium, except *S. aureus* where a slow decline rate was observed (Fig. 7). Other time killing experiment (Datta *et al.*, 2011) showed inhibition in growth at a much higher concentrations (50-100μg/mL).

The extracted active principal from BLS, the petiole - a waste material, showed its antibacterial efficacy in a quite less concentration against a diversed microbial group (Gram positive and negative). Since the extraction procedure and the solvent used are common that makes the steps of purification user friendly. The active principal identified in this study as phenolic, a secondary metabolite from plant waste material, is well accepted for its therapeutic potential. It is further reciprocated while compared a broad spectrum antibiotic with

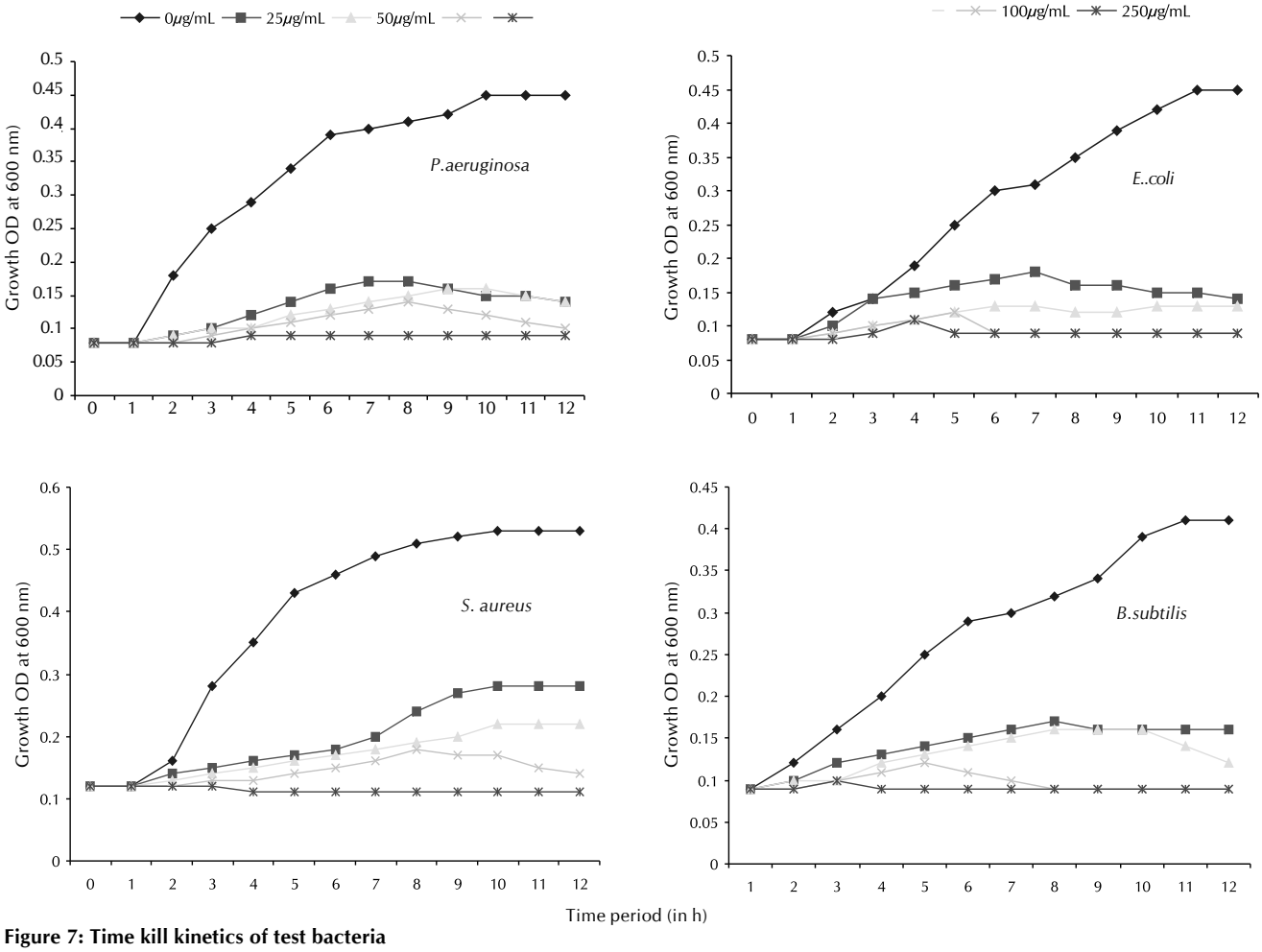

Figure 7: Time kill kinetics of test bacteria

ciprofloxacin). This study carries promise for exploitation of BLS, a renewable resource, for therapeutic use.

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