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Beta lactamase Inhibitors from Indigenous Herbs and Spices.

Sohail Shaikh, Rajiv Lochan, Praneeta Kaul and GD Tandon*

Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Dr. D.Y.Patil Vidyapeeth, Pune 411033, India.

ABSTRACT

Antibiotic resistance being called as the 'ticking time bomb' of the 21st century, as the antibiotic channel is losing sheen and is on a standstill due to lack of initiatives for new antibiotic development, and other novel solutions are required for its revival. Penicillin, the oldest among the antibiotics, obtained from the *Penicillium* mold, was one of the first antibiotics used to treat infectious diseases by inhibiting the growth of gram positive bacteria. Later due to microbial resistance the prominence of this antibiotic lurched. It was due to the ability of some microbial strains to synthesize β-lactamases, which in turn inactivate penicillins by hydrolyzing the β-lactam ring and thereby promoting resistance to the antibiotic. Today several types of β-lactamase enzymes are known to inactivate different classes of antibiotics causing a major crisis in the field of medicine, which requires effective β-lactamase inhibitors to decipher this problem. In this study, Penicillin antibiotic, β-lactamase enzyme and β-lactamase inhibitor activity was analyzed by Iodometry and Bioassay methods. Sulbactum was used as the standard β-lactamase enzyme inhibitor throughout the study. In the current study, the β-lactamase inhibitor activity of 68 extracts from Indian herbs and spices was surveyed. Most promising results of the β-lactamase inhibitor activity *in vivo* and *in vitro* were achieved from the herbal extracts of Baheda (*Terminalia bellerica*), Ginger (*Zingiber officinale*), Brahmi (*Bacopa monnieri*), Garlic (*Allium sativum*), Gurmar (*Gymnema sylvestre),* Satavar (*Asparagus racemosus*) and Pomegranate (*Punica granatum*) peels and seeds against *Staphylococcus aureus* as the test organism. As many microorganisms are becoming resistant to antibiotics, it is indeed necessary to find new β-lactamase inhibitors.

Keywords: Penicillin, β-lactamase, *Staphylococcus aureus*, β-lactamase inhibitor.

Corresponding author

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INTRODUCTION

Traditionally, usage of plants in curing illness has deep roots in man's history [12]. The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products remain the main sources of drugs [6]. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants [27].The search continues to discover safe and effective antimicrobial agents having high therapeutic and prophylactic activity against a wide variety of bacterial infections. Current research on natural molecules and products primarily focuses on plants since they can be sourced more easily and selected on the basis of their ethno-medical use [21].

Most classes of antibiotics, including the β-lactam antibiotics, tetracycline, amino glycosides, and macrolides were originally derived from natural sources, and were further chemically modified to confer better properties on the drug. Microorganisms have evolved at a higher speed than the drug development and current high-end antibiotics have become ineffective in some infections because of drug resistance. Carbapenam is one of the potent but last groups of antibiotics developed world wide. There is a certain need for discovery and development of novel and effective antibiotics to curtail multi-drug resistance. There have been modifications to the available antibiotics but no new drug has come up. *Staphylococcus aureus* is one of the well known notorious gram-positive microorganisms that have been shown to exhibit resistance to a wide range of commonly available antibiotics, especially the penicillins [9,10].

On administration of an antibiotic drug, the defense mechanism of pathogenic bacteria against β-lactam antibiotics becomes active and they produce β-lactamases that hydrolyze the β-lactam ring of antibiotics, rendering it to loosen its activity and providing bacterial resistance to β-lactam antibiotics [2]. To overcome the β-lactamase-mediated resistance, a combination of β-lactam antibiotic and a β-lactamase inhibitor, which protects the β-lactam antibiotic from the activity of the β-lactamase, has been widely used in the treatment of human infections.

Clavulanate, sulbactum and tazobactam are the known irreversible inhibitory molecules for many β-lactamases that form covalent complexes with the β-lactamases which subsequently resist their hydrolysis [4]. The development of β-lactamase inhibitors has allowed clinicians to rely on the well-tolerated, clinically effective antibiotics against a variety of resistant gram-positive and gram-negative bacterial super infections [28].

As the bacteria that cause the infection were resistant to first-line antibiotics, treatment options are usually replaced with a second or third choice of antibiotics, which are generally much more expensive. Therefore, alternative antimicrobial agents are needed to be developed and employed to control multi-drug resistant bacteria. To face this challenge, there has been growing interests to find antimicrobial compounds from medicinal plant extracts as an alternative approach to discover new antimicrobial compounds. The antimicrobial activities of some herbal medicines against different pathogens have been reported from different countries [23,25].

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There is an unrivaled need to uncover newer antibiotics and β- lactam inhibitors. In the present investigation, a survey for the presence of β-lactamase inhibitor activity of different plant products was carried out using precise bioassays and biochemical methods.

MATERIALS AND METHODS

Procurement of herbs and plants

Sixty plants and their products were selected from diverse natural sources, based upon literature survey, medicinal value and traditional indigenous knowledge.

In this study, leaves of Coriander (*Coriandrum sativum*) Pokoranti (*Salacia oblonga*), Curry leaves (*Murraya koenigii*), Fenugreek (*Trigonella foenum*), Bay laurel (*Laurus nobilis*), Waterhyssop (*Bacopa monnieri*) Kutaja (*Holarrhena antidysenterica*),Neem (*Azadirachta indica*). Plumbago (*Plumbago zeylanica*) Holy Basil (*Ocimum sanctum*), Gurmar (*Gymnema sylvestre*), Shankapushpi (*Convolvulus pluricaulis*), Sanjivani (*Selaginella bryopteris*),Chirata (*Gentiana chirata*), Kutki (*Picrorhiza kurroa*), Bala (*Sida cordifolia*), Iceland moss (*Cetraria islandica*), Lemongrass (*Cymbopogon citratus*) and roots of Harda (*Terminalia chebula*), Plumbago (*Plumbago zeylanica*), Indian ginseng (*Withania somnifera*), Satavar (*Asparagus racemosus*), Snakeroot (*Rauvolfia serpentina*), Cypriol (*Cyperus scariosus*), Ginger (*Zingiber officinale*), Turmeric (*Curcuma longa*), White turmeric (*Curcuma zedoaria*) Indian sarsaparilla (*Hemidesmus indicus*), Maca (*Lepidium meyenii*) were used.

The bark of Cinammon (*Cinnamomum zeylanicum*), Indian Sandalwood (*Santalum album*), plant exudates (*gum*) of Asafoetida (*Ferula asafoetidam*) along with seeds of Bahera (*Terminalia bellerica*), Velvet bean (*Mucuna pruriens*), Bishop's weed (*Trachyspermium ammi*), Black pepper (*Piper nigrum*), Cumin (*Cuminum cyminum*), Black mustard (*Brassica nigra*), Fennel (*Foeniculum vulgare*), Coriander (*Coriandrum sativum*), Maize (*Zea mays*), Pomegranate (*Punica granatum*) Wheat (*Triticum aestivum*), Cowpea (*Vigna unguiculata*) Black cardamom (*Amomum subulatum*) and its pod were also used.

Other plants used included bulbs of Onion (*Allium cepa*), Garlic (*Allium sativum*), fruits of Indian gooseberry (*Phyllanthus emblica*), Wood apple (*Aegle marmelos*), Kokum (*Garcinia indica*), Bitter gourd (*Momordica charantia*), Bell peppers (*Capsicum annuum*), Jamun (*Syzigium cuminii*), Drumstick (*Moringa oleifera*), Pomegranate (*Punica granatum*), Bullhead (*Tribulus terrestris*), Shikakai (*Acacia concinna*), Nutmeg (Myristica *fragrans*), Indian long pepper (*Piper longum*) and flowers of Hibiscus (*Hibiscus rosa*), Cloves (*Syzigium aromaticum*), Mace (*Myristica fragans*) and Rosy periwinkle (*Catharanthus roseus*) in this investigation.

The aforementioned plants were selected and used in this study as they possess antioxidant, antibacterial, antidiabetic and antiinflammatory activity [15].

Preparation of extracts from plants and plant products

Methanolic plant extracts in the ratio 1:4 were prepared by pounding 5g of plant material in 20ml methanol. The finely crushed plant material-methanol mixture was

subjected to centrifugation for 15 minutes at 3000rpm for solid-liquid separation. The supernatant (extract) was air dried in sterile petri plates at room temperature, and the dry weights of the solid plant extracts were determined and stored in a refrigerator for further use. The solid plant extracts were resuspended in 1ml phosphate buffer (pH 7.0) and used for the tests as and when required throughout the study.

β-lactamase inhibition activity determination

Iodometry

This method depends upon the reduction of iodine by the hydrolyzed substrate. The standard Iodometric assay measures the time taken for the iodine to be absorbed by a hydrolyzed penicillinase enzyme.1 mol of hydrolyzed penicillin consumes around 3.4 to 4.0 mol of iodine (I_2) using starch as an indicator, accompanied by a change in colour from dark blue to colourless, which can also be determined spectrophotometrically. This assay is frequently used to measure the β-lactamase activity of various substrates. Assimilating this principle, a modified method was designed to determine the β-lactamase inhibition activity of the plant extracts.

Reagent Preparation

Penicillin solution: Benzyl Penicillin Sodium (1660 IU/mg) was procured from Hindustan Antibiotics Ltd., Pimpri, Pune. 60-65mg of Penicillin powder was dissolved in 50 ml phosphate buffer to make Penicillin stock solution.

β-lactamase solution: The vial was procured from Hindustan Antibiotics Ltd., Pimpri, Pune. β-lactamase enzyme was dissolved in 100ml of 0.1M phosphate buffer (pH 7.5-8.0); it was then filtered through 0.2µ membrane to make the β-lactamase stock solution, which is stored in plastic or siliconised glass tubes at 0-4ºC. For experimental use, a 4X dilute solution of enzyme is made by adding 1ml of the enzyme stock solution to 4ml of phosphate buffer. The activity of both Penicillin and the enzyme β-lactamase is checked prior to their usage.

Iodine Reagent: 0.01N Iodine reagent was prepared by dissolving 400mg Potassium iodide (KI) and 127mg Iodine (I_2) in 100ml distilled water and stored in amber coloured bottles for further use.

Starch Indicator: 1g soluble starch is dispensed in 100 ml distilled water and heated until clear to make 1% Starch Indicator.

Gelatin Solution: 1g of Gelatin powder was dissolved in 100ml 0.1M phosphate buffer and heated until clear to make 1% Gelatin solution.

Sulbactum: Sulbactum sodium was used as the standard enzyme inhibitor throughout the investigation. Akin to Penicillin, the same amount of Sulbactum was dissolved in phosphate buffer and used as the positive control. Sterile distilled water was used as the negative control.

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Iodometry method

A modified Iodometry method [11] was employed to compute the cohered enzyme. The chemical reagents viz. Penicillin, starch, iodine, gelatin and phosphate buffer in their aforementioned concentration comprised the substrate mixture in this reaction.

(I) Control tube was prepared with 2.0 ml gelatin, followed by 3 drops of starch and 1.0 ml Penicillin solution, incubated at 30^{o} C for 15 minutes. Post incubation, 2.0 ml iodine solution was added.

(II) 2.0 ml of 1 % gelatin solution was added to each of the following reaction glass test tubes along with 3 drops of 1 % starch solution, and the given test tubes were prepared in the order as given below:

(a) β-lactamase tube: 50µl enzyme solution was added along with 1.0ml of penicillin solution, incubated at 30^{o} C for 15 minutes, 2.0ml iodine solution was added post incubation.

(b) Sulbactum inhibitor tube: 50µl enzyme solution was added followed by 1.0ml of the sulbactum (inhibitor) and incubated for 15 min at 300^{o}C. The incubation time period encourages binding of the enzyme to the inhibitor. After incubation, 1.0ml Penicillin solution was added, followed by quick addition of iodine reagent.

(c) Inhibitor tube with plant extract: 50µl enzyme solution was added followed by 1.0ml plant extract, and incubated for 15 min at 30^{o}C. Post incubation, 1.0 ml Penicillin was added followed by quick addition of 2.0ml iodine reagent. The time taken for decolourization from blue to colourless was recorded for all the sets of test tubes.

Bioassay

Agar Gel Diffusion Bioassay method [14] was employed to determine the β-lactamase inhibitor activity of plant extracts against the test organism, *Staphylococcus aureus* (ATCC-6633).

In this method, 600µl 24h test culture (O.D 0.10 at 540nm) was spread thoroughly on nutrient agar plates, wells were then punctured in the plates using a sterile cork borer having an 8mm diameter. The wells were filled with 100µl test mixture, which was a) penicillin, (b) penicillin+ enzyme; (c) penicillin + enzyme + sulbactum and (d) penicillin+ enzyme + plant extract, and then incubated for 18-24h period at 37°C, and the zone of inhibition obtained on the plates were measured in mm.

RESULTS

The *in vitro* Iodometric method, wherein upon addition of iodine, the time taken for decolourization from dark blue to colourless in the substrate reaction mixture was noted and was compared to that of sulbactum, which was used as a positive control having decolourization time of 6600 seconds (1h 50mins).

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Percentage enzyme inhibition of the plant extracts was deduced as follows:

$$\text{Percentage enzyme inhibition} = \frac{\text{Decolorization time of the plant extract (seconds)} * 100}{\text{Decolorization time of sulbactum (seconds)}}$$

The plant extracts were also screened out for β-lactamase inhibitor activity through the agar well diffusion method. In this bioassay, the plant extracts were tested *in vivo* against *Staphylococcus aureus* to find out their β-lactamase inhibitor activity through their zone of inhibition.

Iodometric analysis

The in vitro Iodometry results are as follows:

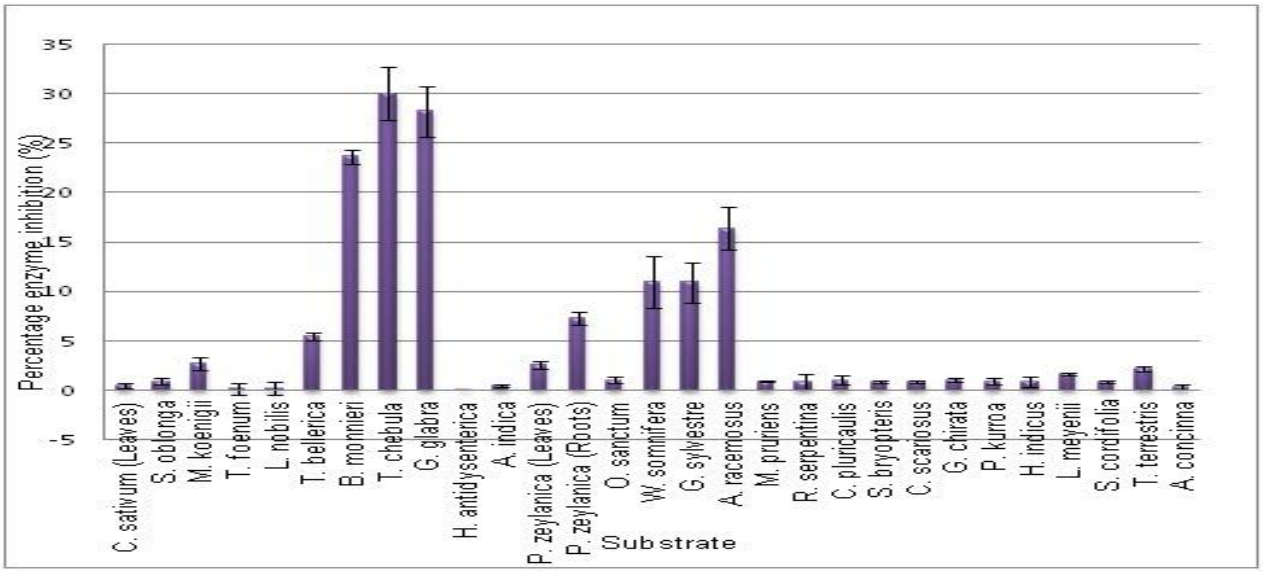

Fig. 1 (a)

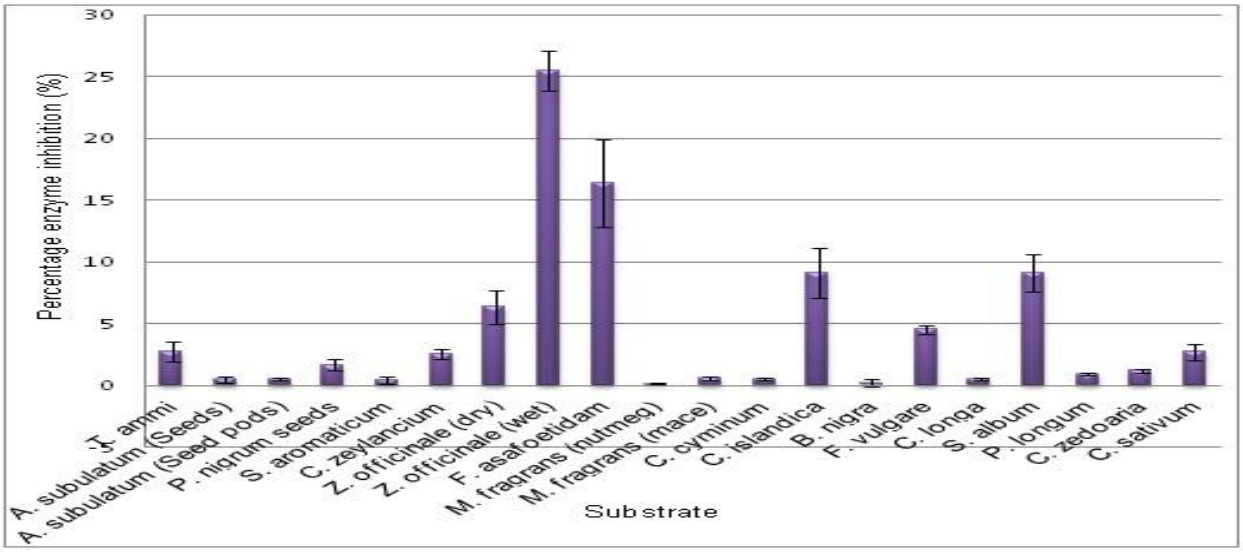

Fig. 1 (b)

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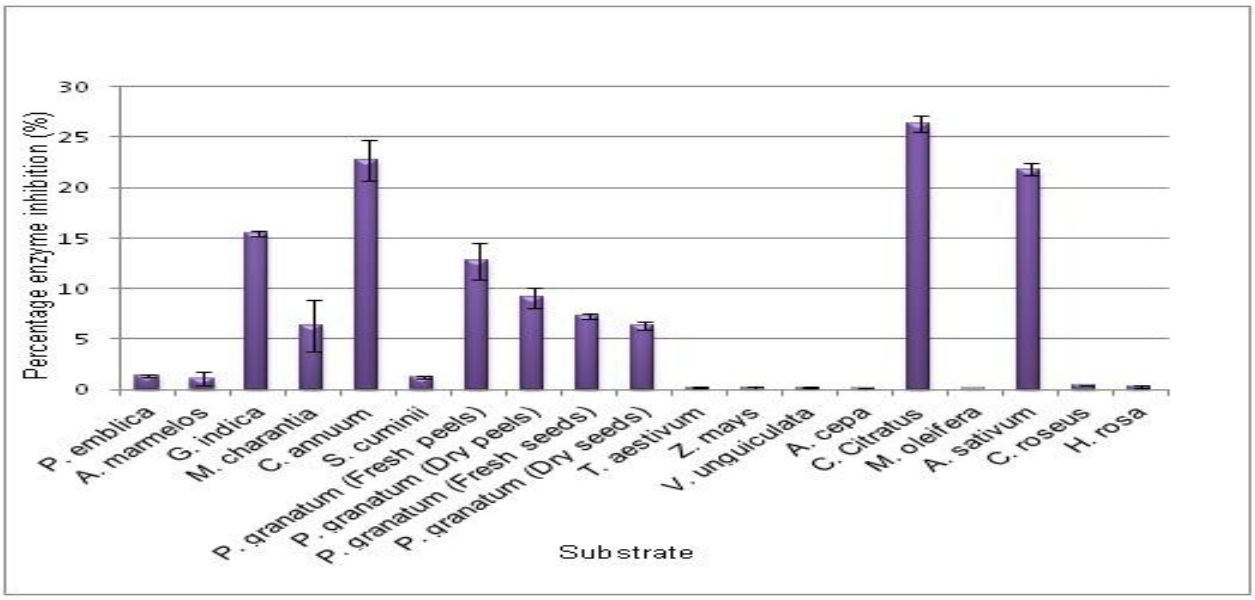

Fig. 1 (c)

Fig. 1 The results of the *in vitro* Iodometry method of β-lactamase enzyme inhibitor activity of the plant extracts, (a) herbs, (b) spices, (c) fruits and flowers.

Bioassay

The agar well diffusion assay method was employed to carry out the *in vivo* testing of the plant extracts. The zone of inhibition of Penicillin (Fig. 3a) and Penicillin + enzyme+ sulbactum (inhibitor standard) (Fig. 3c), which were used as a standard in the bioassay, was 26mm and 22mm respectively.

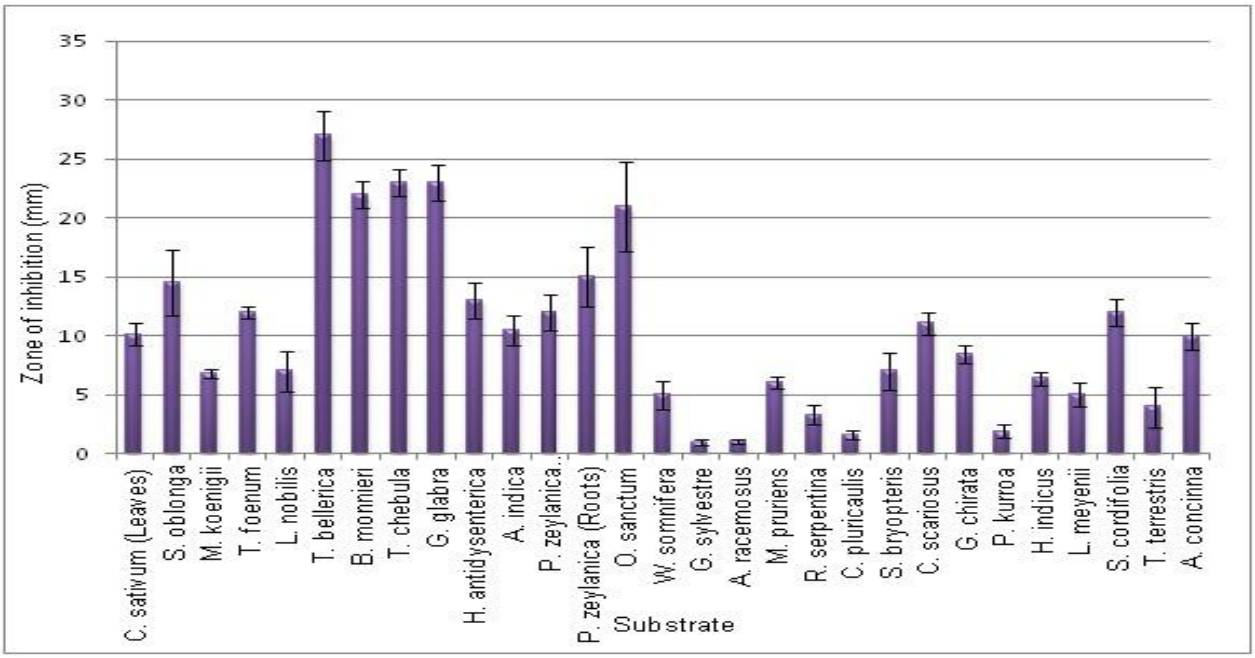

Fig. 2 (a)

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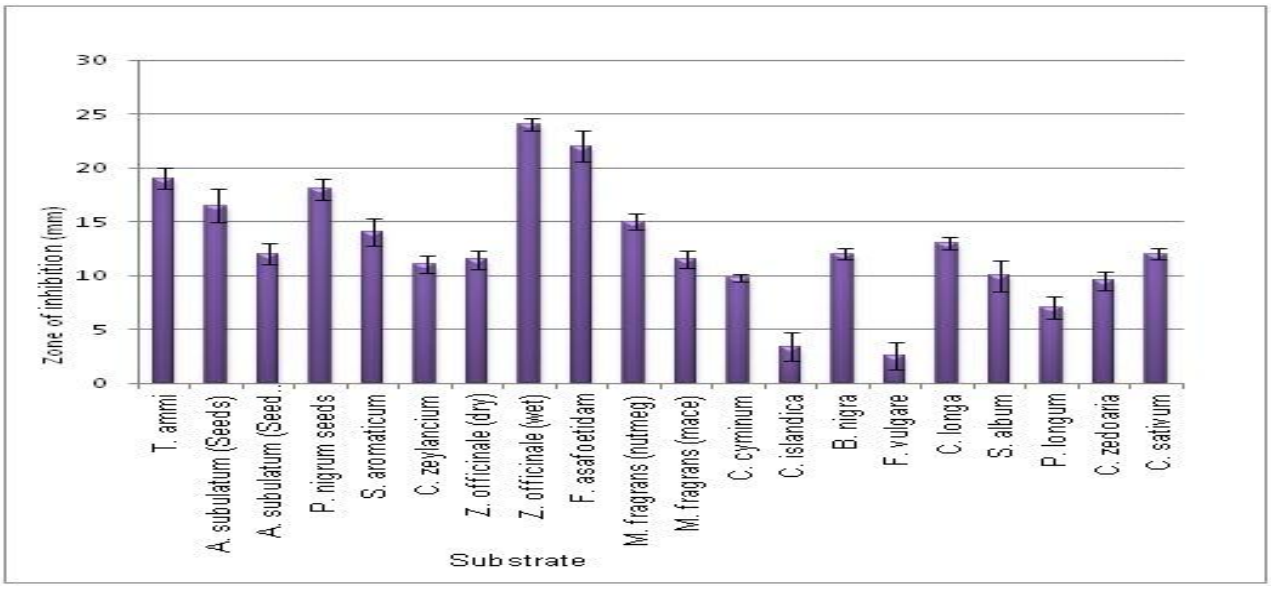

Fig. 2 (b)

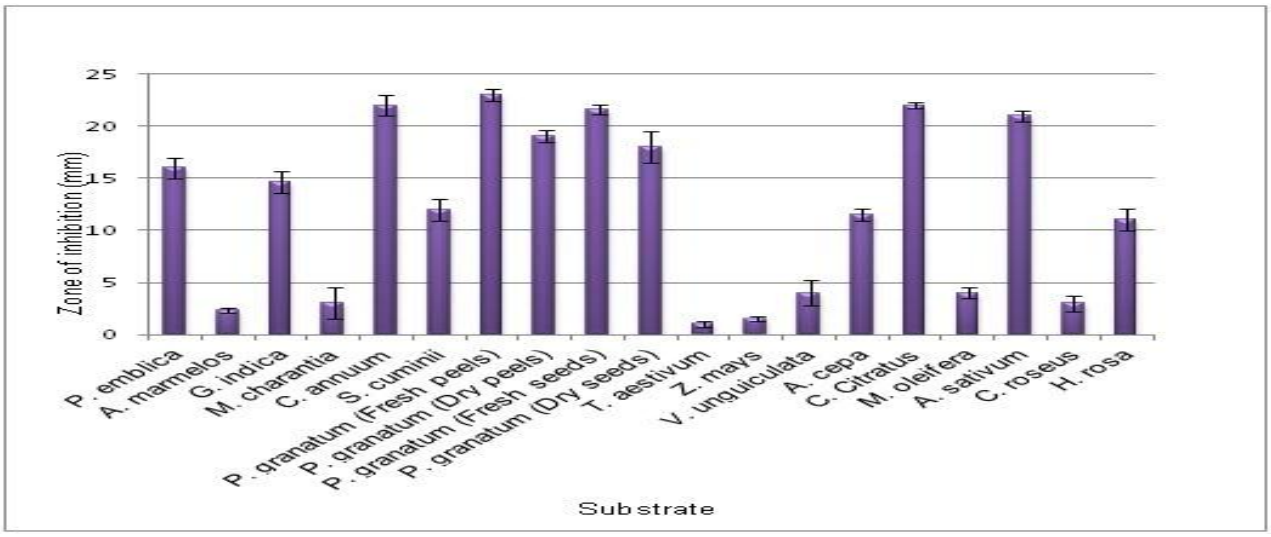

Fig. 2 (c)

Fig. 2 The results of the *in vivo* Bioassay method of β-lactamase enzyme inhibitor activity of the plant extracts, (a) herbs, (b) spices, (c) fruits and flowers

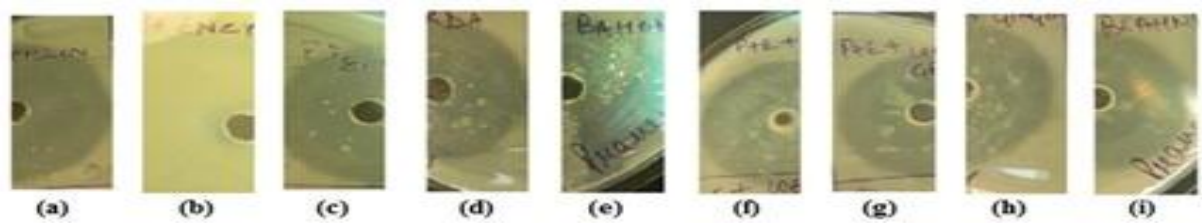

Fig. 3

Fig. 3 The above figure describes the zone of inhibition of some of the selected plant extracts obtained through the bioassay method against the test organism *S. aureus* showing high β-lactamase inhibitor activity. The zone of inhibition obtained is as follows: (a) Penicillin, (b) Penicillin+ Enzyme, (c) Penicillin+ Enzyme+ Sulbactum (Inhibitor standard), (d) Penicillin+ Enzyme+ *T. chebula extract*, (e) Penicillin+ Enzyme+ *T. bellerica* extract, (f) Penicillin+ Enzyme+ *G. glabra* extract, (g) Penicillin+ Enzyme+ *C. citratus* extract, (h) Penicillin+ Enzyme+ *Z. officinale (wet)* extract, (i) Penicillin+ Enzyme+ *B. monneiri* extract.

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In this study, herbal extracts proved to be the most promising β-lactamase inhibitors, followed by spice extracts and fruit extracts, the floral extracts however did not show considerable β-lactamase inhibitor activity via both, *in vitro* Iodometry method and *in vivo* bioassay method.

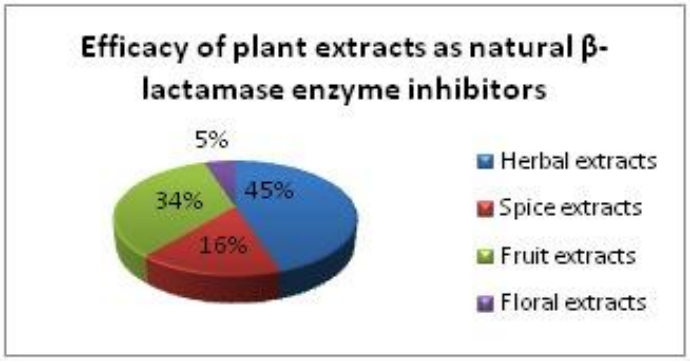

Fig. 4

Fig. 4 The pie chart showing overall efficacy of herbal, spice and fruit and floral extracts as natural β-lactamase enzyme inhibitors

DISCUSSIONS

In the near future, there is an austere possibility that superbugs may expunge medical improvements. Drug resistant microorganisms represent one of the gravest threats in the history of medicine. In the past, drug development kept pace with evolving microorganisms, but currently no new class of drugs has been created since the 1980s. The overuse of antibiotics has resulted into development and persistence of superbugs. Higher the circulation of antibiotics, higher becomes the ability of the bacteria to evolve and become antibiotic resistant. The post antibiotic era is ushering earlier than expected and this developing multiple drug resistance is adequate enough to erase a century of medical and pharmaceutical advances. To curb this crisis, two tasks need to be undertaken concomitantly, i.e. development of novel antibiotics along with development of drug specific enzyme inhibitors.

A simple model was designed in this study to investigate β-lactamase inhibitors from natural sources using the drug Penicillin. This investigation involved screening of natural β-lactamase enzyme inhibitors, from plants and plant parts through their extracts by both *in vitro* (primary screening by Iodometry) and *in vivo* (secondary screening by Bioassay) methods.

The herbal methanolic extracts of *T. bellerica*, *B. monnieri*, *T. chebula*, and spice methanolic extracts of *Z. officinale* (wet) and fruit methanolic extracts of *A. sativum*, and *C. citratus* and *C. annum* showed the highest percentage of β-lactamase inhibitor activity in the *in vitro* Iodometry method.

The herbal methanolic extracts of *T. bellerica* (Fig. 3e), *T. chebula* (Fig. 3d), *G. glabra* (Fig. 3f), *B. monnieri* (Fig. 3i), *O. sanctum* and spice methanolic extracts of *Z. officinale (wet)* (Fig. 3h), *F. asafoetidum* and fruit methanolic extracts of *A. sativum, P. granatum (fresh*

peels), P. granatum (fresh seeds), C. annum and *C. citratus* (Fig. 3g) showed the most promising *in vivo* β-lactamase inhibitor activity through the bioassay method.

It reflects that the extracts contain substance(s) that can inhibit the growth of microorganisms. Other workers have also shown that extracts of some plants inhibited the growth of various microorganisms at different concentrations [1,8,18,17,20]. The observed antibacterial effects on the isolates is believed to be due to the presence of alkaloids, tannins and flavonoids which have been shown to possess antibacterial properties [5,7]. The observed antibacterial properties substantiate its use in traditional medicine. Traditionally, extracts of the plant are used in sore and wound healing and in the treatment of boils. They are also used in the control of diarrhoea and dysentery [16,13]. The large zones of inhibition exhibited by the extracts against *S. aureus*, justified their use by traditional medical practitioners in the treatment of sores, bores and open wounds caused by *S. aureus* [3].

Some reports have indicated the presence of β-lactamase inhibitor activity from certain plants and plant products, while a lot of plant biodiversity is yet to be exploited. In this investigation plants were selected on the basis of their traditional indigenous knowledge and ethno botanical use. The future prospect of this investigation involves screening and isolation of the active component from the plant extracts having high β-lactamase inhibitor activity, chemical structure determination of the promising plant extracts, purification and extraction of the active component, its toxicological studies and efficiency against different classes of β-lactamase inhibitors and its suitability for its commercial utilization as a product.

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