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ORIGINAL ARTICLE **OPEN ACCESS**

Antimicrobial Activities of Leaves and Stem Parts of *Artabotrys odoratissimus R.Br.* (Annonaceae)

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ABSTRACT
Artabotrys odoratissimus R.Br. (Annonaceae) a medium sizes shrub with leaves, hooks, sweet smelling flowers and aggregate fruits, stems was tested for activity against gram positive bacteria and gram negative bacteria. The leaves and stem parts of the experimental plant was extracted with water, chloroform and ethanol. Ethanoloc extract of leaves showed good antibacterial activity and produced zone of inhibition of 32mm. The ethanolic extract of the leaf showed maximum zone of inhibition concentration of 100 mg/mL. Among the crude extracts investigated, Aqueous and Ethanol extracts of leaves demonstrated more potent antibacterial activities against ATCC and clinical strains with zones of inhibition ranging from 8.23±0.25 mm to 13.70±0.26 mm and 7.75±0.25 mm to 13.68 ±0.28 mm respectively. However, all extracts of Stem were found to be less effective in inhibiting the growth of the tested bacteria when compared with leaf extract. Comparatively L. monocytogenes ATCC 15313, shows good zone of inhibition compared to P. vulgaris ATCC 13315 and other stains. The present study clearly indicates that A. odoratissimus had a profound antimicrobial activity and it may be useful in the treatment of various infectious caused by bacteria.
Keywords: *Artabotrys,* Gram positive , Gram Negative Bacteria, Pseudomonas, Zone Of Inhibition

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INTRODUCTION

In India medicinal plants form the backbone of traditional systems of medicine in India, thousands of tribal communities still use folklore medicinal plants for the cure of various diseases. Indian medicinal plants have been studied for potential source of bioactive compounds. The great interest in the use and importance of medicinal plants in many countries has led to intensified efforts on the documentation of ethnomedical data of medicinal plants (1). Earlier there were a few or no synthetic medicine and species of higher plants were the main sources of medicines for the world (2). Medicinal plants are the rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs (3). The use of herbs to treat disease is almost universal among non-industrialized societies and is often more affordable than purchasing modern pharmaceuticals. The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently use herbal medicine for some aspect of primary health care.

MATERIAL AND METHOD

Collection and authentication of plant material

The plant *Artabotrys* odoratissimusbelonging to the family *Annonaceae* were collected from Chittoor district of Andhra Pradesh and was identified and authentified by Dr. Madhavachetty, plant taxonomist, Asst. Prof, Dept. of Botany. The plant voucher No. is 0823 dated 17-12- 2018.

Experimental Plant

Artabotrys odoratissimus is a shrub found in India and its flowers are renowned for its exotic fragrance. The common names include Ylang Ylang Vine, Climbing lang-lang and Tail grape. Leaves are simple and alternate, without hairs. Bisexual flowers are borne singly or in clusters opposite the leaves.
The hexapetalous flowers are scented, and the plant bears fleshy fruits. The yellow colored flowers of this plant are very fragrant. The flowers are greenish in the beginning and turn yellow with age and the

flowers are long lasting with fruity pleasant smell. When young it is a shrub which turns into a climber once attains the height of about 2 meters. Fruits are long when ripe, ovoid and smooth, aggregate of berries. Seeds nearly 2cm in diameter. Here in the present study the fresh leaves and stem parts are taken for the activity shown in figure 1.

ARTABOTRYS ODORATISSIMUS R.Br. FAMILY: (ANNONACEAE)

The leaves and Stem of *Artabotrys odoratissimus*

FIGURE 1. *Artabotrys odoratissimus*

Microbes

In vitro antimicrobial activity was examined for the methanolic extracts of Artabotrys against four bacterial species, *Bacillus subtilis* ATCC 21332 *Listeria monocytogenes* ATCC 15313 *Micrococcus luteus* ATCC 10240 *Proteus vulgaris* ATCC 13315 *Rhodococcus equi* ATCC 33701 *Staphylococcus aureus* ATCC 11632 *Staphylococcus epidermidis* ATCC 12228 *Streptococcus pyogenes* ATCC 19615,*Escherichia coli* ATCC 10536 *Klebsiella pneumoniae* ATCC 13883 *Pseudomonas aeruginosa* ATCC 10145 *Salmonella enteritidis* ATCC 13076 *Salmonella typhimurium* ATCC 14028 ,Gram-positive bacteria *Enterococcus faecalis* Methicillin-resistant *Staphylococcus aureus* (MRSA) Methicillin-sensitive Staphylococcus aureus (MSSA) Oxacillin-resistant coagulase-negative staphylococci (ORCNS) Oxacillin- sensitive coagulase-negative staphylococci (OSCNS) *Streptococcus agalactiae* (Group B Streptococcus, GBS) *Streptococcus pneumoniae* Gram-negative bacteria *Actinobacillus* sp. *Enterobacter* sp. Escherichia coli Extended-spectrum beta-lactamase-producing Escherichia coli (ESBL-EC) Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) *Klebsiella* sp. *Moraxella* sp. Serratia sp. were obtained from Regional Research Institute of Unani,Chennai.

Preparation of plant material

Sequential extraction of plant material

The pulverised leaves (1.30 kg) and Stem (4.79 kg) were extracted sequentially with solvents of increasing polarity starting from Aqueous (Friendemann Schmidt, Australia), chloroform (Friendemann Schmidt, Australia) and 95% (v/v) of ethanol (John Kollin Chemicals, India). Each extraction was performed in triplicate at a solid-to-solvent ratio of 1:5 (w/v) in a 40°C water bath (Julabo, Germany) for three days.

The respective extract was subsequently filtered through qualitative filter papers No. 1 (Whatman International Ltd., England) and the collected filtrate was concentrated to dryness under reduced pressure at 40°C using rotary evaporator (Buchi Labortechnik AG, Switzerland). Eventually, the dried extract obtained was weighed with analytical balance (Sartorius AG, Germany) and stored in glass scintillation vials (Kimble, USA) at –20°C until further use. For stock solutions, each crude extract was dissolved in dimethyl sulphoxide (DMSO) (R & M Chemicals, UK) at a concentration of 100 mg/mL and stored at 4°C.

Phytochemical screening

The phytochemical screenings of crude extracts were carried out using standard procedures. Each crude extract (final concentration of 1 mg/mL) was assayed for the presence of phytochemical constituents such as alkaloids, cardiac glycosides, flavonoids, phenolic compounds, saponins, tannins and terpenoids.

Phytochemical screenings of crude extracts of *Artabotrys odoratissimus*

Prior to pharmacological evaluation of plant extracts, phytochemical screening is the initial and essential step towards understanding the nature of active principles in medicinal plants (Kakpure and Rothe 2012). Based on the preliminary phytochemical analysis of crude extracts presented in Table 3.2–3.9, leaf extracts were found to have more secondary metabolites than stem extracts. Both aqueous and ethanol extracted the widest range of phytochemical constituents including cardiac glycosides, flavonoids, phenolic compounds and terpenoids.

On the other hand, ethanol extract of leaves possessed similar phytochemical constituents to ethanol extract of Stem. However, Aqueous and chloroform extracts of leaves exhibited positive reaction only to Keller-Kiliani test for cardiac glycosides. Among the phytochemical constituents analysed, tannins were absent in all of the tested extracts. This implies that the extracts from leaves and Stem may constitute a different source of secondary metabolites that can serve as a constructive reference for further detailed studies on the pharmacological activities of *Artabotrys odoratissimus*.

IN VITRO* ANTIBACTERIAL ACTIVITY OF *ARTABOTRYS odoratissimus

Bacteria are unicellular prokaryotic microorganisms that exhibit different cellular sizes and shapes ranging from spheres to rods and spirals (4). In the human body, most of the bacteria are rendered harmless or beneficial by the protective effects of the immune system (5). Nevertheless, some species of bacteria are pathogenic and capable of causing infectious diseases such as anthrax, bubonic plague, cholera, leprosy, syphilis and tuberculosis.

In spite of the widespread availability of antibacterial therapies, bacterial infections continue to pose a significant threat to public health worldwide [6]. More importantly, the clinical efficacy of many existing antibacterial drugs is declining precipitously due to the emergence and dissemination of multiple drug resistant pathogens [7]. These bacteria are endowed with the ability to become resistant to antibiotics through mutation or gene transfer [8], which further leads to higher morbidity, prolonged length of stay, increased mortality, and costly healthcare as compared to antibiotic-susceptible microorganisms (9). Therefore, alternative antibacterial agents with diverse chemical structures as well as novel mechanisms of actions are urgently required to combat the new and re- emerging bacterial infections.

Microorganisms and culture media

The microorganisms used in the current study can be categorised into two main groups, namely Gram-positive and Gram-negative bacteria as shown in Table

4.1. Thirty bacterial strains including American Type Culture Collection (ATCC) and clinical strains were procured from the strains were procured from the Regional research institute of Unani Chennai.

Preparation of broth medium

Tryptic soy broth (TSB) was prepared by suspending 30 g of TSB powder in 1 L of sterile distilled water. The solution was mixed thoroughly and warmed slightly to completely dissolve the powder before dispensing into universal bottles. After autoclaving at 121°C for 15 min, the broth was allowed to cool down to room temperature before storing at 4°C until further use.

Preparation of agar medium

Tryptic soy agar (TSA) was prepared by suspending 40 g of TSA powder in 1 L of sterile distilled water. The solution was mixed thoroughly and heated to boiling to dissolve the powder completely, followed by autoclaving at 121°C for 15 min. The autoclaved medium was allowed to cool down by immersing into a 45°C to 50°C water bath (Julabo, Germany) before pouring into sterile Petri dishes (Favorit, India) in laminar flow cabinet (Esco Micro, India). After pouring, the molten agar was allowed to solidify and dried for 30 minutes before covering the plates to prevent formation of water on the agar surface. The prepared agar medium was stored in a 4°C chiller until further use.

Maintenance and storage of stock cultures

Preparation of plate cultures

The streak plate method was employed to obtain pure bacterial cultures. A sterile inoculating loop was dipped into the culture of bacteria and streaked in a pattern over the surface of the TSA plate. The inoculating loop was sterilised following each streak series. As the pattern was traced, bacteria were rubbed off the loop onto the medium. The last cells to be rubbed off the loop were far enough apart to grow into isolated colonies. Streaked plates were incubated 35°C for 24 hrs**.**

Preparation of broth cultures

The bacterial cell suspension was prepared by picking a single isolated colony from freshly streaked plate with a sterile inoculating loop and transferring into universal bottles containing sterile TSB. The prepared cell suspension was vortexed thoroughly and incubated 35°C for 24 hrs**.**

Preparation of glycerol stocks

The glycerol stock was prepared by transferring the bacterial cell suspension into cryovials containing a final concentration of 20% (v/v) of sterile glycerol (R & M Chemicals, UK). The prepared glycerol stock of

bacteria was well-mixed before storing at 2°C for 24 h. Kirby-Bauer disc diffusion assay
The antibacterial activities of crude extracts were evaluated against 30 ATCC and clinical strains using Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute .

Preparation of Mueller-Hinton agar

Mueller-Hinton agar (MHA) (Difco Laboratories, USA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, the agar medium was allowed to cool in a 45°C to 50°C water bath. The freshly prepared and cooled medium was poured into plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm, which corresponded to 25 mL to 30 mL of medium for plates with a diameter of 100 mm. The agar medium was allowed to cool further to room temperature, and unless the plates were used the same day, stored in a 2°C to 8°C refrigerator.

Preparation of impregnated filter paper discs

Qualitative filter paper No. 1 (Whatman International Ltd., England) was used to prepare discs approximately 6 mm in diameter, which were sterilised by autoclaving at 121°C for 15 min. Sterile filter paper discs were impregnated with 10 µL of each crude extract (100 mg/mL) to give a final concentration of 1 mg/disc. Streptomycin sulphate (5 µg/disc) (Fisher BioReagents, China) and DMSO (R & M Chemicals, UK) were served as positive and negative controls respectively. Impregnated discs were left to dry under laminar flow cabinet overnight.

Preparation of inoculum

At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a universal bottle containing sterile TSB. The broth culture was incubated at 35°C for 2 h to 6 h until it achieved or exceeded the turbidity of the 0.5 McFarland standard. The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard using a UV/Visible spectrophotometer (Biochrom Libra, UK) at 625 nm. The absorbance at 625 nm should be in the range of 0.08 to 0.13 for the 0.5 McFarland standard. This resulted in a suspension containing approximately 1×10^8 CFU/mL to 2×10^8 CFU/mL.

Inoculation of test plates

Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the universal bottle above the fluid level to remove excess inoculum from the swab. The dried surface of a MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 min to 5 min, but no more than 15 min, to allow for any excess surface moisture to be absorbed before applying the impregnated discs.

Application of discs to inoculated agar plates

The impregnated disc was placed individually using sterile forceps onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. Eight discs were placed in each plate. The plates were inverted and placed in an incubator (Binder, Germany) set to 35°C within 15 min after the discs were applied.

Reading plates and interpreting results

After 16 h to 18 h of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimetre, using sliding callipers (American Scientific LLC, USA) or a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting background and illuminated with reflected light. Eventually, the sizes of the zones of inhibition were interpreted.

Statistical analysis

Statistical analysis of experimental data was performed using Microsoft Office Excel data analysis software. Data were presented by descriptive analysis as mean and standard deviation of three independent experiments performed in triplicate.

RESULTS AND DISCUSSION

Plants constitute a vast untapped source of medicines with great therapeutic values [10]. The prospects for the development of antibacterial drugs from medicinal plants appear to be rewarding as they can mitigate the adverse effects that are often associated with synthetic antibiotics [11]. In the present study,

Kirby-Bauer disc diffusion assay was conducted to evaluate the antibacterial activities of crude extracts against ATCC and clinical strains. This qualitative method is extensively used for antibiotic susceptibility testing in which filter paper discs impregnated with antibacterial agents are applied on the inoculated agar plate [12]. The efficacy of these agents can subsequently be determined by measuring the diameter of the zones of inhibition that resulting from their diffusion into the agar medium around the discs [13].
The inhibitory effects of crude extracts on the growth of ATCC and clinical bacterial strains are depicted in Figure 2 and 3. Among the crude extracts investigated, Aqueous and Ethanol extracts of leaves demonstrated potent antibacterial activities against ATCC and clinical strains with zones of inhibition ranging from 8.23±0.25 mm to 13.70±0.26 mm and 7.75±0.25 mm to 13.68 ±0.28 mm respectively. However, all extracts of Stem were found to be less effective in inhibiting the growth of the tested bacteria.

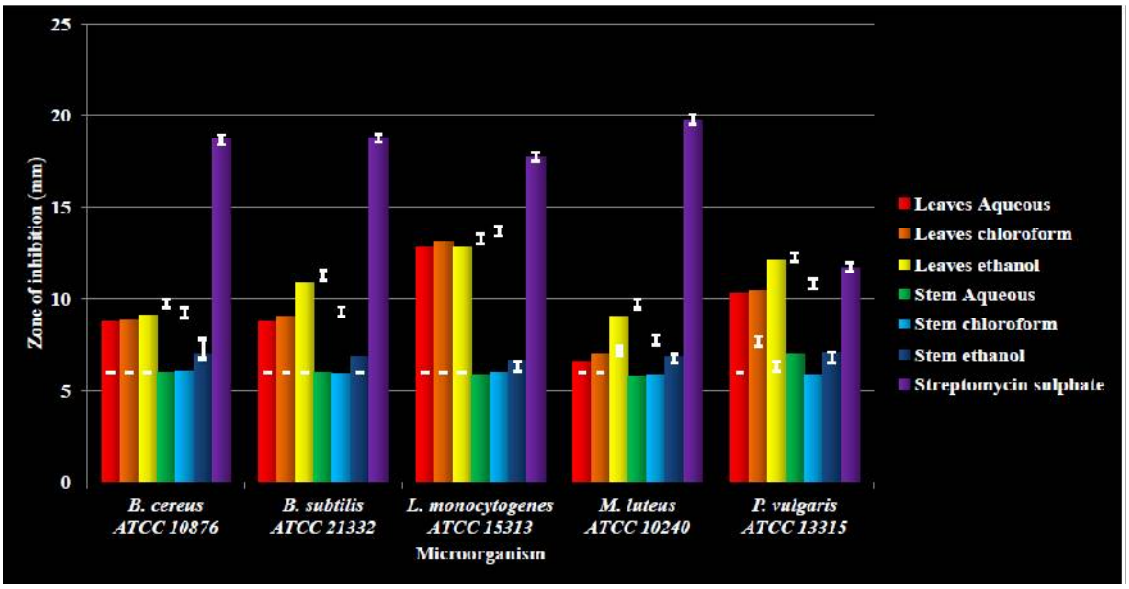

FIGURE 2. Antibacterial activities of crude extracts of *Artabotrys odoratissimus* against ATCC strains. Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

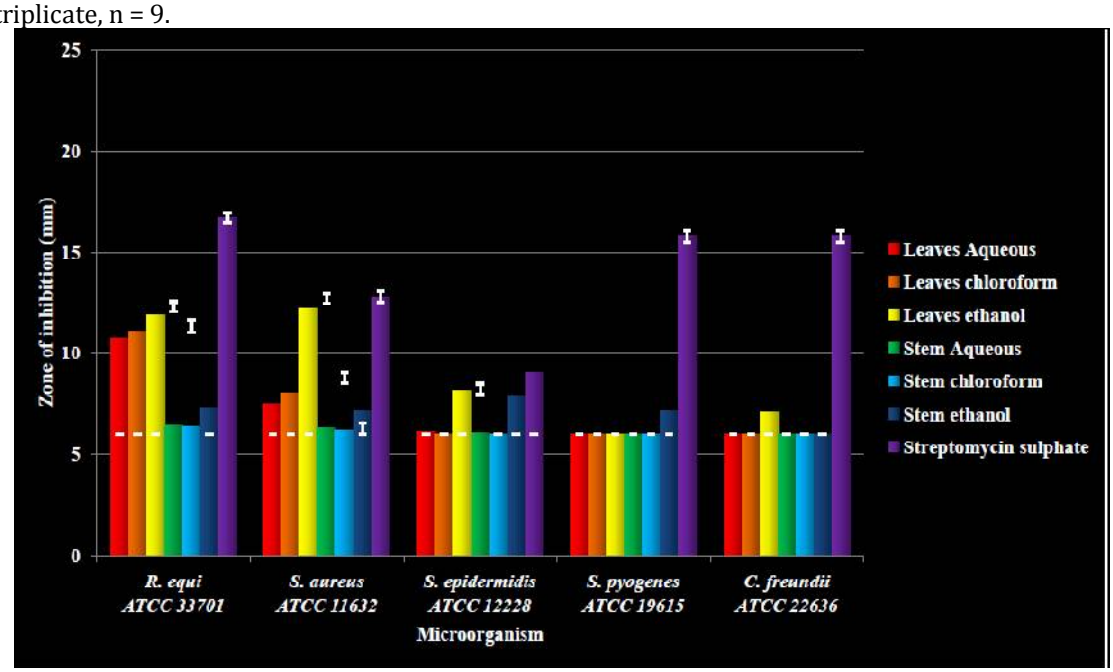

FIGURE 2. Antibacterial activities of crude extracts of *Artabotrys odoratissimus* against ATCC strains (continued). Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

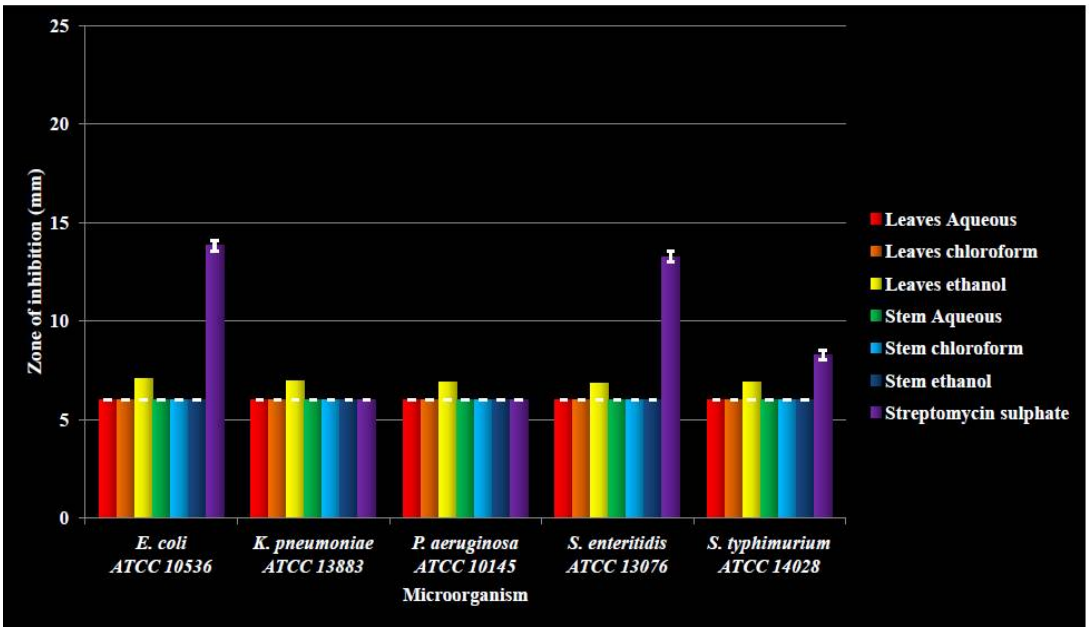

FIGURE 2. Antibacterial activities of crude extracts of *Artabotrys odoratissimus* against ATCC strains (continued). Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

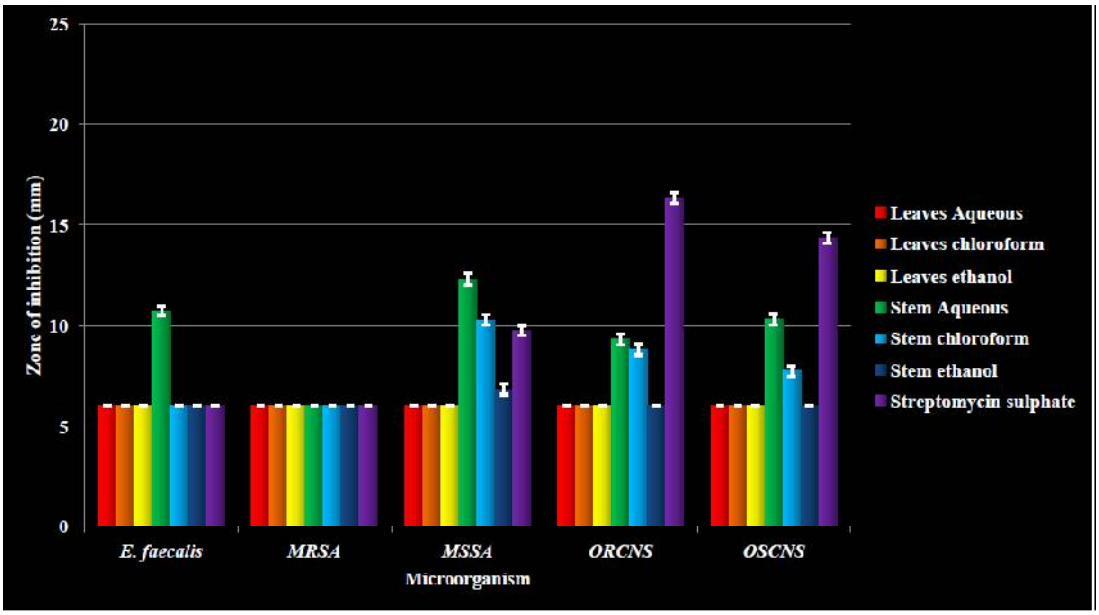

FIGURE 3. Antibacterial activities of crude extracts of *Artabotrys odoratissimus* against clinical isolates. Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

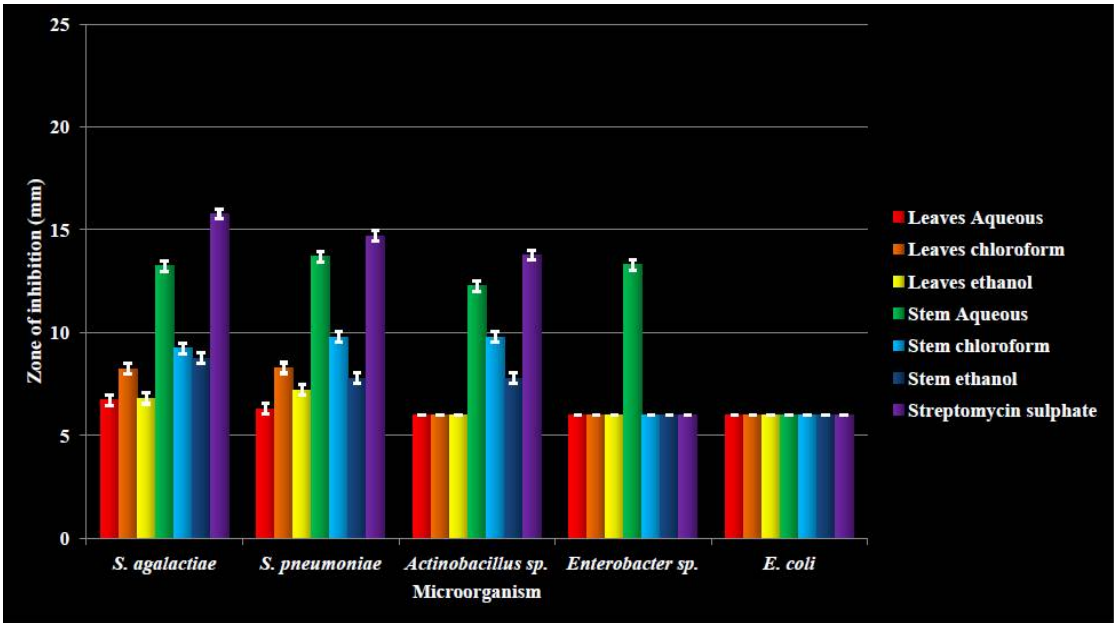

FIGURE 3. Antibacterial activities of crude extracts of Artabotrys odoratissimus against clinical isolates (continued). Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

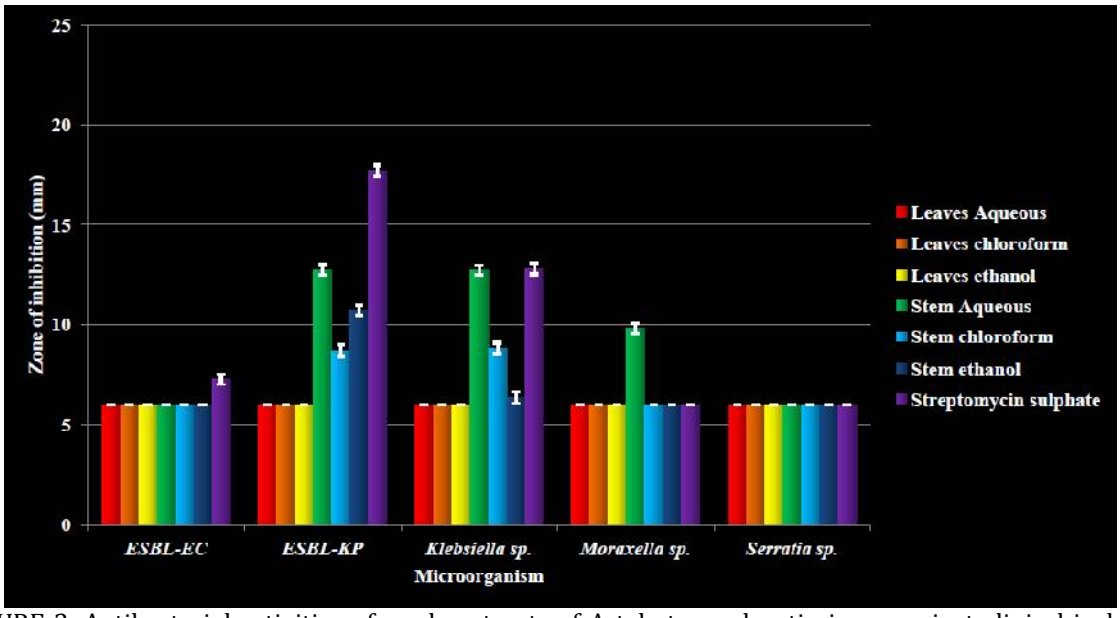

FIGURE 3. Antibacterial activities of crude extracts of Artabotrys odoratissimus against clinical isolates (continued). Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

Moreover, the positive control, streptomycin sulphate, created zones of inhibition ranging from 7.30Â±0.26 mm to 19.79Â±0.26 mm against all of the tested ATCC and clinical bacterial strains, with the exception of *S. epidermidis* ATCC 12228, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 10145, E. faecalis, MRSA, *Enterobacter* sp., *E. coli, Moraxella* sp. and *Serratia* sp. In contrast, no inhibitory activity was observed in the negative control, DMSO. This implies that DMSO, the solvent used for the reconstitution of crude extracts, does not influence the susceptibility of the ATCC and clinical bacterial strains to the corresponding extracts.

Considering the zones of inhibition produced by crude extracts, *S. pneumoniae* and *S. agalactiae* were found to be the most sensitive bacteria, followed by *P. vulgaris* ATCC 13315, *L. monocytogenes* ATCC 15313, ESBL-KP, *Actinobacillus* sp., MSSA, *Klebsiella* sp., *S. aureus* ATCC 11632, *B. cereus* ATCC 10876, M. luteus ATCC 10240, *R. equi* ATCC 33701, B. subtilis ATCC 21332, ORCNS, OSCNS, *Enterobacter* sp., E. faecalis, *Moraxella* sp. and *S. epidermidis* ATCC 12228, with S. pyogenes ATCC 19615, *C. freundii* ATCC

22636, E. coli ATCC 10536, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 10145, *S. enteritidis* ATCC 13076, *S. typhimurium* ATCC 14028, MRSA, E. coli, ESBL-EC and *Serratia* sp. being the least susceptible to crude extracts. This suggests that the respective extracts may be more effective in inhibiting the growth of Gram-positive bacteria.

In general, Gram-negative bacteria are more resistant to plant-based antibacterial agents in comparison to Gram-positive bacteria [14-16]. The susceptibility differences between these two groups of bacteria can be attributed to their distinct cell wall structures [17].

Gram-negative bacteria are characterized by an outer membrane that encloses a comparatively thin layer of peptidoglycan [18-20]. The outer membrane has a phospholipid-rich inner leaflet of similar composition to the cytoplasmic membrane, while the outer leaflet facing the extracellular environment is composed primarily of lipopolysaccharides (LPS), which provide an effective permeability barrier against hydrophobic compounds. In addition to these structural components, the asymmetric lipid bilayer also contains porins, which form water-filled channels that selectively facilitate the passage of hydrophilic compounds based on their molecular weight and ionic charge.

On the other hand, Gram-positive bacteria possess a relatively thick peptidoglycan layer with lipoteichoic acids (LTA) anchored to the cytoplasmic membrane. Nonetheless, they are devoid of a highly impermeable outer membrane, making them more susceptible to antibacterial compounds [10]. These chemical composition and organization of bacterial cell wall may rationalise the variations in the sensitivity of the ATCC and clinical bacterial strains to the crude extracts.

With regard to the phytochemical screening of crude extracts, the occurrence of alkaloids, cardiac glycosides and terpenoids in Aqueous and ethanol extracts of leaves may explain their superior activity as compared to the other stem crude extracts. This warrants further isolation and characterisation of the potentially active principles from the respective crude extracts.

CONCLUSION

The preliminary qualitative phytochemical analysis of crude extracts of *Artabotrys odoratissimus* revealed the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, saponins and terpenoids. Consequently, the chemical profile of crude extracts can help to provide guidance for further investigations in pharmacological properties of *Artabotrys odoratissimus.*

Assessment of the in vitro antibacterial activity of *Artabotrys odoratissimus* revealed that Aqueous and Ethanol extracts of Leaves may be an important source of novel antibacterial compounds in view of their prominent inhibitory activities particularly against Gram-positive bacteria. Hence, further studies are required to isolate and characterise the bioactive compounds responsible for the observed antibacterial properties of *Artabotrys odoratissimus*.

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