International Journal of Agricultural Technology 2021Vol. 17(6):2439-2448
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Functionality of insulin plant (*Costus igneus*) leaf extracts

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Trapse, M. J. L., Solomon, J. R. and Jacob, J. K. S. (2021). Functionality of insulin plant (*Costus igneus*) leaf extracts. International Journal of Agricultural Technology 17(6) : 2439-2448.

Abstract *Costus igneus*, a native of South and Central America, is commonly known as the insulin plant that belongs to family Costaceae. This plant is a perennial, upright, and spreading, growing to a height of two (2) feet with the tallest stems falling and lying on the ground. Studies claim that consumption of its leaves lowers blood glucose levels. Results showed that seven out of nine active phytochemical compounds tested was present in *C. igneus* ethanolic leaf extracts, namely: tannins, saponins, cardiac glycosides, steroids, flavonoids, terpenoids, and alkaloids. Disc diffusion assay revealed that the ethanol extract has exhibited a 20.73 mm and 20.86 mm zones of inhibition against *E. coli and S. aureus* respectively. Furthermore, LC_{50} of 2.88 was observed in lethality assay after 24 hours using probit analysis. Based on these observations, the ethanolic extracts of *C. igneus* contains active biochemical compounds that contributes to its potentiality as antibacterial agent against common food-borne bacteria and can be further studied as anticancer agent due to its high levels of toxicity against *A. salina.*

Keywords: Antibacterial, Brine shrimp lethality assay, *Costus igneus,* Phytochemical

Introduction

Therapeutic plants have provided a supply of innovation for novel healing drugs, as plant-derived drugs have made sizeable contributions to the fitness and welfare of humans (Olowa and Nuñeza, 2013). The use of natural treatments flourished considering that folks believed that natural drugs are better than the commercially available drugs, way more cheaper, and they may be now no longer happy with the results they get with synthetic drugs (Khayyat and AL-kattan, 2017).

Costus igneus, additionally referred to as the Spiral flag, generally grows as a decorative plant. Its leaves are used as a nutritional complement withinside the remedy of diabetes (Hegde *et al.,* 2014). It is a prostate developing plant with spreading root system. Leaves are narrow and lance-

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formed with toothed, scalloped, or lobed margins stained with red pink above and darker pink beneath. The tiny flora develops intermittently during the year. This plant reaches a top of 6 inches and has an indefinite spread (Muthukumar *et al.,* 2019).

Phytochemicals are bioactive compounds received from vegetation broadly carried out in conventional natural medicine (Bansode and Salalkar, 2015). Phytochemicals arise in medicinal vegetation, leaves, vegetables, and roots that need to outline the mechanism and guard against diverse diseases. Phytochemical research has received plenty of hobby amongst plant scientists due to the improvement of modern era and complex outcomes (Muthukumar *et al.,* 2019).

In recent years, research supplied records that the antimicrobial sports of medicinal plants is probably because of the secondary metabolites it produces that supplies medicinal agents. Phytochemicals with enough antibacterial efficacy deals with the bacterial infections (Sardessai *et al.,* 2014). Misusage of antibiotics to cope with infectious illnesses create bacterial pathogenicity and resistant strains (Read and Woods, 2014). Different methods to explore the various medicinal properties of *C. igneus* are developing to make effective and cheaper natural antibiotics with more significant potential, thus possessing minimal side effects. Given the persistence and prolific nature of *C. igneus* and its potential uses, which aimed to determine the phytochemical properties of *C. igneus* and assess its antibacterial activities. Furthermore, the cytotoxic assay was also performed. This study provides a preliminary study that a more specific bioassay can further support after isolating active compounds.

Materials and methods

Collection and extraction of samples

Fresh leaves of the insulin plant were gathered from Pinili, San Jose City, Nueva Ecija, and Matusalem, Roxas, Isabela. The plant leaves had been washed with tap water prior to air-drying at room temperature for approximately five (5) to seven (7) days. After air-drying, the samples were subjected to ethanolic extraction using the standard procedures (Memita *et al*. 2018) with modifications. 100-gram dried samples of *C. igneus* were dispensed on a clean, sterile bottle, added with 500 ml of 95% laboratory-grade ethanol, and stored for 48 hours at room temperature. After which, the mixture was filtered using Whatman filter paper no. 1. The filtrates were refluxed for four (4) hours until a sticky residue was obtained.

Phytochemical analysis of C. igneus leaves

Qualitative phytochemical tests were done to screen and identify the bioactive constituents of *C. igneus* plant using the standard protocols of Sofowara (1993) as cited and modified by Jacob and David (2016). Depending on the change in color and the concentration of reaction, results were indicated as + (traceable amount), ++ (appreciable amount), and – (absent).

The different phytochemical tests undertaken were as follows

Test for alkaloids

Five ml of the *C. igneus* ethanolic extract was dissolved in a 2 ml of 25% hydrochloric acid, filtered and was treated with Wagner's reagent. Reddish-brown precipitate denotes the presence of alkaloids.

Test for saponins

Ethanolic extract of *C. igneus* (2 ml) was boiled with 20 ml distilled water and then filtered. Approximately 10 ml of the filtrate was mixed with five (5) ml distilled water in a test tube and was shaken to produce a stable, persistent froth. The frothing was then mixed with three (3) drops of olive oil and the formation of an emulsion was observed.

Test for tannins

Approximately 0.5 ml of *C. igneus* ethanolic extract was boiled in a 20 ml distilled water in a test tube and then filtered. A drop of $FeCl_3$ (0.1%) was added to observe the existence of tannins. Green and brown coloration signifies gallotannins and pseudotannins respectively.Test for cardiac glycosides.

Approximately one (1) ml of concentrated H_2SO_4 was placed in a test tube. In a separate test tube, five (5) ml of ethanolic extract of *C. igneus* was mixed with 2 ml glacial acetic acid (CH_3CO_2H) containing one (1) drop of $FeCl_3$. The mixture was carefully added to the test tube containing one (1) ml of concentrated hydrogen sulfate so that the concentration of H_2SO_4 was underneath the mixture. The test tube was observed. The formed brown ring at the interphase indicates the presence of cardiac glycosides.

Test for flavonoids

Two to three drops of 1% NH_3 solution were mixed to two (2) ml *C. igneus* ethanolic extract in a tube. The yellow coloration shows the existence of flavonoids.

Test for terpenoids

A 0.2 ml *C. igneus* ethanolic extract were dissolved in a 2 ml chloroform. Concentrated sulfuric acid was then precisely added to create a lower layer. The emergence of reddish-brown color at the interphase shows the deoxy sugar features of cardenolides.

Test of steroids

A 0.5 ml *C. igneus* ethanolic extract was mixed with 2 ml acetic anhydride, then 2 ml of sulfuric acid was added. Alteration in color from violet to blue or green signifies the presence of steroids.

Antibacterial activity of C. igneus

The antibacterial properties of the ethanolic extracts of *C. igneus* were determined by the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol published by Hudzicki (2009) with modifications. Following the aseptic technique, the assay was done inside a laminar flow chamber containing High-Efficient Particulate Air (HEPA) filter to avoid contaminants that may affect the process. Measurement of the zone of inhibition was the basis of assessing the antibacterial property.

Source of test organism

Bacterial strains of *S. aureus* and *E. coli* were acquired from the bacterial culture collection of Microbiology and Bio-Industry Laboratory, Department of Biological Sciences, Isabela State University, Echague, Isabela.

Preparation of Mueller Hilton Agar (MHA)

A total of 38 grams Mueller Hilton agar (MHA) was dispensed in a clean, sterile Erlenmeyer flask with a liter of distilled water. The mixture was heated until homogenized. It was then sterilized on an autoclave for 15 minutes at 121^{o}C at 15psi. After sterilization, it was then allowed to cool and plated on previously sterilized Petri dishes.

Disc diffusion assay

The antibacterial assay was done by employing the disc diffusion method published by Hudzicki (2009). The methodology includes the usage of filter paper discs as a carrier for antimicrobial agents. Circular sterilized discs with six (6) mm diameter were cut from Whatman no. 1 filter paper and soak in liquid treatments. Bacterial cultures of *S. aureus* and *E. coli* were then spread thoroughly onto the MHA plates using a sterile cotton swab in aseptic conditions. Afterward, the saturated discs were arranged equidistantly on the

medium. The plates were incubated at 37ºC and inverted to prevent contamination. Within the 24 hours of incubation, the zone of inhibition was measured using a calibrated digital Vernier caliper every after 8 hours.

Cytotoxicity test using brine shrimp lethality assay

Preparation of brine shrimp nauplii

The Brine Shrimp egg was acquired from the Bureau of Fisheries and Aquatic Resources, San Mateo, Isabela, Philippines. Brine shrimp eggs were hatched in a prepared artificial seawater, as described by Mclaughlin and Rogers (1998). Brine shrimp eggs were added to the artificial seawater where 50g of salt was diluted per liter of water in a glass chamber, and was kept under constant aeration and illumination. After 48 hours of incubation, brine shrimp nauplii were attracted to one side of the vessel using a light source and collected with a pipette.

Cytotoxicity lethality assay

The cytotoxicity property of C. igneus ethanol extract was observed following the brine shrimp lethality test describe by Mclaughlin and Roger (1998) with modifications. Thirty newly hatched nauplii were placed in an Elisa well with ten (10) nauplii per wells. Triplicate per treatment concentration was done. The number of dead nauplii was recorded after the 24^{th} hour. Observations of live and dead nauplii were compared to standard. Live brine shrimps were observed to be actively and constantly moving, while dead nauplii were observed to have been non-motile and floating. Through a stereomicroscope, the number of dead nauplii was identified. Fatality rate was noted, and LC_{50} was found out using Probit Analysis.

Experimental design and treatments

The antibacterial assay and cytotoxicity assay were conducted separately. Each of the tests was done using Completely Randomized Design (CRD) with ten replicates. Recorded data were dealt statistically employing One-Way Analysis of Variance (ANOVA), where means were compared using Tukey's HSD test at $p<0.005$. For the cytotoxicity, the treatment used for the ethanol extract of C. igneus were: 1:1 percent concentration (T1); 1:2 percent concentration (T2); 1:4 percent concentration (T3); 1:8 percent concentration (T4); and 1:16 percent concentration (T5).

Results

Phytochemical constituents of C. igneus

Phytochemicals are bioactive compounds found in plants that offers health benefits for humans. In this study, *C. igneus* was examined for the existence of phytochemical constituents. The phytochemical analysis showed that biochemical constituents such as tannins, cardiac glycosides, steroids, flavonoids, terpenoids, and alkaloids are present on the ethanolic extract of *C. igneus* in a traceable amount while saponins are present in an appreciable amount in the ethanolic extract of *C. igneus* as shown in Table 1.

Table 1. Phytochemical screening on the ethanolic extracts of the *C. igneus*

Constituents	***C. igneus***
Tannins	+
Saponins	++
Cardiac Glycosides	+
Steroids	+
Flavonoids	+
Terpenoids	+
Alkaloids	+

Legend: (+) traceable amount; (++) appreciable amount; (-) absent

Antibacterial properties of C. igneus

C. igneus was extracted for its antibacterial activity against *E. coli* and *S. aureus* using the disc diffusion method. It was found to suppress the growth of *E. coli* and *S. aureus* possesses inhibitory activities against the tested bacteria. Results revealed that after 24 hours of incubation, ethanolic extracts of *C. igneus* have a zone of inhibition with a mean of 20.73 mm against *E*. The highest zone of inhibition was recorded on Streptomycin with a mean diameter zone of 28.88 mm. On the other hand, distilled water did not show any significance among treatments. Furthermore, the ethanolic extract of *C. igneus* against *S. aureus* showed a mean diameter of 20.86 mm. On the other hand, the highest inhibition zone was recorded on Streptomycin with a mean diameter of 30.86 mm which is relatively higher among all the treatments.

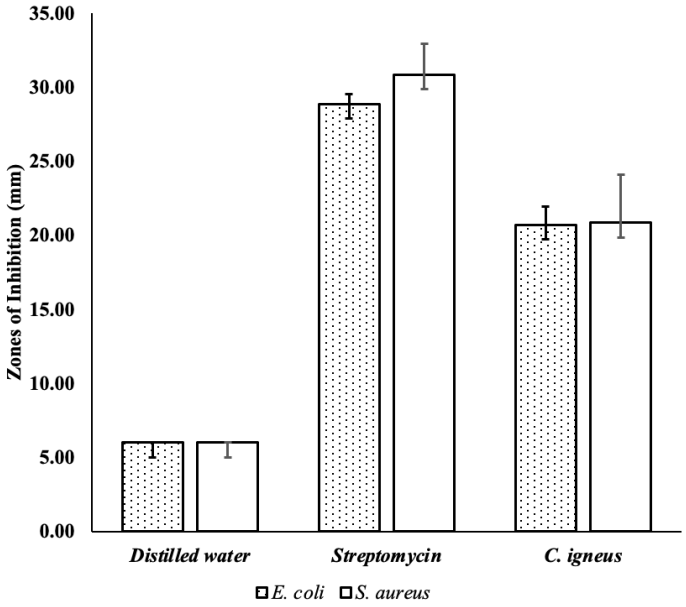

Figure 1. Zones of inhibitions exhibited by the ethanolic extract of *C. igneus* against *E. coli* and *S. aureus* after 24 hours

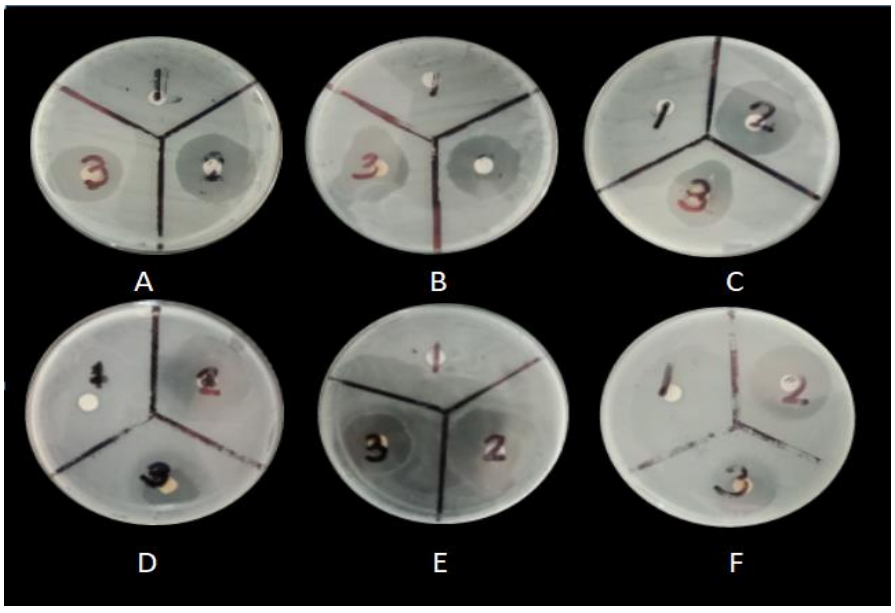

Figure 2. Zones of inhibitions of *C. igneus* after 24 hours (A, B, and C) *E. coli,* (C, D, and E) *S. aureus,* (1) Distilled water, (2) Streptomycin), (3) *C. igneus.*

Cytotoxic activity of C. igneus

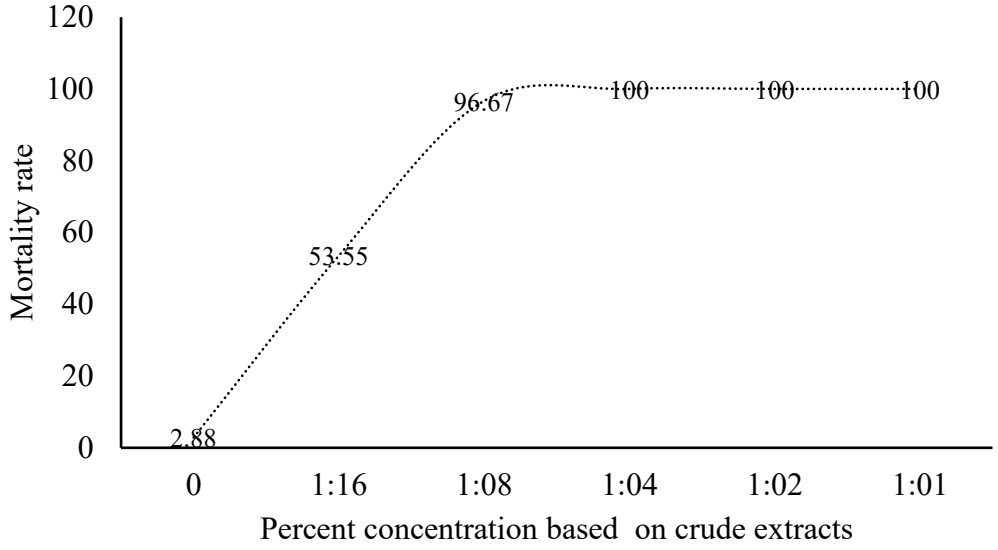

Figure 3. Mortality test of brine shrimp against different concentrations of *C. igneus*

The mortality rate of nauplii exposed to different concentrations of ethanolic extract of *C. igneus* is shown in figure 2. The highest mortality rate in *C. igneus* was recorded at 1:01; 1:02; and 1:04 percent concentration based on crude extract which makes it lethal to 100% of test organisms after 24 hours of observation. Moreover, 1:8 percent concentration based on crude extract exuded 96.67% lethality while 1:16 percent concentration based on crude extract showed 53.55% lethality.

Discussion

Ethanolic extract of *C. igneus* contains traceable to an appreciable amounts of phytochemical components. Phytochemical analysis of plants is extremely important due to its pronounced interest in pharmaceutical companies to create drugs (Wadood *et al.,* 2013). For instance, the presence of tannins has been proven to generate an outer layer on the exposed tissue to lessen infection (Ashok and Upadhayaya, 2012). The toxicity of tannin can be associated to its activity on the membranes of the microorganisms. Tannin's complexation of metal ions explains its toxicity (Akiyama *et al.,* 2001). As reported by Kregiel *et al.* (2013), saponins can increase cleaning/disinfection

processes. In fact, an incorporation of a drop of saponin to a water can be an effective cleanser. Meanwhile, cardiac glycosides are accountable for the production of toxicants. Cardiac glycosides are compounds that aids in the treatment of congestive heart failure (Morsy, 2017). According to Patel and Savjani (2015), plant steroids have interesting activities in the field of medicine which includes anti-tumor, antibacterial, cytotoxic, and cardiotonic. On the other hand, flavonoids are necessary constituent in pharmaceutical applications in view of its anti-oxidative and anti-inflammatory properties (Panche *et al.*, 2016). Based on the study of Mbaveng *et al.* (2014), terpenoids are bioactive compounds known to fight cancer, inflammation, and a variety of infectious diseases. However, some terpenoids can cause gastrointestinal problems. Additionally, alkaloids like cocaine, caffeine, and morphine have strong pharmacologic effects according to Shiel (2018).

The cytotoxicity activity of *C. igneus* revealed that after 24 hours of observation using probit analysis gave LC_{50} of 2.88 against *A. salina*. Therefore, *C. igneus* has the potential antibacterial properties against *E. coli* and *S. aureus* and can be further studied as anticancer agents due to its high levels of toxicity against *A. salina.*

A deeper analysis of cytotoxicity must be undertaken, applying various concentrations of the purified bioactive constituent (phytochemical) to different kinds of animal tissue culture, to generate more conclusive results. Further detailed studies are necessary to confirm the quantitative phytochemical components of *C. igneus* ethanolic extract and determine the antibacterial property of *C. igneus* in a minimum inhibitory concentration.

Acknowledgements

The authors would like to extend their deepest gratitude to Mr. Joe Abucay Jr. for helping them conduct the study. Mrs. Babylin Marcos and Mrs. Juliet Papio for giving them the plant samples. And Mr. Merwin Nuque and Mr. Sean Den Taguba who helped them prepare the samples.

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(Received: 15 August 2021, accepted: 30 October 2021)