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Research Article

Extraction Of Betalains From Red Beet (Beta Vulgaris, L.) And It's Potential Uses As Antimicrobial Agents Against Esbl Producing Isolates

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ABSTRACT

Due to the adverse effects of artificial food additives, pigments from natural sources have begun to increase. This study aimed to examine the antibacterial effects of beetroot containing betalain pigment against ESBL producing clinical isolates. Quantitative measurements of the betalain pigment in beetroot extract were performed using spectrophotometer. The results revealed that, red beet betalains pigment extracted by ethanol extract was more stable at pH 5. In the case of Temperature, 30ºC was suitable of stability of betalains. Meanwhile, confirmed clinical isolates were subjected to with ESBL characterization and then inhibited the potential isolates with betalains pigments by agar well diffusion method. Results revealed that direct crude extract of betalain having the antibacterial activity highly against *K. pneumonia* and *E. faecalis*. From the findings, it can be clearly noted that the betalains is a promising source of natural antimicrobial agent and definitely provides an alternative towards synthetic antimicrobial agents.

Keywords: Betalactamase, ESBL, Beetroot, Betalain, Antibacterial activity.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the major public health problems, especially in developing countries, when compared with developed countries, these countries are relatively easy to obtain and the increase in drug consumption has led to a disproportionately high incidence of inappropriate antibiotic use and resistance levels are also higher [(1)]. India is one of the countries with the highest incidence of bacterial infections in the world, including typhoid fever, cholera, pneumonia, and tuberculosis. AMR occurs when fungi, parasites, bacteria, etc. evolve and stop responding to drugs designed to kill them. Therefore, this phenomenon may erase decades of scientific innovation and medical progress [(2)]. Every year 700,000 people dead due to antimicrobial resistance (AMR) and it is estimated that 10 million people would die from antimicrobial resistance by 2050. AMR alone causes more deaths than cancer and road traffic accidents [(3)].

Extended-spectrum Beta lactamases (ESBLs) are a major resistance mechanism that obstructs antimicrobial therapy of illnesses caused by Enterobacteriaceae and poses a serious threat to the current antibiotic arsenal [(4)]. ESBLs are plasmid-encoded enzymes capable of hydrolyzing the penicillins, narrow- and extended-spectrum cephalosporins and aztreonam. The ESBL can be divided into three main types, called TEM, SHV and CTX-M. ESBL's CTX-M type can be further divided into three groups: CTX-M-1, CTX-M-2 and CTX-M-9. Previously, ESBLs were generally found in *Klebsiella pneumoniae* (TEM or SHV types) and most of the isolates were from nosocomial infections [(5)].

The ESBL producing isolates isn't only resistant to betalactam antibiotics but also it is resistant to other antibiotics. The scientific world is increasingly concerned with the treatment of these multidrug resistant bacteria. The challenge of solving the origin of ESBL-producing organisms worldwide is due to a variety of reasons, including the difficulty and discrepancy in detecting ESBL production [(6 and 4)].

Due to the escalation of the ESBL crisis in the past few years and the limited availability of antibiotics, new antibacterial compounds are needed to counter the emergence of these new antibacterial drugs. For centuries, plants have been used as remedies and treatments of diseases, which containing secondary metabolites are used directly as precursors or as compounds in the pharmaceutical industry [(7)]. The plant pigments have gained a lot of attention among plant products because of their use in food, cosmetics, and pharmacological research.

The recent focus on beetroot is mainly due to the discovery that the source of dietary nitrates may have important implications for health management. The beetroot containing betalains pigments are bioactive compounds and which are water soluble and categorized into betacyanins (red colour) and betaxanthins (yellow colour). The antimicrobial effects of beetroot juice and its pigments have been studied extensively [(8, 9, 10 and 11).] Although various methods for antimicrobial activity of betalains have been studied, the antibacterial activity of betalains against ESBL producing isolates work was not yet studied. It has sparked interest in a possible uses for betalains in antibacterial activity. The present study is thus designed to focus on the evaluation of the antibacterial activity of beetroot (*Beta vulgaris* L) containing betalains pigment against ESBL producing clinical isolates.

METHODS

Separation extraction

About 20 g of red beet was mixed in a blender with 100 ml of ethanol, acetone, and water for 15 min at room temperature (each solvent was done separately). The extract was filtered and adjusted to various pH and left for 24 hours then concentrated under vacuum by a rotary vacuum evaporator at 40ºC. From this stock solution, 1ml was diluted individually with 1ml of relevant solvents (1:1), this solution was used for determination betalain content [(12)].

Estimation of betalain

The amount of betalain in each solvent *viz.,* ethanol, acetone and water was calculated as mg/100g basis. The diluted content was measured at a wavelength of 535 nm and measured as mg betalains/100 g [(13)].

$$\text{Total betalains content (mg / 100 g)} = \quad A \times DF \times MW \times 1000 / €L$$

Collection of clinical bacterial isolates

The clinical isolates of 8 bacterial genera were procured from a clinical laboratory, Coimbatore, India. All isolates were confirmed with cultural characteristics by selective media and biochemical tests.

Determination of Beta lactamase producing bacterial isolates

Penicillin solution was dispensed in 0.5ml volume in small test tubes. Test bacteria were removed with a loop from an overnight culture on a solid medium and suspended in the Penicillin solution to give a density of at least 10^4 CFU/ml. After one hour at room temperature, two drops of the starch indicator were added to the suspension, followed by one drop of Iodine reagent. The positive reaction was indicated by the disappearance of the blue color immediately. Persistence of blue color for longer than 10 minutes constituted a negative test [(14)].

Determination of ESBL producing bacterial isolates by phenotypic method

Confirmation of the ESBL producing isolates was done by the phenotypic confirmatory test according to CLSI recommendation and Polsfussa *et al*., [(15)] method. In this test, first generation of betalactam i.e. Amoxicillin disc (30µg) alone and in combination with clavulanic acid (10 µg) was used. After overnight incubation at 37^{o}C, diameter of zone of inhibition was measured. A 5 mm or more increases in diameter of zone of inhibition for Amoxcillin tested in combination with clavulanic acid versus its zone when Amoxcillin tested alone confirms a ESBLs producing organism.

Determination of antibacterial activity of betalains

The Mueller - Hinton agar plates were inoculated with freshly prepared overnight inoculums, which were swabbed over the entire surface of the medium. Inoculums were of 10^8 CFU/ml of bacteria. The 6mm diameter of the well was made with sterile stainless steel borer on the agar plates. A 100 µl of different concentrations of betalains were filled in well with the help of micropipette and one well filled with ethanol and another well filled with ampicillin (10mcg). The petriplates were kept for incubation at 37 °C for 24 hours. After incubation, the microbial growth was determined by measurement of zone of inhibition around each well and recorded in mm.

RESULTS AND DISCUSSION

In the present study an attempt was made to identify and characterize the betalain pigment present in *Beta vulgaris* L. and their effective utilization in therapeutic areas. During the separation, the pH plays an important role in the stability of betalain pigment. Woo et al., [(16)] reported that betalain is more stable between pH 3 to 7. Another hand of Bastante et al [(17)] reported that betalain color was stable at pH 5 and 6. In this study, it is recorded that highest concentration of betalain observed at pH 5 for all the 2 solvents, *viz.,* ethanol (153.5mg/100mg), and water (132.2±0.14/100mg). The acetone recorded a maximum of 143.8mg/100mg at pH 4. In accordance with findings of the present study, Sabarudin *et al.,*[(18)] reported pH 4-5 as the optimum pH for the extraction of betacyanin from *Bougainvillea* bracts with concentration of 10.67 g/L. Kugler and Carle [(19)] had been reported that betalain are stable between pH ranging from pH 3.0 to 7.0 supports the present study.

The temperature is the most important factor in plant pigment degradation during the extraction and concentration processes [(20)]. The present study recorded an optimum temperature of 30°C, which reported the maximum betalain concentration of 127.5mg/100mg with ethanol. The results of present investigation also corroborate with Tang and Norziah [(21)] and according to these authors, the maximum pigment betalains retention was found between 25 to 30°C and increasing the temperature decreased the betalain retention.

Similarly, Pandey *et al.,* [22] studied the extraction temperature on betalain content. However in this study water extraction of betalain pigment reported the maximum concentration 132.2±0.14/100mg. This can be attributed to the fact that betalain being hydrophilic in nature and a slight increase (25-30°C) in the temperature resulted in an increase in concentration of betalain [23]. Roy *et al*. [24] have reported that the extraction of betalains from red beet was optimal at 40 °C. But however further increase in temperature to 45°C demonstrated a lesser extraction. Earlier study by Garcia-Barrera *et al.,* [25] have reported that betalain degradation occurs with increase in temperatures can be attributed to by isomerization, decarboxylation or cleavage by heat or acids [26].

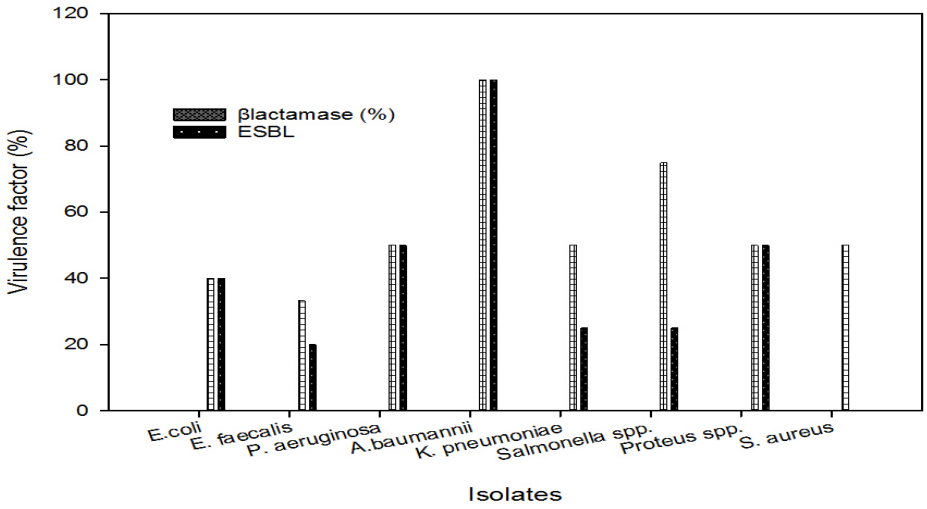
In the present study ethanol proven to be the efficient extraction solvent compared to acetone and water. Study by Ravichandran *et al.,* [27] on the optimal extraction of betalains from freeze-dried roots of *Beta vulgaris* L. had demonstrated that 50% ethanol is the efficient extractant, was in concordance with data of this study. Da Silva *et al*., [28], have reported that the concentration of ethanol in the extraction solvent had the greatest influence on betalain extraction efficiency but however higher ethanol availability did not favor target compounds removal. This can be attributed to the fact that solvent with very high polarity, such as water, or very low solvent strength, such as chloroform, acetone and hexane, did not result in good extraction of bioactive compounds from the plants [29]. Findings of this study are however contrary to the observation by Patil *et al*. [30] who reported that the concentration of betalain in the aqueous extract is higher than in the ethanol extract.

In the present study, 8 bacterial species (18 isolates) were procured from the clinical laboratory and confirmed with chromogenic media. The *E.coli, E. faecalis* and *K. pneumonia* each accounting to 17.65% followed by *P. aeruginosa, Salmonella* sp., *Proteus* sp., and *A. baumannii* each accounting to 11.1% and *S. aureus* accounting to 5%. The 77.7% were identified as gram-negative and 22.5% were found to be gram-positive organisms.

The study revealed that among the individual isolates tested 100% of betalactamase production was demonstrated by *S.aureus* and *E. faecalis*. In India Gajul *et al*., [31] reported that gram positive and gram negative isolates were positive for betalactamase by iodometric method and many authors have reported iodometry is better than other methods. Gajul *et al*. [31] pointed out the iodine test was found to be easy to perform, cheap and effective in detecting β-lactamase (Fig. 1).

Screening of ESBL producing clinical isolates were done by double disc synergy testing which is a simple and cost effective procedure. With respect to ESBL producing isolate, *E.coli* and *K. pneumoniae* demonstrated 33.33%, whereas *E. faecalis* recorded 66.66% and *P. aeruginosa*, *A. boumannii*, *Salmonella* spp. and *Proteus* spp. exhibited 50% activity. *S.aureus* was not subjected to the ESBL characterization. The percentile of the virulence factor was in the range of 33.33 to 100% (Fig.1). Recently Nishanthy and Saikumar [32] found that the impact of ESBL was high on various germs detected on clinical samples and they found that *E.coli* contained high levels of ESBL, which was higher than the prevalence found in the current study but lower than that of other isolates *P. aruginosa* and *K. pneumonia*.

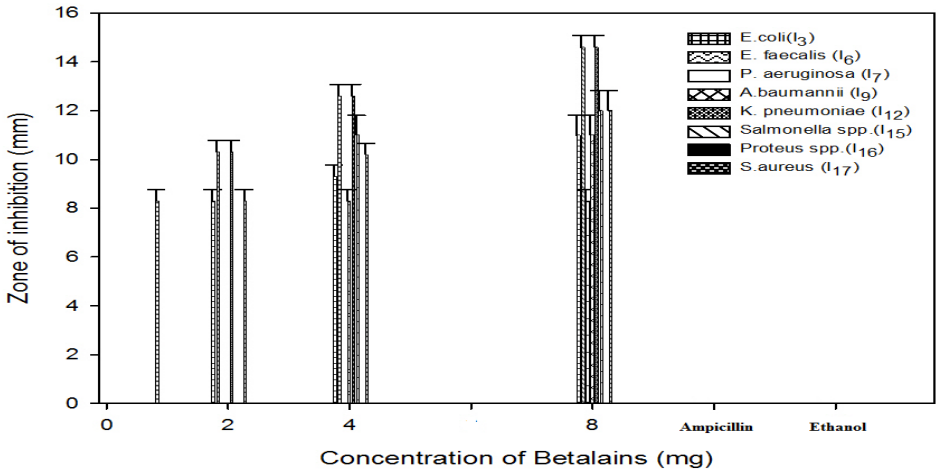
Fig. 1 Prevalence of betalactamase and ESBL isolates



Considering the increase in the ESBL crisis over the past few years and the minimal growth of antibiotics, there is an urgent need for new antibacterial compounds to combat the emergence of these new antimicrobials. Numerous studies have examined the antimicrobial activity of different plant species in different geographical areas in search of new antibiotics. The use of plant pigments as antimicrobials has not been elaborated until recently, since most antibiotics are derived from bacterial or fungal origin [(33)]. This point of view, current study was carryout with beetroot containing betalain pigment.

The betalain was exhibited antibacterial activity against all the test pathogens except *Proteus* spp. (I_{16}). Betalain at 1 mg concentration showed no inhibitory effect on the isolates except, *E. faecalis* (I_6). The findings also revealed that the antimicrobial activity seems to be dose dependent i.e., as the concentration increases there is an increase in bacterial inhibition. The highest zone of inhibition observed was 14.6 ±0.47mm for *K. pneumonia* (I_{12}) and *E. faecalis* (I_6) at 8 mg concentration. The lowest zone of inhibition of 8.3±0.47mm was observed for *E. faecalis* (I_6), *E. coli* (I_3), *A. boumannii* (I_3) and *P. aeruginosa* (I_7) at 1, 2, 4 and 8 mg concentration respectively. *Salmonella* spp. demonstrated a zone of inhibition of 11 ±0.81 and 12 ±0.81 mm at 4 and 8 mg concentration. Negative control ethanol and positive control ampicillin showed no zone of inhibition (Fig.2).

Fig.2 Antibacterial activity of betalains against ESBL producing isolates



The results of a number of studies have proved the beneficial effects of various plants containing betalain [34]. However, research on the antibacterial activity of betalain in beetroot is limited. Jacob *et al*., [35] were suggested that red beet containing betalains play a significant role as an antioxidant, antiviral, antimicrobial, hepatoprotective and anti-cancerous agent. The previous study also suggested that beet containing betalain has been possess the antibacterial activity [36 and 37]. It is speculated that the antibacterial activity of betalain is due to their negative effects on the function, structure and penetration of microbial cell membranes, which ultimately lead to cell death [38].In conclusion, the present study findings add to the increasing evidence of betalain to be utilized as an antimicrobial against ESBL producing isolates. Therefore, the studied plant pigment may represent a valuable therapeutic tool and its active compounds (betalains) as promising alternatives for supplemental therapies for the treatment of several pathological disorders. However, studies on the function of betalains and their properties should continue to reveal their significance of beneficial activity.

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