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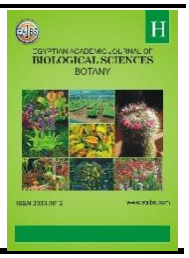

Cytotoxic Potential of Hydroalcoholic Extract of *Annona squamosa* L. Fermented Cotyledon

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ABSTRACT

The present study was aimed to evaluate the cytotoxic potential of hydroalcoholic extract of *Annona squamosa* L. using *Allium cepa* test and Brine Shrimp Lethality assay. Fresh and ripe sugar apple fruits were collected from an orchard at Ota-Efun, Olorunda Local Government, Osogbo, Nigeria (07º 32′ 30.2496″ N, 04º 31′ 41.7036″ E) and their identity authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo, University, Ile-Ife, Nigeria. The seeds were collected, fermented locally for 7days, and oven-dried. The cotyledon was separated from the seed coat, pulverized, defatted, and exhaustively extracted with 80% ethanol. The extract was concentrated and air-dried and named *Annona squamosa* Fermented Cotyledon Hydroalcoholic Extract (AFCHE). The extract was screened for phytoconstituents on GC-MS machine. *Allium cepa* test and brine shrimp lethality bioassay were used to screen for the cytotoxic potential of AFCHE. The root length and protein concentration of the onion root after 7 days of exposure showed a significant decrease with an increase in AFCHE concentration and an increase in percentage growth inhibition of the root. The LC_{50} obtained from the brine shrimp lethality bioassay qualified AFCHE as a highly toxic substance and a good anticancer agent worth screening.

INTRODUCTION

Cancer is the second reason for death worldwide. Conventional therapies for cancer include surgery, cytotoxic chemotherapy, immunotherapy and radiation therapy which are used as single or combinatorial therapy have some side effects (Dutta *et al.*, 2019). Today, many plant-derived compounds "phytochemicals" have been identified that have anti-tumour properties. For example, induction of apoptosis and inhibition of cell proliferation which finally decrease the risk of cancer make these components useful for cancer treatment (Sultana *et al.*, 2021).

A simple search on Google Scholar on 21 June 2018 with the key "Allium test" produced 3170 hits, showing the wide use of the test in a variety of different investigations. A few examples are studies examining the effects of toxic plants (Pesnya *et al*., 2017), nanoparticles (Yekeen *et al*., 2017), synthetic plant hormones such as etephon (Yavuz and Kiliç, 2017), industrial waste (Dutta *et al.*, 2018), aquatic environmental samples (Zotina *et al.*, 2015), etc. The lethality of the test sample in a simple zoological organism like the brine shrimp (*Artemia salina)* has been utilised by many researchers and has proven to be a useful

tool in screening various chemical compounds found in various bioactivities (Azad *et al.,* 2019).

Cytotoxicity testing is preliminary research to the discovery of anti-cancer agents, and cancer is a major global health problem. The majority of anti-cancer drugs that enter clinical trials exhibit little or no therapeutic benefits and fail to obtain regulatory approval. Hence, this study seeks to provide information on cytotoxicity activities of hydroalcoholic extract of fermented cotyledon *Annona squamosa* to be evaluated for potential as an anti-cancer agent. The use of the Allium test (AT) to check the potential genotoxicity of several types of chemical, physical and biological agents has a long history in scientific literature, from the first investigation by Levan (1938) to the more standardized method proposed by Fiskesjö (1985) and later Rank (2003).

MATERIALS AND METHODS

Materials:

1. Fruit Collection and Identification:

Fresh and ripe sugar apple fruit was collected from an orchard at Ota- Efun. Olorunda Local Government, Osogbo, Osun State, Nigeria (07°32' 30.2496" N, 04° 31' 41.7036"E). The Fruit was authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife with number IFE-17805. *Allium cepa* bulbs were purchased from Oja Sanya, Ede, Osun State, Nigeria, and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife with number IFE-17944. Experiments were conducted in the Biochemistry laboratory of Osun state University, Osogbo.

2. Reagents and Chemicals:

All the reagents used in this study were of analytical grade and were procured from various sources.

Methods:

1. Processing of *Annona squamosa* Seed:

The *Annona squamosa* seed was fermented traditional by wrapping the seeds with the pulp on them in banana leaves and kept inside a dark cupboard for seven (7) days for fermentation (Dare *et al.,* 2013). These seeds were shelled by separating the seed from the cotyledon and the cotyledon was milled separately into powder using QBL Grinder for easy extraction.

2. Defatting *Annona squamosa* Seeds:

Typically, 20g of the cotyledon was extracted with 400ml diethyl ether, using a soxhlet extractor. This extracted the non-polar components (oil) from the samples. The extracted oil was kept for future use and the residue was dried and weighed.

3. Preparation of Hydroalcoholic Extract of *Annona squamosa*:

The defatted sample was exhaustively extracted with 80% ethanol. The hydroalcoholic extract was concentrated on a rotary evaporator and termed *Annona squamosa* fermented cotyledon hydroalcoholic extract (AFCHE). The extract was air-dried and kept in the freezer for further use.

4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of AFCHE:

After extraction, gas chromatography-mass spectrometry (GC-MS) was employed in the analysis of the compounds present in AFCHE. This was done according to the procedure described by Akther *et al*. (2017), using a 7820A gas chromatograph coupled to 5975 inert mass spectrometer (with triple-axis detector) and electron impact source (Agilent Technologies). The separation of the compounds was carried out on HP-5 Capillary Column coated with 5% Phenylmethyl siloxane (30 m length x 0.32 mm diameter x 0.25 μm film thickness) (Agilent Technologies). The carrier gas was helium used at a constant flow rate

of 1.573 ml/min, an initial nominal pressure of 1.9514 psi and at an average velocity of 46 cm/s. sample (1µl) was injected in split less mode at an injection temperature of 260 ^{o}C. Purge flow was 1ml/min at 0.50 min with a total gas flow rate of 23.355 ml/min and the gas saver mode was switched on. The oven was initially programmed at 60 ^{o}C for 1 min, and then the temperature was gradually increased at the rate of 4 ^{o}C/min to 110 ^{o}C for 3 min. run time was 56.25 min with a 3 min solvent delay. The mass spectrometer was operated in electron-impact ionisation mode at 70 eV with the ion source temperature of 230 ^{o}C, quadrupole temperature of 150 mode at 70 eV with an ion source temperature of 280 ^{o}C. Scanning of possible compounds was from m/z 30 to 550 Da at 2.62 s/scan rate. The relative percentage amount of each component was calculated by comparing peak area to the total areas, and interpretation on the mass spectrum of GC-MS was done using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The unknown compounds were identified by comparing measured mass spectral data with those in NIST14 Mass Spectral Library.

5. Root Growth Inhibition Assay:

The root growth inhibitory assay was carried out as reported (Sharma and Vig, 2012) using *A. cepa*. Typically, outer scales and root remnants of the onion bulbs (30) were carefully removed in order not to destroy the root primordia. The *A. cepa* bulbs were exposed for 96 h to varying concentrations (0, 100, 200, 300, 400 and 500 µg/ml) of AFCH while distilled water served as control. The test solution was replaced every 24 h with fresh solutions until the roots were fully developed. The best developed 10 roots of each onion in each group were collected, measured with a ruler and the mean root length was calculated. The percentage growth inhibition was calculated using the expression:

$$\text{Percentage Inhibition} = \left(\frac{Root\ length_{control} - Root\ length_{sample}}{Root\ length_{control}} \text{ x}100\right)$$

6. Preparation of Onion Roots Homogenates:

The (*Allium cepa)* Onions roots (0.5g) were homogenized with 5ml (0.85% v/v) normal saline with mortar and pestle. The homogenates were centrifuged at 3000 rpm for 15 minas described earlier. The supernatants were collected, stored and the residues were washed with 0.1 M NaOH and further centrifuged at 3000 rpm for 15 min. The supernatants were combined and used for the estimation of total protein.

7. Estimation of Total Protein Concentrations:

The protein concentrations in the (*Allium cepa*) Onions roots homogenates were estimated as described by Schacterle and Pollack (1973), using bovine serum albumin as standard. The onions roots homogenates (1ml) were pipetted into different clean dried test tubes. One (1ml) Alkaline Copper reagent (10% (w/v) Na_2CO_3, 0.1% (w/v) K-Na-tartrate and 0.05% $CuSO_4$. $5H_2O$ in 0.1 M NaOH) was added and mixed thoroughly. The mixture was left to stand for 10 min at room temperature undisturbed after which 2ml of Folin-Ciocalteu's Phenol reagent (1:10 dilution) was added carefully. It was then incubated at 55 °C for 15 min in a water bath, tubes were collected and allowed to cool. The blank was treated similarly with distilled water in place of the sample. The absorbance was read at 650 nm against the blank. A standard calibration curve was prepared using bovine albumin 0.0, 40, 80, 120, 160 and 200 µg/ml. The protein concentrations were extrapolated from the standard calibration curve and expressed as mg/g fresh weigh of plant sample (mg/g AFCHE).

8. Cytotoxic Test - Brine Shrimp Lethality Bioassay:

The cytotoxicity activity of AFCHE was carried out according to the method of Chatterjee *et al*. (2013). Typically, 1 g of *Artemia salina* (Linn) cysts were aerated in a 400 ml capacity specially constructed container containing 300 ml filtered seawater (pH 8.2) collected from the Atlantic Ocean (Bar Beach), Ikoyi, Lagos, Nigeria. After 48 hr incubation

at room temperature, under continuous illumination of fluorescent lamp, newly hatched free-swimming (pink-coloured) nauplii were harvested.

The assay system contained varying concentrations of AFCHE (0, 20, 40 60, 80, and 100 µg/ml) and control in sterilised transparent plastic containers. To each container was transferred 20 nauplii and incubated for 24 hr, under constant illumination with a fluorescent lamp. The number of survived nauplii was counted with a hand lens at 3 hr intervals. Replicates (5) were prepared for each concentration of AFCHE. Then, percentage mortality was estimated after 12 hr as:

$$\text{Percentage mortality} = \frac{Number\ of\ survived\ nauplii\ after\ 24hr}{Number\ of\ nauplii\ at\ the\ start\ of\ the\ experiement}\ x\ 100$$

The effectiveness or the concentration-mortality relationship of AFCHE was expressed as a median lethal concentration (LC_{50}) value.

RESULTS

1. GC-MS Profile of AFCHE:

The spectrum of AFCHE as revealed by GC-MS analysis is shown in Figure 1. More than a hundred phytoconstituents were detected and the reported biological activities of some of the phytoconstituents are anti-inflammation, antioxidant, cytotoxic, anti-cancer, anti-fungal, bactericidal, antiviral, antileishmanial, wound healing, hypotensive, etc. The most abundant constituents in AFCHE include chalcones (~3%), vitamin E (~5%), glycerine (~4%), octadecanoic acid (~7%), oleic acid (~7%), linoleic acid ethyl ester (~9%), sterols (campesterol, stigmasterol and lupeol, sitosterol ~10%).

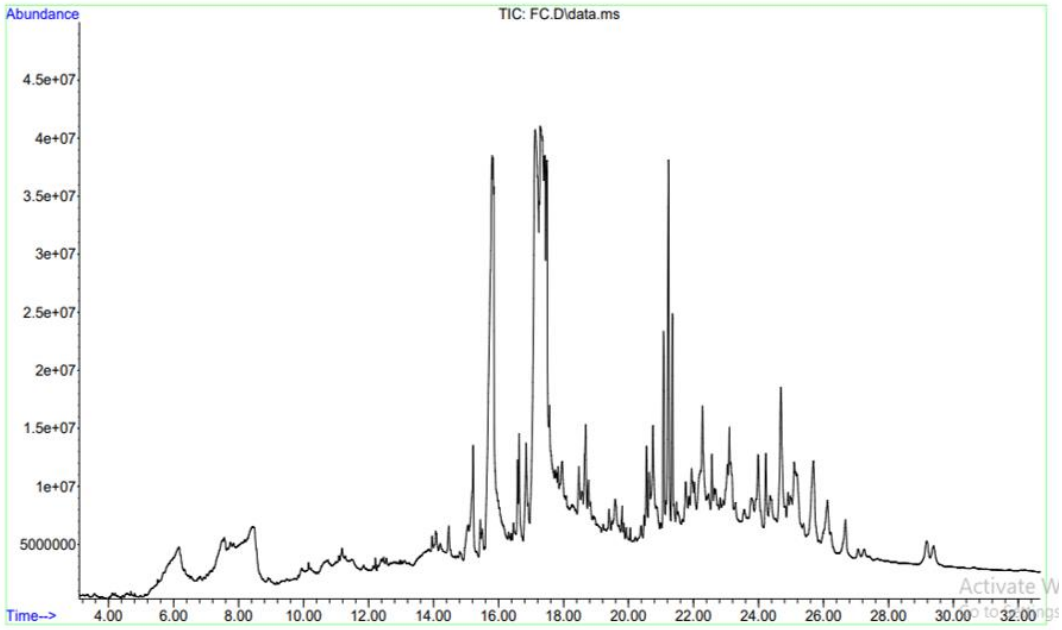

Fig. 1: GC-MS Spectrum of AFCHE

2. *Allium cepa* Test:

From the *Allium cepa* test, there was observed significant decrease in the root length of the onion bulb with an increase in the concentration of AFCHE (Fig. 2). The percentage inhibition of the root growth also increased as the concentration of AFCHE increased (Fig. 3) and the IC_{50} was 97.745±3.76µg/ml. There was a significant decrease in the concentration of protein in the root with an increase in AFCHE concentration (Fig. 4).

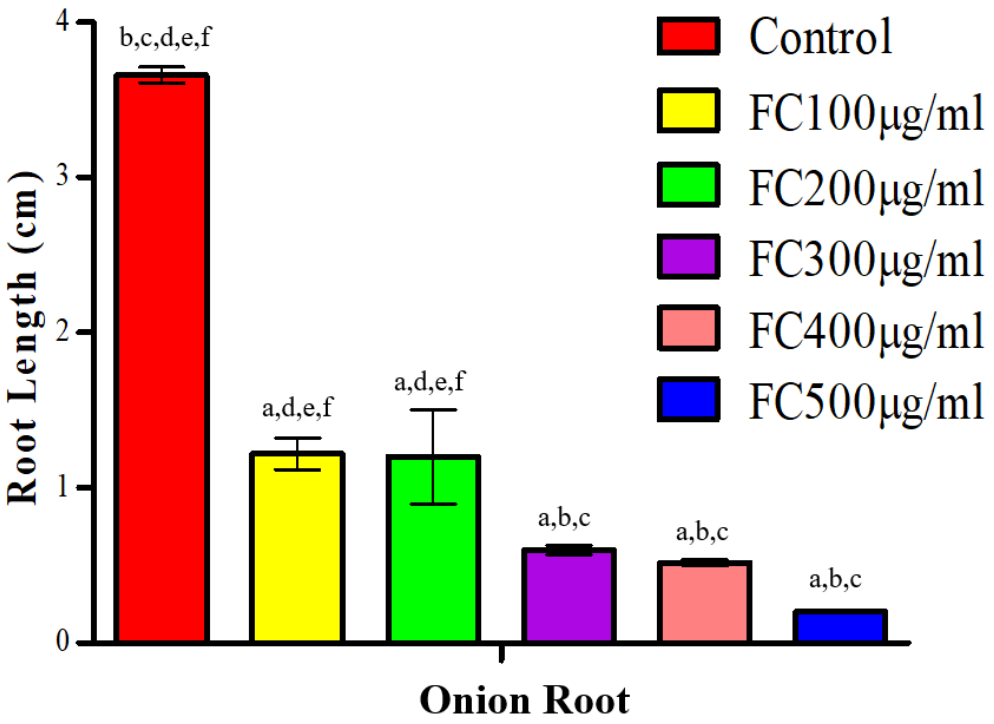

Fig. 2: Onion Root Length

Each value represented Mean ± SEM of n = 5. The values with alphabetic superscripts are statistically significant at P<0.05. a compares Control with others, b compares AFCHE 100 µg/ml with others, c compares AFCHE 200 µg/ml with others, d compares AFCHE 300 µg/ml with others, e compares AFCHE 400 µg/ml with others, f compares AFCHE 500 µg/ml with others.

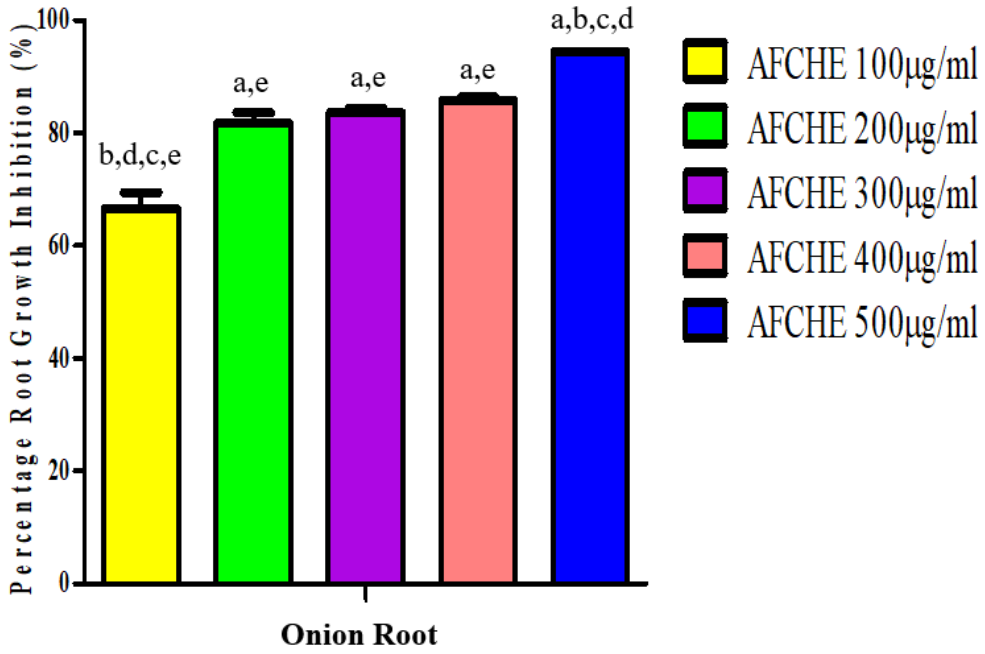

Fig. 3: Percentage Onion Root Growth Inhibition

Each value represented Mean ± SEM of n = 5. The values with alphabetic superscripts are statistically significant at P<0.05. a compares AFCHE 100 µg/ml with others, b compares AFCHE 200 µg/ml with others, c compares AFCHE 300 µg/ml with others, d compares AFCHE 400 µg/ml with others, e compares AFCHE 500 µg/ml with others.

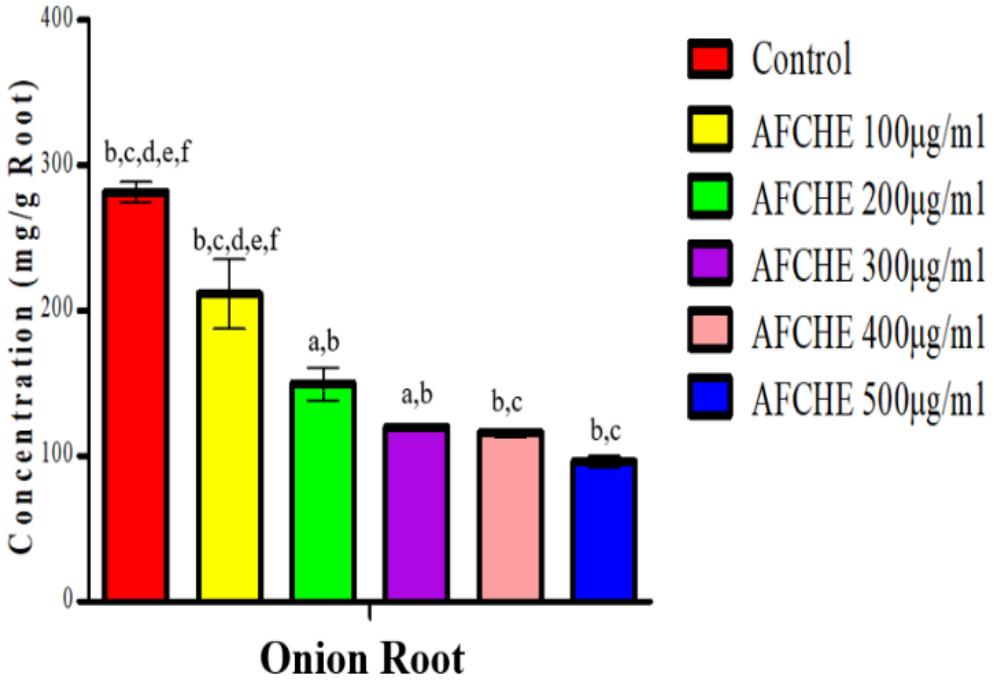

Fig. 4: Protein Concentration in Onion Root

Each value represented Mean ± SEM of n = 5. The values with alphabetic superscripts are statistically significant at P<0.05. a compares Control with others, b compares AFCHE 100 µg/ml with others, c compares AFCHE 200 µg/ml with others, d compares AFCHE 300 µg/ml with others, e compares AFCHE 400 µg/ml with others, f compares AFCHE 500 µg/ml with others.

3. Brine Shrimp Lethality Bioassay:

As the time progressed at 3 hr intervals, the nauplii died at a logarithmic rate from the highest concentration of the extract to the lowest (i.e. from 100µg/ml - 20µg/ml) except control group of the experiment (Table 1). The experiment was stopped after 12 hr against 24 hr it was designed for because all the nauplii in the test had died as at 12 hr.

There was an increase in percentage mortality of the nauplii with an increase in the concentration of AFCHE (Table 2). The observed LC_{50} value was as low as 25.939 ± 1.32 µg/ml and portrayed the extract as highly toxic.

Table 1: Brine Shrimp Lethality

USCP (µg/ml)	Naupli No 0hr	Naupli No 3hr	Naupli No 6hr	Naupli No 9hr	Naupli No 12hr
Control (0)	20.00±0.00	20.00±0.00	19.60±0.25	18.25±0.25	17.75±0.29
20	20.00±0.00	8.20±0.97	6.33±0.33	1.50±0.50	0.00±0.00
40	20.00±0.00	8.20±0.97	4.50±0.50	1.50±0.50	1.00±0.00
60	20.00±0.00	7.50±0.98	3.00±1.00	1.00±0.00	0.00±0.00
80	20.00±0.00	6.60±0.40	2.50±0.50	1.33±0.33	0.00±0.00
100	20.00±0.00	3.60±0.40	0.75±0.25	0.00±0.00	0.00±0.00

Each value represented Mean ± SEM of n = 5 replicates.

Table 2: Percentage Mortality of Nauplii

Concentration (µg/ml)	**Percentage Mortality**
20	67.700 ± 1.00
40	77.041 ± 1.70
60	84.694 ± 2.62
80	87.245 ± 4.48
100	96.173 ± 1.44
LC_{50} (µg/ml)	25.939 ± 1.32

Each value represented Mean ± SEM of n = 5 replicates.
The control group change rate (%) was taken as 100 %, and the other concentrations were compared to this group.

$$\% \text{ Inhibition} = \left(\frac{Naupli\ number_{control} - Naupli\ number_{sample}}{Naupli\ number_{control}} \text{ x100}\right)$$

DISCUSSION

The use of medicinal plants as an approach in the prevention and treatment of cancer has been followed for many years and many therapeutic plants with anticancer activity are reported in the literature (Hekin *et al.*, 2018). As the interest in organic and simple lifestyles grows, the interest in plant-based medicine also increases (Schultz *et al*., 2020). In addition, adverse effects and drug interactions are major restrictions in synthetic anticancer drugs; therefore, plants have been investigated across the world to exploit novel and potential sources of anticancer agents (Ntungwe *et al*., 2020).

The use of parameters related to *Allium cepa* root growth to assess the toxicity of several substances has been increasing, because data about *Allium cepa* can provide information about lethal effects of the compounds, and delay in root growth can provide information about sub-lethal effects (Pantano *et al.*, 2021). With root growth data of all treatments and negative control of a bioassay, the concentration at which 50% inhibition of root growth occurs when compared with negative control (IC_{50}) can be estimated.

There was a significant reduction in the root lengths of onion bulbs exposed to different concentrations of AFCHE in a dose-dependent manner. The root growth inhibition could have occurred as a result of the inhibition of cell division and it is thus an index for estimating general toxicity. It occurs when roots are exposed to extreme pH, or to substances that prevent nutrient uptake (Owolarafe *et al.*, 2020). The inhibitory effects could also be due to cell extension, that is, cessation of root elongation which is correlated with the decrease in protein concentration observed. Metal stress can act at different sites to inhibit a large number of enzymes having functional sulfhydryl groups. It results in a deleterious effect in the normal protein form by disrupting the pathways and protein synthesis (Chen *et al.*, 2021). This implies that the hydroalcoholic extract was toxic to the onion bulbs and inhibited the growth of the onion root as the concentration increased.

Brine shrimp lethality bioassay is an efficient, rapid and inexpensive assay for testing the bioactivity of plant extracts. It is an excellent choice for elementary toxicity investigations based on the ability to kill laboratory-cultured *Artemia* nauplii (Sharma *et al.*, 2013; Naidu *et al.*, 2014). Studies have demonstrated a positive correlation between the brine shrimp lethality and oral lethality test in mice in medicinal plant research.

Brine shrimp lethality assay after 12 hr of exposure to the hydroalcoholic extract of *Annona squamosa* L. and positive control were investigated. As the time progresses at each 3 hr interval, the number of nauplii decreased at a logarithmic rate from the highest concentration of the extract to the lowest (i.e. from 100µg/ml - 20µg/ml) except control group of the experiment

In the present study, different measures of lethality were observed with exposure to different concentrations of FACHE (20, 40 60, 80, and 100 µg/ml) and control. According

to Meyer's toxicity index, hydroalcoholic extracts with LC_{50} < 1000 µg/ml are considered as toxic, while extracts with LC_{50} > 1000 µg/ml are considered non-toxic (Meyer *et al*., 1982; Rahman *et al*., 2020). Clarkson's toxicity criterion for the toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC_{50} above 1000 µg/ml are non-toxic, LC_{50} of 500 - 1000 µg/ml are low toxic, extracts with LC_{50} of 100 - 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson *et al*., 2004; Asghar *et al*., 2020). From the results obtained, LC_{50} value of 25.939 µg/ml of AFCHE qualified it as a highly toxic substance according to the toxicity indices by Meyer and Clarkson, and it is a highly recommended anti-cancer agent worth screening.

Many of the phytoconstituents detected in GC-MS analysis have proven biological activities many of which are responsible for the cytotoxic activities observed in this study and also presented AFCHE as a good lead in search of anticancer drugs. For example, chalcones (Chavan *et al.,* 2016), hexadecanoic acid (Nagata *et al.,* 2015), squalene (Kelly, 1999; Minhas *et al.,* 2020), plant sterols such as campesterol, stigmasterol and lupeol have also proven to exhibit anti-tumour properties (Luo *et al.,* 2015).

Conclusively, the anticancer activities of AFCHE should be evaluated on cancer cell lines for their potential as an anti-cancer agent.

Declaration of interest: None

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