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Halochromic properties and antimicrobial potential of crude extracts from five species of ornamental plants

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ABSTRACT: Introduction: the colours of flowers are a result of secondary metabolites that have long been used in the medical and textile industries, and those that are halochromic are used in colour display because they change color according to pH changes, but many species are yet to be studied in detail. **Objective:** to explore the halochromic properties and the antimicrobial potentials of the crude extracts of several ornamental plants. **Methods:** we used aqueous and organic solvents to extract pigments from petals of five fascinating flowers planted around International Institute of Tropical Agriculture station, Cotonou, Benin: *Allamanda blanchetii*, *Cascabela thevetia*, *Eichhornia crassipes*, *Ixora casei* and *Thunbergia erecta,* followed by an investigation into their halochromic properties. Antibacterial potentials of the extracts were tested on important rice pathogens: *Xanthomonas oryzae* pv. *oryzae, and Pantoea agglomerans*, which are gram-negative bacteria; and on *Bacillus subtilis*, a gram-positive bacterium. **Results:** The crude extracts of *T. erecta and A. blanchetii* have good halochromic properties within pH 2 – 12, exhibiting distinct colours. The chromophores of the *C. thevetia*, *E. crassipes*, and *I. casei* are not halochromic as the colours of the crude extracts remain the same at the pH range except pH 12 which is similar for the five extracts. Crude extracts of *T. erecta* inhibited growth of *P. agglomerans* without development of resistance, whereas the bacteria developed resistance against *Penicillin* after 18 hrs of incubation. *T. erecta and A. blanchetii* were able to inhibit growth of *X. oryzae* and both inhibited *B. subtilis.* **Conclusion:** Pigments from both *T. erecta and A. blanchetii* are good pH indicators; however, *T.* erecta is a better antibacterial agent than *A. blanchetii* because it has broad-spectrum activities against bacteria.

Key words: antimicrobial, halochromism, *Thunbergia erecta,* anthocyanin, *Xanthomonas oryzae* pv. *Oryzae, Pantoea agglomerans*, *Bacillus subtilis.*

RESUMEN: "Propiedades halocrómicas y potenciales antimicrobianos de extractos crudos de cinco plantas ornamentales". **Introducción:** los colores de las flores son el resultado de metabolitos secundarios, que se han utilizado durante mucho tiempo en las industrias médica y textil, y los que son halocrómicos se usan en la visualización a color porque cambian de color según los cambios de pH, pero muchas especies aún no se han estudiado en detalle. **Objetivo:** explorar las propiedades halocrómicas y los potenciales antimicrobianos de los extractos crudos de plantas ornamentales. **Métodos:** utilizamos disolventes acuosos y orgánicos para extraer pigmentos de pétalos de cinco flores fascinantes plantadas alrededor de la Estación Internacional de Agricultura Tropical, Cotonou, Benin: *Allamanda blanchetii*, *Cascabela thevetia*, *Eichhornia crassipes*, *Ixora casei* y *Thunbergia erecta*, seguidas de una investigación de sus propiedades halocromáticas. Los potenciales antibacterianos de los extractos se probaron en importantes patógenos del arroz: *Xanthomonas oryzae* pv. *oryzae, Pantoea agglomeran* que son bacterias gram-negativas y en *Bacillus subtilis* una bacteria gram-positiva. **Resultados:** Los extractos crudos de *T. erecta* y *A. blanchetii* tienen buenas propiedades halocrómicas dentro del pH 2 - 12, mostrando colores distintos. Los cromóforos de *C. thevetia, E. crassipes* y *I. casei* no son halocrómicos ya que los colores de los extractos crudos permanecen iguales en el rango de pH, excepto el pH 12, que es similar para los cinco extractos. Los extractos crudos de *T. erecta* inhibieron el crecimiento de *P. agglomerans* sin desarrollo de resistencia, mientras que las bacterias desarrollaron resistencia contra la penicilina después de 18 horas de incubación. *T. erecta* y *A. blanchetii* pudieron inhibir el crecimiento de *X. oryzae* y ambos inhibieron *B. subtilis.* **Conclusión:** los pigmentos de *T. erecta* y *A. blanchetii* son buenos como indicadores de pH. Sin embargo, *T. erecta* es un mejor agente antibacteriano que *A. blanchetii* ya que tiene actividades de amplio espectro contra las bacterias.

Palabras clave: antimicrobiano, halocrómico, *Thunbergia erecta,* antocianina, *Xanthomonas oryzae* pv. *Oryzae, Pantoea agglomerans*, *Bacillus subtilis.*

The colours of flowers are a result of pigments produced by plants, which are secondary metabolites. These metabolites have auxiliary functions other than for metabolism and growth of plants (Neilson, Goodger, Woodrow, & Møller, 2013). The synthesis of secondary metabolites by plants has been attributed to survival mechanisms (Akula & Ravishankar, 2011). These metabolites have been shown to address specific needs in the evolution of plants (Pichersky & Gang, 2000; Michael Wink, 2003) and because of the uniqueness of the synthesis of these secondary metabolites in each plant, they are useful as taxonomic markers (Wink & Mohamed, 2003). Furthermore, the age-long chemical warfare between plants and their pests has necessitated the production of these metabolites some of which serve as toxic substances in defense against pathogens and herbivores, and others to aid territory colonization by inhibiting growth of other plants (Wink, 1988). Conversely, some are colourful with aroma and sweetness to attract animals for seed dispersal (Koes, Verweij, & Quattrocchio, 2005).

These plants' secondary metabolites have been used as raw materials for the production of medicines since prehistory (Cowan, 1999). Particularly useful as antimicrobial agents that have saved many lives. Since 1928 when Alexander Fleming discovered penicillin from mould, antibiotics production has relied heavily on bacterial and fungal sources, with plant sources virtually ignored (Cowan, 1999). In the past few decades however, there has been a rise in number of antimicrobial resistance (AMR) cases reported worldwide (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015) against these antibiotics. The most significant case of antibiotics resistance reported is that to the antibiotics of last resort, colistin (Gao et al., 2016). For this reason, developing new antimicrobial agents is important to stem the growing trend of superbugs spreading across the globe. Promising among the sources of antimicrobial agents is therefore the secondary metabolites from plants (Cowan, 1999). The pigmentation of the secondary metabolites of plants also has usefulness in the textile industry, and those that are halochromic (i.e. change color according to pH changes) are used in colour display and pH indicators (Forster, 1978; Sharifabad & Bahrami, 2016).

In this study, in order to explore the halochromic properties and the antimicrobial potentials of the crude extracts of several ornamental plants, we examined both in flowering plants in the gardens around International Institute of Tropical Agriculture, Benin Republic. The characteristic shape of the petals of the flowers were used to identify the selected flowers. Crude extracts from the petals were tested against common pathogens of rice and the halochromic properties of each flower established.

MATERIALS & METHODS

Sample collection: the flower samples used in this experiment were collected from the garden at International Institute of Tropical Agriculture (IITA), Cotonou, Benin. The bacteria pathogens were provided and earlier characterized by the Pathology Unit of Africa Rice, Cotonou (Lee, Hong, & Kim, 2010; Afolabi et al., 2016; Kini et al., 2017).

Identification of flowers: The characteristic shape of leaf, number of petals and colour were used to identify the flowers by comparing them with images of documented flowers.

Extraction of pigments from flower petals: Pigments were extracted from the petals of all the flowers except in *E. crassipes*, where the thick, glossy leaves were used. Pigments were extracted by crushing 1g of petals in 1mL of solvent with the use of a mortar and pestle. The slurry generated were transferred into 1,5mL Eppendorf tubes and vortexed (Fisher Vortex Genie 2TM– from Fisher Scientific) for 10mins for maximum extraction of the pigments. The resultant mixtures were then centrifuged (Eppendorf Centrifuge Model no 5415D) at 11 000RPM for 30mins to recover the solvents, with the supernatant collected into fresh Eppendorf tubes using a micropipette and stored at 4˚C.

Investigating the halochromic properties of the flower extracts: In order to establish the halochromic properties of the extracts from the flowers, aqueous solutions with pH ranging from 2–12 were prepared by titrating sodium hydroxide with hydrochloric acid. pH of the titrations was monitored with a pH meter and titration stopped at each desired pH points. Transparent 96-well plates were used as colour plates. Seven wells for the pH range and five columns for the different flowers were used. Each well contained 20µL of flower extract, with 20µL of each of the pH range solutions added to the wells on a row. Colour change of the extracts in each well was observed and recorded.

Antimicrobial screening: This screening was carried out in order to investigate the antibacterial property and rate of bacteria inhibition of the crude extracts of the flowers using two techniques respectively: Agar well diffusion method and growth inhibition in liquid culture, Luria Broth (LB).

Agar Well Diffusion Method: Lysogeny broth solidified with agar was prepared using, 2g of peptone (BactoTM Peptone), 2g of NaCl (Sodium Chloride Certified ACS Crystalline from Fisher Scientific), 1g of yeast extract (BactoTM Yeast Extract from Becton Dickinson microbiology systems) and 3g of agar (DifcoTMAgar, granulated solidifying agent from Becton Dickinson microbiology systems) in 200mL distilled water and then autoclaved (Napco® Model 8000-DSE Autoclave). About 20mL of the autoclaved nutrient agar was then poured into petri dishes and allowed to solidify. The bacterial samples were applied to the plates using top agar gel. Briefly, 2% agar was made in distilled water and then autoclaved. Secondary culture of the bacterial samples were inoculated at 1:100 in LB medium and allowed to grow to mid log phase. The 2% agar solution was allowed to cool down to 40°C over water bath and the bacterial growth at mid log phase was quickly added at 1:1 and spread on the nutrient agar plates. A 2mm sterile cork borer was used to punch wells 2cm apart on the nutrient agar plates. The wells were labelled and filled with 80µL of the crude extracts. Known penicillin concentrations, autoclaved distilled water and acetonitrile were used as positive and negative controls respectively. The plates were then incubated at 37°C overnight after the crude extracts have diffused into the plates.

Bacterial growth rate assay: Luria Broth (LB) medium was prepared using 2g of peptone, 2g of NaCl and 1g of yeast extract in 200mL of distilled H_2O. The mixture was split into five aliquots, 500mL each in conical flasks to hold 40mL of the medium and autoclaved. Secondary culture of the bacterial samples was inoculated at 1:100 in LB medium and allowed to grow to mid log phase. Thereafter, 40 µL of penicillin (100mg/mL) and 40µL of flower plant extracts (*T. erecta*) were added to two of the five labelled flasks respectively. Then, 200µl of the bacteria strain was added to the two conical flasks with the antibiotic and flower extract. A third flask was also inoculated as control. The three flasks were mounted on a shaker (Stuart Orbital Shaker SSL1) at 27˚C and 200RPM. The starting OD_{600} was recorded and the growth rate monitored at time interval of 30min at OD_{600} using a spectrophotometer (Jenway 6 300 Spectrophotometer).

Ethical, conflict of interest and financial statements: the authors declare that they have fully complied with all pertinent ethical and legal requirements, both during the study and in the production of the manuscript; that there are no conflicts of interest of any kind; that all financial sources are fully and clearly stated in the acknowledgements section; and that they fully agree with the final edited version of the article. A signed document has been filed in the journal archives.

RESULTS

Identification of the flowery plants: The brightly colored petals and leaves of the flowers enabled easy identification when compared with documented flowers. The Fig. 1 shows the images of the flowers collected at IITA Benin's garden and their corresponding names based on the physical characteristic of the leaves and petals.

Extraction of the pigments with aqueous and organic solvents: The pigments in the various petals of the selected ornamental plants exhibited different solubility in water, methanol and acetonitrile. For each flower, the most compatible solvent on the basis of level of solubility and retention of original colour, was selected as shown in Table 1.

Halochromic properties of the crude extracts in pH range of 2-12: the halochromic properties of the various crude extracts from the flowers were examined at pH range of 2–2. The Fig. 2 shows that *T. erecta* and *A. blanchetii* extracts have distinct halochromic properties.

Top agar diffusion screening of the antimicrobial activity of the extracts on Gram-negative bacterium, *Pantoea agglomerans*: the antibacterial activities of the

TABLE 1
Solvents used for active component extraction

Sample	Flower name	Colour of petal	Extraction solvent
A	*Thumbergia erecta*	Purple	Water
B	*Allamanda blanchetii*	Light pink	Acetonitrile
C	*Ixora casei*	Red	Water
D	*Cascabela Thevetia*	Yellow	Acetonitrile
E	*Eichornia crassipes*	Green leaf	Methanol

Fig. 1. Flowers used for the halochromic and antimicrobial screening: *Ixora Casei* (Rubiaceae), *Allamanda blanchetii* (Apocynaceae), *Cascabela Thevetia* (*Apocynaceae*), *Thumbergia erecta* (*Acanthaceae*), *Eichornia crassipes* (*Pontederiaceae*).

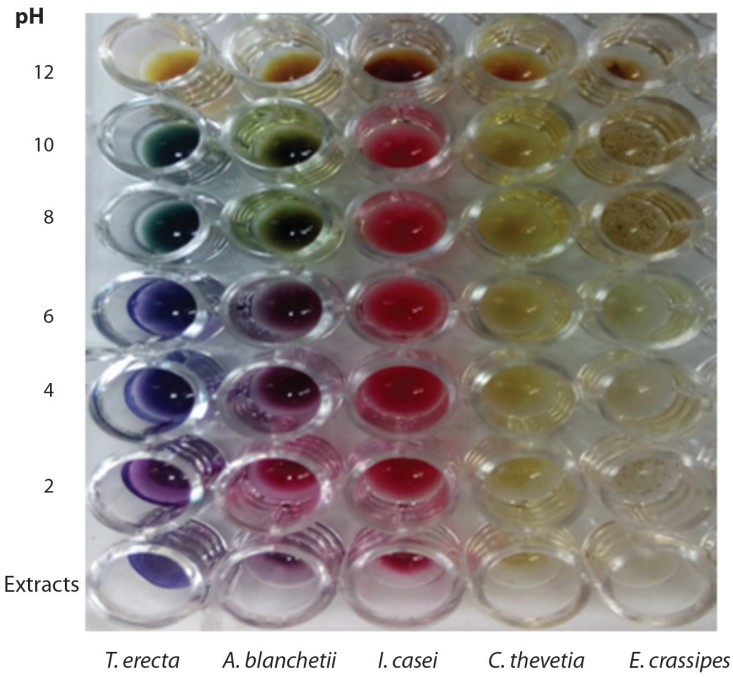

Fig. 2. Halochromic properties of the various flower extracts: Two of the extracts (*T. erecta* and *A. banchetti)* show different colours at the various pH range examined.

extracts were examined on *P. agglomerans* and a purified crystalline penicillin at three concentrations of 200mg/mL, 100mg/mL and 50mg/mL were used as positive control while sterile distilled water was used as negative control. The Fig. 3 shows the top agar plates of the incubation and bacterial growth inhibitions by the extracts and controls observed at 6hrs and 18hrs. Out of all the crude extracts only *T. erecta* shows antibacterial activities against the *P. agglomerans*. Penicillin also inhibited the growth of the gram-negative bacterium as shown in Fig. 3.

We, however, observed resistance being developed by the bacterium after 18hrs of incubation and a noticeable resistance ring forming against penicillin. Fig. 3b shows the various ring formations and the inhibition rings represented as Bar Chat in Fig. 4. The highest concentration of penicillin inhibited the growth of the bacterium to

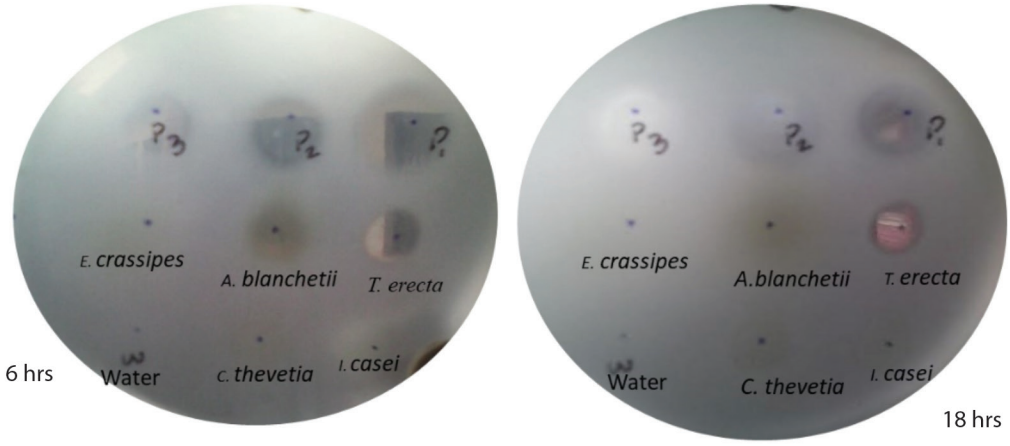

Fig. 3A & B. Top agar diffusion experiment of the antimicrobial activities of the crude extracts on *P. agglomerans.* Concentrations of the penicillin used were 200mg/mL, 100mg/mL and 50mg/mL represented as P1 – P3 respectively on the plates.

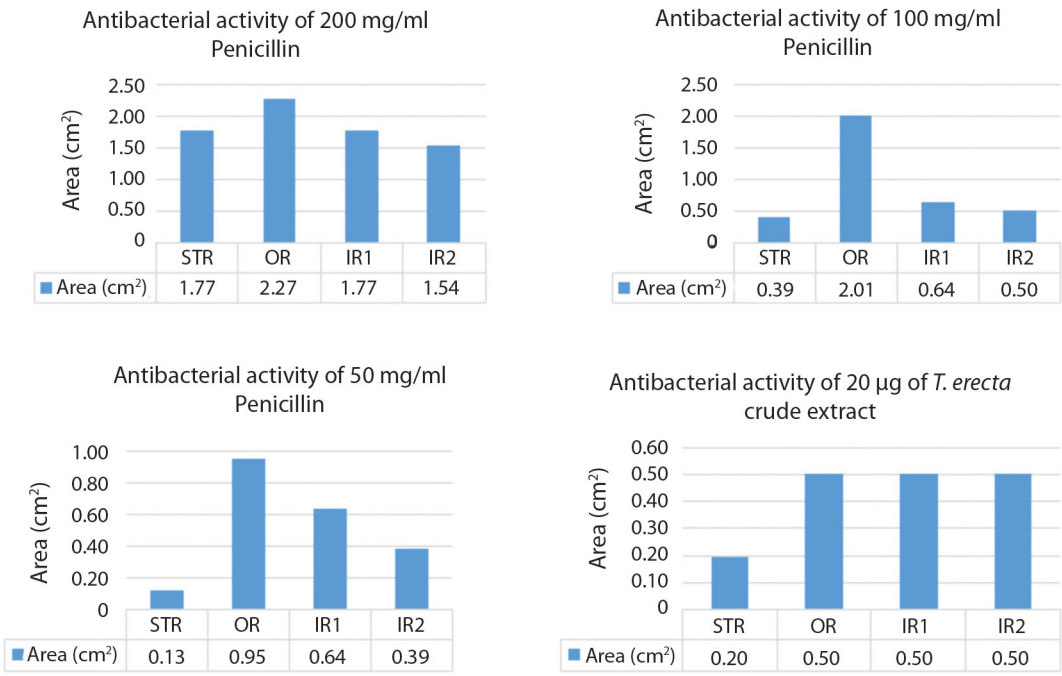

Fig. 4. The 24hr incubation with the antimicrobial agents comparing growth rate and resistance of the bacteria over time. The diameters of the inhibitions were taken, and the area of inhibition plotted as Bar-Chats. It was observed that the bacteria quickly developed resistance to penicillin as indicated by various rings seen on the plate: STR- Start (6hrs incubation; OR- outer ring; IR1 1st inner ring; IR2- 2nd inner ring).

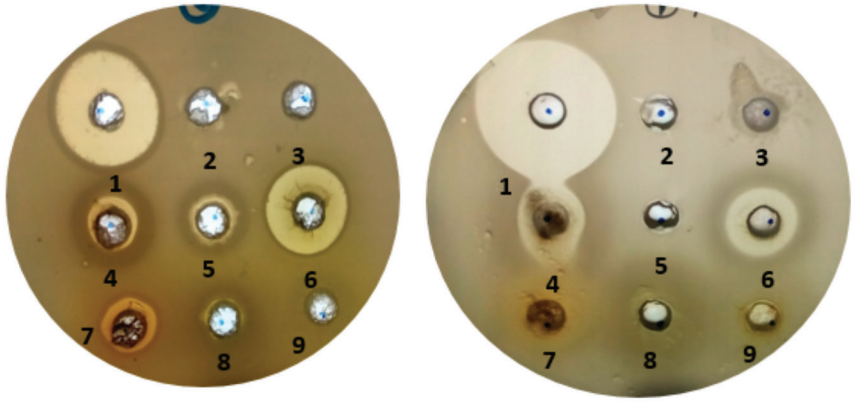

Fig. 5. Top agar diffusion experiment of the antimicrobial activities of the crude extracts on *B. subtilis* and *X. oryzae*.1- Penicllline (200 mg/mL), 2- ethanol, 3-phosphate buffer, 4- *A. blanchetii*,, 5- *I. casei* 6- *T. erecta*, 7-Propolis, 8- *E. crassipes*, 9- *C. Thevetia*

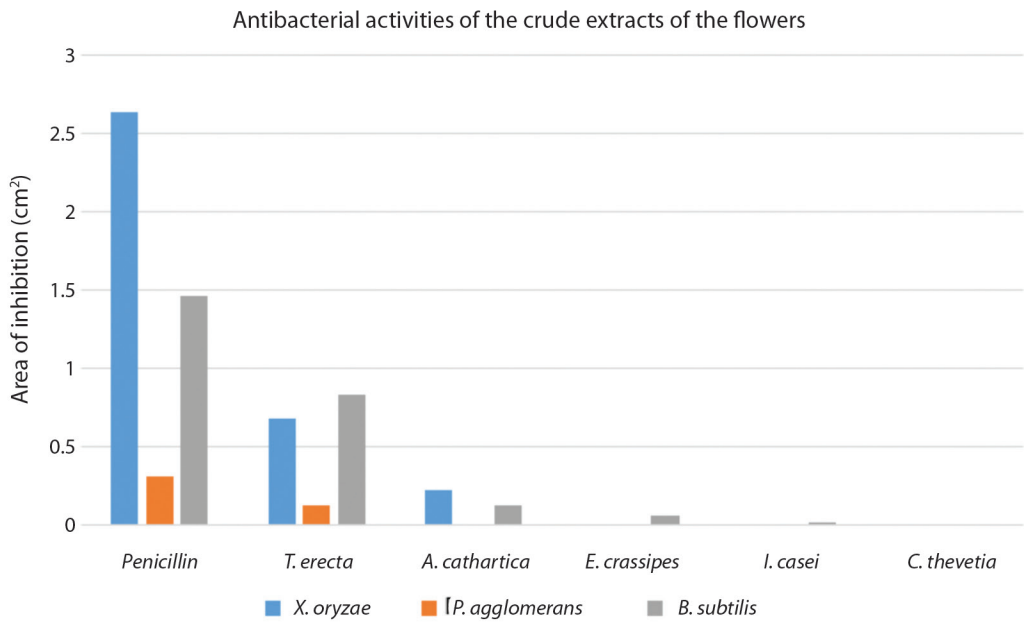

Fig. 6. Summarised data of the top agar diffusion experiments of the antimicrobial activities of the extracts against the three bacteria used. Purified known concentration (200mg/mL) of penicillin used as control.

2,27cm² in 24hrs but the bacterium resisted by reducing the inhibition to 1,77cm² forming the first inner ring and then to 1,54cm² forming the second inner ring. No resistance rings were formed with the growth inhibition by *T. erecta*.

Comparison of the extracts on gram-positive *Bascillus subtilis* and gram-negative bacterium *Xanthomonas oryzae* pv. ***oryzae* using top agar diffusion:** all the extracts have some levels of inhibition against the gram-positive bacterium except *C. thevetia*. However, only T. erecta and *A. blanchetii* have antimicrobial activities against *X. oryzea* as shown in Fig. 5.

We further examined the ability of the *T. erecta* in bacterial inhibition growth in LB medium culture comparing it with penicillin.

DISCUSSION

Halochromic properties of the crude extracts: purple pigmentation in flowers are associated with the

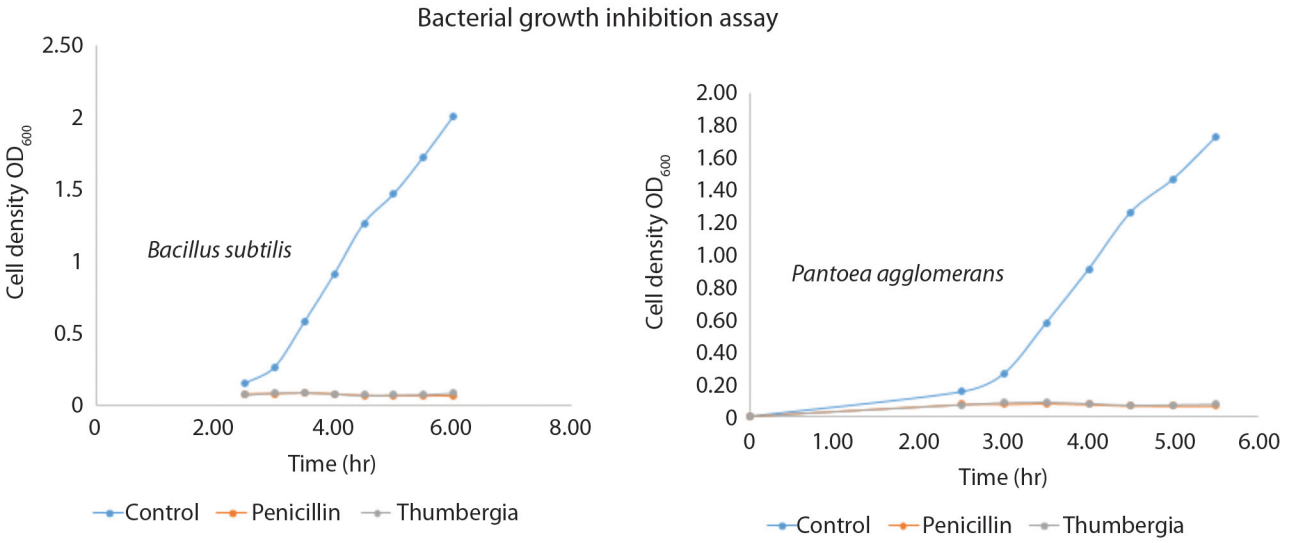

Fig. 7. (A) Bacterial growth monitored at OD_{600} in the presence of 200µg/mL of penicillin and 180µL of *T. erecta*. Penicillin and *T. erecta extract* were added into 40mL LB and a primary culture of both *B. subtilis* and *P. agglomerans* grown to 0.5 OD_{600} was inoculated into the LB. *T. erecta* extract was first incubated 1:1 (200µL) with the bacterium of OD_{600} 0.5 for 1hr before starting the secondary culture in 40mL LB. Control was treated same way but with deionised water. This was done to increase rate of interaction of the extract with the bacterium because of its low concentration.

synthesis of anthocyanins by plants (Archetti et al., 2009). Anthocyanins are a subclass of phenolic phytochemicals in the forms of anthocyanidin glycosides and acylated anthocyanins (Khoo et al., 2017). These are water soluble secondary metabolites (Geissman, 1955; Khoo et al., 2017) and their colour depend on the pH of the solution because of the ionic charge of their molecular structure (Turturica, Oancea ,Râpeanu, & Bahrim, 2015). They have been found in all the tissues of higher plants and are derivatives of anthocyanidins (Andersen & Jordheim, 2010). Halochromic property of anthocyanidins have been proposed to be a good pH indicator (Michaelis, Schubert, & Smythe, 1936) and antimicrobial activities established in berries (Miceli et al., 2009; Genskowsky et al., 2016). The halochromic property of anthocyanin extracted from raspberry juice is being used as colour indicator to monitor the pH change of wound which depends on healing stages and presence of infection (Coomber, 2018). The exhibition of halochromic property observed in *T. erecta and A. banchetti* indicated they have pigments with ionic molecular structure. Further work will be required to confirm the identity of these charged pigments.

Antimicrobial activity: *Thunbergia erecta* is one of the common species of *Thunbergia* (Sultana, Chatterjee, Roy, & Chandra, 2015). Members of these species have been found to have antimicrobial activities against both gram positive and gram negative bacteria (Jeeva, Johnson, Aparna, & Irudayaraj, 2011; Jenifer, et al., 2014; Kosai, Jiraungkoorskul, & Jiraungkoorskul, 2015). The anti-microbial activities of *T. erecta* against *Panteoa agglomeran* and *Xanthomonas oryzae* are remarkable. *Pantoea agglomerans* and *Xanthomonas oryzae* pv. *oryzae* are both pathogens of rice cultivation (Salzberg et al., 2008; Lang et al., 2010; Lee et al., 2010) . They are rod-shaped, gram-negative bacteria found in both temperate and tropical parts of the world and cause 10-50% yield losses. Recently, *Pantoea agglomerans* were discovered to be the plight of farmers on wild rice *Oryza longistaminata* plants near Tanguiéta town, at Pendjari National Park, northwest Benin republic (Afolabi et al., 2016; Kini et al., 2017).

The mode of action of antimicrobial agents are classified into six: cell wall synthesis inhibition, protein synthesis inhibition, DNA synthesis inhibition, RNA synthesis inhibition, mycolic acid synthesis inhibition and folic acid inhibition (Hugo, 1967; Hahn, 2012) . Penicillin is a cell wall inhibitor (Wise & Park, 1965). Antimicrobial resistance of bacteria to penicillin is the production of enzymes capable of destroying the b-lactam ring of penicillin (Tenover, 2006). Once the penicillin is destroyed, resistance ring will form signifying clearance of the antibiotics and demonstrating the resistance status of the bacteria. The additional outer membrane layer of gram-negative organisms differentiates them from gram-positive organisms. This provides additional protection and thereby enhancing survival during stress conditions (Schwechheimer & Kuehn, 2015). The Fig. 3 and 5 show the gram-negative *P. agglomerans* and *X.*

oryzae respectively resisting some of the flower extracts while the gram-positive, *B. subtilis,* was susceptible to all the extracts except *C. thevetia*.

Based on the results obtained from this research, it can be concluded that the secondary metabolites from the crude extract of *T.erecta* is a promising antimicrobial candidate whose bioactive component can be exploited to aid in the fight against the rising problem of antimicrobial resistance. The absence of resistance ring by bacteria against *T. erecta* shows that the mode of action of this antimicrobial agent is different from that of penicillin. Isolation, purification and identification of the pigments from the crude extract of *T.erecta* would give better understanding of the active pigment responsible for these useful properties and add to the arsenal of molecules to fight antimicrobial resistance.

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