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Anticancer and Apoptotic Potentials of *Gliricidia sepium* Leaf Extract on Breast HCC1395, Prostate DU145 and Colorectal CT26 Cell Lines

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Authors' contributions

This work was carried out in collaboration among all authors. Author AOM designed the research work did the literature search and wrote the manuscript. Author IN supervised the whole work proofread and corrected the manuscript writing. Author DO supervised data analysis; he also reviewed and edited the manuscript. Author AMA monitored the conduct of the BSLA and plant extraction. Author RWM participated in the conduct of the In vitro cancer studies and proofread the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Plants have become a household name in the quest for effective and safe cancer chemotherapy in the Pharmaceutical industry. We studied the Brine shrimps lethality, antiproliferative and apoptotic potentials of *Gliricidia sepium* leaf extract against some selected cancer cell lines.

Methodology: Plant leaves extraction was done with 70% ethanol and petroleum ether. Twenty four hours old shrimp's larvae exposure to different concentrations (1 mg/ mL, 100 µg/ mL, 10 µg/ mL and 1 µg/ mL) of the extracts were used to evaluate the cytotoxicity in Brine shrimps lethality assay (BSLA). Three cancer cell lines: Breast (HCC1395); Prostate (DU145); Colorectal (CT26) and one normal cell line (Vero E6) were used for the in-vitro cytotoxicity testing using MTT assay. IC_{50}, CC_{50} and Selectivity Index (SI) were determined respectively, using standard methods. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out on HCC1395 and DU145 cell lines. Primers for the genes p53 and BAX were generated and amplified for apoptotic evaluation. The fold change relative to GADPH, the housekeeping gene, was calculated using double delta Ct analysis [$2^{-}\Delta\Delta CT$].

Results: The results of BSLA showed that both extracts of *G. sepium* are strongly toxic (LC_{50} < 100 µg/ml). However, petroleum ether extract (PGS) exhibited the highest toxicity to the shrimps, with LC_{50} of 11.95 µg/ml. This is about 8 times more cytotoxic than cyclophosphamide (LC_{50} of 98.76 µg/ml). On cancer cells, PGS exhibited varying antiproliferative activities; it was high on prostate (IC_{50} = 12.76 µg/ml), antiproliferative (IC_{50} = 23.55 µg/ml) and moderately antiproliferative (IC_{50} = 77.58 µg/ml) on breast and colorectal cancer respectively. PGS CC_{50} value was greater than 100 µg/ml. Ethanol extract (EGS) showed very high toxicity to all the tested cancer cells (IC_{50} <20µg/ml) with low cytotoxicity (CC_{50} = 41.81 µg/ml) to the normal cells. It exhibited a significant difference and high selectivity index across all the cell lines used in this study. EGS also upregulated both p53 and BAX biomarkers in the qRT-PCR apoptotic study.

Conclusion: The leaf extracts of *G. sepium* is a potential anticancer agent. The 70% ethanol extract selectively induced antiproliferative activities on cancer cells and upregulated apoptotic genes. The Petroleum ether extract also showed very low cytotoxicity to the normal cells. The plant should be considered a novel candidate for further studies.

Keywords: P53; cancer cell lines; Bax; Gliricidia sepium; brine Shrimps; apoptosis.

1. INTRODUCTION

The menace of cancer is overwhelming and it is consuming the world financial and human resources every day. Many of the world intellectuals have succumbed to the cold hands of death due to cancer, unfortunately, the tragedy still continues without a definite solution [1]. The number of deaths due to cancer increased from 6 million in the year 2000 to 7.6 million in 2007 [2]. In the year 2007, cancer alone was responsible for 13% of all deaths around the world [3]. In 2008, 1 in 4 deaths in the United States was said to be due to cancer [4]. In the year 2012, the worldwide burden of cancer involved 14 million new cases [5]. Projections for the year 2030, based on the demographic growth and the ageing process of populations, indicate the occurrence of 26 million new cases and 17 million deaths by cancer, most of which will occur in the least developed regions of the planet [6]. Presently, there is no confirmed cure for all types of cancer and thus, the efforts to get safe and effective remedies are commendable everywhere on the planet earth, especially, cures from natural plant sources.

In many underdeveloped and developing countries, most of the population does not have access to a well-organized and well-coordinated cancer management system. A diagnosis of cancer is like a death sentence, often leads to catastrophic personal health expenditures [7] and may threaten social stability [8,9]. Cancer has become one of the most prominent diseases in humans with a considerable scientific and commercial interest in the discovery and development of new anticancer agents from natural product sources [10].

Brine shrimp (*Artemia salina*) lethality assay (BSLA) is a test commonly used to check the

cytotoxic effect of bioactive materials. The mechanism of action of the assay is based on the killing capability of test compounds on Brine shrimps [11]. This assay is a preliminary toxicity screening of plant extracts [12-15], nanostructures [16], cyanobacteria toxins [17], cytotoxicity testing of dental material [18], screening of heavy metals [19], fungal toxins [20], and pesticides [21].

In cancer research, tumor-derived cell lines are the most frequently utilized in-vitro tumor models [22]. Over the past decades, their use has helped immensely in the understanding of cancer biology in research [22]. They have been adjudged to be an indispensable tool in the genetic approach, and their characterization reveals that they are an excellent model for the study of the biological mechanisms in cancer processes [22]. Extract of *G. sepium* was subjected to cancer cell lines study to ascertain the presence or otherwise, of the antiproliferative property of the plant leaf extract.

To measure the effectiveness and grand function of any therapeutic agents, the mechanisms of action must be known and clearly documented. When cells lost their functional/genetic integrity, they become a threat to the body by growing uncontrollably. P53 tumor suppressor thereby functions to prevent the propagation of such cells [23]. Most of the genetically programmed cancer in animal models were developed majorly based on a p53-deficient background. Defects in the germline of the TP53 gene can exposed organisms to the early development of cancer [24]. In-vivo investigation of p53-dependent tumor suppression showed apoptosis as its main effector mechanism [25].

Apoptosis is a programmed cell death used by the body system to eradicate cells that can cause pathological or physiological unrest to the organism. Studies have revealed that apoptosis may be involved in cell death due to chemotherapy and researchers have identified the act of suppression of apoptosis by the tumor-promoting agents in pre-neoplastic cells as a major role player in tumor promotion. So, the ability to induce apoptosis has become a major consideration factor in assessing the efficiency of chemopreventive agents [26]. Bax, a pro-apoptotic gene, is a p53 target, as a member of the Bcl-2 family, it has shown to be up-regulated in series of systems during p53-mediated apoptosis [26]. In this study, we have selected p53 and its pro-apoptotic oncogene, Bax, to determine the mechanisms of action of this extract.

Gliricidia sepium is a medium-sized leguminous tree, known as Agunmoniye among the Yoruba-speaking people of Nigeria. It belongs to the family Fabaceae with Central America as the origin. It has, however, spread to many other continents, including Africa. The uses of this plant have been reported in many countries for different purposes [27]. The aqueous extract of the leaf of this plant was reported to have anti-sickling effects on red blood cells [28]. Extracts of the leaf, flower and bark of *Gliricidia sepium* have been used in the treatment of patients with pathogenic bacterial infections [29], skin diseases [30] and nematodes [31]. Its antioxidant and antitumor activities have also been documented [32,33] and it also has free radical scavenging potentials [34].

However, we are not aware of any cancer screening on this plant extract in the whole of Sub-Saharan Africa, though, people are using it every day for unorthodox medication.

2. MATERIALS AND METHODS

2.1 Collection and Extraction of the Plant Leaves

G. sepium leaves were collected, identified and authenticated with Voucher number UILH/002/2019/752 and were deposited at the herbarium. Air-dried leaves were blended into powder and 100g each was soaked in 1 liter of 70% ethanol and PE respectively at room temperature for 48 hours, on a pre-timed shaker. Extracts were filtered through filter paper and concentrated on a rotary evaporator (Bibby RE100).

2.2 Brine Shrimps Lethality Assay

This assay was carried out following the procedure of Quazi et al., [35]. Brine shrimp (*Artemia salina*) eggs (Bay Brand, USA) were hatched in natural seawater and incubated for 48 h in 3.8 g/L seawater. Ten mg of dried ethanol extract was dissolved in 1ml of distilled water to prepare a stock solution in a clean test tube, PE extract was first dissolved in 1ml of DMSO [11]. Concentrations of 1 mg/ mL, 100 µg/ mL, 10 µg/ mL and 1 µg/ mL were prepared by serial dilution from the stock solution. One ml of each

concentrated solution was dispensed into five test tubes which contains 10 nauplii in 1ml of seawater. The negative control group contains only 10 nauplii each in 1ml of seawater and DMSO respectively, while cyclophosphamide was used as the positive control. The number of dead nauplii was counted after 24 hours of incubation at room temperature (25°C). Mortality endpoint was taken as the absence of controlled forward motion during 30 sec of observation. Percentage death was calculated using the formula below:

% Death = {Number of dead nauplii / (Number of dead nauplii + Number of live nauplii)} x100

2.3 Antiproliferative Assay

2.3.1 Preparation and culturing of HCC1395, DU145, CT26 and Vero E6 cells

The three cancer cell lines; HCC1395, DU145, CT26 and the normal cell line (Vero E6) used for this study were obtained from ATCC and stored in liquid nitrogen before the commencement of the study. The Minimum Essential Medium (MEM) used was supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% antibiotic (Penicillin/Streptomycin).

Five ml of the growth medium pipetted into a 15 ml centrifuge tube and the thawed cell vial content was carefully transferred into the medium using a 1 ml pipette. The cells were re-suspended by up and down pipetting and the mixture was centrifuged at 3000rpm for 5 minutes. The supernatant was carefully decanted leaving only the cells as the sediment. Twelve ml of the growth medium was added to the sediment and re-suspended by gentle up and down pipetting. Initial cell density count was done with 10 µl of the cell suspension using a hemocytometer and the remaining was plated at 40,000 viable cells/10 ml in a T-75 cm² flask. The flask was then incubated at 37 ˚C, 5% CO_2 for 5 days when the confluence was achieved.

2.3.2 Cytotoxicity testing

When the cell growth has attained confluence, cells were washed with saline phosphate buffer and harvested by trypsinization [36]. The cell density counting was done by Trypan blue exclusion method (TBEM) [37]. An aliquot of 100µl containing 2.0 $\times 10^4$ cells/ml suspension was seeded into a 96-well plate and incubated at 37^{o}C and 5% CO_2 for 24hrs.

After 24hrs, the media were carefully changed by pipetting out and replaced with fresh ones. One hundred (100) µl of sample extracts serially diluted in 3 folds to seven different concentrations (100 to 0.137 µg/ml) were added on Row H-B of the plate. Only cells and the medium were left in Row A which served as the negative control. Ten (10) µg/ml of doxorubicin serially diluted (10 to 0.1563) was used as the positive control. The experiment was done in triplicate and the cells were incubated for 48hrs. Ten µl of [3 (4, 5 Dimethyl-2-thiazolyl)-2, 5 diphenyl-2H-tetrazolium bromide)] (MTT dye) was added and the plate was further incubated for 2 hours at 37˚C in a 5% CO_2 saturated incubator.

The addition of MTT dye was to measure cell metabolic activity. The viability was measured in terms of reductive activity, as an enzymatic conversion of the tetrazolium compound into water-insoluble formazan crystals, by dehydrogenases of the mitochondria of living cells [38,39].

The amount of formazan formed is directly proportional to the number of live cells [38] and was confirmed using an inverted light microscope before solubilized with 50µl of 100% DMSO. ELISA multiplex reader (Applied Biosystem) was used to read the optical density (OD) at 540nm and a reference wavelength of 720nm.

The effect of the test extracts on cancer and normal cells was expressed in IC_{50} values (the extract concentration which kills 50% of the cancer cells) and CC_{50} values (concentration of extract that exerted cytotoxic effects to 50% of the normal cells) respectively [40,41]. Selectivity Index (SI) which determines the ability of the extract to exert selective toxicity to cancer cells while sparing the normal cells was calculated using:

$SI = CC_{50} / IC_{50}$

Where;

CC_{50} = Concentration of extract that exerted cytotoxic effect to 50% of the normal cells.
IC_{50} = Concentration of extract that inhibited the growth of cancer cells by 50%

2.4 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

2.4.1 RNA extraction from HCC1395 and DU145 cells

The confluence HCC1395 and DU145 were used for the qRT-PCR and RNA extraction was done using a commercial extraction kit (BioFactories, 5 min DNA) as instructed by the manufacturer. Cells were treated with the extract concentration equivalent to their IC_{50}, a negative control was exposed to fresh media only and the set was incubated for 48 hours. Cells were disrupted by carefully scraping with the aid of a pipette. The homogenate was then collected into a tube and kept at room temperature for 5 minutes.

Media was aspirated and 700 µl of cell RNA solution was added with gentle mixing for 30 seconds. The content was vortexed for 15 minutes at 10,000rpm after being transferred to a microtube at room temperature. The lysate was transferred to the column and centrifuged for 15 seconds, flowthrough was discarded and 700 µl of RNA washing solution was added, centrifuged for 15 sec and the flowthrough discarded. 400 µl of RNA washing solution added, centrifuged for 30 sec and flowthrough again discarded. The collection tube was replaced with a clean microcentrifuge tube and 100 µl of RNA elution buffer was added, closed and vortexed for 15 seconds. It was further centrifuged for 15 seconds and the flowthrough was reloaded and centrifuge for 30 seconds. The concentration of the extracted RNA was determined using a Nanodrop spectrophotometer (Thermo Fischer).

2.4.2 cDNA synthesis (Reverse transcription)

The cDNA synthesis was carried out from the prepared 50 ng/µl concentration of each RNA sample. According to the manufacturer's instruction; FIREScript RT Complete Oligo(dT) cDNA synthesis kit (Solis Biodyne, Tartu, Estonia) was used to reverse-transcribe 10 µl of the sample. The reaction mixture was incubated at 45˚C for 30 minutes and the RT enzyme was inhibited by heating at 85˚C for 5 minutes. The synthesized cDNA was kept at -20˚C.

2.4.3 PCR Amplification

The following primers for the genes p53 and Bax were generated using the primer designing tool of NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast).

p53

Forward 5'-CTTCCTGCAGTCTGGGACAGC C-3';
Reverse 5'-GCAGCTGGGCCTACAGCACACG -3'

BAX

Forward 5'-CAGGATGCGTCCACCAAGAA-3';
Reverse 5'-AGTCCGTGTCCACGTCAGCA-3'

HOT FIREPol® EvaGreen® (no ROX) qPCR mix kit (Solis BioDyne, Estonia) was used for the quantitative polymerase chain reaction and was performed in a LightCycler® 480 SYBR Green I Master instrument (Roche Applied Science, USA). Five microlitres of the cDNA solution was PCR-amplified with the forward and reverse primers of each of the genes. The total reaction volume for the amplification was 20 µl (PCR mix, gene-specific primers (10 pmol for each) and 5 µl of cDNA). For target genes expression normalization, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The three-step PCR cycling conditions were used: initial cycle for polymerase activation at 95˚C for 10 minutes. Forty cycles of denaturation at 95˚C for 15 seconds, annealing at 60˚C for 60 seconds and a third extension step at 72˚C for 20 seconds. The fold change relative to GADPH, the housekeeping gene, was calculated using double delta Ct analysis [$2^{-}\Delta\Delta CT$].

2.5 Data Analysis

Data obtained were analyzed using a linear regression model to get the LC_{50} and IC_{50} of the extracts and the values were represented as Mean ± Standard Error of Mean (SEM). Student T-test at 95% confidence interval was used to evaluate the significance of the difference between the mean values of the measured parameters in the respective test and control groups. A mean difference was considered significant at $p < 0.05$.

3. RESULTS

3.1 Effect of *G. sepium* Extracts in Brine Shrimps Lethality Assay

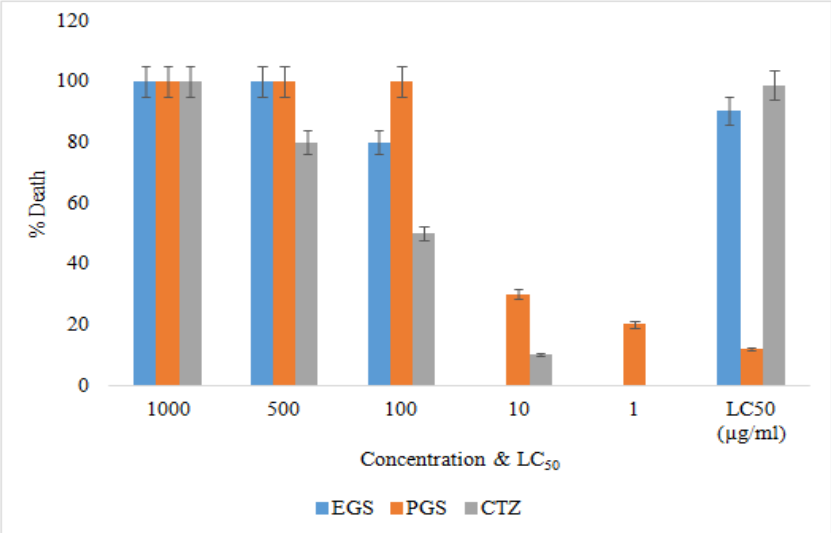

Fig. 1. Result of brine shrimps lethality assay for *G. sepium* ethanol leaf extract

The percentage of death and the LC_{50} values calculated were put together on the same chart. EGS= Ethanol extract of Gliricidia sepium, PGS = Petroleum ether extract of Gliricidia sepium, CTZ= Cyclophosphamide

3.2 *In-vitro* Antiproliferative Study

Table 1. The IC_{50} value (µg/ml) for HCC1395, DU145, CT26 and CC_{50} value for VERO E6 Cell Lines after Exposure to *G. sepium* extracts for 48 hours

Cell type	Breast Cancer	Prostate Cancer	Colon Cancer	Normal cell
Cell line	HCC 1395	DU 145	CT 26	VERO E6
PGS	23.55±0.02[a]	12.76±0.17[b]	77.58±0.22	ND
EGS	3.55±0.01[a]	5.81±0.03[b]	3.41±0.00[c]	41.81±0.06[d]
DOX	0.54±0.03[a]	0.24±0.03[b]	2.95±0.04[c]	0.98±0.01[d]

Values are expressed as Mean±SEM, Significantly different values carry the same superscript letter with the control, PGS = Petroleum ether extract of G. sepium, EGS= Ethanol extract of G. sepium, DOX= Doxorubicin ND=Not Determined

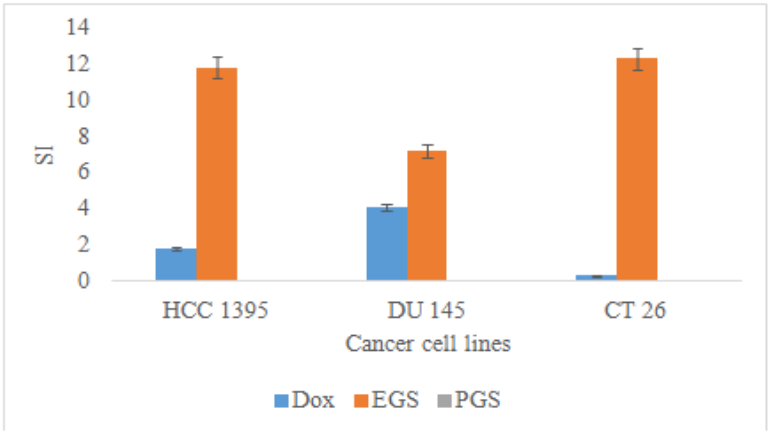

Fig. 2. Selectivity Index (SI) of EGS and PGS

3.3 Apoptotic Study

Table 2. The Fold change calculation for P53 and BAX genes

Gene	Cancer Cell	TGCCt	HGCCt	ΔCCt	TGTCt	HGTCt	ΔTCt	ΔΔct	2-(ΔΔct)	2-(ΔΔct)/2	SEM
P53	DU145	38.741	34.168	4.573	32.874	34.740	-1.866	6.439	86.80	61.26	25.54
					33.696	34.281	-0.585	5.158	35.72		
	HCC 1395	38.741	34.168	4.573	35.349	35.829	-0.48	-5.053	33.18	22.56	10.625
					35.349	34.353	0.996	-3.577	11.93		
BAX	DU145	31.591	34.168	-2.577	31.765	34.740	-2.975	-0.398	1.32	1.17	0.15
					31.672	34.281	-2.609	-0.032	1.02		
	HCC 1395	31.591	34.168	-2.577	32.999	35.829	-2.83	-0.253	1.19	1.05	0.135
					31.899	34.353	-2.454	-0.124	0.92		

Where: ΔCCt = TGCCt – HGCCt, ΔTCt = TGTCt – HGTCt, TGCCt = ct value of gene of interest for control (untreated cells), HGCCt = ct value of housekeeping gene (GADPH) for untreated cells, TGTCt = ct value of gene of interest for treated cells, HGTCt = ct value of housekeeping gene (GADPH) for treated cells. Double delta ct value (ΔΔct) = ΔTCt - ΔCCt. Fold change = $2^{-(\Delta\Delta ct)}$

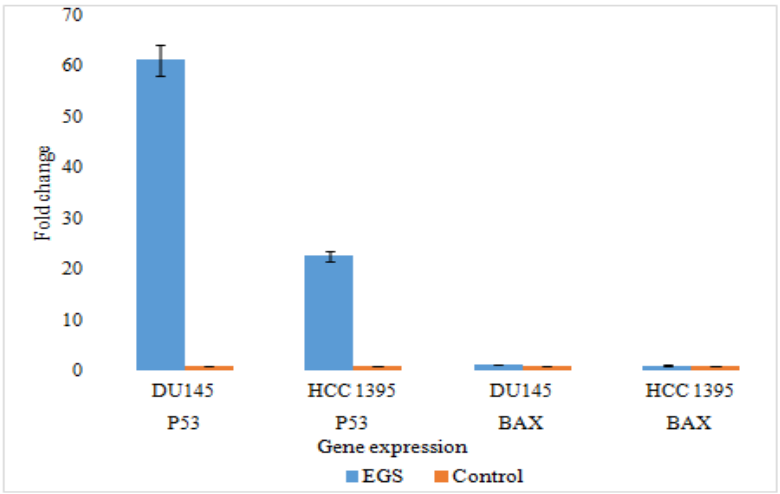

Fig. 3. Apoptotic potential of EGS on P53 and BAX biomarkers

4. DISCUSSION

Plants have been the best companion of Human beings on the planet earth. Apart from the continuous clearing of the planet's CO_2, it has served as medication for various illnesses since time immemorial, and it's still serving some protective functions as our green neighbors. Many generations in the past have depended solely on plant materials to solve their medical problems [42]. Plants have been used for treating various diseases throughout the ages and they continue to be a critical source of potent anticancer agents due to their contents [43]. Some of the plant-derived compounds such as vincristine, vinblastine and paclitaxel are still in use as chemotherapeutic agents in clinical cancer treatment [44]. Most of the available cancer therapy has serious side effects [45] it is, therefore, important to develop new-selective anticancer agents to combat the disease. Anticancer potentials of *G. sepium* have been invested for this same purpose.

4.1 Brine Shrimps Lethality Assay

The lethality of the extracts on the brine shrimps was classified according to the method of Padmaja et al., [46] in the BSLA of this study, where LC_{50} value ≥ 1000 µg/mL was considered to be non-toxic, LC_{50} of 500 to 1000 µg/mL as weakly toxic, LC_{50} of 100 to 500 µg/mL as moderately toxic and LC_{50} ≤ 100 µg/mL as strongly toxic.

Fig. 1 showed the result of BSLA. Both ethanol and PE extracts are strongly toxic with LC_{50} ≤ 100 µg/ml. Based on the grading, they might be

having anti-cancer properties. PE extract (LC_{50} of 11.95 µg/ml) demonstrated the highest toxicity to the shrimps and it's about eight (8) times more cytotoxic than the cyclophosphamide (LC_{50} of 98.76 µg/mL) which is the control and a potent anticancer drug. The negative controls have zero percentage death.

4.2 *In-vitro* Antiproliferative Study

MTT assay results of growth inhibitory rate were used to categorize the antiproliferative activities of the extracts based on their Median Inhibitory Concentration (IC_{50}). Using the criteria for *in vitro* cytotoxicity after the exposure time of 48 hours as established by the U.S. National Cancer Institute (NCI); an IC_{50} < 20µg/ml for crude extracts is considered to be highly antiproliferative. An IC_{50} value of between 20-29µg /ml is said to be antiproliferative, between 30µg/ml and 100µg /ml is termed moderately antiproliferative while above 100µg/ml is considered inactive [47].

The result from this research was comparatively compared with a popular anticancer drug, Doxorubicin, as there is a dearth of literature as regards *in vitro* anticancer potential of this plant extract. The result of the IC_{50} for HCC 1395, DU 145, CT 26 and CC_{50} for Vero cells is represented in Table 1. PGS showed varying antiproliferative activities; it was high on prostate cancer, antiproliferative and moderately antiproliferative on breast and colorectal cancer respectively. Its concentration that exerted cytotoxic effect on 50% of the normal cells was greater than 100 µg/ml. All the cancer cell lines showed a significantly different increase in the IC_{50} values when compared with the control drug. The EGS result also showed a very high antiproliferative capability (IC_{50} <20µg/ml), a highly significant increase in the IC_{50} values across the groups ($p < 0.05$) and high selectivity indices that were constant across all the tested cell lines. With a CC_{50} of 41.81µg/ml, EGS could be far more selective than doxorubicin in it crude form. This indicates that the extracts are not only capable of inhibiting cancer growth, but can also spare the normal cells in their cytotoxicity activity.

Chemotherapy is one of the most reliable methods of treating cancers. While it is desired that low doses of drugs should eliminate the cancer cells, the inability of those drugs to differentiate between the normal and cancerous cells posed a far bigger concern [48]. These side effects make it difficult to rely only on the anticancer activity of drugs, but also their ability to select their target amongst the conglomerates of body cells. High selectivity with low activity is therefore preferred to the otherwise, in cancer drug development and this depicts high therapeutic value [48]. Throughout the experimental studies, EGS displayed selective activities and has specifically proved to be a potential anticancer drug with a high therapeutic index (high activity and high selectivity).

4.3 Apoptotic Study

Apoptosis, also referred to as self-induced cell death, plays a key role in cancer cell death and growth regulation [49]. In normal cells, the apoptosis machinery works perfectly by helping the system to get rid of malfunctioning and aged cells, which might have impaired cellular functions [49,50]. However, in cancer, which are abnormal cells that grow uncontrollably at a rapid rate and multiply into multifold, their apoptosis machinery is impaired. Therefore, an agent capable of activating or inducing apoptosis in cancer cells is a potential cancer cell death inducer and anticancer chemotherapeutic agent [51]. The earlier stated results of in-vitro antiproliferative studies revealed that EGS exhibited strong cytotoxic effects against HCC 1395 and DU 145 cancer cells. Hence, the effects of EGS extract on apoptosis of the cancer cells were determined by observing the regulation of the apoptotic biomarkers, Bax and p53 before and after treating the cancer cells with the extract using PCR analysis. The results revealed that EGS slightly upregulated pro-apoptotic Bax in the cancer cells, which depicts that the extract induced apoptosis in the cancer cells via intrinsic mitochondrial-dependent apoptotic pathway [52,53]. Furthermore, EGS also upregulated pro-apoptotic protein p53 in the two cancer cells with a high fold change (Fig. 3), which indicated that the extract activated p53 mediated cell death pathway [54,55]. The activation of the p53 mediated pathway resulted in alteration of cell cycle progression and subsequent arrest of cell growth mechanism [52,56].

5. CONCLUSION

The leaf extracts of *G. sepium* possess an anticancer agent based on the significant activity observed in this study. Both ethanol and petroleum ether extracts displayed possession of antiproliferative potentials. The plant has been penned down for further studies that will include

bio-guided isolation of anticancer compound, purification and characterization. The mechanisms of action discovered for EGS is a guide to subsequent studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Scott HR, McMillan DC, Brown DJF, Forrest LM, McArdle CS, Milroy RA. Prospective study of the impact of weight loss and the systemic inflammatory response on quality of life in patients with inoperable non-small cell lung cancer. Lung Cancer 2003;40:295-299.
   DOI: 10.1016/s0169-5002(03)00077-1.
2. World Health Organization. Global burden of cancer among young adults aged 20–39 years. International agency for research on cancer. Lyon France; 2017.
   Available: https://www.iarc.who.int/news-events/global-burden-of-cancer-among-young-adults-aged-20-39-years/
3. Yılmaz HH, Yazıhan N, Tunca D, Sevinç A, Olcayto EÖ, Ozgül N. et al. Cancer trends and incidence and mortality patterns in Turkey. Jpn. J. Clin. Oncol. 2011;41:10-16.
   DOI: 10.1093/jjco/hyq075.
4. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J. Clin. 2011;61(2) :69-90.
   DOI: 10.3322/caac.20107.
5. Stewart BW, Freddie B, David F, Hiroko O, Kurt S, Andreas U, Wild CP. Cancer prevention as part of precision medicine: plenty to be done. Carcinogenesis. 2016;37:2-9.
   DOI:10.1093/carcin/bgv166.
6. Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: priorities for prevention. Carcinogenesis. 2010;31:100-110.
   DOI: 10.1093/carcin/bgp263.
7. Engelgau MM, Karan A, Mahal A. The economic impact of non-communicable diseases on households in India. Global Health. 2012;25:8-9.
   DOI: 10.1186/1744-8603-8-9.
8. Ramsey S, Blough D, Kirchhoff A, Kreizenbeck K, Fedorenko C, Snell K. et al. Washington state cancer patients found to be at greater risk for bankruptcy than people without a cancer diagnosis. Health Affairs (Millwood). 2013;32:1143-1152.
9. Pramesh CS, Badwe RA, Borthakur BB, Chandra M, Raj EH, Kannan T. et al. Delivery of affordable and equitable cancer care in India. Lancet Oncol. 2014;15:223-233.
10. Kinghorn AD, Farnsworth NR, Soejarto DD, Cordell GA, Swanson SM, Pezzuto JM. et al. Novel strategies for the discovery of plant-derived anticancer agents. Pharm. Biol. 2003;41:53-67.
    DOI: 10.1080/1388020039051744.
11. Chao W. An important player in brine shrimp lethality bioassay: The solvent. J Adv Pharm Technol Res 2014;5:57-58.
    DOI: 10.1016/S1470-2045(14)70117-2.
12. Syahmi ARM, Vijayaratna S, Sasidharan S, Latha LY, Kwan YP, Lau YL. et al. Acute oral toxicity and brine shrimp lethality of Elaeis guinennsis Jacq. (oil palm leaf) methanol extract. Molecules. 2010;15:8111-8121.
13. Ghosh A, Banik S, Islam M. In vitro thrombolytic, anthelmintic, anti-oxidant and cytotoxic activity with phytochemical screening of methanolic extract of Xanthium indicum leaves. Bangladesh J. Pharmacol. 2015;10:854-859.
    DOI: 10.3329/bjp.v10i4.23829.
14. Sufian M, Haque M. Cytotoxic, thrombolytic, membrane stabilizing and anti-oxidant activities of Hygrophila schulli. Bangladesh J. Pharmacol. 2015;10:692-696.

15. Kibiti C, Afolayan A. Antifungal activity and brine shrimp toxicity assessment of Bulbine abyssinica used in the folk medicine in the Eastern Cape Province, South Africa. Bangladesh J. Pharmacol. 2016;11:469-477.
DOI: 10.3329/bjp.v11i2.24405.
16. Maurer-Jones MA, Love SA, Meierhofer S, Marquis BJ, Liu Z, Haynes CL. Toxicity of nanoparticles to brine shrimp: An introduction to nanotoxicity and interdisciplinary science. J. Chem. Educ. 2013;90:475-478.
DOI.org/10.1021/ed3005424.
17. Hisem D, Hrouzek P, Tomek P, Tomšíčková J, Zapomělová E, Skácelová, K, Lukešová A, Kopecký J. Cyanobacterial cytotoxicity versus toxicity to brine shrimp Artemia salina. Toxinol. 2011;57:76-83.
DOI: 10.1016/j.toxicon.2010.10.002.
18. Pelka M, Danzl C, Distler W, Petschelt AA. New screening test for toxicity testing of dental materials. J. Dent. 2000;28:341-345.
DOI: 10.1016/s0300-5712(00)00007-5.
19. Saliba LJ, Krzyz RM. Effect of heavy metals on hatching of brine shrimp eggs. Marine Pollution Bulletin. 1976;7:181-182.
doi: 10.1016/0025-326X(76)90036-9.
20. Harwing J, Scott PM. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl. Microbiol. 1971;21:1011-1016.
DOI: 10.1128/am.21.6.1011-1016.1971.
21. Michael AS, Thompson CG, Abramoritz M. Artemia salina as a test organism for bioassay. Science. 1956;123:464.
DOI: 10.1126/science.123.3194.464.
22. Dhanalakshmi G, Sangeetha K, Lokesh U, Aleemasahada DA. Review on Cancer Cell Line Studies. Int. J. Pharm. Sci. Rev. Res. 2016;41:220-224.
Available:www.globalresearchonline.net.
23. Chipuk JE, Maurer U, Green DR, Schuler M. Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer cell. 2003;4:371-381.
DOI: 10.1016/s1535-6108(03)00272-1.
24. Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: new online mutation analysis and recommendations to users. Hum. Mutat. 2003;19: 607-614.
DOI: 10.1002/humu.10081
25. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions in vivo. Cancer Cell. 2002;1:289-298.
DOI: 10.1016/s1535-6108(02)00047-8.
26. Choudhuri T, Pal S, Agwarwal ML, Das T, Sa G. Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. FEBS Letters. 2002;512:334-340.
DOI: 10.1016/s0014-5793(02)02292-5.
27. Beckstrom-sternberg SM, James AD, Wain KK. The Ethnobotany Database. http://probe.nalusda.gov.8300kg:-bin//browse/ethnobotdb. ACEDB version 4.3-data version.1994.
28. Oduola T, Dallatu MK, Muhammed AO, Ndakotsu MA, Adebisi IM, Hassan SW. Gliricidia sepium aqueous leaf extract possesses antisickling property. IBRR. 2016;5:1-6.
DOI: 10.9734/ibrr/2016/25181.
29. Reddy LJ, Jose B. Evaluation of antibacterial activity of the bark, flower, and leaf extracts of Gliricidia sepium from South India. Int. J. Curr. Pharm. Res. 2010;2:177-179.
Available:https://innovareacademics.in/journal/ijap/Vol2Issue2/5.pdf.
30. Rojas JJ, Ochoa VJ, Ocampo SA. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric Medicine: a possible alternative in the treatment of non-nosocomial infections. BMC Complem. Altern. Med.2006;6:1-6.
DOI: 10.1186/1472-6882-6-2.
31. Akharaiyi FC, Boboye B, Adetuyi FC. Antibacterial, Phytochemical and Antioxidant activities of the leaf extracts of Gliricidia sepium and Spathodea campanulata. World Appl. Sci. J. 2012;16:523-530.
Available:https://www.scinapse.io/papers/2185980879.
32. Nazil R, Sohail T, Nawab B, Yaqeen Z. Insecticidal, nematicidal and antibacterial activities of Gliricidia sepium. Pak. J. Botany. 2008;40:2625-2629.
Available: https://agris.fao.org/agris-search/search.do?recordID=PK2011000498.
33. Rasterelli L, Caceres A, De Simone F, Aquino R. Studies on the constituents of Gliricidia sepium leaves and roots: isolation and structure elucidation of new triterpenoid, saponins and aromatic compounds. J. Agric. Food Chem. 1999;47:1537-1540.
DOI: 10.1021/jf9808731

34. Sinha SN. Phytochemical profiles and antioxidant activities of the leaf extracts of Gliricidia sepium. Intl. J. Innov. Bio-Sci. 2013;3:87-91.
Available: http://www.parees.co.in/ijibs.htm
35. Quazi SS, Chowdhury FA, Misbahuddin M. Brine shrimp lethality assay. Bangladesh J. Pharmacol. 2017;12:186-189.
Available: https://www.banglajol.info/.../22168.
36. Chintalapani S, Swathi MS, Narasu ML. Antiproliferative and apoptosis-inducing potential of whole plant extracts of Sesuvium portulacastrum against different cancer cell lines. J. Appl. Pharm. Sci. 2019;9:38-43.
DOI: 10.7324/JAPS.2019.90205.
37. Strober W. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 2015;111.
DOI: 10.1002/0471142735.ima03bs111.
38. Daniel Z, Howard DT, Michael B. New Anticancer Agents: In vitro and in vivo Evaluation. In-vivo. 2005;19:1-8.
Available:https://pubmed.ncbi.nlm.nih.gov/15796152.
39. van Tonder A, Joubert AM, Cromarty AD. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Res. Notes. 2015;20:47-49.
DOI: 10.1186/s13104-015-1000-8.
40. Nemati F, Dehpouri AA, Eslami B, Mahdavi V, Mirzanejad S. Cytotoxic Properties of Some Medicinal Plant Extracts from Mazandaran, Iran. Iranian Red Crescent Med. J. 2013;15:e8871.
41. Offermanns S, Rosenthal W. IC_{50} Values. Encyclopedia of Molecular Pharmacology. Berlin Heidelberg. Springer; 2008.
DOI: 10.1007/978-3-540-38918-7_5943.
42. Yusuf AS, Ibrahim S, Yusuf H, Ibrahim LK. Phytochemical Screening and Antibacterial Activity of Acalypha wilkesiana and Maerua angolensis. J. Pharm. Chem. Biol. Sci. 2017;5:103-107.
Available:https://www.jpcbs.info/2017_5_2_04_Amina.pdf.
43. Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. Biochem. Biophys. Acta. 2013;1830:3670-3695.
DOI: 10.1016/j.bbagen.2013.02.008.
44. Kintzios SE. Terrestrial Plant-Derived Anticancer Agents and Plant Species Used in Anticancer Research. CRC Crit. Rev. Plant Sci. 2006;25:79-113.
DOI: 10.1080/07352680500348824.
45. Unnati S, Ripal S, Sanjeev A, Niyati A. Novel anticancer agents from plant sources. Chin. J. Nat. Med. 2013;11:16-23.
DOI: 10.1016/S1875-5364(13)60002-3.
46. Padmaja R, Arun PC, Prashanth D, Deepak M, Amit A, Anjana M. Brine shrimp lethality bioassay of selected Indian medicinal plants. Fitoterapia. 2002;73:508-510.
DOI: 10.1016/S0367-326X(02)00182-X
47. Fadeyi SA, Fadeyi OO, Adejumo AA, Okoro C, Myles EL. In vitro anticancer screening of 24 locally used Nigerian medicinal plants. BMC Complement Altern. Med. 2013;13:1-10.
DOI: 10.1186/1472-6882-13-79
48. Peters GJ, Govaerts A, Hendriks HR. The role of pharmacology in anticancer drug development. ADMET & DMPK. 2018;6:4-14.
DOI: 10.5599/admet.6.1.496
49. Karagül MI, Aktaş S, Yetkin D, Bayrak G, Çelikcan D. P53, Bcl2 and Bax Expression and Apoptosis in Perifosine and Vitamin D-Treated Endometrial Cancer Cell Line (HEC1A). Proceedings. 2018;2:1564-1569.
DOI:10.3390/proceedings2251564.
50. Karna P, Yang L. Apoptotic signaling pathway and resistance to apoptosis in breast cancer stem cells. In: Chen GG, Lai PB, editors. Apoptosis in carcinogenesis and chemotherapy. Dordrecht. Springer; 2009.
DOI: 10.1007/978-1-4020-9597-9_1
51. Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression?. Cell Death Differ. 2018;25:104-113.
DOI: 10.1038/cdd.2017.169
52. Chen J. The Cell-cycle arrest and apoptotic functions of p53 in tumor initiation and progression Cold Spring Harb. Perspect. Med. 2016;6:a026104.
DOI: 10.1101/cshperspect.a026104.
53. De Bruin EC, Medema JP. Apoptosis and non-apoptotic deaths in cancer development and treatment response. Cancer Treat. Rev. 2008;34:737-749.
DOI: 10.1016/j.ctrv.2008.07.001.

54. Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nat. Rev. Cancer. 2014;14:359-370.
DOI: 10.1038/nrc3711.

55. Fridman JS, Lowe SW. Control of apoptosis by p53. Oncogene. 2003; 22:9030-9040.
DOI: 10.1038/sj.onc.1207116.

56. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993;75 :805-816.
DOI: 10.1016/0092-8674(93)90499-g.

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