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Original Research Article

Unfolding the Cytotoxic Potential of *Cassia siamea* L. (Fabaceae) Stem via a Combination of Cost-Effective Anticancer Screening Templates

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ABSTRACT

Cassia siamea (SB) is a common medicinal plant in Southwestern Nigeria, known for its usage in the management of cancer among locals. The study aimed to unveil the claimed anticancer property of SB using cytotoxic bench-top bioassays. Cytotoxicity potential was investigated using *Raniceps raninus* and *Saccharomyces cerevisiae* bioassay models, and radical scavenging strength against DPPH stable radical was also unfolded. SB fractionation resulted in three bulked column fractions (SB1 – 3). SB1 was observed to induce severe DNA damage with a percentage mortality of 75.21±5.85%, which is significantly different from 21.85±7.30% $p<0.05$ observed with 5% DMSO (NC; negative control). The concentration-dependent increase in the mortality of *R. raninus*, which is an indication of its cytotoxic influence, was noted with SB1 at 10 – 50 µg/mL with 100.00±0.00% mortality observed at 40 and 50 µg/mL. SB1A obtained from SB1 showed the presence of 16 compounds (GC-MS analysis), with caffeic acid found to be the most abundant. Further fractionation of SB1B (obtained from SB1) resulted in pure compound SB1B3 (15.78 mg), which exhibited about 69.82±8.82% mortality of the *S.cerevisiae* $p<0.05$ relative to the NC. SB1B3 at 5 – 25 µg/mL also showed 100.00±0.00% mortality of *R. raninus* at $p<0.05$ relative to NC. Although the identity of the SB1B3 is yet to be ascertained. The findings of this research confirm that SB possesses cytotoxic potential as claimed by locals.

***Keywords*:** *Cassia siamea*, Chromatography, Cytotoxicity, *Raniceps raninus*, *Saccharomyces cerevisiae*

Introduction

Medicinal plants have been known since time immemorial to be rich in intriguing chemicals, which are usually known for their effective therapeutic compounds that are used in the development of pharmaceuticals.[1,2] The active ingredient(s) found in different morphological organs possess varying therapeutic influence on the living cells, hence their ability to be utilized as medicines to treat many ailments in both humans and animals, including cancer.[1] However, cancer is regarded as a group of diseases characterized by the growth of carcinoma cells, which are frequently recognized for their propensity to migrate or spread from one part of the body to another.[3] Contrarily, benign tumors are restricted to a single area of the body and have no propensity to spread to other sections of the body.[4] In 2020, there will likely be 1.3 million fatalities from cancer and 2.7 million new cases (all forms, excluding non-melanoma skin cancer). According to these statistics, men are marginally more affected by cancer than women, accounting for 54% of new cases and 56% of deaths.[5]

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In developing countries, 8.8 million cancer cases have recently been reported (15.7 percent of deaths).[5] Nigeria had 124,815 new cases in total in 2020. Of them, 51,398 were in males, with prostate cancer being the most prevalent kind at 29.8%, and 73,417 were in females, with breast cancer being the most common type at 38.7%, followed by cervix cancer at 16.4%.[5] Among the options for management include hormone therapy, palliative care, surgery, radiation therapy, chemotherapy, and targeted therapy. The patient's preferences, overall health, and the location, kind, and stage of the cancer all affect the available treatment options.[2] The high cost of cancer medications and treatment options, in addition to the lack of local access to anticancer agents and corresponding treatments, as well as their toxic side effects (such as abdominal cramps, memory impairment, weakness, fatigue, nausea, hair loss, vomiting, etc.), allow for the potential application of related medicinal plants and/or promising natural compounds with a history of ethnomedical usage in the management or treatment of cancer.[2]

A popular evergreen ornamental tree of average size, *Cassia siamea* Lam. (Fabaceae; SB), commonly referred to as the Cassia tree in English, Bakin raskata in Hausa, and Odan in Yoruba, native to Southeast Asia and other tropical regions of the world, including Nigeria. It can reach a height of 20 m and has a straight trunk with a maximum diameter of 30 cm.[6] The herb has reportedly been used as a treatment in ethnomedicine to cure cancer, diabetes, hypertension, asthma, typhoid fever, constipation, and other conditions.[7]

Screening of plant extract, compounds, and other potential anticancer samples in a resource-limited environment has been a challenge due to the unavailability of cancer cell lines, shortage of power supply, limited technical skills of the scientists in the usage of modern cancer screening

methods, unavailability of needed chemicals and equipment have necessitated the search of novel methods for the screening of potential anticancer drug candidates. These methods include the use of *Saccharomyces cerevisiae*, brime shrimp, *Raniceps ranninus*, *Sorghum bicolor*, etc. These methods have been reported to be reproducible, and predictive due to the genetic similarities between the human cells and the cells of these lower organisms.[8,9,10]

In this study, various anticancer bench-top test templates were combined to assess the cytotoxic potential of crude and bulked column fractions of SB.

Materials and Methods

Collection and identification of plant material

On April 4, 2022, *Cassia siamea* stem bark (SB) was collected at Afe Babalola University in Ado Ekiti, Ekiti State (coordinates: 7.6709° N, 5.3071° E). Also, at the Department of Pharmacognosy and Natural Products, College of Pharmacy, Afe Babalola University, Ado Ekiti, Ekiti State, the plant was botanically identified and validated. The voucher specimen was deposited at this location, and the herbarium number ABUAD/021 was assigned.

Extraction of plant material

About 500 g of SB was extracted with 5 L of absolute methanol (Anal. grade) using the Soxhlet apparatus. The SB crude extract was concentrated in a vacuum chamber and the resulting extract was refrigerated at 4^0C.

Phytochemical screening of the crude extract

The phytochemical screening was done according to standard protocol.[7]

Fractionation of SB using column chromatography

About 50 g of SB was subjected to open-column chromatography. Moreover, 200 mL of each of the solvent systems (varying polarities ranging from n-hexane to methanol) was utilized as the mobile phase, using the gradient elution pattern (to enhance peak resolution, faster analysis times, and better detectability). This resulted in 150 column fractions (anal. Thin Layer Chromatography, TLC (Silica gel G; n-Hexane: Ethyl acetate 1:4, 2:3; Concentrated tetraoxosulphate (IV) acid was used as spray reagent). The collected column fractions were further bulked into three main bulked column fractions, based on the similarities in the TLC pattern of each fraction, namely; SBI - SB3 as shown in Table 2.[2,8]

Cytotoxicity assay of SB1 – 3 using Saccharomyces cerevisiae cell culture model

A preliminary cytotoxicity investigation was conducted on the extract of crude SB and bulked column fractions of SB using the *Saccharomyces cerevisiae* cell culture assay model. About 1.0x107 cells per mL of *S. cerevisiae* cell cultures was realized via inoculation of *Saccharomyces cerevisiae* cells on 20 mL of Yeast extract peptone-dextrose broth (YPD; yeast extract 1%, peptone 0.5%, and glucose 2%), followed by incubation at 30ºC for 16 – 20 hours. To achieve a grand volume of 2.2 mL, about $0.5x10^6$ cells were placed into 4 mL disposable cuvettes containing YPD medium, and different concentrations (7.81 – 500 µg/mL) of test samples were added. An organism-free YPD medium was employed as a negative control. For five hours, the full set of loaded disposable cuvettes was incubated in a Heidolph Inkubator 1000 with a shaker set to 30^0C and 230 rpm/min. On the other hand, during the 300 minutes, the absorbance was first measured at 0 minutes and then every 60 minutes, for every concentration, three duplicates of the assay were run. To assess the method's repeatability and ascertain the percentage growth inhibition rate of *S. cerevisiae* in the presence of the test samples, a covariance analysis was performed on the growth curves' slopes.[9,16]

Cytotoxicity assay of SB1 – 3 using Raniceps raninus model

The following procedure was used to perform the *Raniceps raninus* cytotoxicity assay model. Ten *R. raninus* that were roughly the same size and age were chosen, and they were carefully placed into beakers with distinct labels that had 30 mL of natural water and 15 mL of distilled water (which was taken from the organism's natural habitat). One millitre of the test samples (concentration 20 - 400 µg/mL) were added to make up the volume to 50 mL using 5% DMSO. The assay was carried out in triplicate using fifty millilitres of 5% DMSO in distilled water. After a 24-hour observation period, mortality was measured for cytotoxicity.[2,10]

Radical scavenging assay of SB1- 3

Using a technique outlined by Oluwasegun *et al*.,[11] the radical scavenging ability of the SB, bulked column fractions, and the isolated molecule was tested against the 2, 21-Diphenyl-1-picryl hydroxyl radical (Sigma-Aldrich).Two millilitres of 0.1 mM DPPH in methanol were mixed with 20–100 µg/mL of test samples and vitamin C individually and the absorbance was measured at 517 nm. Moreover, the control (5% DMSO) was subjected to similar treatment. The following formula was used to compute the radical scavenging activity:

% inhibition = {[Ab-Aa]/Ab} x 100

where Aa represents the test sample's absorption and Ab represents the blank sample's absorption.[11,12]

Purification of bioactive SB1

Further purification of bioactive SB1 using a combination of column and planar chromatography was carried out. This resulted in 24 fractions, which were pooled into two bulked fractions namely; SB1A and SB1B as shown in Table 3.

Biological activity of SB1A and SB1B

Benchtop cytotoxicity screening of the two bulked fractions resulted in the bioactive compound SB1B3, which was obtained from Prep. TLC of bioactive SB1B. SB1A was subjected to GC-MS analysis using the method described below.

GC-MS analysis of SB1A

An HP-5 MS fused Silica column (5% phenyl methyl siloxane, 30.0 m 250 M, film thickness 0.25 M) was used in the GC-MS analysis, which was performed in a combined 7890A gas chromatograph system (Agilent 19091-433HP, USA) and mass spectrophotometer interfaced with a 5675C Inert MSD with Triple-Axis detector. The column velocity flow of 1.0 mL/min was achieved by adjusting the helium gas, which was utilized as the carrier gas. Additional GC-MS parameters include: 250°C for the ion-source; 300 °C for the interface; 16.2 psi for pressure; 1.8 mm for out-time; and 1 µL injector in split mode with split ratio of 1:50 and injection temperature of 300°C. After five minutes at 36°C, the temperature in the column increased to 150 °V at a rate of 4 °C per minute. At a rate of 20°C per minute, the temperature was increased to 250°C and maintained there for five minutes. Elution took 47.5 minutes in total. By comparing the average peak area of each component to the total areas, the relative percent amount of each component was determined. The supplier's MS solution software was utilized to operate the system and collect the data.[13,14]

Compound identification

The National Institute of Standards and Technology (NSIT) database was used to analyze the mass spectra and identify the components based on their retention indices. There are around 62,000 patterns of recognized compounds in the database. The standard mass spectra of known components kept in the NIST collection (NISTII) were compared with the acquired spectra of the unknown components of SB1A.[13,14]

Statistical analysis

GraphPad Prism 7 was used to analyze all of the study's data. The relevant findings of the investigation were presented using appropriate tables, charts, and descriptive statistics. The mean ± SEM was used to express the data. The One-Way Analysis of Variance (ANOVA) test and the non-parametric Krustal-Wallis test were used in the statistical analysis of the data.

Results and Discussion

Extraction yield

The powdered sample of SB (1.26 kg) extracted with methanol (Anal. grade) using Soxhlet extraction yielded 69.45 g extract corresponding to 5.56%. However, a better yield of the plant material might be obtained if a more advanced extraction method like ultrasonic extraction, cold continuous extraction, etc were used. These improved methods are known to be faster, produce a high extractive yield, and have a minimal destructive tendency on the chemical integrity of the bioactive principles in the medicinal plant.[14]

Preliminary Phytochemical Screening

A preliminary analysis of the phytochemical composition showed that condensed tannins are lacking, but flavonoids, saponins, steroids, reducing sugars, glycosides, terpenoids, and phenolic compounds are present. A summary of the screening is shown in Table 1 Different classes of compounds have been reported by several authors to possess cytotoxic activity, among them are flavonoids, which are ubiquitous among plants and exhibit anticancer effects due to their unique structure and concentration. Other classes include: terpenoids, alkaloids saponins have been reported to also exhibit cytotoxic potential in both normal and cancer cells.[6] The phytochemical result obtained from this research was observed to confirm the previous findings of Mohammed *et al*.[15] which also explained that *C. siamea* leaves contain flavonoids, steroids, saponins, terpenoids, etc.

Result of fractionation of SB using column chromatography

Bulking of SB resulted in three bulked column fractions, namely; SB1 – 3 as shown in Table 2.

Result of cytotoxicity screening of SB1 – 3 using Saccharomyces cerevisiae cell culture model

The genomes of *Saccharomyces cerevisiae* and humans are substantially similar, these basic eukaryotes have been used to test the potential of various chemical and physical agents to cause cytotoxic, mutagenic, or genotoxic lesions. Moreover, yeast is a helpful tool for researching the mechanism of DNA repair. Reactive oxygen species have the ability to cause DNA damage.[9] ROS-induced DNA damage has been linked to various diseases and biological processes, including mutagenesis and carcinogenesis.

The primary mechanism of repair for nuclear and mitochondrial DNA is called base excision repair (BER), which has been referred to as a tool for fixing broken DNA bases. Furthermore, the lack of BER typically causes mitochondrial dysfunction, an increase in DNA double breaks, and stoppage of the cell cycle, all of which lead to cell death or the fixation of mutations that may help cause the onset of neurological or cancerous disorders.[9] Consequently, Figure 1 below displays the index of oxidative lesion produced in *S. cerevisiae's* genomic and mitochondrial DNA by the various bulked column fractions of SB (SB1–3).

Table 1: Preliminary phytochemical screening of *C. siamea* bark

Class of phytochemical	*C. siamea*
Phenolic compounds	+
Hydrolysable tannin	-
Condensed tannin	+
Saponins	+
Flavonoids	+
Steroids	+
Reducing sugars	+
Glycosides	+
Terpenoids	+
Alkaloids	-

Key –indicate absent; + indicate present

Table 2: Chromatographic bulking pattern of SB

S/N	Bulked sample	Bulking pattern
1.	SB1	1 – 36
2.	SB2	37 – 75
3.	SB3	76 – 150

SB1 was observed to induce severe DNA damage with a percentage mortality of 75.21±5.85% significantly different from 21.85±7.30% $p<0.05$ observed with 5% DMSO (NC; negative control). A weak activity was observed with bulked column fractions SB2 and SB3. In addition, nystatin (PC; positive control) also exhibited nuclear and mitochondrial DNA damage, which is expected from the compound, based on its established antifungal potential, with a percentage mortality of 91.50±7.98% $p<0.05$ relative to NC as shown in Figure 1. The findings of this research also support the works performed by other researchers, stating that *C. siamea* leaves exhibited a cytotoxic effect using the brine shrimp lethality bioassay method with an LD_{50} value of 68.633 µg/mL.[15,16] In addition, no literature has shown the cytotoxic effect of SB using S. cerevisiae. However, the cytotoxic evaluation using cancer cell lines has been reported by previous researchers.[16,17,18,19] Yang *et al*.[20] reported that Cassanthraquine A cytotoxic impact was seen against NB4, SH-SY5Y, PC3, A549, and MCF7 cancer cells with IC_{50} values of 6.2, 7.8, 3.5, 8.6, and 5.4 µmol/mL, respectively, in a 95% ethanol extract of *C. siamea* twigs. Yueh-Hsiung *et al*.[21] also showed that 5-(hydroxymethyl) -2-methyl-6-prenylisoindolin-1 -one, isolated from SB, had a cytotoxic effect against NB4, A549, SHSY5Y, PC3, and MCF7 cancer cell lines with IC_{50} values of 3.2, 4.6, 2.8, 6.4, and 2.5, respectively.

Result of cytotoxicity assay of SB1 – 3 using Raniceps raninus model

Researchers have employed a variety of methods to find plants that may have anti-tumor effects.[17] In situations when cancer cell lines are not easily accessible, alternative straightforward benchtop assays that anticipate the anticancer impact might be employed. These include cytotoxicity against specific zoological species, such as *R. raninus*, mosquito larvae, and Artemia salina.[2,10] Plant extracts have been shown to have the capacity to impede the growth of tumor-producing cells based on their ability to inflict cytotoxicity on these organisms.[10] However, the cytotoxic effect of SB1 – 3 at different concentrations using bench top model involving *R. raninus* model is shown in Figure 2 below.

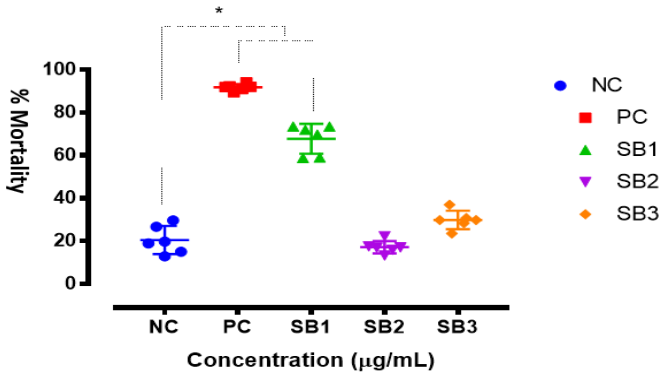

Figure 1: Cytotoxic effect of bulked SB1-3 using *Saccharomyces cerevisiae*

The values above are the mean of six replicates n = 3 X ± SEM. Values with superscript (*) indicate a significant difference at $p< 0.05$ when compared with negative control (distilled water) using one-way ANOVA (Dunnette). NC – negative control

The concentration-dependent increase in the mortality of *R. raninus*, which is an indication of its cytotoxic influence, was noted with SB1 at 10 – 50 µg/mL with 100.00±0.00% mortality observed at 40 and 50 µg/mL. Also, the cytotoxic activity is significantly different $p<0.05$ relative to 0.00±0.00% observed in NC (negative control; 3% DMSO). This activity confirms the potential cytotoxic and genotoxic effect of some of the constituents present in SB1 as shown in Figures 1 and 2.
As reported earlier, the cytotoxic potential of *C. siamea* using *R. ranninus* has not been verified in the literature. Other biological activity related to *C. siamea* has been reported by Adebayo *et al.* [23] which also state that the leaves of *C. siamea* exhibit antimicrobial, antioxidant, and antiproliferation potential in a significant manner. *C. siamea* has also been shown to possess cytotoxicity against human oral epidermal carcinoma (KB), breast adenocarcinoma (MCF-7), and small cell lung carcinoma (NCI-H187). Other species of Cassia have been reported to possess cytotoxic potential as described.20,21

Result of radical scavenging assay of SB1- 3 using in vitro model
Increased oxidative stress, which suggests an imbalance between reactive oxygen species (ROS) and antioxidants, is a hallmark of cancer. Targeting oxidative stress in cancer treatment may make sense because increased ROS accumulation brought on by metabolic disruptions and abnormalities in signalling might accelerate the development of cancer by triggering pro-oncogenic signalling and causing gene alterations.[21-23,26-29] The radical scavenging potential of SB1 – 3 using DPPH is shown in Figure 3.
SB1 was observed to exhibit radical scavenging potential against DPPH as shown in Figure 3. A concentration non-dependent increase in radical scavenging potential was associated with the most promising bulked fraction (SB1), with no significant difference between the observed activity and that which was observed with L-ascorbic (standard drug) $p<0.05$ as shown in Figure 3. Findings suggest that the constituents in SB1 (most active) might be responsible for the donation of electrons to scavenge the oxidative stress that was created due to the imbalance between ROS and endogenous antioxidants.[21,22,24] The constituents in SB1 ultimately repair mutations in the altered genes and ameliorate pro-oncogenic signaling.

The outcome of this research also supports the previous findings that *C. siamea* exhibited radical scavenging potential at different concentrations.[23,30] Gurpreat *et al.*[25] revealed that at a concentration of 250 µg/mL, the *C. siamea* extract demonstrated a significant reducing ability and a high quantity of polyphenols. Other data also suggest that *C. siamea* and other species of Cassia exhibit anticancer property.[25,30]

Result of purification of bioactive SB1
The bulking pattern of the bioactive bulked column fraction SB1 is shown in Table 3.

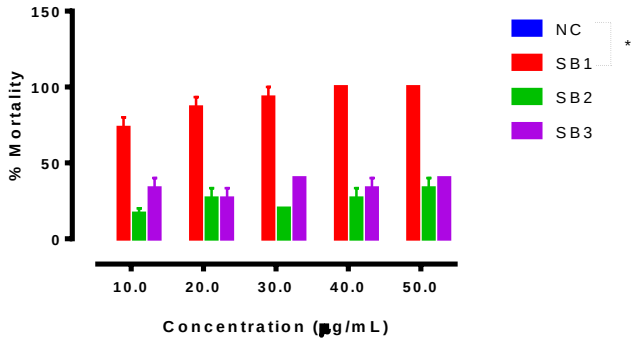

Figure 2: Cytotoxic effect of SB1 – 3 using bench top model involving *Raminus raninus* model
The values above are the mean of six replicates n = 3 X ± SEM. Values with superscript (*) indicate a significant difference at $p< 0.05$ when compared with negative control (distilled water) using one-way ANOVA (Dunnette)

Table 3: Bulking pattern of bioactive SB1

S/N	Bulked sample	Bulking pattern
1.	SB1A	1 – 17
2.	SB1B	18 – 24

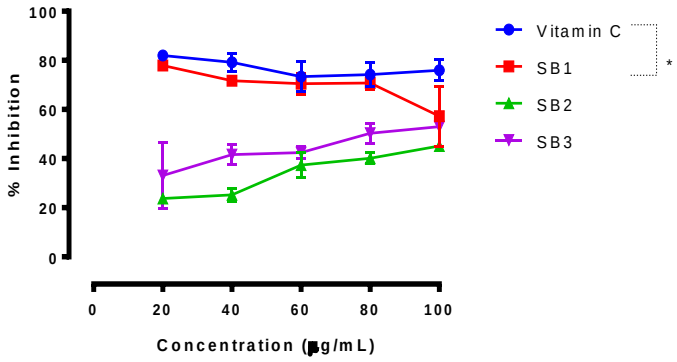

Figure 3: Radical scavenging potential of SB1 – 3 using DPPH radicals
The values above are the mean of six replicates n = 3 Mean ± SEM. Values with superscript (*) indicate a significant difference at $p< 0.05$ when compared with negative control (distilled water) using one-way ANOVA (Dunnette)

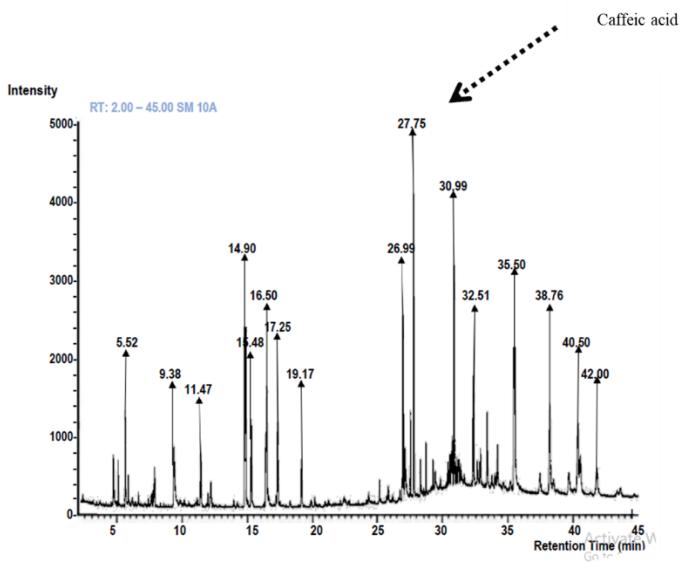

Figure 4: SB1A chromatogram

Result of GC-MS analysis of SB1A
SB1A was subjected to GC-MS analysis because of its small quantity (11.21 mg; Figures 4 and 5). The GC-MS analysis of sample SB1A indicated the presence of caffeic acid as the most prevalent compound in the bulked column fraction, with a retention factor of 27.75 and a fragmentation pattern shown in Figure 5. Caffeic acid is described as a common phenolic present in numerous plant species. Although the compound has not been reported in *C. siamea*, but literature search suggested the compound and its derivative possess antioxidant, anti-inflammatory, and anti-cancer properties.[31] Some research data showed that caffeic acid exhibited *in vitro* and *in vivo* cytotoxic effects on hepatocarcinoma cells, this cytotoxic activity might be linked to its pro-oxidant and antioxidant potentials due to the OH- group in the catechol group and the double bond in the carbonic chain.[31] The cytotoxic and antioxidant activities in C. siamea might be linked to caffeic acid.[31]

Result of further purification of SB1B
Preparative TLC analysis of SB1B resulted in SB1B1 (5 mg), SB1B3 (15.78 mg), and SB1B4 (4.67 mg). SB1B3 was further purified and the NMR analysis will be done. Based on this, the identity of the compound is yet to be ascertained.

Result of cytotoxicity screening of isolated compound SB1B3
The effect of the isolated compound SB1B3 using *S. cerevisiae* and *R. raninus* models are shown in Figures 6 and 7 respectively. The cytotoxic influence of compound SB1B3 was ascertained using the S. cerevisiae model. The compound exhibited about 69.82±8.82% mortality of the organism $p<0.05$ relative to negative control (NC). Based on this, the isolated compound (SB1B3) was subjected to another preliminary cytotoxicity screening using different concentrations.

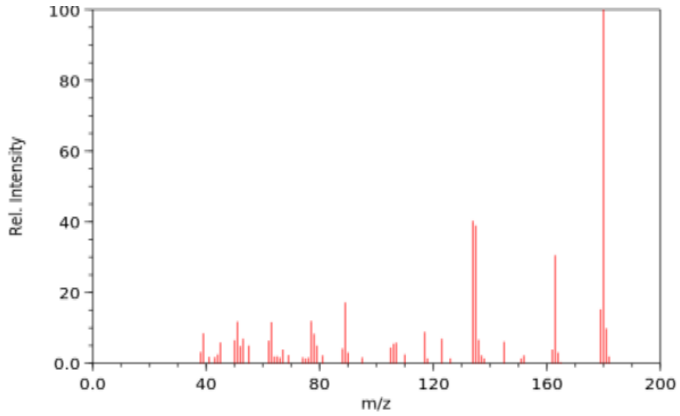

Figure 5: Mass spectrum of cafffeic acid

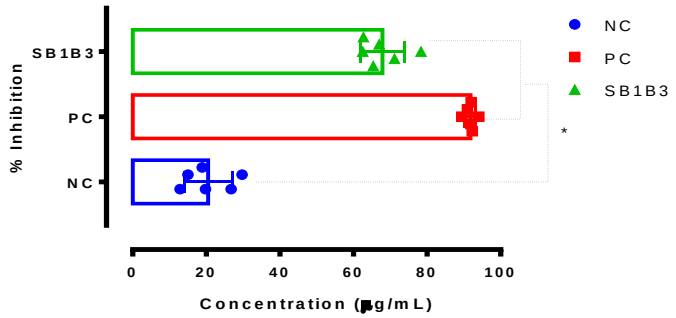

Figure 6: cytotoxic effect of compound SB1B3 using *S. cerevisiae* bench-top mode
The values above are the mean of six replicates n = 3 Mean ± SEM. Values with superscript (*) indicate a significant difference at $p< 0.05$ when compared with negative control (distilled water) using one-way ANOVA (Dunnette)

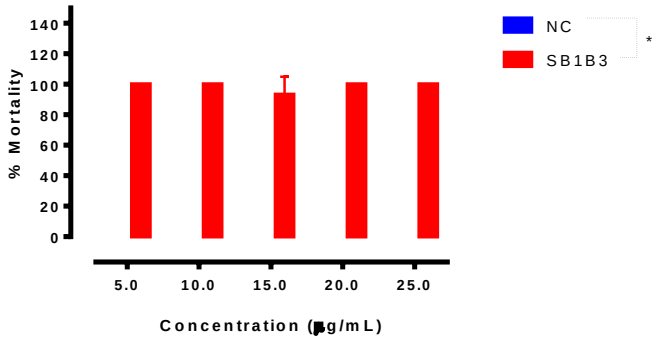

Figure 7: Cytotoxic effect of compound SB1B3 using *R. raninus* bench top model

The compound was noted to be exceedingly active at a lower concentration range of 5 – 25 µg/mL, with a 100.00±0.00% mortality of *R. raninus* at $p<0.05$ relative to 3% DMSO observed in NC. It is noteworthy to state that although the nomenclature of the isolated compound is yet to be ascertained, the compound is expected to be 12.5% active more than the most active bulked column fraction (SB1) by exhibiting 100% cytotoxicity at 5 µg/mL while similar activity was noted at 40 µg/mL of SB1

Conclusion

The cytotoxicity ethnomedicinal claim of *Cassia siamea* stem was investigated in this research using the combination of two cost-effective cytotoxicity screening models. The data generated confirmed the claim of usage of SB among locals for the treatment of cancer and related ailments. Also, the two cytotoxicity models used for the evaluation can be utilized in resource-limited environments or as complementary screening templates for the evaluation of medicinal plants with cytotoxic potential

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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