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Effectiveness of *Piper betel* leaf extracts against *Acinetobacter* species isolated from bronchitis and pharyngitis patients

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Key words: Bronchitis; pharyngitis; *Acinetobacter*; *Piper betel* leaf; GC-MS analysis.

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Abstract

Acinetobacter species is associated with healthcare-associated infections especially chronic cough and other related complications. They are becoming increasingly antibiotic resistant. In the present work, the antimicrobial activity of different extract (water, ethanol, methanol, and chloroform) of *Piper betel* leaves were tested against *Acinetobacter* sp. SZ-1 and *Acinetobacter baumanii* TM-1 isolated from clinical samples collected from the patients suffering from pharyngitis, bronchitis and sore throat. For the antibacterial activity, different plant extracts were used in which *Piper betel* leaf stand out to be the most effective. Six solvents were used for this study in which only chloroformic extract showed the zones of inhibition. TLC analysis showed five spots with R_f 0.896,0.973,0.747,0.574 and 0.482. SDS-PAGE revealed proteins of different molecular weight when the bacterial cells were treated with ethanolic extract. It was also confirmed by Bradford analysis. GCMS analysis depicted different bioactive compounds including phytol; phenol,2,2-methylenebis[6-(1,1-dimethylethyl)-4-methyl; spirost-8-en-11-one,3-hydroxy-,(3β,5α,14β,20β,22β,25R) and 2,2,4-trimethyle-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol. Furthermore, *in silico* analysis can enhance the existing knowledge about establishing the significance of *P. betel* leaves as an effective drug to treat the acute and chronic upper respiratory tract infections.

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Introduction

The genus *Acinetobacter* was discovered in the eighteenth century when it was considered as a pathogen of the soil (Doughari *et al*., 2011). Since its discovery, the genus and its originating species have long been characterized and re-characterized under many genera, before being finally distinguished from other closely affiliated (on the basis of morphological and genetic grounds) genera (Jung and Park, 2015). The species belonging to this genus are reported to be aerobic, Gram negative in their Gram morphology and are typically present in the form of pairs or chains. Their growth characteristics and morphological patterns are highly dependent upon the type of media used for their isolation. Although the genus is home to many species, the most commonly known, studied and well identified species of the genus is *Acinetobacter baumanii*. It is usually found to inhabit water bodies and soil, but is also typically found in healthcare settings, environments and medical equipment (Villegas and Hartstein, 2003). There have been various studies that report the incidence of multidrug resistant *A. baumanii*, which has been the causative agent of many nosocomial and hospital acquired infections in European countries (Kamolvit *et al.,* 2015). Over the years, it has emerged as a serious pathogen, as being reported as the causative agent of many skin, tissue, wound, and blood infections, sepsis, meningitis, and hospital acquired pneumonia (Howard *et al.,* 2012). *A. baumanii* responsible for hospital acquired infections are usually isolated from different environmental settings like hospital walls, roofs, beddings, curtains, medical equipment, door knobs and handles, bin stands, as well as dispensers. It has the ability to sustain on living and non-living sources for long periods of time, and its persistence in hospitals and healthcare facilities is the leading factor behind its resistance to antibiotics and other disinfectants (Evans *et al*., 2013). It mainly targets exposed tissues and organs like the mucous membranes and those areas which have been exposed open by any trauma or wound injury (Sebeny *et al.,* 2008). These infections can take a turn for the worse if these are left untreated, by leading to septicemia and eventual death (Howard *et al.,* 2012). The other reason may be the exposure to the pathogen and its acquiring from environment of the infected hospital as well as the exposure and contact of the healthcare personnel with an infected patient (Rodríguez-Baño *et al*., 2009).

The other species of *Acinetobacter* such as *A. pittii* and *A. nosocomialis* also cause infection in the patients of intensive care unit that is reported around the world while *A. calcoaceticus* causes bacteremia and pneumonia. The other species like *A. lwoffii*, and *A. junii* also have been observed to cause infections in patients with compromised immune systems (Al-Atrouni *et al*., 2016). The affected groups of people can range from all age periods and ranges, but *A. baumannii* can particularly affect those people who are hospitalized and are immunocompromised, which may or may not be hospitalized. The patients who have a protracted stay at the hospitals are also susceptible to infection. Interestingly enough, the peculiar group with an increased risk of its infection are the soldiers and the armed forces who have been sent to conflicted war zones, especially those environments which are dry and humid. The desert areas provide an ideal setting for its growth which is reported to be the main causative agent of infection in wounded armed personnel (CDC, 2004).

The incidence of multiple drug resistant *A. baumannii* is usually reported in patients which are kept in the ICU of hospitals, where the estimated fatality rate is often high (Seifert *et al.,* 1995). Therefore, it is often difficult to ascertain the fatality strictly related to it, and not depending upon the patients' other root causes of disease. However, it would not be wrong to say that the presence of *A. baumannii* certainly elevates the risk of high fatality (Abbo *et al.,* 2007).

The other probable cause in the case of *A. baumannii* is that it acts as a biomarker or a precursor of an increased rate of mortality in the case of bacterial infections associated with patients admitted under critical care in hospitals (Eliopoulos *et al.,* 2008).

The use of plants in the form of drugs has been in use since the advent and enlightenment of disease. The origin of this phenomenon is most probably the result of basic instinct and interest, as the case with animals and their first interaction with humans as a food source. The knowledge was beginning to take shape at the time, in the light of inadequate information regarding the pathogenesis of disease and the use of plants as their cure. The passage of time resulted in the discovery of specific plants which were solely used for the treatment of specific diseases (Petrovska, 2012). The medicinal plants may be defined as those plants that have been associated with herbal treatments since ancient and recent times, for the treatment of established and new infections and diseases, as well as for use in simple herbal and therapeutic concoctions that provide relief against simple ailments like insect bites, headache, nausea etc (Schulz *et al*., 2001).

There have been many plants that are reported for their beneficial properties, countless therapeutic and commercial applications, due to which they have been in use since ancient times, long before their beneficial properties were scientifically proven and researched about. *Piper betel* (*Piper betle* Linn.) plant is one such example. It is one of the most widely known and used plants worldwide. It belongs to the family of Piperaceae, which is known by various names across many countries of the world. It is known as 'ikmo' in the Philippines, where it is largely cultivated (Quisumbing, 1978). It is also grown in many other Asian countries like China, Taiwan, India, Pakistan, Indonesia and Malaysia (Guha, 2006). It has been reported to be effective against various bacterial strains like: *Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes, Micrococcus luteus, Staphylococcus aureus, Aeromonas hydrophila, Escherichia coli, Salmonella Enteritidis, Pseudomonas aeruginosa, Streptococcus mutans* (Khan and Kumar, 2011), *Enterococcus faecium, Actinomycetes viscosus, Streptococcus sanguis, Fusobacterium nucleatum* as well as *Prevotella intermedia* and *Streptococcus pyogenes* (Datta *et al.,* 2011).

The aims of the current study were to find the *Acinetobacter* species associated with pharyngitis, bronchitis and sore throat. The biochemical and molecular characterization of *Acinetobacter* sp., screening of the medicinal plants against it, selection of the medicinal plant on the basis of its bioactivity, thin layer chromatography (TLC), qualitative and quantitative assays of proteins, estimation of antioxidative enzymes including superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APOX), glutathione reductase (GR), catalase (CAT) and gas chromatography mass spectrophotometry (GC-MS) will be ascertained.

Materials and methods

Isolation and biochemical characterization of bacterial colonies

The samples were collected from the patients suffering from pharyngitis, bronchitis and sore throat from different tertiary care hospitals. These samples were proceeded on basic agar medium (Luria Bertani medium). The cultural characterization i.e., size, shape, smell, color, texture, margin and elevation of the isolated colonies was performed. The morphological identification was done by Gram staining. On the bases of cultural and morphological characterization, these colonies were proceeded on differential media (blood agar, MacConkey agar and chocolate agar) and biochemical media (urease, citrate, indole, motility, TSI agar etc) (Cheesbrough, 2006).

Molecular identification of bacterial strains

The genomic DNA isolation was carried out according to the method of Wilson (2001). The isolated and purified DNA was analyzed by agarose gel electrophoresis (Sambrook and Russell, 2001). The bacterial colonies were preserved in glycerol (Cheesbrough, 2006). The 16S rRNA ribotyping of the selected bacterial strain was done.

Screening of medicinal plants against Acinetobacter species

For this study, ten plants were selected, namely *Matricaria chamomilla* (Babona), *Achyranthus*

aspera (Charchitta), *Syzygium aromaticum* (Long), *Piper cubeca* (Kabab Cheeni), *Myristica fragrans* (Jaiphal), *Achillea mellifolium* (Branjasf), *Saussurea lappa* (Qist-e-Shireen), *Anacylus pyrethrum* (Aqarqarha), *Commiphora myrrha* (Marmaki), and *Piper betel* (Paan leaf). The powder of the plants (50 g) was dissolved in one litre of each of the respective solvent (ethanol, methanol, n-hexane, petroleum ether, chloroform, and distilled water) for 6-12 days. The solution was then evaporated using rotary evaporator. The material obtained was lyophilized and stored in vials. At the time of experiment, its solution was made by dissolving it in DMSO (Ishnava *et al.*, 2013).

Antimicrobial activity of selected plants

The antimicrobial activity of the selected plant was determined by using well diffusion and disc diffusion method. The experiments were performed according to the methods of Bauer *et al.* (1966) and Shamim and Khan, (2017).

Quantitative determination of antioxidative enzymes

The activity of antioxidative enzymes was determined according to the method of Shamim and Rehman, (2013). The activity of five antioxidative enzymes (catalase, ascorbate peroxidase, peroxidase, superoxide dismutase and glutathione reductase) was evaluated and the results were observed.

Qualitative and quantitative analysis of proteins

The qualitative and quantitative analysis of proteins was performed by SDS-PAGE (Laemmili, 1970) and Bradford assay (Bradford, 1976) respectively.

TLC (Thin-layer chromatography)

The analysis of TLC was performed according to Dwivedi and Mehta, (2011) and the R_f value of the compounds was calculated.

Gas Chromatography-Mass Spectrometry (GC-MS)

For GC-MS analysis, the sample was injected into the GC chamber of the equipment and the results were obtained in the form of retention time, which was represented on the graph by peaks of various lengths (Rukhsana *et al.*, 2017).

Results

Collection of samples

The samples were collected from fifty patients suffering from acute and chronic bronchitis, acute pharyngitis, chronic pharyngitis and sore throat from four different tertiary care hospitals of Lahore (Table 1). Out of total samples, the positive samples were eighty-five.

Isolation and biochemical characterization of microbial flora

For the purpose of isolation and purification of microbial flora, all swabs were proceeded on Luria Bertani growth medium. The bacterial strains selected for this study were designated as SZ-1 and TM-1. The results of biochemical tests were also observed (Table 2).

Table 1. Sample collection from different hospitals in Lahore, Pakistan.

Sr. No.	Infections	Hospital-1	Hospital-2	Hospital-3	Hospital-4
1.	Acute bronchitis	07	04	05	-
2.	Chronic bronchitis	03	08	04	-
3.	Sore throat	08	03	08	-
4.	Acute pharyngitis	09	10	-	06
5.	Chronic pharyngitis	15	19	-	11
Total no. of samples				120	

Molecular characterization of bacterial strains

The bacterial strains (SZ-1 and TM-1) demonstrated the most frequent number of colonies (CFU/plate) due to which they were selected for 16S rRNA sequencing. The biochemical results demonstrated the presence of *Klebsiella* sp., *Pseudomonas aeruginosa*, *Acinetobacter* sp., and *Bacillus cereus*. The 16s rRNA sequencing elucidated the bacterial strains as *Acinetobacter* sp. SZ-1 and *Acinetobacter baumanii* TM-1.

Table 2. Biochemical characteristics of the bacterial strains.

Sr. No.	Biochemical test	Strain SZ-1	Strain TM-1
1.	Gram staining	Negative	Negative
2.	Catalase	Positive	Positive
3.	Coagulase	Negative	Negative
4.	Urease	Negative	Negative
5.	Citrate	Positive	Positive
6.	MacConkey	Negative	Negative
7.	Motility	Negative	Negative
8.	Oxidase	Negative	Negative
9.	Indole	Negative	Negative

Antimicrobial activity of P. betel leaf extracts against Acinetobacter spp

The bioactivity of ten medicinal plants was evaluated against *Acinetobacter* sp. SZ1 and *A. baumanii* TM-1. The plant extract was used at the concentration of 100 mg/mL. No zones of inhibition were observed in the case of any plant, except ethanolic extract of *Piper betel* leaf (Figure 1).

Profiling of antioxidative enzymes

The results of antioxidative enzymes are demonstrated in Figures 2-5. The APOX activity was expressed the most in the ethanolic extract. Followed by the control sample. Its activity was not observed in the methanolic extract (Fig. 2). The activity of GR was observed to be the highest in the case of the ethanolic extract, followed by the chloroformic extract, whereas the activity of the control sample and the methanolic extract was found to be equally expressed (Fig. 3).

The POX enzyme was most expressed in the control sample, whereas no activity in the ethanolic extract was observed (Fig. 4). The SOD enzyme was observed to be strongly expressed in all extracts, with the highest activity being reported in the case of the ethanolic extract (Fig. 5).

Table 3. Estimation of proteins by Bradford assay.

Samples	Protein concentration (µg/ml)	
	Strain SZ-1	Strain TM-1
Control	0.532	0.372
Bacteria + Chloroformic extract	0.311	0.315
Bacteria + Ethanolic extract	0.334	0.11
Bacteria + Methanolic extract	0.334	0.392

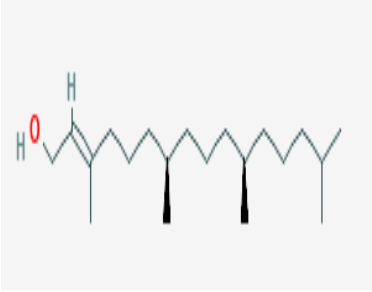
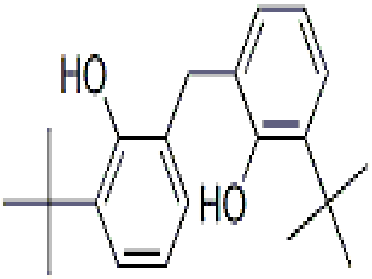
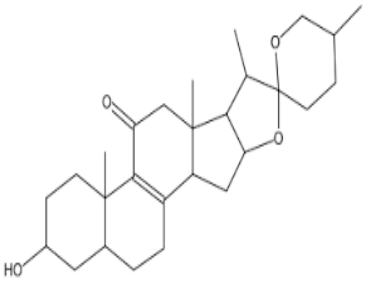
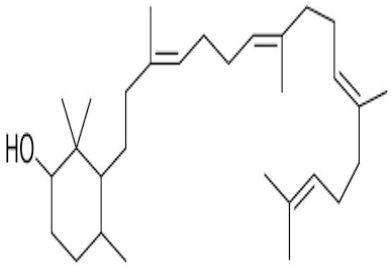
Protein profiling

The results of SDS-PAGE elucidated the presence of several bands in case of control sample, the methanolic and the chloroformic extracts, whereas in the case of the ethanolic extract the presence of only one band (20 kDa) was observed. The other bands which were visible in the other extracts were found to be suppressed (60, 70, 80, 100, 120 kDa), as shown in Figure 6. Bradford assay revealed that protein concentration was least expressed in the chloroformic extract whereas maximum expression was observed in the ethanolic and methanolic extract, as shown in Table 3.

TLC

TLC results are shown in Figure 7. From left to right, three spots are demonstrated depicting the chloroform, ethanol, and methanol extract respectively. In spot 1, Rf values was calculated to be 0.929 and 0.729, whereas the Rf values of spot 2 and 3 was calculated as 0.905, 0.682 and 0.941, and 0.741, respectively.

Table 4. Bioactive components of *Piper betel* L. extract (ethanol) as demonstrated by GC/MS analysis.

Sr. No.	Retention time (min)	Component	Molecular formula	Molecular weight	Structural formula
1	18.329	Phytol	$C_{20}H_4O$	296	
2	21.109	Phenol,2,2-methylenebis[6-(1,1-dimethylethyl)-4-methyl	$C_{23}H_{32}$	34	
3	22.955	Spirost-8-en-11-one,3-hydroxy-,(3β,5α,14β,20β,22β,25R)	$C_{27}H_{40}O_4$	428	
4	23.905	2,2,4-trimethyle-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	$C_{30}H_5O_2$	428	

GC-MS analysis of P. betel extract

The GC-MS analysis of the chloroformic extract revealed the presence of phytocomponents like pregnane -3; 20β-diol,14α,18α-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl]-; diacetate; azulene, 1, 2, 3a, 4, 5, 6, 7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-[1R-(1α,3aβ,4α,7β)]-; alfa-caryophyllene and alfa-copaene; spirost-8-en-11-one,3-hydroxy-(3β,5α,14β,20β,22β,25R); phenol,2,2-methylenebis (1,1-dimethylethyl)-4-methyl-,2,2,4-trimethyle-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol and phytol. Among them phytol; phenol,2,2-methylenebis[6-(1,1-dimethylethyl)-4-methyl; spirost-8-en-11-one,3-hydroxy-,(3β,5α,14β,20β,22β,25R) and 2,2,4-trimethyle-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol were found to be bioactive against *Acinetobacter* sp. (Table 4).

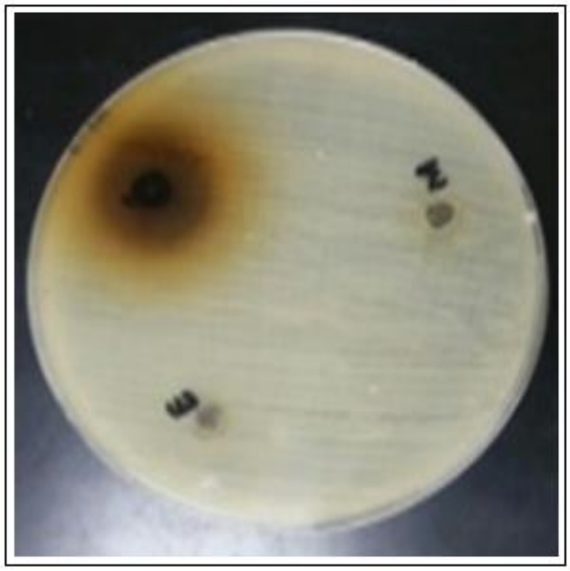


Fig. 1. Well diffusion assay for Piper betel leaf (Paan leaf) extracts. A clear zone of inhibition for ethanolic extract was observed (3.6 mm) against strain TM-1.

Discussion

The pathogenesis of bacterial, viral or fungal pathogens results in the development of infections in the upper respiratory regions, like bronchitis and pharyngitis. The incidence of bronchitis or acute bronchitis is characterized as the inflammation of the bronchioles. The regular symptom which occurs is cough (Albert, 2010). The cough with or without sputum usually lasts for one to three weeks (Wenzel and Fowler, 2006). The typical pathogens that are responsible for the pathogenesis are viruses, which explains the reason of the lesser efficiency of antibiotics in this case (Park *et al.,* 2016). Over the years, there have been many studies that have demonstrated the causative agents of acute bronchitis to the viral agents like influenza virus, adenovirus, and rhinovirus, among many others (Louie *et al.,* 2005). In stages occurring after acute bronchitis, there are more chances of the patient to develop chronic bronchitis, which is characterized by the protracted appearance of aggravated signs and symptoms over long periods of time (Kim and Criner, 2013). According to our study, bacteria were the causative agent of bronchioles infection which contradict with the previous study of Boldy *et al.* (1990). The incidence of pharyngitis for a long period of time may lead to its development at a chronic stage, which can further lead to many complications including otitis media and severe sinusitis (Hildreth *et al.*, 2015). The *Acinetobacter* spp. are Gram negative bacteria that are naturally found in ubiquitous habitats including water and soil, and may routinely be found to inhabit food sources as well as the human body including skin, mucosal membranes, open wounds, and the respiratory and gastrointestinal tracts (Albrecht *et al.,* 2006).

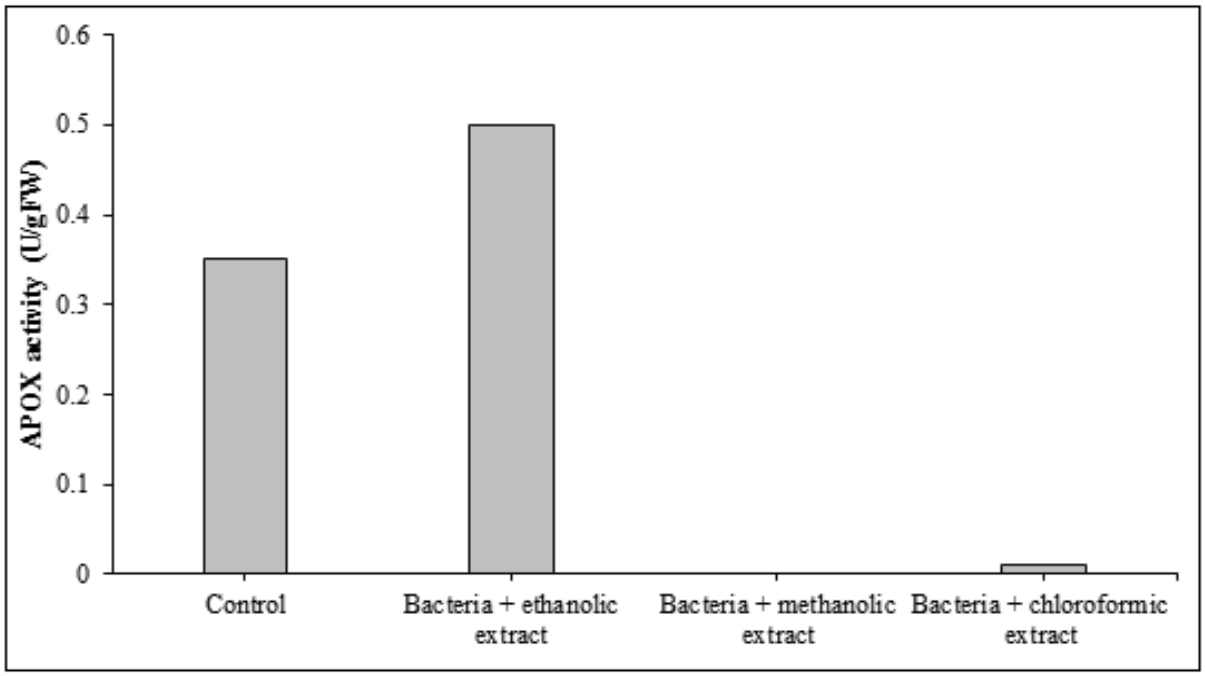


Fig. 2. APOX activity in the ethanolic, chloroformic, and methanolic extract of *P. betel* leaf, and control.

The epidemiology associated with *Acinetobacter* is broad, and encompasses a wide range of infections that are associated with both the community and clinical settings, with infections occurring in tropical environments, war zones, natural calamities and even hospital outbreaks in moderate environments (Lolans *et al.,* 2006).

The composition of antibiotics is often made up of synthetic compounds whereas plant-based medicines are composed of different compounds which are extracted from various reported plants and their metabolites. This factor is also very imperative in the case of antibiotic resistance, as the plant-based medicines are relatively less impervious to the development of resistance in microorganisms against them. The wide variety of phytochemical compounds found in plants have been in use since ancient times for their action against many human pathogens of bacterial, fungal and viral origins.

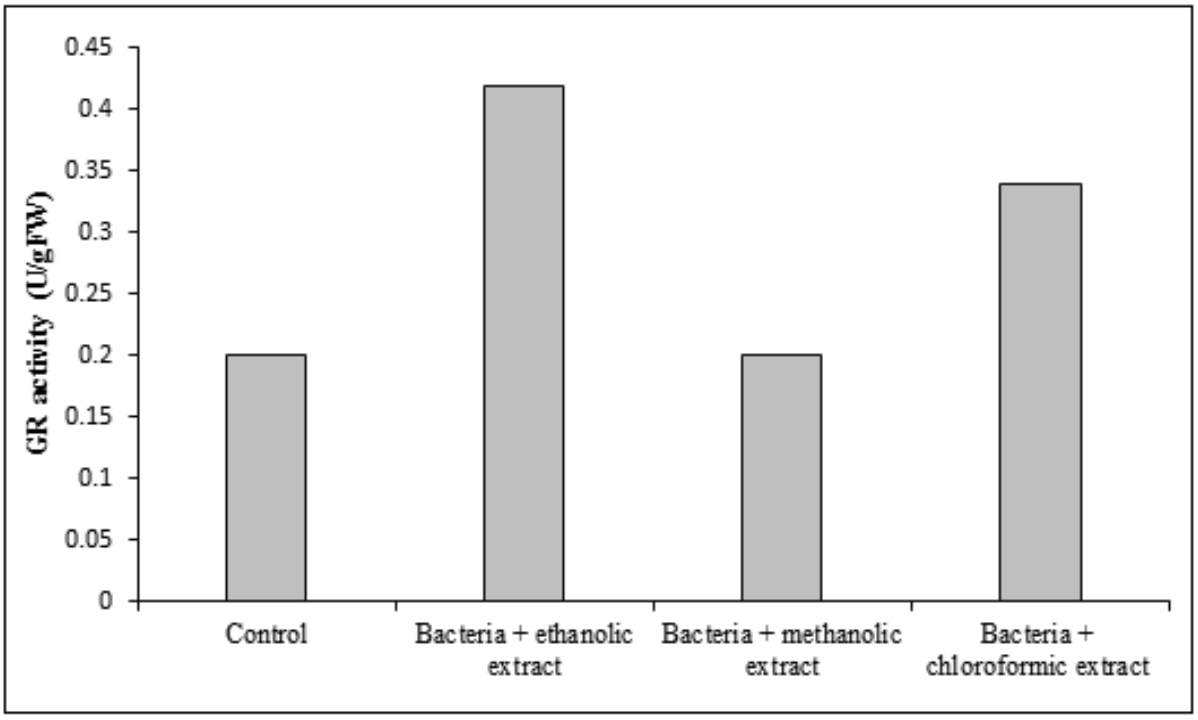


Fig. 3. GR activity in the ethanolic, chloroformic, and methanolic extract of *P. betel* leaf, and control.

The *P. betel* plant is an enriched source of proteins, minerals, antioxidants and phytochemicals. It has been long used as a medicine in the treatment of boils, abscesses, halitosis, wound abrasion, constipation, and oral ailments like mouth, buccal cavity ulcers, and dental cavities. Its leaf is often used in conjunction with other condiments like areca nut, slaked lime, and tobacco which is collectively known as betel quid. It is a home to more than hundred different varieties, with the most significant and popular varieties of *P. betel* known in India as Kauri, Mysore, Bagerhati, Salem, Ghanagete, and Banarasi, among many others (Chauhan *et al.,* 2016). In this research work, different extracts of *P. betel* leaf, along with other plants extracts were prepared. Their antibacterial activity was determined by disc and well diffusion methods. The results elucidated that there was no zone of inhibition observed in any of the extracts but a zone of inhibition in the case of ethanolic extract of *P. betel* leaf against *A. baumannii* TM-1 and *Acinetobacter* sp. SZ-1. The study conducted by Khan and Kumar (2011) also investigated the effectiveness of the ethanolic and methanolic extract of *P. betel* leaf, where it was found that the methanolic extract was more effective than the ethanolic extract against many reported pathogens, which contradicted with our study findings. However, the study findings of Kaveti *et al.* (2011) agreed with our study, as according to them the potential antibacterial activity of the ethanolic

extract of *P. betel* leaves against the multiple antibiotic resistant bacterial species cannot be ignored. According to the study of Valle *et al.* (2016), the ethanol extract of *P. betel* leaf demonstrated the zones of inhibition ranging from 20-30 mm, whereas the methanol extract gave the zones of inhibition of approximately 20-25 mm in range.

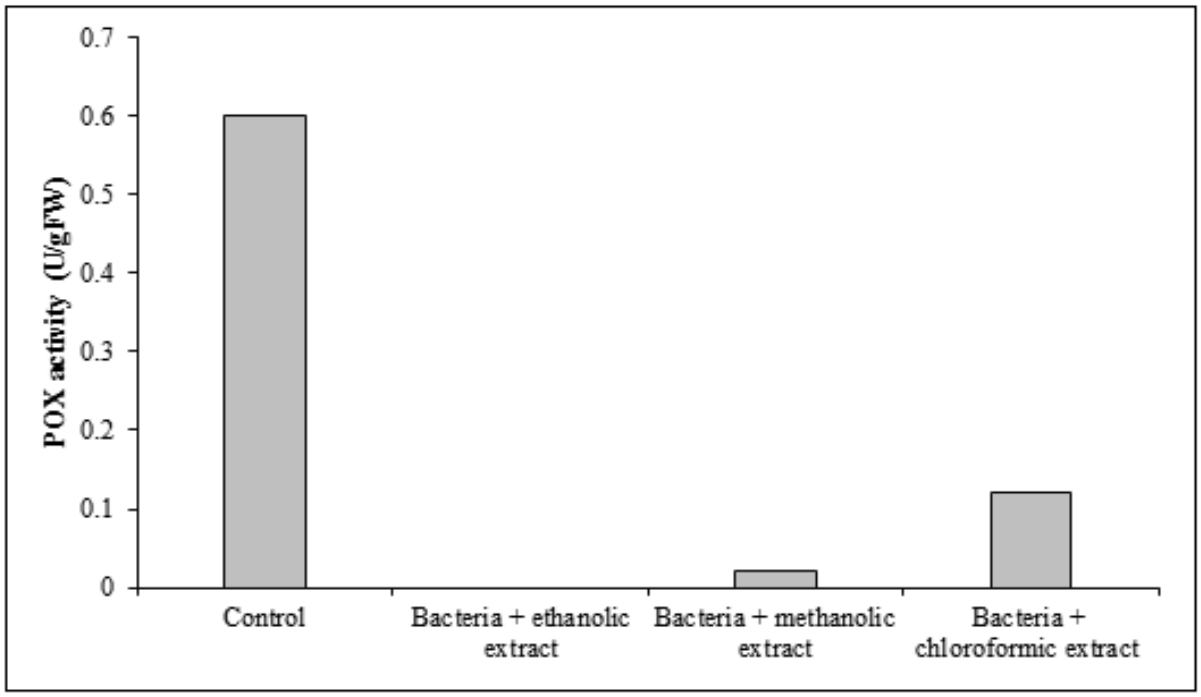


Fig. 4. POX activity in the ethanolic, chloroformic, and methanolic extract of *P. betel* leaf and control.

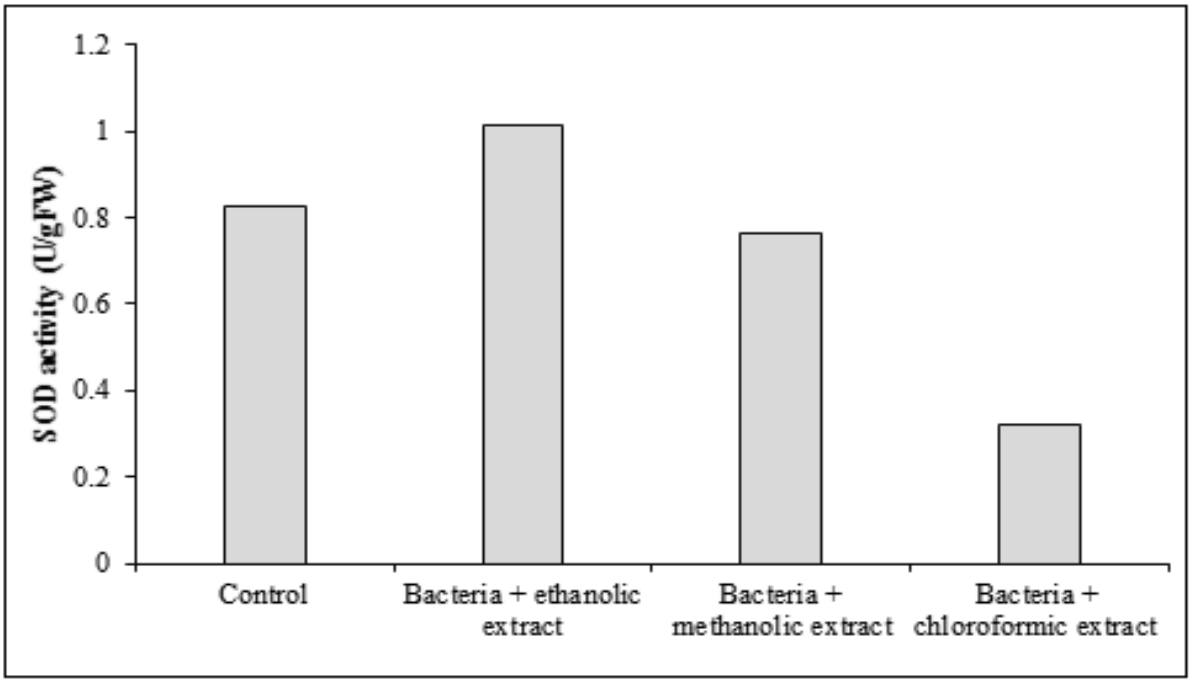


Fig. 5. SOD activity in the ethanolic, chloroformic, and methanolic extract of *P. betel* leaf, and control.

The similar results were reported in the study of Khan and Kumar, (2011), where the different concentrations of methanolic and ethanolic extracts were observed to be effective against several bacterial isolates like *S. aureus*, *E. coli* and *P. aeruginosa*, with the methanolic extract showing larger zones (10-30

mm) as compared to the ethanolic extract (15-20 mm) in the agar well diffusion method. The minimum inhibitory concentration (MIC) ranged from 1-10 µg/ml for both of the extracts used in the study. The study conducted by Agarwal *et al.* (2012) reported the antimicrobial activity of aqueous and acetate extracts from different varieties of *P. betel* leaves. The results of our study were in agreement with the results of Datta *et al.* (2011), which reported the efficient activity of *P. betel* chloroformic and ethanolic extract against Gram negative bacteria. The antibacterial activities have also been widely reported in various studies conducted over the years (Ramji *et al.*, 2002; Nalina and Rahim, 2007; Row and Ho, 2009; Chakraborty and Shah, 2011; Subashkumar *et al.*, 2013; Nouri *et al.*, 2014).

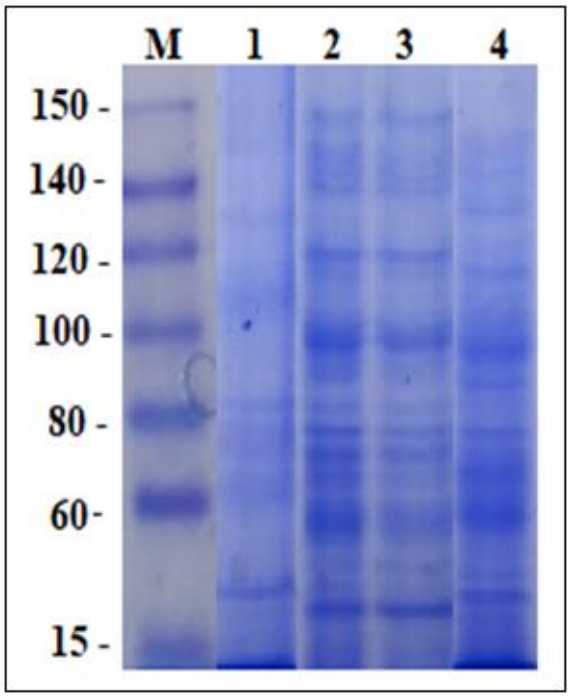


Fig. 6. Bands of proteins of various molecular weights observed in SDS-PAGE, where 1= ethanolic extract (+ bacteria), 2= methanolic extract (+ bacteria), 3= chloroformic extract (+ bacteria) of *P. betel* leaf, 4= control (only bacteria), and M= protein marker.

The research study of Liu *et al.* (2015) expressed the protein compounds present in *P. betel* leaf and its extracts, which were found to be responsible for the remarkable antioxidative ability of *P. betel* leaf reported against many bacterial and fungal pathogens. The antioxidative enzymes are nature's soldiers with a superlative defense mechanism.

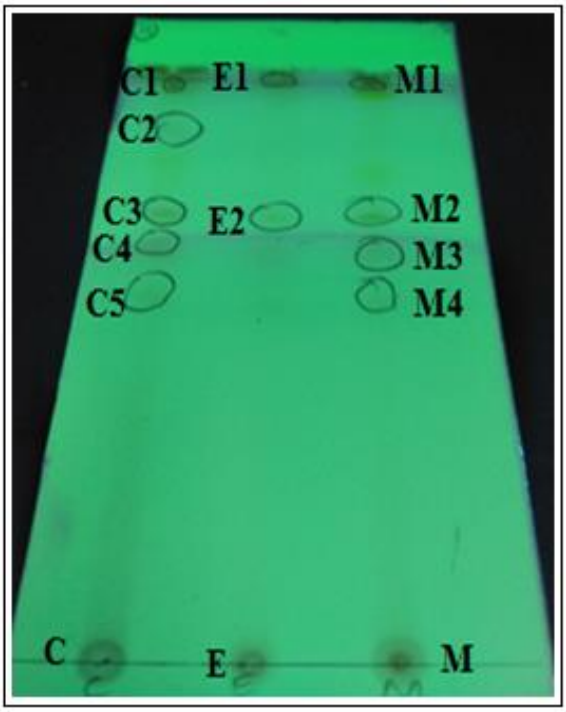


Fig. 7. Visualization of components in TLC analysis of *P. betel* extracts.

They are the defendants of the living organisms as they combat the oxidative stress that tends to be produced naturally in the bodies. The results revealed the expression of different antioxidative enzymes in the presence of all extracts, but the most activity was reported in the case of SOD enzyme. The activity of the APOX was most reported in the case of the ethanolic extract, followed by the chloroformic and methanolic extract, respectively.

The results of our study agreed with the study findings of Abrahim *et al.* (2012), where the elevated expression of SOD was observed in the presence of *P. betel* leaf extract. The elevated expression of the SOD enzyme demonstrates their ability to remove or scavenge superoxide anions, leading to the alleviation of reactive oxygen species (ROS).

The elevated activities of CAT, SOD and glutathione peroxidase was also reported in the study work of Aliahmat *et al.* (2012). The analysis and determination of the bioactive compounds present in the ethanolic extract of *P. betel* leaf revealed the

presence of several potential bioactive compounds (Table 4). The presence of these compounds was also elucidated in the study of Vikrama *et al.* (2018), where the presence of these compounds was also determined by GC-MS technique. These bioactive compounds can be used to affirm the bioactive potential of *P. betel* leaf, which maybe attributable to the cumulative list of potential beneficial properties that are attributed to *P. betel* leaf and its extracts.

Conclusion

(Key findings and recommendations)

The medicinal importance of *P. betel* leaf is one of the most promising aspects of its use, as reported by the many literatures that reveal that the leaf contains several vitamins, antioxidants, antibacterial compounds, etc. The leaf acts as a natural antioxidant that is related with different biological activities like hepatoprotective, antidiabetic and anticancer properties, since free radicals are involved in all these diseases. The leaf produces enzymes that help in digestion and possess the broad-spectrum antimicrobial activity against various bacterial strains especially against *Acinetobacter* sp. SZ-1 and *Acinetobacter baumanii* TM-1. All these properties ascertain the imperative biologically active properties of *P. betel* leaf. Furthermore, in depth analyses like *in silico* studies can elucidate the active role that the compounds serve in the effectivity and antimicrobial action of *P. betel* leaf.

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