The American University in Cairo

Promising antitumor therapeutics of herbal origin: Exploring cytotoxic activity of glycoalkaloids and unraveling underlying mechanisms.

A Thesis Submitted by
Noha Nagdy Farrag

To the Biotechnology Graduate Program
Fall / 2014
In partial fulfillment of the requirements for
The degree of Master of Science in Biotechnology

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Fall / 2014
DEDICATION

This work is dedicated to my Mother (Soheir Hamdy), Father (Nagdy Farrag) and to the kindness on earth: Prof. Suher Zada, who has been my compensation for a lot of challenges that I have encountered throughout my research experience. Also to my dearest who has provided great support to me throughout the tough journey and was there whenever I needed a shoulder to lean on: Eman Abdul-Ghany.
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The American University in Cairo

ABSTRACT

Promising antitumor therapeutics of herbal origin: Exploring cytotoxic activity of glycoalkaloids and unraveling underlying mechanisms.

By Noha Nagdy Farrag

Under the supervision of: Professor Suher Kamal Zada, Professor Eman El-Ahwany and Dr. Mai Fathy Tolba

To evaluate the anticancer potential of the extracts of the two Solanaceae family plants: Solanum macrocarpon (African Eggplant) and Solanum seaforthianum (Brazilian Nightshade), cytotoxicity screening was performed. The extract exhibiting the most potent effect; Total glycoalkaloid extract of Solanum seaforthianum (TGASS) was then investigated further, through screening of individual compounds fractionated from it and comparing their cytotoxicity with each other and with other standard Solanum alkaloids. Further investigations were performed to define the underlying mechanisms by which each of the three most potent alkaloids function on hepatocellular carcinoma (HepG2) cell line. The hypothesis involves that alkaloids fractionated from the Solanaceae family of plants posses a promising antitumor activity. The two main aims were to: i) Determine the most potent compounds through cytotoxicity screening Sulforhodamine B (SRB) assay on both of HepG2 and urinary bladder cancer (SCaBER) cell lines. ii) Explore the molecular mechanisms by which these compounds exert their cytotoxic effect.

The most potent alkaloids: (Solanine, Solamargine and Nitroso-Solamargine) were investigated further, by estimation of cell cycle induced alterations via flowcytometry. Expression of cyclin D1 was then evaluated by immunocytochemistry. Ki67, survivin, caspase-3, caspase-9 gene expression was evaluated through quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). B cell lymphoma 2-associated X (Bax) protein expression was evaluated by western blot. Caspase-3, caspase-9, Bax and B cell lymphoma-2 (Bcl-2) protein expression were estimated using ELISA (Enzyme-Linked Immunosorbent Assay).

On HepG2: the three alkaloids produced an increase in the Pre-G phase, a reduction in the proliferation phases: S-phase and G2/M phase and a reduction in the G0/G1 phase. Nitroso-Solamargine has produced the greatest reduction in the S-phase and the highest increase in the Pre-G. CyclinD1 was reduced only with Solamargine and Nitroso-Solamargine; Ki67 and survivin were reduced by the three alkaloids. Nitroso-Solamargine exhibited the highest reduction. Bax, caspase-3 and caspase-9 were upregulated, with Nitroso-Solamargine exhibiting the highest upregulation, whereas Bcl-2 was a little upregulated opposite to what is anticipated.

In conclusion, The three glycoalkaloids: Solanine, Solamargine and Nitroso-Solamargine has exhibited the lowest IC\textsubscript{50} values on both of HepG2 and SCaBER cell lines, with Nitroso-Solamargine exhibiting the lowest IC\textsubscript{50} values as well as the most prominent upregulation of intrinsic apoptotic markers and downregulation of proliferation markers, indicating an important role of the Nitroso moiety in enhancing the cytotoxic activity of Solamargine.
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<td>HER2</td>
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<td>Sulforhodamine B</td>
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<td>SS</td>
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1. Introduction
Cancer occupies the second rank worldwide in mortality causes. It is distinguished by genetic mutations that lead to the loss of ability to control growth of cells, leading to unregulated proliferation of normal cells and continuous division without a halt, leading to tumor development that gradually metastasizes and spreads to adjacent organs in the body (Anand et al., 2008). Cancer represents a serious economic burden, having 64% of cancer caused mortalities occurring in 2008, in the developing countries, with 12.7 million emerging cases, of which 7.6 million tumors associated mortalities existing only in the year 2008. In developing countries, tumors survival rates are estimated to be lower, for reasons related to delayed diagnosis that usually takes place at late stages of the tumor, time, treatment facilities and therapeutic limitations (Jemal et al., 2011).

Cancer acquires no more than 10% genetic related factors for incidence, whereas the remaining 90% incidence factors are environment related as: pollution, exposure to radiation, infections and stress, or lifestyle related as: unhealthy food, obesity, smoking and alcohol overconsumption (Anand et al., 2008). Cancer is one of the principal causes of mortality worldwide in which the common treatment approaches are either chemotherapeutics and/or radiation. Chemotherapeutics include utilizing drugs that specifically eradicate cancer cells or restrict their division. Many challenges exist in the current chemotherapeutic treatments approaches, including resistance, low selectivity and high toxic margins of current chemotherapeutics (Nussbaumer et al., 2011). This has called for the need to continuously explore new chemotherapeutics that would hopefully overcome the drawbacks of currently existing therapy.

Hepatocellular carcinoma (HCC) has resulted in a death rate of over a million per year and ranked as the fifth most prevalent cancer among different types of cancers, with the highest frequency rates in Asia followed by Africa (Srivatanakul et al., 2004; Parkin et al., 2005; Perry et al., 2005). HCC has recently acquired growing rates with double occurrence frequencies during the past 10 years in Egypt. This is related to various biological predisposing factors such as hepatitis B, hepatitis C and liver cirrhosis all of which can progress into hepatocellular carcinoma. Other contributing factors as smoking
cigarettes, drinking alcohols and schistosomiasis can also lead to HCC development. Increased aflatoxin poisoning, which is found in elevated levels recently in contaminated food has also been found as one of the main predisposing risk factors of HCC in Egypt (Anwar et al., 2008). HCC is the cause of 40% of the deaths taking place worldwide of all HCC cases, with 137,000 new cases per year (Skolnick, 1996).

In the light of the increasing prevalence of HCC worldwide with Egypt’s frequencies increasing in particular, an emerging need arises of exploring new therapeutic drugs with better efficiency against cancer. Resection of the hepatic tumor has been the regular therapeutic approach of HCC, as chemotherapy remained inefficient in counteracting metastasis and recurrent progress of HCC, posing the need for novel therapeutic tactics, enhanced diagnosis and efficient treatment of HCC (Feitelson, 2002). Hence a growing interest of exploring various natural herbal products as an innovative therapeutic approach began to arise.

With HCC representing around 4.7% of the total chronic liver diseases in Egypt, the lack of efficient therapeutic approaches to it and the fact that it is a widely spread lethal disease that yet lacks a radical cure with increasing deaths rate worldwide put an essential need for developing novel therapeutic approaches for HCC, especially with the liver resection being inefficient for treating HCC. The poor prognosis rate of HCC also poses a crucial need for developing novel and enhanced diagnostic approaches for HCC (Liu et al., 2009).

Due to the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) in a large segment of the Egyptian population, as well as the common exposure of Egyptians to pesticides due the agricultural nature of several regions and aflatoxins which cause DNA (Deoxyribonucleic acid) damage and mutation and hence deactivation of the tumor suppressor gene p53; all are predisposing factors for the development of HCC among Egyptians and represent central significant factors that mainly contribute to the continuously increasing numbers of HCC in Egypt (Ezzat et al., 2005).
Urinary Bladder cancer as well, is one of the most commonly spread types of cancer, worldwide and especially in Egypt. Among the most frequent tumors worldwide, urinary bladder cancer acquires the ninth rank, with a share of 3.3% of newly identified cases of cancer, and resulting in 2.1% of cancer caused mortalities worldwide (Parkin, 2008), which is raised to 8.7% of cancer caused mortalities in Egypt, occupying the fifth rank in causes cancer associated mortalities in Egypt, possessing a frequent incidence rate of 37.1% (El Attar, 2005). In 2008, Bladder cancer had acquired newly emerging cases of about 386,000 and around 150,200-bladder cancer caused mortalities worldwide. Its incidence was majorly reported in males, acquiring death frequency of 16.3 in each 100,000 cases of Egyptian males. The main contributing factor for incidence rates in developing countries, with around 50% probability of causation and specifically in Africa, is infection with Schistosoma hematobium, in addition to smoking. Bladder cancer represents the most frequent type of cancer spread among Egyptian males (Jemal et al., 2011).

Resistance to chemotherapy and radiation as well as the non-specific reactions and damaging side effects that current therapies have on patients suffering from cancer, have called for continuous exploration of novel antitumor drugs in hope of overcoming the drawbacks of existing therapies (de Melo et al., 2011). Hence the use of “ethnomedicine” has been a growing trend among researchers. Using plant extracts and their sub-fractions (as alkaloids, flavonoids, isothiocyanates and other bioactive derived molecules) and screening for their potential anticancer activity, is a common approach among researchers that has led to the discovery of several novel effective potential antitumor drugs, with several other promising herbal extracts awaiting to be extensively studied (Fabricant & Farnsworth, 2001).

One of the principal resources for discovering and developing novel antitumor drugs is natural herbs. Several countries rely to a great extent on the traditional remedies and folk medicine, mainly consisting of natural herbs and plant extracts. Exploring the bioactivity of natural products against different ailments and identifying their mechanism of action, would facilitate development of novel therapeutics (Briskin, 2000).
Epidemiological research has proposed that ingestion of fruits, vegetables and phytochemicals rich nutrients might greatly decrease the chances of tumors’ incidences. Several Plant products were reported to stimulate apoptosis in cancer but not in normal cells (Reddy et al., 1997). Examples of drugs developed from natural herbs include; morphine (from opium), atropine (from solanaceae plants) and vincristine and vinblastine (from *Catharanthus roseus*) (Nelson, 1982).
2. Literature review

Over the years, the Brazilian plants have been considered as a valuable source for extracts of potential pharmacological activity. The reported anticancer activity of several Brazilian plants (Mans et al., 2000; Pessoa et al., 2006; dos Santos Júnior et al., 2010) is an intriguing target for researchers to explore the potential for Brazilian herbs to provide for a promising source of novel antitumor drugs. African plants were as promising with many of them exhibiting potent antitumor activity against diverse kinds of cancer, and are considered an essential pool for several plants to be nominated for their significant antitumor activity (Fadeyi et al., 2013). Hence, This has encouraged us to explore the anticancer activity of Solanum seaforthianum (Brazilian origin) and that of Solanum macrocarpon (Nigerian origin).

2.1 The Solanum genus

Both plants originate from the Solanum species of plants, whose extracts has been frequently reported to exhibit antitumor activity over various types of cancer. Solanum genus represents a main source for several extracts that exhibit important antitumor activity against various types of cancer, rendering it an essential intriguing resource for researchers to continuously explore the antitumor potency of different extracts derived from various Solanum genus of plants for the development of potential novel potent antitumor therapeutics. Solanum aethiopicum (Ethiopian Nightshade) extracts were reported to exhibit cytotoxic effect with reduced mitotic index on the growth of root cells of Allium cepa that is probably be attributed to DNA synthesis suppression (Yekeen et al., 2011). Moreover, Solanum aethiopicum was reported to contain several beneficial extracts (as glycoalkaloids, flavonoids, steroids and phytosterols) that are present in greater variety and quantity than those of Solanum macrocarpon, whose extracts are fewer and lack steroids (Chinedu et al., 2011). This would encourage future further investigations in order to explore the potential cytotoxic effect of the promising extracts of Solanum aethiopicum, on cancer cells.

Cytotoxic action of flavonoidal and alkaloidal extracts of Solanum crinitum and Solanum jabrense (a perennial shrub) were estimated on murine Ehrlich cancer using MTT (3-
(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and was found to have remarkable cytotoxic effect, with IC50 (Half maximal inhibitory concentration) values of; 69.50 µM for the tirilosiide flavonoid derived from Solanum crinitum, 19.5 µg/mL for the glyacoalkaloid fraction of Solanum crinitum and 74.20 µM for the solasonine rich glyacoalkaloid extract obtained from Solanum jabrense. On Leukemia K562 cells, the flavonoid tirilosiide extracted from Solanum crinitum exhibited an IC50 of 118.4 µM, the glyacoalkaloid rich extract of Solanum crinitum showed an IC50 of 13.65 µg/ml, whereas solasonine derived from Solanum jabrense had an IC50 of 60.35 µM (Esteves-Souza et al., 2002).

Other Solanum species that were reported to have an efficient antitumor effect over cancer cells were; Solanum tuberosum (potato), Solanum melongena (British eggplant) and Solanum lycopersicum (tomato), from which 18 compounds were extracted and examined for cytotoxicity via MTT assay against colon cancer (HT29) and hepatocellular carcinoma (HepG2) cell lines and have demonstrated anti-proliferative action (Lee et al., 2004). Solanum lycopersicum extract was reported to reduce number and frequency of mice skin papilloma, when applied topically. When combined with cyclophosphamide, Solanum lycopersicum extract demonstrated 37.7% inhibition of tumor, compared to 25.9% inhibition rate of cyclophosphamide alone, in B16F10 melanoma mouse model (Agrawal et al., 2009). Solanum aculeastrum (Soda Apple) extracts demonstrated anti-proliferative action against (HeLa) cervical cancer cells, (MCF-7) breast cancer cells and (HT29) colon cancer cells, stimulating cell cycle arrest and activating the apoptotic pathway for cell death (Koduru et al., 2007). Solanum jamesii (wild potato) various extracts were reported to possess an anti-proliferative effect on both colon cancer (HT29) and prostate cancer (LNCaP) cell lines, with colon cancer being more sensitive to lower doses of extracts (as low as 5 µg/ml), whereas, prostate cancer demanded a higher dose of 10 µg/ml of the extracts to exhibit the extracts’ cytotoxic effect (Nzaramba et al., 2009).

Another well-researched Solanum genus belonging plant is; Solanum nigrum that was reported to have anti-proliferative action against liver cancer (HepG2) cells (Lee et al., 2004; Ji et al., 2008), colon cancer (HT29) (Lee et al., 2004), cervical cancer (U14) (Li et
al., 2008) and breast cancer (MCF-7) cells (Son et al., 2003). Solanum nigrum (European Black-berry nightshade) has been extensively utilized for hepato-protective, anti-carcinogenic, antipyretic and anti-inflammatory effects. It has captured researchers’ attention due to its glycoalkaloid rich content that includes solanine and solamargine (Jain et al., 2011). Extracts of Solanum nigrum has caused overexpression of JNK (c-Jun N-terminal Kinase) and consequent overexpression of the pro-apoptotic Bax (Bel-2-associated X protein), as well as caspases that will eventually lead to apoptosis (Son et al., 2003). An extracted phyto-glycoprotein of Solanum nigrum has caused down-regulation of NF-kB (nuclear factor-KappaB) and PKCα (protein kinase C alpha) (Heo et al., 2004), both of which play a central role in the progression of cancer. Organic and aqueous extracts of Solanum nigrum were reported to exhibit anti-proliferative action against human leukemic cell lines; (Jurkat and HL-60), with the methanol organic extract displaying a more potent effect rather than the aqueous extract and with both extracts demonstrating a stronger cytotoxic action on Jurkat acute leukemic cell line of T-cells, rather than on the HL-60 acute promyelocytic (immature white blood cells) cell line (Gabrani et al., 2012). Methanolic extract was also reported to exhibit an anticancer activity on HeLa cervical cancer cell line, with a much lower anti-proliferative action demonstrated on Vero normal monkey kidney cell line, and hence nominating this methanolic extract of Solanum nigrum as a good candidate for development of anticancer drug, since its proliferation inhibition activity is greater on cancer rather than normal cells (Patel et al., 2009).

A study by Nawab et al. (2012), has demonstrated the cytotoxic and apoptotic effect of poly-phenolic rich extract of Solanum nigrum on prostate (PC-3, CA-HPV-10 and DU145) cancer cells, with much lower anti-proliferative action of this extract over normal virally transfected prostate epithelial cells (PZ-HPV-7), demonstrating a selective potent cytotoxic action of the poly-phenolic Solanum nigrum extract against prostate cancer cells versus prostate normal cells, as well as a remarkable DNA fragmentation, indicating the apoptotic inducing activity of the poly-phenolic extract in treated versus untreated control prostate cancer cells. A cell cycle alteration induced by the Poly-phenolic extract were also evaluated and was found to pose a G2/M cell cycle arrest in
prostate cancer cells, with induced accumulation of greater number of cells in the G2/M phase in treated prostate cancer cells Vs. the untreated “control” cells after 24 h (Hu et al., 1999).

Other Solanum genus belonging plant extracts that were reported to exhibit remarkable anticancer activity included; Ethyl acetate extract of *Solanum anguivi* (Forest bitterberry), was found to have anti-proliferative action against Hepatocellular carcinoma (HepG2) and breast cancer (MCF-7) cell lines, and has induced DNA fragmentation ladder pattern, that is indicative of apoptosis (Gandhiappan & Rengasamy 2012). Ethanolic extracts of *Solanum spirale* (a small fruiting shrub) that has shown anti-proliferative action against lung (NCI-H187), breast (MCF-7) and KB oral cavity tumors, demonstrating the greatest anticancer activity possessing the lowest IC\textsubscript{50} on breast cancer cell line (MCF-7) (Keawsa-ard, Liawruangrath, et al., 2012). The chloroform extract has also shown anti-proliferative action against the latter mentioned three cell lines, with the most potent anti-tumor action also against breast cancer cell line. Both extracts cytotoxic potential were evaluated with the Resazurin micro-titer assay (Keawsa-ard, Natakankitkul, et al., 2012). *Solanum pseudocapsicum* (Jerusalem cherry) alkaloidal component fractionated from methanol extract was reported to acquire anticancer activity as demonstrated in the Lymphoma ascites mouse model, where life span and average survival time of Dalton lymphoma bearing mice, were remarkably prolonged (Badami et al., 2003). *Solanum trilobatum* (Purple-fruited pea eggplant) was another Solanum genus belonging plant whose saponin-extracted portion was examined for anticancer activity against laryngeal HEp-2 carcinoma cell line, which demonstrated anti-proliferative action using MTT assay (Kanchana & Balakrishna, 2011). *Solanum muricatum* (Sweet Pepino) extracts were demonstrated to posses an antitumor action over colon, gastric, lung, hepatic, breast, prostate and ovarian cancer cells, via stimulation of apoptosis and consequently degrading PARP (poly-ADP ribose polymerase) and generating the characteristic apoptotic DNA ladder (Ren & Tang, 1998).

Glycoalkaloids are the focus of numerous research papers, abundant in several diets and has widely reported toxicity. Glycoalkaloids belong to a class of steroidal glycosides that
was reported to function in plants’ resistance to infections and pests, rather than plant growth. Glycoalkaloids’ toxicity is attributed to either their membrane damaging ability or to their suppression of acetylcholine-esterase action. They are composed of two main elements: the aglycone part which is mainly a 27 carbons structure with a nitrogen atom integrated, and the hydrophilic glycosidic part consisting of a carbohydrate chain with a 3-hydroxyl group integrated within the side chain (Milner et al., 2011).

2.1.1 Solamargine
Solamargine is one of the most frequently extracted glycoalkaloids, fractionated from the Solanaceae family of plants. Solamargine was nominated to be a promising candidate for development of novel potent antitumor therapeutics. Solamargine was reported to function through infiltration of cell membrane by diffusion (Alzérrreca & Hart, 1982). Solanum nigrum derived steroidal glycosides and namely; solamargine was also suggested to be the main potent cytotoxic component of the plant extracts showing IC\textsubscript{50} ranges of a maximum of 3 µM on prostate (PC-3 and LNCaP), colon (HT29) and breast cancer (T47D and MDA-MB-231) cell lines (Hu et al., 1999). On colorectal cancer (HCT-8) cells, solamargine extracted from Solanum nigrum demonstrated a potent cytotoxic action producing an IC\textsubscript{50} value of (10.63 µM) as examined via MTT assay (Milner et al., 2011).

Lee et al. (2004) has also reported isolation of solamargine from Solanum melongena (Dutch eggplant), which has demonstrated cytotoxic activity against both Hepatocellular carcinoma (HepG2) and colon cancer (HT29) cell lines, with a stronger activity against liver cancer cells. Solamargine exhibited a higher antitumor activity than its aglycone; solasodine, indicating an essential role of the carbohydrate side chain within the antitumor activity of solamargine.

Solamargine derived from other Solanum species as; Solanum sodomaeum (Apple of Sodom) was reported to have antitumor activity against human skin cancer (Cham et al., 1987). Solanum incanum (bitter-apple) derived solamargine demonstrated cytotoxicity on (Hep3B) hepatoma cell line and has induced apoptosis (Kuo et al., 2000). It also
exhibited potent cytotoxic activity in lung cancer cells with IC\textsubscript{50} values that were all less than 7.5 µM, in four lung cancer cell lines, surpassing IC\textsubscript{50} values of current chemotherapeutics as; paclitaxel, cisplatin and etoposide (Liu et al., 2004). Solamargine has also exhibited cytotoxic action against; leukemic K562, KB squamous carcinoma, prostate PC-3 cancer and breast MCF-7 cancer cells line, with the most potent effect on prostate cancer cell line demonstrating the least IC\textsubscript{50} value of 5.9 µM. A more promising finding was the low IC\textsubscript{50} values demonstrated on the multiple drug resistance cell lines; K562/A02 and KB/VCR reporting IC\textsubscript{50} values of 5.4 and 7.1 µM respectively, with a much higher IC\textsubscript{50} value on normal RPE1 cell line of 23.4 µM, indicating a promising cytotoxic selectivity of solamargine against cancer cells versus normal cells. Solamargine treatment on both leukemic and squamous carcinoma cells, has also induced lactate dehydrogenase discharge from the cytoplasm as a result of loss of membrane integrity induced by the solamargine disrupting activity on the cellular membrane, along with enhanced penetration of propidium iodide on both of leukemic and squamous cell cancer cells, as shown by increased DNA intercalation by the propidium iodide dye (Sun et al., 2011). Moreover, glycoalkaloids (under which solamargine is classified) were reported to function through integrating the aglycone unit within the 3-β-hydroxyl groups of plasma membrane cholesterol, forming an immobile complex at the outer membrane, resulting in budding of the complex containing portion of the membrane, and hence leading eventually to membrane disintegration (Keukens et al., 1996). Further actions as microtubules formation was greatly decreased, with a concurrent increase in actin stress fibers, as demonstrated by immunofluorescence and immunoblotting were also observed (Sun et al., 2011).

Solamargine cytotoxic potential was greatly attributed to its rhamnose carbohydrate moiety. When rhamnose moiety was eliminated yielding a khasianine alkaloid, IC\textsubscript{50} was greatly raised from 3 µg/ml for solamargine to 20 µg/ml for khasianine on Hep3B hepatoma cell line. Khasianine (opposite to solamargine) also failed to induce the Pre-G1 peak indicative of apoptosis, as detected by flow cytometry. This suggested a strong attribution of the apoptotic inducing action and the cytotoxic function, to the rhamnose moiety of solamargine (Chang et al., 1998). On osteosarcoma cell line (U2OS),
solamargine exhibited cytotoxic action examined via MTT assay. The more promising finding was the reported relatively higher IC\textsubscript{50} of solamargine on normal liver cell line (HL7702), and on retinal epithelial cell line (RPE1), as compared to the much lower IC\textsubscript{50} demonstrated on osteosarcoma; MG-63, Saos-2 and U2OS cell lines (Li et al., 2011). This provides a promising selective anticancer potential of solamargine on cancer cells vs. normal cells that requires further investigation.

Solamargine fractionated from the Brazilian \textit{Solanum lycocarpum} (Wolf Apple), was reported to exert potent anti-proliferative action against several cancer cell lines using Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium (XTT) cyto-toxicity assay including; hepatocellular carcinoma (HepG2) with IC\textsubscript{50} value of 4.58 µg/ml, colon cancer (HT29) with IC\textsubscript{50} value of 9.88 µg/ml, glioblastoma (MO59J) with IC\textsubscript{50} value of 9.59 µg/ml. Solamargine toxic effect on normal cell lines as; human lung fibroblasts (GMO7492A) with IC\textsubscript{50} value of 26.66 µg/ml was much less than that on cancer cells. Among different cancer types, solamargine exhibited the most potent activity against HepG2, demonstrating a promising potential of solamargine as an anticancer therapeutic, especially as anti-hepatocarcinoma potential therapy (Munari et al., 2014).

Solamargine exhibited membrane-disrupting ability at concentrations not less than 50 µM, through examination on liposomes and evaluation of peroxidase action detected in the supernatant. Furthermore, the combination of solamargine and solanine has produced a synergistic phosphatidylcholine membrane lysis effect on liposomes at concentration of 75 µM for both. At concentrations also not less than 50 µM, solamargine has greatly induced lysis of protoplasts at a concentration of 70 µM. On bovine erythrocytes, 10 µM of solamargine has caused no significant percentage of hemolysis after 2 h of treatment, whereas, at 20 µM concentration, solamargine has resulted in total 100% of hemolysis (Roddick et al., 1990).

Solamargine uptake into cancer cells is reported, through endogenous lectins located within tumor cells and not within normal non-cancerous cells. In a way this provides an
explanation of the selective cytotoxicity of solamargine against ovarian cancer cells, compared to normal fibroblast cells, with much enhanced selectivity for cancer cells, more than that acquired by vinblastine and cisplatin against ovarian cancer cells. A six times less concentrations of solamargine, compared to other cytotoxic compounds, were also reported suggesting a potent anticancer potential of solamargine (Daunter & Cham, 1990).

Ding et al. (2012) has reported a cytotoxic action of solamargine on SMMC-7721 hepatoma cell line, with an IC\textsubscript{50} of (9.21 µg/ml), using MTT assay, whereas IC\textsubscript{50} of solamargine on HepG2 almost yielded double value of (19.88 µg/ml). Further investigation on the mechanism by which solamargine inhibits proliferation on the hepatoma cell line with the more potent IC\textsubscript{50} (9.21 µg/ml); SMMC-7721 was conducted, and has demonstrated up-regulation of capsase-3 (a key executioner of apoptosis and DNA fragmentation), with an overexpression that is proportional to the increased concentrations of solamargine treatment. This has strongly suggested that solamargine cytotoxic action on SMMC-7721 hepatoma cells was done at least partly through induction of apoptosis. Whether solamargine induced cytotoxicity on HepG2 follows the same mechanism or not, remains to be explored.

Solamargine was reported to be more cytotoxic to lung cancer than currently used chemotherapeutics as; cisplatin and Taxol (Cham, 2013).

2.1.2 \(\alpha\)-Solanine

Another most commonly extracted glycoalkaloid from the \textit{Solanaceae} family of plants is solanine. Solanine extracted from \textit{Solanum nigrum} exhibited cytotoxic effect on hepatocellular carcinoma HepG2, gastric carcinoma SGC-7901 and large intestine cancer LS-174 cell lines, with IC\textsubscript{50} values of; 14.47 µg/ml for HepG2 and above 50 µg/ml values for both of gastric and intestinal carcinoma cell lines, via MTT assay (Ji et al., 2008).

Solanine exhibited anti-proliferative action on melanoma A2085 cell line, with enhanced action proportional to increasing doses of solanine. A drawback however was that;
although solanine has exhibited cytotoxicity on melanoma cells at concentration of 23uM, yet it has exhibited a similar cytotoxicity with the same concentration (23uM) on normal fibroblast and keratinocyte cells. At a lower concentration of 18.4 uM, solanine had shown no cytotoxic effect on either melanoma or normal cells (Lu et al., 2010). This puts solanine effectiveness as an anticancer in question and requires further research to validate.

Anti-migratory activity of solanine was hence examined at the lower non-cytotoxic concentration of solanine 18.4 µM and lower on melanoma cells. Significant inhibition of migration was observed on melanoma cells via the wound healing assay, as well as inhibition of invasion through the matrigel surface of the Boyden chamber was detected. On the molecular level, Under-expression of the metastatic markers; matrix metalloproteinases (MMP-2 and MMP-9), (which are responsible for breaking down the extracellular matrix, which is a primary main step of the metastatic process) was also detected via RT-PCR and western blotting. This was also confirmed by the downregulation of proMMP-2 and proMMP-9 that is detected via gelation zymography of the conditioned medium of melanoma cells, post 24 h treatment with solanine, revealing a suppressing role of solanine on metastasis on A205 melanoma skin cancer cells. This has indicated an anti-metastatic ability of solanine that is not correlated to its cytotoxicity, as it was applied on melanoma cells with the non-toxic concentration (Lu et al., 2010).

Solanine has also repressed JNK, PI3K (phos-phatidylinositide-3 kinase) and Akt signaling pathway, via inhibiting their activating phosphorylation. Suppression of the nuclear factor kappa B (NF-kB) was also reported. These reported downregulations have confirmed the proliferation and metastatic inhibitory action of solanine (Lu et al., 2010). Cytotoxic action of solanine on several other cancer cell types as; gastric and cervical cancer as well as lymphoma cell lines, were also reported (Hu et al., 1999; Lee et al., 2004; Friedman et al., 2005).

Solanine isolated from Solanum tuberosum, was examined on colon cancer HT-29 cells.
It has stimulated apoptosis via suppression of the phosphorylation of ERK (extracellular signaling regulating kinase) and has upregulated caspase-3 (S.-A. Yang et al., 2006). On pancreatic cancer cells, solanine caused cytosolic shrinkage and rounding of cells. It has also suppressed proliferation of SW1990 and Panc-1 pancreatic cancer cell lines. Solanine has also down regulated the metastasis markers; MMP-2 and MMP-9, as detected by quantitative RT-PCR and immunoblotting, in a concentration dependent pattern, indicating an anti-metastatic action of solanine on pancreatic cancer cells. In a xenograft nude mice model, solanine was also reported to reduce the tumor weight of pancreatic cancer cells injected (Sun et al., 2014).

Solanine was reported to posses an inhibitory action on the proliferation of both of HepG2 and HT-29 cell lines. Moreover, elimination of glucose moiety from α-solanine yields β2-solanine which exhibit lower cytotoxicity on both liver and colon cancer cells, with the larger effect on colon cancer cells. On normal liver (Chang) cells, solanine has also exhibited a cytotoxic effect, indicating that safety should be taken into account while using solanine as a potential anticancer therapy (Lee et al., 2004). Contradictory results were reported by Friedman et al. (2005), whose study has exhibited that solanine had greater cytotoxic effect on HepG2 hepatocarcinoma cells rather than normal liver Chang cells. A previous study has also reported no toxic side effects of 200mg/kg glycoalkaloids that were ingested by volunteers via potatoes (Mensinga et al., 2005), which favors the safety reports rather than the toxic reports, in the ongoing safety considerations debate regarding glycoalkaloids.

2.2 Cell Cycle
The cell cycle is a process governing cell growth and division. It consists of four main phases; The G0 phase which is the quiescent phase of the cell in which the cell have a diploid 2N nucleus and is in a resting state and hence not growing nor proliferating, The G1 phase (also 2N nuclei) which is the first gap, in which the cells are getting ready for the DNA synthesis, repair any possible errors in DNA prior to DNA replication and during which the cells decide whether to progress to the next round of cell cycle or not i.e. decide to commit to DNA replication or not. This is followed by the S-phase during
which DNA replication takes place, and hence cells at this phase nuclei vary between 2N and others as 4N in an aneuploid state. This is followed by the G2 phase, which is the second gap, during which the cells are able to correct any possible replication errors that might have occurred during the previous S-phase, prior to chromosomal separation and hence prevent passage of faulty DNA to daughter cells. G2 phase is the phase following DNA replication and hence contains a tetraploid DNA content. Cells in this phase prepare for mitosis taking place in the following M phase, during which cells divide, rendering the cellular DNA content into the original diploid 2N state (Figure 1) (Schafer, 1998; Vermeulen et al., 2003; Senderowicz, 2004).

In breast cancer cells, Solamargine has generated a pre-G1 peak (also referred to as sub-G1 peak) detected by PI flowcytometry, post 16h treatment with the IC\textsubscript{50} concentration estimated by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. A simultaneous reduction in the G2/M phase was reported, suggesting solamargine cytotoxic action on breast cancer were conducted through stimulating apoptosis in G2/M phase cells, whereas no other significant cell cycle alterations were reported (Shiu et al., 2007). A similar effect of solamargine was reported on Hep3B hepatoma cells, regarding elevated sub-G1 peak, after propidium iodide staining, as well as simultaneous inhibition in the G2/M phase suggesting that solamargine greatly influences Hep3B cells in the G2/M phase in particular, driving them into apoptosis, as demonstrated by the apoptotic pre-G1 peak (Kuo et al., 2000). On SMMC-7721 hepatoma cell line, solamargine has also induced a cell cycle arrest at the G2/M phase, which was exhibited by the reduced number of cells detected in the G2/M phase, with a remarkable shift and increase in the number of cells in the pre-G1 phase, that is indicative of increased apoptosis (Ding et al., 2012). Flow cytometry has also demonstrated that solamargine has not induced cell-cycle arrest on K562 leukemic cells rather than an increased preG phase indicative of dead cells (Sun et al., 2011).

\textit{Solanum nigrum} derived solanine was reported to cause cell cycle alterations post 48 h of treatment of HepG2, causing an increase in the percentage of cells in the sub-G0 peak, diminishing G2/M phase and increasing percentage of cells in the S-phase, in a dose
dependent manner (Ji et al., 2008). On HT-29 colon cancer cells, solanine has induced emergence of the subG0 phase indicative of apoptosis post 48 h of treatment, in a dose dependent pattern (S.-A. Yang et al., 2006).

Figure 1a. Diagram illustrating Cell cycle phases and events taking place in each phase. Adopted from Malumbres et al. (2008). Figure 1b. Representative diagram of flow cytometry chart. Adopted from Cooper et al. (2000). It depicts how flow cytometer distinguishes between different phases of cell cycle according to the 2n or 4n nuclear content of the cell.

2.2.1 CyclinD1
Cyclin D1 upregulation is associated with several types of cancer as; lymphoma (Weisenburger et al., 1987), parathyroid adenoma (Motokura et al., 1991), lung, bladder and esophageal cancer (Hall & Peters, 1996), as well as, leukemia, breast cancer and squamous cell carcinoma (Peters, 1994; Xu et al., 1994; Zhang et al., 1994). Cyclin D1 elevated levels are maintained through G1 phase. It is required for the initiation of the S phase, after which it declines rapidly during the whole S-phase by degradation via proteasomes, as a result of phosphorylation of threonine at position 286 of the cyclin D1
(Diehl et al., 1997), in order to permit DNA synthesis, which is suppressed in the presence of cyclin D1, via inhibitory binding of cyclin D1 to the crucial DNA synthesis regulator; PCNA (Proliferating cell nuclear antigen) (Xiong et al., 1992; Pagano et al., 1994; Fukami-Kobayashi & Mitsui, 1999). PCNA is a part of the DNA polymerase essential for DNA replication during the S-phase and hence is expressed once the cyclin D1 levels starts declining, relieving its inhibitory action on PCNA expression, once the cell enters the S-phase (Schafer, 1998).

Cyclin D1 binds to cyclin dependent kinase 4 or 6 (CDK4/6), and together, they perform inhibitory phosphorylation to the retinoblastoma protein (Rb), diminishing proliferation-suppressing action of Rb, and hence releasing the E2F transcription factors essential for cell cycle progression (Sherr & Roberts, 1999; Olashaw & Pledger, 2002). This promotes stimulation of cyclin E-CDK2, as well as cyclin A-CDK2 complexes, that are crucial for the initiation and progression of the S-phase (Girard et al., 1991). After the S phase, cyclin D1 is elevated again in the G2 phase, to ensure progression of cell cycle (Figure 2) (Stacey, 2003). This is confirmed by the exogenous overexpression of cyclin D1, which causes lengthening of the S-phase and accordingly the whole cell cycle, as a result of impeding cell cycle progression by the elevated exogenous cyclin D1 levels during the S-phase (K. Yang et al., 2006).

The crucial decision of proceeding proliferation occurs at the G2 phase, after ensuring complete and proper DNA synthesis is finished in the previous S-phase. This is performed via the Ras-induced, cyclin D1 expression during the G2 phase, which maintains cell cycle progression into the M phase and hence cellular proliferation (Figure 2) (Hitomi & Stacey, 1999, 2001). In case of defective DNA synthesis during the S-phase, cyclin D1 remains low during the G2 phase. Accordingly cell fails to commit to the next cell cycle division and consequently cellular proliferation is halted (Stacey, 2003).
Inducing apoptosis has grown to be a novel focus while developing new mechanism-dependent therapeutic approaches. Known antitumor drugs as cisplatin and Taxol were reported to execute their cytotoxic action on cancer cells through induction of apoptosis.

2.2.2 Ki67

Ki67 is expressed continuously in cells undergoing active continuous cell cycle, and is upregulated during the G1, S, and G2/M phases in an ascending manner (Manoir et al., 1991) but not in the G0 resting phase. It plays an essential role in cellular proliferation and cell cycle progression, rendering it as an excellent indicator of the continuous growth of the cells and cellular division (Schafer, 1998; Scholzen & Gerdes, 2000). This has revealed an important role of Ki67 in tumor growth and progression as well as, its vulnerability as a growth marker to chemotherapeutics (Scholzen & Gerdes, 2000). Indeed, Ki67 has been associated with several tumors as; non-Hodgkin’s lymphomas (Gerdes et al., 1984), prostate cancer (Cher et al., 1996), breast cancer (Weikel et al., 1994; Archer et al., 1995; Molino et al., 1997), soft tissue sarcoma (Heslin et al., 1998) and multiple myeloma (Miguel-Garcia et al., 1995), with essential prognostic and cancer recurrence values were reported, as well as association with metastasis (Molino et al., 1997; Heslin et al., 1998).

2.3 Triggering apoptotic pathway

Inducing apoptosis has grown to be a novel focus while developing new mechanism-dependent therapeutic approaches. Known antitumor drugs as cisplatin and Taxol were reported to execute their cytotoxic action on cancer cells through induction of apoptosis.
Apoptosis is defined as the main fundamental process by which cell achieves death and elimination. It is considered as the tuning process by which cell proliferation is controlled (Kerr, 1971). Apoptosis is an essential regulating homeostatic mechanism, balancing between tissues development and proliferation in all organisms (Ankarcreona et al., 1996; Brown & Attardi, 2005). Apoptosis involves cleavage of substrates that play an important role in DNA repair, proper assembly of cytoskeleton, maintaining nuclear entity and life span of cell (Goyal, 2001). Suppression of the apoptotic pathway is an approach used by cancer cells to evade the corrective immune response that result eventually in the destruction of cancer cells (Chouaib et al., 2002). In response to antitumor radiation therapy and cytotoxic chemotherapy, cancer cells undergo apoptosis and diminish, instead of continuing to proliferate and flourish. Thorough research of the molecular mechanism by which tumor cells undergo apoptosis, is essential for understanding and optimizing novel approaches to counteract cancer progression through enhancing treatment approaches (Kerr et al., 1994).

Macrophages perform phagocytosis to fragmented dead cells, to prevent dispersion of cellular remains and hence, consequent stimulation of immune inflammatory reaction (Martin & Green, 1995). Apoptosis grew into a major interest for cancer researchers, since unregulated apoptosis may lead to unregulated proliferation and consequent malignancy (Sarraf & Bowen, 1988; Carson & Ribeiro, 1993). Evaluating the strength of antitumor activity of alkaloids from the apoptosis context has grown to be an essential evaluating parameter for researchers determining the potency and efficiency of proposed antitumor products.

### 2.3.1 Pathways of apoptosis

Two major directions of apoptotic pathway; the intrinsic pathway achieved through mitochondria and the extrinsic pathway achieved through cellular membrane including Tumor Necrosis Factor-alpha (TNF-α) and its receptor; Tumor Necrosis Factor alpha Receptor-1 (TNFR-1), Fas and its receptor. Multiple caspases are in charge of apoptosis, having caspase-3 as the main widespread executioner of apoptosis in the cell, through
processing various substrates that eventually result in the apoptotic morphology. Initiator caspases as; caspase-8 and caspase-9, act upstream cleaving and hence activating caspase-3 in a cascade manner. Caspase-3 degrades the inhibitor of the CAD/DFF40 nuclease (Caspase-activated Dnase/ DNA fragmentation factor), and hence permitting the nuclease to perform its cleaving function at the linker sites on chromatin amidst nucleosomes, that would produce the distinguished apoptotic DNA oligonucleosomes fragmentation ladder (Kerr et al., 1994; Zimmermann & Green, 2001). Caspase-3 also cleaves and inactivates the enzyme PARP, which functions in DNA repair, as well as the DNA-dependent protein kinase (DNA-PK), functioning in restoring DNA with double strand breaks (Lazebnik et al., 1994; Smith & Jackson, 1999), and hence impeding cell recovery from DNA degradation, directing cells in only an irreversible route of dying through undergoing apoptosis. The DNA distinguished ladder is about 180-200 base pairs that could be extracted from few cells as low as $10^6$. However, DNA fragmentation takes place at a later stage of apoptosis and is one of the eventual incidences of apoptosis and hence, its absence does not completely deny the incidence of apoptosis, as cells not exhibiting the fragmented DNA hallmark, could possibly be just starting an earlier phase of apoptosis. A potential drawback however, is that DNA fragmentation could take place during processing of cells, rendering it difficult to generate the distinguished nucleosome ladder characteristic of apoptosis (Elmore, 2007).

Necrosis could also result in DNA fragments; however, DNA degradation is random and generates a much diffused and randomly sized smear after DNA gel electrophoresis than the characteristic and size specific ladder produced by apoptosis (Kerr et al., 1994). Necrosis is an alternative death process to apoptosis, where the cells neither receive an external stimulus that would induce apoptosis intrinsically, nor their membrane receptors would be activated by their ligands, directing the cells to extrinsic apoptosis. It is rather a passive, uncontrolled, non-programmed and non-energy involving mode of death that includes cell swelling and degradation that does not include either caspases or ATP (adenosine triphosphate). It is usually as a result of massive and direct cell injury, like blunt and gross injury to the cellular membrane or impairment of the cellular energy source, that would force the cell to end its life immediately without the passing through.
systematic, controlled and organized pathways of death. As opposed to apoptosis, necrosis involves an inflammatory reaction induction, as a result of cell membrane rupture that leads to efflux of cytosolic contents into the neighboring tissues, post development and swelling cytosolic vacuoles and lysosomes and mitochondrial eruption. This leads to eventual triggering and recalling of macrophages at the site of necrotic-ruptured cells through a chemotactic inflammatory signaling leading eventually to engulfment of the released cellular debris by phagocytosis (Savill & Fadok, 2000; Kurosaka et al., 2003; Elmore, 2007). Apoptosis on the other hand, involves degradation of the cytoskeletal and protein content, which are then contained within apoptotic bodies (along with the degraded nuclear DNA, for protecting leakage of active nuclear DNA of apoptotic cells into macrophages or neighboring cells during phagocytosis of apoptotic cells), condensation of the cytosol, increase in cellular density and shrinkage of cellular contents that are maintained in an intact plasma membrane. An additional and rather remarkable feature of apoptosis is the translocation of inner cell membrane markers as phosphatidylserine from the inner direction of the cell, to the outer surface of cell membrane, to facilitate identification of the apoptosis undergoing cells by macrophages and hence allowing swift phagocytosis of apoptotic cells, without compromising the neighboring tissues (Kerr et al., 1994; Elmore, 2007). The latter mentioned differences between apoptosis and necrosis, calls for the careful examination of the effect of antitumor therapeutics on cancer cells, to ensure the occurrence of potential apoptosis or not, as a molecular organized active response to the anticancer therapy, distinguishing it from the uncontrolled passive necrosis process.

During the extrinsic apoptotic pathway; activation of the membrane receptors; TNFR-I, TNF-Related Apoptosis Inducing Ligand (TRAIL) and Fas by their ligands, induce a downstream signaling cascade, through Fas associated death domain (FADD), which interact and dimerize the initiator caspase-8 and hence activating the latter, which in turn cleaves and activates the executioner caspases; caspase-3 and caspase-7. This activation cascade of caspases leads eventually to apoptosis (Zimmermann & Green, 2001; Tait & Green, 2010).
Intrinsic pathway is different; it is usually induced through an apoptotic stimuli as; DNA damage, stress or virus attack. It involves mitochondria, which when stimulated by the apoptotic stimuli; Bax is shifted from the cytosol to the outer surface of the mitochondrial membrane, where it is recruited with Bcl-2 (B cell lymphoma 2) antagonist or killer (Bak) and develops pores of oligomers, causing permeablization of the mitochondrial outer membrane and consequently an efflux of cytochrome c into the cytoplasm, which in turn binds to and induces Apaf-1 (apoptotic protease activating factor-1), through stimulating a conformational change in Apaf-1, permitting its oligomerization and hence development of the cytochrome c/Apaf-1 heptamer complex responsible for caspases stimulation, known as the apoptosome. This apoptosome then binds and induces the initiator pro-caspase-9, leading to its dimerization into the active caspase-9 form, which eventually activates the apoptosis executioner caspases; caspase-3 and caspase-7 (Figure 3) (Zimmermann & Green, 2001).

A central antiapoptotic protein is the B cell lymphoma 2 (Bcl-2), which acts on mitochondria during the intrinsic apoptotic pathway to inhibit Bax oligomerization with Bak and hence inhibit the intrinsic apoptotic pathway through limiting mitochondrial permeability. Therapy resistant tumor cells would usually display overexpression of the antiapoptotic Bcl-2 and under-expression of the proapoptotic Bax. The Bax and Bcl-2 relative expression is usually governed by the tumor suppressor player which also acquires an essential role cell cycle regulation; p53 (Figure 3) (Elmore, 2007). Hence drugs that cause either or both of upregulation of Bax and downregulation of Bcl-2 would be promising therapeutics that would overcome anticancer therapy resistance.

Cross talk between extrinsic and intrinsic apoptotic pathways is done through the activation of (Bcl-2 homology 3 (BH3)-interacting domain death agonist); BID through its activation via cleavage by the initiator extrinsic caspase-8. BID then induces Bax/Bak stimulation either by direct interaction with the complex or through inhibitory interaction with the Bcl-2 and hence releasing the activated Bax/Bak to perform their pro-apoptotic function (Figure 3) (Willis et al., 2003).
Inhibitors of apoptosis (IAPs) are protein family that are essential for governing proliferation, cell division and regulating apoptosis, which has been extensively researched for cancer therapy (Elmore, 2007). They have a common domain called; Baculovirus IAP repeat domain (BIR) and their overexpression were reported to delay apoptotic induction (Dubrez-Daloz et al., 2008). Survivin and X-linked Inhibitor of Apoptosis Protein (XIAP) are the most prominent family members; They directly suppress executioner caspases as; caspase-3 and caspase-7 as well as initiator caspases as; caspase-9 and hence diminish apoptosis (Figure 3) (Zimmermann & Green, 2001).

Figure 3. Diagram illustrating Signaling pathways of apoptosis. Image modified from Khan et al. (2010).
2.3.2 Survivin

Survivin is the smallest molecule among IAPs with a 16.3 kDa sized protein composed of 142 amino-acids, coming from a 15kb gene (Dubrez-Daloz et al., 2008). Survivin forms a homodimer that has a crucial function of maintaining cell viability and ensuring regular process of mitosis. The dual function of survivin as antiapoptotic and cell cycle and hence cell division-regulating molecule was clearly demonstrated on HepG2 cells, where survivin was overexpressed located mainly in the nucleus (Ito et al., 2000; Dai et al., 2012). An essential role of survivin in promoting cell cycle progression was reported (Ambrosini et al., 1998). Survivin is highly expressed in embryonic and cancer tissues but not in normal tissues (Chiou et al., 2003), being classified as the fourth most frequently expressed gene in human cancer cells (Velculescu et al., 1999). Tumor suppressor genes as p53 and PTEN (Phosphatase and tensin homolog), were reported to inhibit survivin expression, whereas, tumor promoting genes as c-myc and STAT-3 (Signal transducer and activator of transcription 3), were reported to enhance survivin upregulation. Survivin is a molecule of nodal character which provides a crucial criteria, for chemotherapeutic drugs targeting survivin as they have greater potential to overcome potential resistance, through suppression of multiple pathways, rather than chemotherapeutics that target single-pathway molecules in tumor cells (Ryan et al., 2009). Survivin upregulation was correlated with resistance to several chemotherapies and radiotherapy as well, which is also consistent with the reports of increased susceptibility of cancer cells to chemotherapy upon inhibition of survivin activity (Asanuma et al., 2000; Rödel et al., 2003; Chakravarti et al., 2004; Pennati et al., 2008). This is clearly demonstrated by the enhanced susceptibility of cancer cells for antitumor drugs; cisplatin, vincristine, etoposide, doxorubicin and imatinib, upon knocking down of survivin via antisense oligonucleotides (Jiang et al., 2006; Yonesaka et al., 2006; Ryan et al., 2009). Suppression of tumor proliferation, reduction in tumor weight and angiogenesis inhibition of gastric cancer cells in murine xenograft model, was also reported upon survivin inhibition through its specific antisense oligonucleotides (Tu et al., 2003). Reports about survivin role in enhancing healing process of injuries and its role in promoting cell division has rendered it a promising target molecule for researchers developing anticancer therapies. Survivin expression was reported to increase
proportionally with cancer progression, revealing an important role of survivin in carcinogenesis (Chiou et al., 2003). It was found to be overexpressed in various cancer types as; lung, liver, gastric, breast, ovarian, pancreatic cancers and many others (Altieri, 2001). In clinical trials, using survivin inhibitor molecules on patients having prostate cancer, lymphoma, leukemia and lung cancer, were reported to exhibit reduced and/or shrunk tumors and stabilized cases with encouraging low toxicity margin and minimal side effects (Altieri, 2013).

Survivin was also reported to be upregulated in breast, pancreatic, ovarian, brain and several other types of cancers (Tanaka et al., 2000; Satoh et al., 2001; Yoshida et al., 2001; Chakravarti et al., 2002). In survivin transgenic mice, skin papilloma were less frequently developed, as compared to control mice, revealing a crucial role of survivin in promoting cancer progression (Allen et al., 2003).

Moreover, Survivin was reported to contribute in apoptosis inhibition through suppression of apoptosis in endothelial cells and hence promoting survival of endothelial cells which play a key role in angiogenesis (Ryan et al., 2009). Survivin upregulation mediated the antiapoptotic function of vascular endothelial growth factor (VEGF), suggesting that chemotherapeutic function of survivin could possibly target angiogenic and vasculature properties of cancer cells (Tran et al., 2002).

2.3.3. Solamargine and apoptosis

Previous studies on osteosarcoma (U2OS) cell line, solamargine has also induced chromatin aggregation, development of apoptotic bodies, and displaying the remarkable apoptotic marker phosphatidylserine on the outer surface of cellular membrane as shown by annexin V flowcytometry. Up-regulation of the Pro-apoptotic markers; p53 and the downstream Bax, as well as caspase-3, caspase-9, and cytochrome c release were reported. Down-regulation of the anti-apoptotic; Bcl-2 was also demonstrated in U2OS osteosarcoma cells post solamargine treatment, indicating the cytotoxic potential of solamargine via stimulating p53 and Bax mediated intrinsic apoptosis (Li et al., 2011). Solamargine derived from Solanum incanum on hepatoma Hep3B cells has caused
condensation of nuclear chromatin and demonstrated fragmented DNA, both of which are characteristic markers for apoptosis. Solamargine has also caused up-regulation of tumor necrosis factor alpha receptors (TNFR-I and TNFR-II) that were demonstrated to play an important role in solamargine induced apoptosis, that was greatly alleviated by TNFR-I and TNFR-II neutralizing antibodies (Kuo et al., 2000). On lung cancer, Solanum incanum derived Solamargine, caused stimulation of the apoptotic pathway was demonstrated by phosphatidylserine translocation on outer surface of cellular membrane, detected by annexin V as well as the pre-G1 peak, revealing percentage of cancer cells undergoing apoptosis detected by PI staining, via flow cytometry. Other apoptosis hallmarks induced by solamargine treatment on lung cancer cells included; liberation of mitochondrial cytochrome c, stimulation of caspase-3 and under-expression of the anti-apoptotic Bcl-2. This was also associated with fragmentation of DNA into oligonucleosomes. The quenched expression of TNFR-I and TNFR-II in lung cancer progressing cells was reported to be reversed upon solamargine treatment of lung cancer cells which exhibited overexpression of TNFR-I and TNFR-II, indicating increase in TNF-α binding sensitivity of lung cancer cells and consequently TNF-mediated apoptosis. Solamargine has also up-regulated the downstream TNFR-I-associated death domain (TRADD) and Fas-associated death domain (FADD) markers involved in activated extrinsic apoptotic pathway (Liu et al., 2004).

Furthermore Shiu et al. (2007) has reported solamargine as the most potent anticancer drug against breast cancer, among several current antitumor therapeutics as; cisplatin, cyclophosphamide, methotrexate and 5-fluorouracil. Solamargine cytotoxic action on breast cancer was performed through stimulation of extrinsic and intrinsic apoptotic pathway as demonstrated by the up-regulation of the extrinsic apoptotic hallmarks; Fas, Fas-associated death domain (FADD) and tumor necrosis factor receptor I (TNFR-I), as well the intrinsic apoptotic hallmarks; Bax, caspase-3, caspase-9 and cytochrome c discharge from the mitochondria as well as the down-regulation of the anti-apoptotic Bcl-2. Moreover, a combinatorial treatment of solamargine and cisplatin was reported to overcome cisplatin resistance rendering cisplatin resistant breast cancer cells more vulnerable to cisplatin, with a synergistic cytotoxic action on breast cancer.
On leukemia K562 cells, solamargine has exhibited potent antitumor activity, causing rupture of the lysosomes and hence consequent pH increase and cathepsin B (which is an important mediator of caspase involved apoptosis) discharge within the cytoplasm post lysosomal rupture and membrane loss, as verified through western blot. Apoptotic hallmark of cytochrome c discharge, as well as Ca\(^{2+}\) accumulation post solamargine treatment upon leukemic cells was also reported. This was associated with overexpression of the intrinsic apoptotic marker; Bax, as well as caspase-3 and caspase-9, with a simultaneous down-regulation of the anti-apoptotic Bcl-2 (Sun et al., 2010).

2.3.4 Solamargine and multiple drug resistance

Multiple drug resistance (MDR) has always posed a challenge rendering several antitumor therapies ineffective. MDR cancer cells as; breast cancer, ovarian cancer and leukemia had exhibited efficient cytotoxicity upon solamargine treatment, which has also demonstrated a much higher cytotoxicity on cancer cells vs. normal liver HL7702 and H9C2 cells. Solamargine efficiency against MDR cells could be explained by its ability to be up-taken by the endogenous lectins of tumor cells, which could possibly counteract the drug efflux action of the p-glycoprotein, characterized in MDR cancer cells (Cham, 2013). On A549 lung cancer cells, solamargine treatment has also caused overexpression of caspase-8 and Bax, in addition to a synergistic up-regulation of caspase-3, caspase-9 and caspase-8 that was reported upon combination treatment of both solamargine and cisplatin, surpassing the A549 cells’ resistance to cisplatin (Liang et al., 2004). Solamargine was reported to cause overexpression of Fas as well as down-regulate HER2 (Human epidermal growth factor receptor 2) -whose up-regulation usually contributes in chemotherapeutic drugs resistance- in non-small cancer lung cell line H441 and A549 lung cancer cell line. Applying a combinational treatment of low concentrations of; solamargine and TOP2A (topoisomerase II α) inhibitor upon H441 and A549 lung cancer cells, was reported to enhance solamargine-induced apoptosis (C. H. Liang et al., 2007). Nevertheless, it was also reported to up-regulate HER2 in human large cell lung cancer (H661) and small cell lung cancer (H69) cell lines. Solamargine was thus utilized to enhance sensitivity of human large cell lung cancer (H661) and small cell lung cancer.
(H69) cell lines to the anti-HER2 “Trastuzumab” chemotherapy, through up-regulation of the intrinsic oncogene HER2 possessing tyrosine kinase function, without affecting the anticancer apoptosis-inducing activity of solamargine individually against lung cancer cells. HER2 was reported to contribute in the resistance of cancer cells against chemotherapeutic drugs induced apoptosis, as well as cancer progression, angiogenesis and metastasis. HER2 induced resistance was manipulated by the solamargine-induced up-regulation of HER-2, which has rendered H661 and H69 lung cancer cells more vulnerable to the antitumor inhibitory action of “Trastuzumab”, which selectively suppresses proliferation of HER2 over-producing cancer cells, rather than HER2 low-producing normal cells. Hence, a combinatory antitumor treatment of solamargine and trastuzumab produces a synergistic anti-proliferative effect against H661 and H69 lung cancer cells (C.-H. Liang et al., 2007). In breast cancer, HER2 up-regulation, which induces resistance to anticancer drugs, was diminished by the effect of solamargine on ZR-75-1 breast cancer cell line, via inhibition of HER2 expression that was demonstrated by RT-PCR and immunocytochemistry. In this context, Solamargine has exhibited an enhanced potent antitumor action on HER2-drug resistant overexpressing breast cancer cell line, over commonly used antitumor drugs as cisplatin and methotrexate. Moreover a combinatorial treatment of each of these drugs with solamargine has exhibited an increased vulnerability of the breast cancer cell line to each of these antitumor drugs in the presence of solamargine, indicating a promising potential of solamargine to be utilized in combinatorial therapy as an effective approach to counteract anticancer drug resistance (Shiu et al., 2008).

2.3.5. Solanine and apoptosis
Solanum nigrum derived Solanine has stimulated apoptosis in HepG2 cells in a dose dependent manner. Western blot exhibited reduction in the antiapoptotic Bcl-2 protein expression in a dose dependent manner, and hence avoid alteration of mitochondrial permeability by Bcl-2 and permit cytochrome c mitochondrial release for progression of apoptosis (Ji et al., 2008). On Hepatocarcinoma HepG2 cells solanine has also caused cytosolic and nuclear shrinkage, chromatin condensation and fragmentation and development of apoptotic bodies, all characteristic of apoptosis. It has also reduced the
weight of tumor developed in $S_{180}$ tumor bearing mice and lengthened survival duration of $H_{22}$ tumor bearing mice. Phosphatidylserine externalization was also detected; post 24 h treatment of HepG2 by solanine, via annexin V flow cytometry. Calcium release from mitochondria was also reported, that has increased proportionally with increased solanine concentrations. Using immunofluorescence, Bax was found to be upregulated, with a concurrent downregulation of Bcl-2, was detected, with a consequent reduction in the Bax/Bcl-2 ratio post 24 h treatment of HepG2 with solanine. Caspase-3 upregulation was observed post solanine treatment, as examined by the active caspase-3 assay, using its substrate; p-nitroaniline on a microplate (Ji & Gao, 2012).

On pancreatic cancer cells, solanine has induced apoptosis as detected by annexin V and propidium iodide flow cytometry, in a concentration dependent pattern. Upregulation of P53 and Bax as well as downregulation of Bcl-2 were also detected. In addition to the consequent cytosolic increase of cytochrome c, as a result of increased permeability of the mitochondrial membrane due to the increased Bax/Bcl-2 ratio, were also observed. Moreover, Caspase-3 was overexpressed post solanine treatment as confirmed by immunoblotting. In a xenograft nude mice model, downregulation of Bax and upregulation of the antiapoptotic Bcl-2, was also reported upon solanine treatment to the pancreatic tumor injected cells, confirming a role of solanine in inducing the intrinsic mitochondrial apoptotic pathway (Sun et al., 2014).
3. The objectives of this study;

This study tested the hypothesis that glycoalkaloids extracted from Solanaceae family have potential cytotoxic activity against hepatocellular and urinary bladder carcinoma, through induction of apoptosis and inhibition of proliferation. This was investigated through the following specific aims;

1- Performing general screening for the main extracts of Solanum seaforthianum and Solanum macrocarpon. This was achieved through the use of five different concentrations with one log cycle difference (10 fold serial dilutions), for determining the potential cytotoxicity against HepG2 hepatocellular carcinoma cell line.

2- Selecting the extracts with highest potency and performing general screening to compare the cytotoxic potency of compounds fractionated from those extracts with those of several standard Solanum alkaloids. This was achieved through the use of five different concentrations with one log cycle difference (10 fold serial dilutions), for determining the potential cytotoxicity against HepG2 hepatocellular carcinoma cell line.

3- Selecting the most potent compounds and comparing their anticancer potential through performing cytotoxic screening using eleven different concentrations with half log cycle difference (3 fold serial dilutions), for accurate and more precise determination of IC$_{50}$ values in both hepatocellular carcinoma (HepG2) and urinary bladder (SCaBER) cell lines.

4- Defining the underlying molecular mechanisms responsible for the cytotoxic effect of the most potent compounds selected in specific aim 3 by:

i) Evaluation of cell cycle-induced alterations.

ii) Evaluation of expression of cyclinD1 as a cell cycle progression marker.

iii) Evaluation of expression of proliferative markers; Ki67 and survivin.

iv) Evaluation of expression of apoptotic markers; Bax, caspase-9, caspase 3 and the antiapoptotic marker; Bel-2.
4. Materials and Methods

4.1 Preparation of extracts, fractions and pure compound:

I. Plant extracts

A. Ethanol extracts

The air-dried aerial parts of *Solanum seaforthianum* Andr. and *Solanum macrocarpon* L. (1000 g, each) were separately extracted with ethanol 90% by cold maceration till exhaustion. The solvent was then removed by vacuum distillation at a temperature not exceeding 40°C and the residue, in each case, saved for successive liquid-liquid fractionation. The process is described in details by Alsherbiny (2014).

B. Fractions of the ethanol extracts

The dry ethanol extracts of the two species under investigation were, separately, suspended in water and subjected to successive fractionation using solvents of increasing polarities viz., Hexane, chloroform, ethyl acetate and *n*-butanol saturated with water. The solvent, in each case, was removed by distillation under vacuum at a temperature not exceeding 40°C. The solvent-free extractives were then weighed and saved for further examination. The process is described in details by Alsherbiny (2014).

C. Fractions of Total glycoalkaloid

Acid base extraction method was adapted from Bushway *et al.* (1980) for mass extraction of glycoalkaloids, where 100 g. of the total alcohol extracts prepared from the dried aerial parts of *Solanum seaforthianum* Andr. and *Solanum macrocarpon* L. were acidified with 300 ml. 10% acetic acid then successively extracted with 20ml. ethyl acetate for several times. The acidic aqueous layer was separated and then alkalinized with 200ml. ammonia solution where precipitation take place, filtered and the residues were further purified by dissolution in acetic acid and re-precipitation by concentrated ammonia solution.
The precipitated glycoalkaloid was then filtered and dried by distillation under vacuum at a temperature not exceeding 40°C, where the solvent-free residues were collected as total glycoalkaloid of *Solanum seaforthianum* Andr. and *Solanum macrocarpon* L. assigned as Total glycoalkaloid extract of *Solanum seaforthianum* (TGASS) and Total glycoalkaloid extract of *Solanum macrocarpon* (TGASM) respectively.

**D. Isolation of pure alkaloid**

TGASS fraction was subjected to Vacuum Liquid Chromatography-Reverse Phase (VLC–RP) column, on gradient mobile phase starting from water till complete elution with methanol through 5% methanol increment gradually and fractions collection upon screening on RP-TLC (Reverse Phase-Thin Layer Chromatography) plates using P-anisaldehyde spraying reagent and MeOH: H₂O:NH₄OH (9:1:drops) as mobile phase were three major alkaloids were separated and identified as Solasodine, Solasonine and Solamargine by NMR (Nuclear Magnetic Resonance) analysis comparing with published data and using Co-TLC compared with authentic alkaloid sample. The process is described in details by Alsherbiny (2014).

**II- Individual standard alkaloids**

i) **Synthesis of Solanidine**: Solanidine was isolated from *Solanum tuberosum* as described by Attoumbré *et al.* (2013).

ii) **Synthesis of α-Solanine**: α-Solanine was isolated from; *Solanum chacoense* Bitter (wild potato) as described by Väänänen (2007).

iii) **Synthesis of Tomatidine**: Tomatidine was isolated from *Solanum chacoense* Bitter as described by Hunter *et al.* (1976).

iv) **Synthesis of Solasodine glucoside and Solasodine galactoside**: Using condensation of Solasodine with 2,3,4,6-tetraacetyl-α-bromo-D glucose or 2,3,4,6-tetraacetyl-α-bromo-D galactose respectively, as described in details by Pańczkowski & Wojciechowski (1994).

v) **Synthesis of N,O-Diacetyllosasodine**: Acetylation of solasodine was performed with Acetic anhydride as described in details by Bird *et al.* (1979).

vi) **Synthesis of N-Nitroso solamargine**: Solamargine was dissolved in acetic acid and treated with Sodium nitrite, as described in details by Shabana (1969).
All plant extracts and individual Standard alkaloids were kindly provided by Muhammed Als Alsherbiny, from the Pharmacognosy department. Faculty of Pharmacy. Cairo University.

4.2 Cell culture
The hepatocellular carcinoma cell line HepG2 and the urinary bladder cancer cell line SCaBER, were obtained from American Type Culture Collection (Rockville, Maryland, USA). HepG2 were grown in RPMI 1640 medium and SCaBER were grown in DMEM medium (GIBCO, Grand Island, NY), supplemented with 10% v/v heat inactivated fetal bovine serum and 5% penicillin/streptomycin, and were incubated at humidified 5% CO2 and maintained 37°C. HepG2 and SCaBER cells were cultured as a monolayer in T-25 flasks and were seeded to attain 70% confluency prior to seeding, propagation or treatment.

4.3 SRB Cytotoxicity assay
A- Principle
Sulforhodamine B (SRB) is a red fluorescent aminoxanthine dye containing two sulfonic moieties that binds to basic amino acids of cellular proteins fixed by trichloroacetic acid (TCA), within moderate acidic setting. TCA’s main function is fixation of cells via binding to their protein composition. SRB fluorescence is detected within a linear range at wavelength 540 nm. High fluorescence intensity of SRB dye, allows detection of number of cells, up to 1000 cells/well. On a more practical note, cells fixed with TCA and stained with SRB, are dried and provide the option of long term storage of the dried dye bound cells, until a suitable time for measurement, where dye is then dissolved with Tris-base, forming a stable red colored dye solution and the optical density is measured spectrophotometrically using plate reader (Vichai & Kirtikara, 2006). SRB is usually compared with MTT cytotoxicity assay. Although SRB is reported to have a limitation of binding to both viable and dead cells, as opposed to MTT assay (Skehan et al., 1990) which depends on cellular metabolism (specifically the action of succinate dehydrogenase in mitochondria) for reduction of the yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the purple formazan
precipitate that is solubilized later on for measuring the color spectrophotometrically (Plumb et al., 1989). Yet this limitation has not discredited SRB efficiency to evaluate cytotoxic action of different drugs, as several studies has confirmed that concurrent IC\textsubscript{50} values for both assays on the same compounds, with SRB demonstrating a marginal increase in the IC\textsubscript{50} values (Rubinstein et al., 1990; Perez et al., 1993; Haselsberger et al., 1996). This drawback in SRB has been totally eliminated in our study, since media was discarded and hence any dead floating cells were discarded, leaving only attached viable cells to bind to TCA for fixation, before application of the SRB dye. SRB surpasses MTT, as several compounds might interfere with the reduction activity of MTT, without having any contribution to cell viability (Plumb et al., 1989). SRB stain is insensitive to such interference and its binding and formation is not attributed to cellular metabolism and hence usually requires fewer optimization, providing a more efficient and sensitive assay for screening of cytotoxic drugs (Vichai & Kirtikara, 2006).

**B- Experimental method**

Cells were cultured in T-25 flasks as a monolayer, till 70 % confluency. Cells were then seeded into a 96 well microtiter plates, at a concentration of 3000 cells/well contained in 100 µl. Cells were left 24 h to attach and then treated with different concentrations of each main extract/drug, prepared in serial dilutions of 10 folds for general screening (0.1-1000 µg/ml or µM), or serial dilutions of 3 folds (from 0.001-100 µM) for accurate IC\textsubscript{50} value determination. Cells treated with equivalent amounts of Dimethyl Sulfoxide (DMSO) (not exceeding 0.1%, vol/vol) were used as controls. Cells were then incubated for 72 h, after which media was discarded and cells were fixed with 150 µl per well, 10% trichloroacetic acid (TCA) for 1 h at 4°C. Cells were then washed 3 times to remove any residual TCA, which hinders SRB protein interaction. Seventy microliters of 0.4% SRB (prepared through dissolving SRB powder in 1% acetic acid) were then added and cells were incubated at dark in room temperature for 10 minutes. Washing to discard excess unbound dye was then performed for three times by 1% acetic acid, until a colorless drainage is obtained. Microtiter plates were air dried for 24 h after which, the SRB-protein complex is solubilized with 150 µl/well of 10mM Tris base (pH 7.4), and plates were shaken at 1600 rpm for 5 minutes. Optical density is then assessed via spectrophotometric plate reader at 545nm. Wells containing DMSO were used for control
cell viability and represented 100% cell survival, and wells without cells for blanking the spectrophotometer. IC$_{50}$ values (which is the concentration at which 50% of the cells are dead) for each cell line were evaluated at a dose of drug causing 50% absorbance reduction in comparison with DMSO-treated control cells. The IC$_{50}$ values are then calculated via sigmoidal concentration-response curves that are produced using GraphPad Prism software, version 5.00 (GraphPad Software, La Jolla, CA).

4.4 Flow Cytometry

A- Principle
Flow Cytometric analysis of HepG2 post treatment was conducted using FACScalibur flowcytometer (BD biosciences). Cells were detached using trypsin, then cells were collected and lysed with nonionic detergent. RNA cellular content was eliminated by Ribonuclease A. Nuclear chromatin was then stained with propidium Iodide (PI). DNA-PI bound complex was detected using a flow cytometer, according to the diploid, tetraploid and aneuploid DNA content that distinguishes different phases of the cell cycle (Figure 1b) (Cooper et al., 2000).

B- Experimental method
HepG2 cells were cultured in T-25 flasks at a density of 5X10$^5$ cells and left to attach for 24 h, after which different treatments were applied at a concentration equivalent to their IC$_{50}$ values, for 72 h. Cells were then trypsinized and collected, centrifuged at 400 xg for 10 minutes at room temperature and media discarded. Pellet was re-suspended in PBS (Phosphate buffered saline) as a washing step, and then re-centrifuged at 400 xg for 10 minutes, after which PBS supernatant is discarded and pellet is dissolved in 250 µl of solution A (trypsin buffer) and is incubated at room temperature for 10 minutes. Two hundred microliters of solution B (trypsin inhibitor and RNase buffer), was then added and cells were incubated at room temperature for 10 minutes, after which 200 µl of cold solution C (propidium iodide stain) and cells were then incubated at 4°C for 10 minutes in the dark. Samples were then run on the flow cytometer (BD FACSCalibur, BD Biosciences) for DNA content analysis.
4.5 Immunocytochemistry

A- Principle

Cells seeded on sterile glass slides coated with poly-L-lysine to improve cell adhesion. Different treatments were applied as described above. At the end of exposure, cells were fixed with absolute ethanol and washed with PBS (pH 7.4). Permeabilization of cell membrane was done by 0.01% Triton X-100 in PBS for 1 min, after which cells were incubated with freshly prepared 10% H$_2$O$_2$ in methanol for 30 min to quench endogenous peroxidase activity and then washed with PBS. Blocking was then done with 5% normal horse serum in Tris-Buffered Saline plus Tween-20 (TBST), for 30 minutes was then done, to prevent non-specific binding of the secondary antibody. The slides were then incubated with the primary antibody followed by incubation with the biotin-linked secondary antibody. Streptavidin horseradish peroxidase (HRP) complex was then applied and binds through its streptavidin portion to the biotin portion of the secondary antibody. A colorimetric substrate specific to the HRP enzyme portion was then added, yielding colored cells expressing the protein examined, which was counted using Image J software (Javois, 1999).

B- Experimental Method

HepG2 cells were seeded on charged slides (Menzel Gläser, Braunschweig, Germany), pre-coated with sterile-filtered poly L-lysine, at a density of 5x10$^5$ cells/ml. After exposure to different treatments, cells were fixed with absolute ethanol, washed in phosphate buffered saline (PBS), incubated with 0.01% Triton X-100 in PBS for 1 min, followed by incubation with freshly prepared 10% H$_2$O$_2$ in methanol for 30 min and then washed with PBS (pH 7.4). Blocking was then performed using 5% normal horse serum in TBST for 30 min, followed by incubation with primary cyclin D1 rabbit polyclonal antibody (1:50 dilution, Thermo Fisher Scientific, UK) (100 µl- 200 µl of the diluted antibody added to each slide) overnight at humid chamber at 4ºC. In the following day the slides were incubated with the corresponding conjugated anti-rabbit IgG (1:2000 dilution, Santa Cruz Biotechnology Inc., Dallas, TX, USA). Cells were treated afterwards with streptavidin horseradish peroxidase complex (1:100 dilution, ABC/HRP;
Vector Laboratories, Burlingame, CA, USA) in TBST for 50 min. The color reaction was developed for 5 min in 3,3′-diaminobenzidine (DAB) solution (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Percent of DAB-positive cells per high power field was calculated as [(number of DAB-positive cells/ total number of cells) x100] using Image J software.

4.6 Real time PCR

A- Principle

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) is performed in a thermal cycler that will apply a light beam on each sample at a certain wavelength ($\lambda_{\text{max}} = 497$ nm), for detection of different fluorescence emission levels, released from each sample, that is corresponding to different mRNA (Messenger ribonucleic acid) expression levels in each sample (Rychlik et al., 1990). The qRT-PCR requires SYBR-Green mixture that contains dNTPs and polymerase within. SYBR-Green is cyanine dye that would bind to the nucleic acids of the cDNA (Complementary deoxyribonucleic acid) templates. When the cDNA templates are amplified by the action of the polymerase and primers, SYBR-Green dye becomes enclosed within double stranded DNA products. The dsDNA-dye complex absorbs light at the wavelength emitted by the thermal cycler ($\lambda_{\text{max}} = 497$) and consequently emits green light at wavelength ($\lambda_{\text{max}} = 520$), which is then detected and translated into amplification curve by the thermal cycler (Zipper et al., 2004). The more cDNA templates (corresponding to the mRNA levels expressed originally in the cells), the more green light emissions that will display as greater number of amplicon copies, at earlier cycles in the amplification curve, compared to less cDNA templates with lower green light emissions displaying as lower number of amplicon copies, at later cycles in the amplification curve. Green light emissions are measured with a value known as; fold change (RQ “Relative Quantification”), which will be explained shortly in details. The thermal cycler then captures through an internal camera, the green fluorescence emitted by the SYBR-Green dye that is equivalent to the quantity of the amplified cDNA product.
The delicacy and precision of qRT-PCR depends on accurate detection of SYBR-Green-dsDNA complex. One of the main limitations identified with recognition of the SYBR-Green fluorescence, is the detection of non-specific double strands as primer dimers or a non-desired amplicon produced as a result of non-specific primer annealing. Non-specific nucleic acid duplexes add up to the total SYBR-Green fluorescence and hence affect the precision of quantification. This has called for essential need to perform a melting curve, after the execution of the qRT-PCR amplification reaction (Ririe et al., 1997). The melting curve displays the negative first derivative of fluorescence change as a function of temperature. Temperature is increased to denature the double strand products formed as a result of the qRT-PCR, and thus reducing the SYBR-Green fluorescence produced, which results in increments in the negative first derivative of fluorescence change. Evaluation of the melting curve enables the assessment of the specific binding and dissociation between cDNA templates and primers and hence offering a precise estimation of the melting temperature (Tm) for each primer, which is the temperature at which 50% of the amount of template-primer duplex is separated, seen at the peak of the melting curve (Ririe et al., 1997). Other non-specific nucleic acids duplexes melt at other temperatures and thus produce another peak in the melting curve indicating their presence, which would interfere with the fluorescence signal contributing to the total fluorescent signal and hence affecting accuracy of quantification of the qRT-PCR nucleic acid duplex products, using the produced fluorescence signal. Hence, a melting curve showing a single peak (as illustrated in the Figure 4) referring to the double stranded amplicons produced by the qRT-PCR amplification reaction, with no other additional peaks, indicate a high quality qRT-PCR, free of non-specific double stranded nucleic acids products, ensuring that the fluorescence signal detected entirely indicates and equally corresponds to the quantity of the nucleic acids duplexes products generated by the qRT-PCR reaction.

B- Experimental Method
i) RNA extraction and cDNA synthesis
Quantitative evaluation of mRNA expression levels involves a four-step procedure. In the first step; total RNA was extracted using Qiagen RNeasy extraction kit (cat no: 74104).
After 48 h of treatment with; solanine, solamargine and nitroso-solamargine, HepG2 cells were collected with media post trypsinization, centrifuged at 10,000 rpm and the pellet was then rinsed with PBS, centrifuged at 6000 rpm and PBS was discarded. Pellet was then treated 350µl of RLT Lysis buffer and 3.5µl of β-mercaptoethanol, vortexed for 2 sec and then 350µl of 70% ethanol (prepared in RNase free water) is added. All contents of the tube are then transferred to the spin column, which is centrifuged at 10,000rpm for 15 sec, after which 700µl of RW1 buffer is added to the spin column, and again centrifuged at 10,000 rpm for 15 seconds. 500µl of RPE buffer is then added followed by a final centrifuge at 10,000 rpm for 15 sec, after which RNA at the spin column is eluted using 30 µl of RNase free water. Detailed RNA extraction procedure is further elaborated per manufacturer’s kit instructions. The second step involves RNA quantification using NanoDrop2000 Spectrophotometer. Reverse transcription was then performed in order to obtain the cDNA corresponding to the mRNA isolated from the treated HepG2 cells. cDNA synthesis was conducted using the (applied biosystems High Capacity cDNA Reverse Transcription kit). Each reaction consisted of; 2µl Reverse Transcriptase Buffer, 0.8ul dNTPs, 2µl random primer, 1µl Reverse Transcriptase Enzyme, 1µl RNase Inhibitor, 1µg of total RNA and completed with RNase-free water to form a total reaction of 20µl. cDNA was obtained using Biometra TProfessional Thermocycler (Germany), under the following conditions; 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and finally reaction stopped at 4°C.

ii) Quantitative reverse transcription real time PCR (qRT-PCR)
The qRT-PCR reaction consists of; 2µl of the cDNA template products, 12.5 µl RT² SYBR Green ROX qPCR mastermix (cat no: 330520, QIAGEN), 1µl of each of the forward and reverse primers, 8.5 µl of RNase free water, in a final volume of 25 µl reaction. Cycling conditions were as follows; initial denaturation step at 95°C for 10 minutes, followed by 45 cycles, each consisting of; denaturation at 95°C for 15sec, annealing step at 60°C for 1 min, and a final extension step at 60°C for 10 min. Melting curve conditions are started right after qRT-PCR completion and are as follows; 95°C for 15 sec, 60°C for 1 minute and 95°C for 15 sec.
For endogenous control, β-actin was used in each experiment as the housekeeping gene. Relative quantification for expression levels of each gene was determined using values of the cycle threshold (C_T), which is defined as the cycle at which the sample surpasses all background noise. (C_T) of different samples is then compared to determine different expression levels in different samples. The cycle threshold is inversely proportional to the amount of template (Rychlik et al., 1990). In brief; C_T values for β-actin were subtracted from C_T values of each of the following genes; Ki67 and Survivin, for both of the control and the treated samples. ΔΔ C_T is then obtained through subtracting Δ C_T of the control from Δ C_T of the treated sample for each of the genes. The ΔΔ C_T values are then translated into fold differences called RQ values, compared to untreated (control) cells, by raising 2 to the power of −ΔΔ C_T. Fold change measurements were performed at least twice independently, with each of the two experiments having triplicate wells for each gene. Real time PCR was performed using Applied Biosystems 7500 instrument (Life Technologies, USA) that has calculated fold change according to the previously mentioned equations. Sequences of primers used are provided in the following table;

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>F-5’-CTTGGGTGCGACTTGACG-3’ R-5’-GTCGACCCCGCTCCTTTT-3’</td>
</tr>
<tr>
<td>Survivin</td>
<td>F-5’-TGCCCCGACGTTGCC-3’ R-5’-CAGTTCTTGAATGAGATGCAGT-3’</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F-5’-TTCAGAGGGGATCGTTGTAG-3’ R-5’-CAAGCTTGTCGGCATACTG-3’</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>F-5’-GCAGACTAACAGGGCGAGC-3’ R-5’-ACCACGAGCAAGGAGC-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>F-5’-GCCATCCTGACGCTGAAGTA-3’ R-5’-GGGGTGTGAGCTCCTCAA-3’</td>
</tr>
</tbody>
</table>

**Table1. Sequence of primers used:** all having annealing temperature of 60°C.
**Figure 4.** Melting curve of qRT-PCR, showing single peak for the primer of each gene expressed, and thus demonstrating high quality of the qRT-PCR performed. The Y-axis represents the negative first derivative of fluorescence change, displayed as a function of the x-axis presenting the temperature in degree Celsius. The Tm of each primer is at the peak of its melting curve.
4.7 Western Blot

A- Principle
Western blot is a technique used to separate proteins according to their molecular weight on an SDS-polyacrylamide gel. Proteins run on two types of gels casted as one on top of the other. SDS is added to proteins to give them a net negative charge and proteins are denatured by heating and then loaded into the casted gel which consists of: The stacking gel; consisting of low polyacrylamide content that yields a gel with large pores, performing poor protein separation, yet allows proteins to stack into thin and sharp bands. Then the resolving gel, which consists of higher polyacrylamide content and hence yielding gel of narrow pores, resolves proteins according to their molecular weights efficiently. Proteins are loaded to the stacking gel and run throughout the whole resolving gel form negative to positive charge direction. Protein bands with smaller weights migrate faster in the gel than those with larger molecular weights. Resolved proteins on the polyacrylamide gel are then transferred to PVDF membrane again from the negative to the positive charge direction, within the assembled transfer sandwich. Protein containing-membrane is then blocked with 5% non-fat dry milk, diluted in TBST. Primary antibody is diluted in TBST and then added to the membrane for overnight incubation, after which membrane is washed three times with TBST and the secondary antibody (also diluted with TBST), is added for 1 h incubation at room temperature. After washing three times, chemiluminescent substrate that is specific to the secondary antibody is added and specific bands are detected through developing this specific chemiluminescence on an X-ray film (Mahmood & Yang, 2012).

B- Experimental Method
i) Protein extraction and quantification
HepG2 cells were seeded in T-75 flasks and incubated until reached 70% confluency, and then treated with IC$_{50}$ values of each of; solanine, solamargine and nitroso-solamargine, for 72 h. Cells were then collected with trypsin, washed with PBS and then lysed with ice-cold RadioImmunoPrecipitation lysis (RIPA) buffer (0.5 M Tris–HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA), for 30 minutes at 4°C shaking thermoblock. Centrifugation was done at 14000 rpm for 10 minutes at 4°C, and
the supernatant was transferred to a clean new microcentrifuge tube to collect lysate.

**ii) Protein Quantification and loading**

Protein Quantification of lysate was then performed using Pierce BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Scientific, USA) (cat no: 23227). Standard BSA with known concentrations was serially diluted and then 20µl of each standard concentration is added to a microcentrifuge tube containing 1ml reagent A and 20µl reagent B, which is then incubated at 37°C in a thermoblock for 30 minutes and then left to cool to room temperature. Optical densities of standard BCA concentrations are read spectrophotometrically at 562nm, and a standard curve is generated using excel software (*Figure 5*). To quantify protein lysate of each treatment, 20µl lysate is added to a microcentrifuge tube containing 1ml reagent A and 20µl reagent B, which is then incubated at 37°C in a thermoblock for 30 minutes and then left to cool to room temperature. Optical density measured at 562 nm and concentration is determined using the equation generated from the standard curve (*Figure 5*). Each sample is quantified in triplicates and the average is calculated.

**iii) Running and blotting**

Fifty micrograms of each protein lysate was combined with loading dye (10% glycerol, 0.5M Tris pH 6.8, 10% SDS, 5% β-mercapto-ethanol, bromophenol blue specks) and completed with RIPA buffer to 30µl, and boiled at 99°C for 10 minutes. 25 µl of each sample was then loaded and 5 µl of protein ladder (cat no: 26616, Thermo Scientific, USA), into wells of a 12% SDS PAGE gel using electrophoresis buffer (0.192 M glycine, 25mM Tris, 0.1% SDS). After electrophoresis, the gel was transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc., CA, USA) using transfer buffer (0.192 M glycine, 25mM Tris, 0.025% SDS, 10% methanol). Membranes were blocked in TBST with 5% non-fat dry milk and incubated overnight with the primary anti-Bax (1:1000). In the following day the membranes were washed and then incubated with secondary HRP-linked antibody (1:5000) (KPL Inc., USA). Detection was done by Abcam Optiblot ECL Detect Kit (Abcam, USA). Anti-β-tubulin antibody (1:20,000) (Sigma-Aldrich Co., St. Louis, MO, USA) was used to for loading correction.
**4.8 ELISA**

**A- Principle**

ELISA kits with microtiter plates pre-coated with primary antibody specific to **Caspase-3, Caspase-9, Bax and Bcl2** respectively. Standards or samples are then added to the appropriate microtiter plate wells. Then a secondary biotin-conjugated antibody specific to the protein captured by the primary well-coating antibody is added to bind the captured protein, forming a “sandwich” of specific antibodies around the desired protein in cell lysate. Streptavidin-HRP complex is then used to bind to the biotin linked secondary antibody through its streptavidin portion and to react with the TMB substrate, which is added subsequently, through the HRP portion. The colored product is measured at wavelength of 450nm ± 10nm, after reaction termination via the addition of sulfuric acid solution. The concentration of the protein evaluated in each sample is then determined by comparing the O.D. of the samples to the standard curve.

**B- Experimental method**

Cells are seeded in T-25 flasks until 70% confluency and then treated with IC<sub>50</sub> concentration of each treatment for 72 h, after which cells are trypsinized and collected by centrifugation in a conical tube at 300 xg for 10 min. Supernatant was discarded and
pellet of each treatment is lysed with 100 µl ice cold RIPA lysis buffer, incubated for 30 minutes in ice and then centrifuged at 14,000 rpm for 10 min, after which supernatant lysate is transferred to a new clean tube.

Total protein content of the lysate was estimated using Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then 100 µl of each of; standard dilutions, blank and samples are added into the appropriate wells. The wells were covered with the plate sealer. Incubate for 2 h at 37°C, after which liquid in wells was discarded and 100 µl of Detection Reagent A working solution is added to each well. Plate is covered with a seal and incubated for 1 h at 37°C. Solution was aspirated and wells were then washed with 350 µl of 1X Wash Solution, incubated for 2 min and then discarded. Washing is done three times after which plate is inverted onto absorbent paper, to remove any remains of washing buffer. Hundred microliters of solution B are then added to each well, plates were covered with a seal and incubated for 30 min at 37°C. The aspiration/washing steps were repeated for three times, after which 90 µl of substrate solution is added to each well and plate is incubated for 20 min at 37°C in the dark. Optical density of the colored product formed is measured at 450 nm using spectrophotometer plate reader, after stopping the reaction by addition of 50 ul of stop solution to each well, which will turn the liquid color into yellow.

The plate coated with the primary antibody and Reagent A (which is the secondary antibody), will vary according to the protein being quantified by the four kits; Capsase-3 (cat no: E90626Mu, Uscn, China), Caspase-9 (cat no: CSB-E08862H, Cusabio, China), Bax (cat no: ADI-900-138, Abnova, USA) and Bcl-2 (cat no: ADI-900-133, Abnova, USA).

4.9 Statistical Analysis
Experiments were done in triplicates. Data are represented as mean with standard deviation or standard error of mean (results of real time PCR and western blot). Statistical comparisons were performed using one-way ANOVA (analysis of variance) with Tukey HSD as post hoc test. P values: < 0.05, <0.1 and <0.001 were regarded as statistically significant.
5. Results

![Diagram of scheme illustrating sequence of screening of the potential cytotoxic compounds for cytotoxicity and IC<sub>50</sub> value of each. But SM = Butanol extract of Solanum macrocarpon, Total Alc SM = Total alcoholic extract of Solanum macrocarpon, TGAS = Total glycoalkaloid extract of Solanum macrocarpon, But SS = Butanol extract of Solanum seaforthianum, Total Alc SS = Total alcoholic extract of Solanum seaforthianum and TGASS = Total glycoalkaloid extract of Solanum seaforthianum.]

5.1 Cytotoxicity screening of the six main extracts of Solanum macrocarpon and Solanum seaforthianum

SRB has shown that among the six main extracts, TGASS has exhibited the lowest IC<sub>50</sub> values of 1.55±0.046 µg/ml, followed by But SS (Butanol extract of Solanum seaforthianum) with IC<sub>50</sub> values of 17.9±1.155 µg/ml. The total alcoholic extracts of both SS and SM, has exhibited the lowest cytotoxicity activity, with the highest IC<sub>50</sub> values of 53.4±0.349 and 44.7±1.105 µg/ml, respectively. TGAS and But SM (Butanol extract of Solanum macrocarpon) acquired IC<sub>50</sub> values, lying between the previously mentioned highest and lowest IC<sub>50</sub> values. TGA SS IC<sub>50</sub> value was 23 folds lower than that of TGA SM (Table 2, Figure 6 and Figure 7).
5.1.1 Cytotoxicity screening of individual compounds, including those fractionated from TGASS extract.

Three compounds were fractionated from TGASS; Solamargine, Solasonine and Solasodine. Cytotoxicity screening in HepG2 cells was performed for these compounds as well as other, standard alkaloids that are frequently derived from the Solanum genus of plants; Solanidine, α-solanine, Tomatidine. This is in addition to the nitroso-derivative; Nitroso-Solamargine, as well as Solasodine modified compounds; Solasodine galactoside, Solasodine glucoside and O.N-diacyl solasodine.

Solanidine had the weakest cytotoxic effect, with the highest IC$_{50}$ value (777.8 µM) followed by O.N-diacyl solasodine with IC$_{50}$ value (226.7 µM), then by Tomatidine with IC$_{50}$ value (130.22 µM) and then Solasodine with IC$_{50}$ value (97.93 µM). Solasonine has exhibited a considerably low IC$_{50}$ value of (42.9 µM), that were comparable with those exhibited by Solasodine galactoside and Solasodine glucoside (37.3 µM and 45.2 µM), respectively. Solasodine galactoside had a 2.6 folds (61.9%) reduced IC$_{50}$ value, than that of Solasodine, whereas, Solasodine glucoside exhibited a 2.2 folds (53.8%) reduction of the IC$_{50}$ value than that of Solasodine. As opposed to the latter reductions of the IC$_{50}$ values, that took place upon modifications on the solasodine, O, N diacetyl Solasodine has exhibited a 2.3 folds (56.8%) increase in the IC$_{50}$ values than that of Solasodine (Table 2, Figure 6 and Figure 8). Three compounds were found to acquire the most potent cytotoxic activity on HepG2; α-Solanine, Solamargine and Nitroso-Solamargine, exhibiting very low IC$_{50}$ values of; (2.54 µM, 3.21 µM and 2.41 µM) respectively (Table 2, Figure 6 and Figure 8).

5.1.2 IC$_{50}$ Determination for the most potent compounds; α-Solanine, Solamargine and Nitroso-Solamargine on hepatocellular carcinoma (HepG2) and urinary bladder (SCaBER) cell lines.

The tested agents were evaluated using eleven different concentrations with half log cycle difference (serial dilutions of 3 folds) in the range 0.001 µM to 100 µM, in order to get more accurate values for IC$_{50}$ of the tested agents.
Nitroso-solamargine exhibited significant (p < 0.01) lower IC$_{50}$ values of (7 µM and 5.91 µM), than those of Solamargine (11.9 µM and 8.9 µM), on both of HepG2 and SCaBER cell lines respectively (figures 9 and 10). IC$_{50}$ value of Nitroso-Solamargine was reduced by 1.7 folds (41.2%) than that of solamargine on HepG2 cell line (figure 9). On SCaBER cell line as well, 1.5 folds reduction (33.6%) of nitroso-solamargine IC$_{50}$ value than that of solamargine was also recorded (figure 10). IC$_{50}$ values of Solanine (6.6 µM and 6.5 µM) were comparable on both cell lines; HepG2 and SCaBER cell lines respectively (figure 11), and were significantly lower than that of solamargine (p <0.01) (figures 9 and 10), whereas Solamargine had exhibited a more potent cytotoxic effect on urinary bladder cancer SCaBER, with IC$_{50}$ of (8.9 µM), that is significantly lower (p < 0.01) than that exhibited on Hepatocellular carcinoma HepG2 cell line (11.9 µM), demonstrating a 1.3 folds reduction (25.2%) in the SCaBER cell line IC$_{50}$ value (figure 11). Nitroso-solamargine had exhibited concurrent cytotoxic activity, with lower IC$_{50}$ value on SCaBER (5.91 µM) than that on HepG2 (7 µM), demonstrating a 1.2 folds reduction (15.6%) in the SCaBER cell line IC$_{50}$ value (Table 2 and figure 11).
Table 2: IC₅₀ values of the tested agents using SRB cytotoxicity assay in HepG2 and SCaBER cell lines.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average IC₅₀±SD (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Main Extracts</td>
<td></td>
</tr>
<tr>
<td>ButSM</td>
<td>36.82±1.32</td>
</tr>
<tr>
<td>Total Alc SM</td>
<td>44.66±1.11</td>
</tr>
<tr>
<td>TGASM</td>
<td>35.77±1.22</td>
</tr>
<tr>
<td>ButSS</td>
<td>17.92±1.16</td>
</tr>
<tr>
<td>Total Alc SS</td>
<td>53.36±0.35</td>
</tr>
<tr>
<td>TGASS</td>
<td>1.55±0.05</td>
</tr>
<tr>
<td>B) Individual compounds</td>
<td></td>
</tr>
<tr>
<td>Solanidine</td>
<td>777.8±1.23</td>
</tr>
<tr>
<td>α-solanine</td>
<td>2.54±0.13</td>
</tr>
<tr>
<td>Tomatidine</td>
<td>130.22±1.18</td>
</tr>
<tr>
<td>Nitroso-solamargine</td>
<td>2.41±0.16</td>
</tr>
<tr>
<td>Solamargine</td>
<td>3.21±0.25</td>
</tr>
<tr>
<td>Solasonine</td>
<td>42.93±0.62</td>
</tr>
<tr>
<td>Solasodine</td>
<td>97.93±1.33</td>
</tr>
<tr>
<td>Solasodine galactoside</td>
<td>37.34±1.33</td>
</tr>
<tr>
<td>Solasodine glucoside</td>
<td>45.21±1.14</td>
</tr>
<tr>
<td>O.N-diacetyl solasodine</td>
<td>226.75±1.45</td>
</tr>
<tr>
<td>C) Three most potent on HepG2</td>
<td></td>
</tr>
<tr>
<td>α-solanine</td>
<td>6.6±0.8</td>
</tr>
<tr>
<td>Solamargine</td>
<td>11.87±1.46</td>
</tr>
<tr>
<td>Nitroso-solamargine</td>
<td>7±0.3</td>
</tr>
<tr>
<td>D) Three most potent on SCaBER</td>
<td></td>
</tr>
<tr>
<td>α-solanine</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td>Solamargine</td>
<td>8.9±0.4</td>
</tr>
<tr>
<td>Nitroso-solamargine</td>
<td>5.91±0.11</td>
</tr>
</tbody>
</table>

**Table 2: Collective IC₅₀ values after cytotoxicity screening via SRB assay.**

A) General screening of the main extracts of the two plants; HepG2 cells were treated with main extracts in a range of five concentrations, with one log cycle difference (serial dilutions
of 10 folds), between each concentration and the following one (0.1-1000 µg/ml), for 72 h. But SM = Butanol extract of *Solanum macrocarpon*, Total Alc SM = Total alcoholic extract of *Solanum macrocarpon*, TGASM = Total glycoalkaloid extract of *Solanum macrocarpon*, But SS = Butanol extract of *Solanum seaforthianum*, Total Alc SS = Total alcoholic extract of *Solanum seaforthianum* and TGASS = Total glycoalkaloid extract of *Solanum seaforthianum*. B) General screening of individual compounds including those fractionated from the main extracts; HepG2 cells were treated with individual compounds in a range of five concentrations, with one log cycle difference (serial dilutions of 10 folds), between each concentration and the following one (0.1-1000 µM), for 72 h. The three most potent compounds were then screened further for more accurate determination of IC$_{50}$ values; C) HepG2 cells were treated with the three most potent compounds, in an extended range of eleven concentrations, with half log cycle difference (serial dilutions of 3 folds), between each concentration and the following one (0.001-100 µM), for 72 h. D) ScaBer cells were treated with the three most potent compounds in an extended range of eleven concentrations, with half log cycle difference (serial dilutions of 3 folds), between each concentration and the following one (0.001-100 µM), for 72 h. IC$_{50}$ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 7a. Concentration-response plots obtained after exposure of HepG2 cell line to the main extracts of *Solanum macrocarpon* (0.1-1000 µg/ml) for 72 h. But SM = Butanol extract of *Solanum macrocarpon*, Total Alc SM = Total alcoholic extract of *Solanum macrocarpon* and TGASM = Total glycoalkaloid extract of *Solanum macrocarpon*. The cells were then fixed and stained with Sulforhodamine B (SRB). IC\(_{50}\) values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 7b. Concentration-response plots obtained after exposure of HepG2 cell line to the main extracts of *Solanum seforthianum* (0.1- 1000 µg/ml), for 72 h. But SS = Butanol extract of *Solanum seforthianum*, Total Alc SS = Total alcoholic extract of *Solanum seforthianum* and TGASS = Total glycoalkaloid extract of *Solanum seforthianum*. The cells were then fixed and stained with Sulforhodamine B (SRB). IC$_{50}$ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>But SS</td>
<td>17.92±1.16µg/ml</td>
</tr>
<tr>
<td>Total Alc SS</td>
<td>53.36±0.35 µg/ml</td>
</tr>
<tr>
<td>TGA SS</td>
<td>1.55±0.05 µg/ml</td>
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Figure 7c. Comparison of the general Screening of Main Extracts. Comparison of IC$_{50}$ values of the main extracts of, *Solanum macrocarpon* (SM) and *Solanum seaforthianum* (SS) in HepG2 cell line (0.1- 1000 µg/ml) for 72 h treatment. But SM = Butanol extract of *Solanum macrocarpon*, Total Alc SM = Total alcoholic extract of *Solanum macrocarpon*, TGASM = Total glycoalkaloid extract of *Solanum macrocarpon*, But SS = Butanol extract of *Solanum seaforthianum*, Total Alc SS = Total alcoholic extract of *Solanum seaforthianum* and TGASS = Total glycoalkaloid extract of *Solanum seaforthianum*. 
Figure 8a. Concentration-response plots obtained after exposure of HepG2 cell line to Solanidine, α-Solanine, Tomatidine and Nitroso-Solamargine (0.1-1000 µM), for 72 h. The cells were then fixed and stained with Sulforhodamine B (SRB). IC$_{50}$ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 8b. Concentration-response plots obtained after exposure of HepG2 cell line to Solamargine, Solasonine, and Solasodine (0.1-1000 µM), for 72 h. The cells were then fixed and stained with Sulforhodamine B (SRB). IC$_{50}$ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 8c. Concentration-response plots obtained after exposure of HepG2 cell line to Solasodine galactoside, Solasodine glucoside, and O, N-diacyl solasodine (0.1-1000 µM), for 72 h. The cells were then fixed and stained with Sulfurhodamine B (SRB). IC<sub>50</sub> values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 8d. Chart comparing the average IC$_{50}$ values of the screened alkaloids. Solanidine, α-Solanine, Tomatidine, Nitroso-Solamargine, Solamargine, Solasonine, Solasodine, Solasodine galactoside, Solasodine glucoside and O, N-diacetyl solasodine in HepG2 cell line. The compounds were screened at a concentration range of (0.1- 1000 µM), for 72 h.
Figure 9a. Concentration-response plots obtained after exposure of HepG2 cell line to α-Solanine, Solamargine, and Nitroso-Solamargine (0.001-100 µM), for 72 h. The cells were then fixed and stained with Sulforhodamine B (SRB). IC₅₀ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 9b. Chart comparing the average IC$_{50}$ values of α-Solanine, Solamargine, and Nitroso-Solamargine in HepG2 cell line. The compounds were tested at concentrations (0.001-100 µM), for 72 h. ** significantly different at P <0.01.
Figure 10a. Concentration-response plots obtained after exposure of SCaBER cell line to α-Solanine, Solamargine, and Nitroso-Solamargine (0.001- 100 µM), for 72 h. The cells were then fixed and stained with Sulforhodamine B (SRB). IC$_{50}$ values were expressed as the mean ± SD of three independent experiments, each carried out in triplicate wells (n=3).
Figure 10b. Chart comparing the average IC$_{50}$ values of α-Solanine, Solamargine, and Nitroso-Solamargine in SCaBER cell line. The compounds were tested at a concentration range of (0.001-100 µM), for 72 h. ** Significantly different at P <0.01.
Figure 11. Comparing IC\textsubscript{50} values of the three most potent compounds; \(\alpha\)-Solanine, Solamargine and Nitroso-Solamargine in both of the tested cell lines (HepG2 and SCaBER). ** Significantly different at P <0.01.
5.2 Cell Cycle alterations

Examining possible cell cycle alterations as a result of HepG2 treatment with the three most potent compounds; Solanine, Solamargine and Nitroso-Solamargine, for 72 h, has yielded the following; all of the three have induced a Pre-G peak, indicative of dead cells. Nitroso-Solamargine has produced the most significant (P < 0.001) increase of Pre-G percentage of cells by 56.1 folds (99.4%), compared to control. This was followed by the significant (P <0.001) increase in the pre-G induced by solamargine producing increase by 47.6 folds (97.9%), compared to control. The least pre-G induction was generated by α-Solanine, demonstrating also a significant (P < 0.001) 37 folds (97.3%) increase of the percentage of cells at pre-G (figures 12, 13a and Table3). Nitroso-Solamargine pre-G percentage of cells was significantly (P < 0.01) greater than that of Solamargine by 1.2 folds (15%) and significantly (P <0.001) greater than that of α-Solanine by 1.5 folds (34%). Solamargine has also induced a significant (P < 0.001) increase in the pre-G than that of α-Solanine by 1.3 folds (22.3%) (Figures 12, 13a and table3).

This was also associated with a simultaneous significant (P < 0.001) reduction of G0/G1 phase by 1.8 (45.4%), 1.7 (42.1%), 2 folds (51.4%) for α-solanine, nitroso-solamargine and solamargine, respectively compared to control. No significant difference was observed in the G0/G1 cells’ percentage between the three treatments (Figures 12, 13b and table3). The S-phase was also reduced by the three glycoalkaloids, with Nitroso-solamargine exhibiting the least S-Phase with significant (P <0.001) reduction by 2.25 folds (55.6%), followed by a significant (P < 0.001) reduction by 1.9 folds (46.4%) for solamargine; compared to control. α-Solanine has exhibited a 1fold (4.96%) reduction compared to control. Nitroso-Solamargine has exhibited a significant (P < 0.05) reduction in S-phase by 1.2 fold (17.3%) compared to Solamargine. α-Solanine had a significant (P <0.001) increase in the percentage of cells at S-phase by 1.8 folds (43.6%) and 2 folds (53.3%) compared to Solamargine and Nitroso-Solamargine, respectively (Figures 12, 13c and table3).

The percentage of cells at G2/M phase also exhibited a significant (P <0.001) reduction by 2 folds (52.1%), 1.9 folds (48%) and 1.6 folds (36.9%) after treatment with α-
Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. No significant difference was recorded between the G2/M phase cell percentages, between the three treatments (Figures 12, 13d and table3).

Overall, the three compounds: α-Solanine, Solamargine and Nitroso-Solamargine have inhibited the G0/1 and G2/M phases, as well as increased the Pre-G phase. Whereas, only Solamargine and Nitroso-Solamargine have inhibited the S-phase, with Nitroso-Solamargine exhibiting the greatest inhibition (Figure 13e). This was further confirmed by estimating the proliferation index (PI) according to the equation PI (%)= (S+G2/M)/(G0/G1+S+G2/M) x 100, as indicated by Li et al. (2004). α-Solanine, Solamargine and Nitroso-Solamargine exhibited significant (P < 0.001) reduction in the proliferation index by 1.2 folds (13.4%), 1.9 folds (46.7%) and 2.1 folds (52.3%), respectively compared to control. Nitroso-Solamargine exhibited a significant (P < 0.05) reduction in PI by 1fold (10.5%) compared to α-Solamargine, whereas, α-Solanine exhibited a significant (P <0.001) increase in PI by 1.6 folds (38.5%) and a 1.8 folds (44.9%) compared to α-Solamargine and Nitroso-Solamargine, respectively (Figure 14).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pre-G (%)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.93 ± 0.14</td>
<td>63.26 ± 0.43</td>
<td>29.42 ± 0.24</td>
<td>6.39 ± 0.61</td>
</tr>
<tr>
<td>α-Solanine</td>
<td>34.41 ± 3.28</td>
<td>34.57 ± 4.49</td>
<td>27.96 ± 1.6</td>
<td>3.06 ± 0.39</td>
</tr>
<tr>
<td>Solamargine</td>
<td>44.31 ± 1.18</td>
<td>36.6 ± 0.98</td>
<td>15.77 ± 0.005</td>
<td>3.32 ± 0.2</td>
</tr>
<tr>
<td>Nitroso-Solamargine</td>
<td>52.18 ± 0.49</td>
<td>30.73 ± 0.25</td>
<td>13.05 ± 0.27</td>
<td>4.03 ± 0.27</td>
</tr>
</tbody>
</table>

Table 3. Percentage of cells in each phase of the cell cycle at 72 h after treatment of the HepG2 cell line with α-Solanine, α-Solamargine and Nitroso-Solamargine. Control: untreated HepG2 cells. Data are expressed as cell percentage in each phase (means ± SD) of three independent determinations. *** Significantly different from control at P <0.001.
Figure 12. Effect of α-Solanine, α-Solamargine and Nitroso- Solamargine on the cell cycle phases distribution at 72h treatment in HepG2 cells. Control: untreated HepG2 cells. a) Flow Cytometry charts. b) Cumulative bar chart of cell cycle phases. The cells were treated with concentration equal to IC$_{50}$ of each compound. Data are expressed as cell percentage in each phase (means ± SD) of three independent determinations.
Figure 13a. Percentage of HepG2 cells in Pre-G phase at 72 h of treatment with; Solanine, Solamargine and Nitroso-Solamargine. Control: untreated HepG2 cells.

** Significantly different at P <0.01 and *** Significantly different at P <0.001. Data are expressed as cell percentage (mean ± SD) of three independent determinations.
Figure 13b. Percentage of HepG2 cells in G0/G1 phase, at 72 h treatment with; Solanine, Solamargine and Nitroso-Solamargine. Control: untreated HepG2 cells. ***Significantly different at P<0.001. Data are expressed as cell percentage (mean ± SD) of three independent determinations.
Figure 13c. Percentage of HepG2 cells in S-Phase, at 72 h treatment with; Solanine, Solamargine and Nitroso-Solamargine. Control: untreated HepG2 cells.

*Significantly different at P <0.05 and ***Significantly different at P <0.001. Data are expressed as cell percentage (mean ± SD) of three independent determinations.
Figure 13d. Percentage of HepG2 cells in G2/M phase, at 72 h treatment with; Solanine, Solamargine and Nitroso-Solamargine. Control: untreated HepG2 cells. ***Significantly different at P<0.001. Data are expressed as cell percentage (mean ± SD) of three independent determinations.
Figure 13e. Comparing distribution of cells in each phase of the cell cycle at 72 h treatment of HepG2 with; Solanine, Nitroso-Solamargine and Solamargine. Control: untreated HepG2 cells. Data are expressed as (mean ± SD) of three independent determinations.
Figure 14. Proliferation index of HepG2 cells, at 72 h of treatment with; Solanine, Solamargine and Nitroso-Solamargine. Data are expressed as (mean ± SD) of three independent determinations. Control: untreated HepG2 cells.

* Significantly different at P <0.05 and *** Significantly different at P <0.001.
5.3 Cyclin D1 Immunocytochemistry
Examining the expression of the cell cycle inducer, cyclin D1 was performed by evaluation of percentage of cyclin D1 positive cells at 72 h treatment with Solanine, Solamargine and Nitroso-Solamargine. This has yielded significant (P < 0.001) reduction of cyclin D1 by 2 folds (50%) and 2.6 folds (60.9%) for Solamargine and Nitroso-Solamargine, respectively compared to control. Solamargine also exhibited a significant (P <0.01) reduction of cyclinD1 expression by 1.7 folds (41.2%) compared to that of Solanine. Nitroso-Solamargine exhibited a more significant (P <0.001) reduction of cyclin D1 by 2.2 folds (53.9%) compared to that of solanine. Solanine reduced cyclin D1 by 1.2 folds (15.1%) compared to that of control. Nitroso-Solamargine reduced the abundance of cyclin D1 by 1.3 folds (21.7%) compared to that of Solamargine (Figure 15).
Figure 15. Immunocytochemical evaluation of the effect of Solanine, Solamargine, and Nitroso-solamargine on cyclin D1 expression in HepG2 cells at 72 h. Control: untreated HepG2 cells. Data are expressed as average percentage of cyclin D1 positive cells (mean ± SD) of three independent determinations (n=3). ** Significantly different at P <0.01 and *** Significantly different at P <0.001.
5.4 Real time PCR

Evaluation of the levels of mRNA transcripts of caspse-9, -3, ki67 and survivin was done using reverse transcriptase q-RT-PCR. Our data revealed that caspase-3 was upregulated by (2.6, 1.47 and 1.78 folds) with (61.5%, 32% and 43.8%) after treatment with Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Only Solanine fold change compared to control was statistically significant (P <0.05). Nitroso-Solamargine exhibited higher fold change value of caspase-3, with (14.7%) increase than that of Solamargine (Figure 16a and 16b). Caspase-9 was also significantly upregulated by 77.4%, 67.8% and 74% increase in the fold changes in cells treated with solanine, solamargine and nitroso-solamargine, respectively compared to control. Although Nitroso-Solamargine exhibited higher upregulated RQ values than that of Solamargine, yet no significant difference was recorded in caspase-9 levels between the three treatments (Figure 17a and 17b).

The gene expression of proliferation markers as Ki67 and survivin was also evaluated at 72 h of treatment. For Ki67; Solanine, Solamargine and Nitroso-Solamargine have significantly reduced fold change by 42%, 40% and 77%, respectively compared to control. Nitroso-Solamargine has exhibited a significant (P < 0.01) highest reduction of Ki67 expression by 61.7% and 60.3% than that of Solamargine and Solanine, respectively (Figure 18a and 18b). Survivin exhibited comparable results with significant (P < 0.001) reduction by 55%, 55% and 75% after treatment with Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Nitroso-Solamargine also exhibited the most significant (P <0.01) reduction of survivin gene expression by 44.4% compared to both of Solamargine and Solanine (Figure 19a and 19b).

Fold changes (RQ) mentioned above were calculated automatically by the real time PCR machine, after normalizing against β-actin $C_T$ (Figure 20), according to the following equations:

- $\Delta C_T$ of control = $C_T$ of gene of interest in control sample - $C_T$ of housekeeping gene (β- actin) in control sample.
• $\Delta C_T$ of treated Sample = $C_T$ of gene of interest in treated sample - $C_T$ of housekeeping gene (β-actin) in treated sample.

• $\Delta\Delta C_T = \Delta C_T$ of treated Sample - $\Delta C_T$ of control

• $\text{RQ (fold change)} = 2^{\Delta\Delta CT}$

A comprehensive comparison of fold change (RQ) values of each treatment on HepG2 cells, revealed an upregulation of caspase-3 and caspase-9 by Solanine, Solamargine and Nitroso-Solamargine. Nitroso-Solamargine exhibited higher fold change values than that of Solamargine for both apoptotic markers. A reduction of fold change values for both of Ki67 and survivin was observed with the three treatments. Nitroso-Solamargine exhibited the least fold change values for both proliferation markers that are lower than those of Solamargine (Figure 21).
Figure 16a. Effect of Solanine, Solamargine and Nitroso-Solamargine on caspase-3 gene expression. HepG2 cell line was exposed to the IC$_{50}$ of each compound for 72 h. Control: untreated HepG2 cells. Data are expressed as (mean ± SEM) of RQ values of three independent determinations, each carried out in triplicate wells (n=3). *Significantly different at P <0.05.
Figure 16b. qRT-PCR quantification curve for caspase-3, representative of three independent experiments. The X-axis represents the number of cycles of the PCR reaction and the y-axis represents the number of amplicons in the form of log (ΔRn). Rn is the fluorescence emitted by the reporter dye (SYBR green), normalized to the fluorescence of the passive reference dye (ROX). The threshold line determines accurate quantification; the intersection point between the threshold line and the amplification curve of each sample produces the $C_T$ (threshold cycle) of each sample for caspase-3 gene.
Figure 17a. Effect of Solanine, Solamargine and Nitroso-Solamargine on caspase-9 gene expression. HepG2 cell line was exposed to the IC$_{50}$ of each compound for 72 h. Data are expressed as (mean ± SEM) of RQ values of three independent determinations, each carried out in triplicate wells (n=3). *Significantly different at P <0.05, **significantly different at P <0.01 and *** significantly different at P <0.001.
Figure 17b. qRT-PCR quantification curve for caspase-9, representative of three independent experiments. The X-axis represents the number of cycles of the PCR reaction and the y-axis represents the number of amplicons in the form of log (ΔRn). Rn is the fluorescence emitted by the reporter dye (SYBR green), normalized to the fluorescence of the passive reference dye (ROX). The threshold line determines accurate quantification; the intersection point between the threshold line and the amplification curve of each sample produces the $C_T$ (threshold cycle) of each sample for caspase-9 gene.
Figure 18a. Effect of Solanine, Solamargine and Nitroso-Solamargine on Ki67 gene expression. HepG2 cell line was exposed to the IC₅₀ of each compound for 72 h. Data are expressed as (mean ± SEM) of RQ values of three independent determinations, each carried out in triplicate wells (n=3). ** Significantly different at P <0.01 and *** significantly different at P <0.001.
Figure 18b. qRT-PCR quantification curve for Ki67, representative of three independent experiments. The X-axis represents the number of cycles of the PCR reaction and the y-axis represents the number of amplicons in the form of log (ΔRn). Rn is the fluorescence emitted by the reporter dye (SYBR green), normalized to the fluorescence of the passive reference dye (ROX). The threshold line determines accurate quantification; the intersection point between the threshold line and the amplification curve of each sample produces the $C_T$ (threshold cycle) of each sample for Ki67 gene.
Figure 19a. Effect of Solanine, Solamargine and Nitroso-Solamargine on Survivin gene expression. HepG2 cell line was exposed to the IC$_{50}$ of each compound for 72 h. Data are expressed as (mean ± SEM) of RQ values of three independent determinations, each carried out in triplicate wells (n=3). ** Significantly different at P <0.01 and *** significantly different at P <0.001.
Figure 19b. qRT-PCR quantification curve for survivin, representative of three independent experiments. The X-axis represents the number of cycles of the PCR reaction and the y-axis represents the number of amplicons in the form of log (ΔRn). Rn is the fluorescence emitted by the reporter dye (SYBR green), normalized to the fluorescence of the passive reference dye (ROX). The threshold line determines accurate quantification; the intersection point between the threshold line and the amplification curve of each sample produces the CT (threshold cycle) of each sample for survivin gene.
Figure 20. qRT-PCR quantification curve for β-actin, representative of three independent experiments. The X-axis represents the number of cycles of the PCR reaction and the y-axis represents the number of amplicons in the form of log (ΔRn). Rn is the fluorescence emitted by the reporter dye (SYBR green), normalized to the fluorescence of the passive reference dye (ROX). The threshold line determines accurate quantification; the intersection point between the threshold line and the amplification curve of each sample produces the $C_T$ (threshold cycle) of each sample for β-actin gene.
Figure 21. Comprehensive chart displaying fold change (RQ) values of the tested genes; Caspase-3, caspase-9, Ki67 and survivin. Data are expressed as (mean ± SEM) of RQ values of three independent determinations, each carried out in triplicate wells (n=3).
5.5 Western Blot

Evaluation of the protein expression of the intrinsic apoptotic marker Bax was performed using western blot. The three treatments; Solanine, Solamargine and Nitroso-Solamargine has induced significant (P <0.001) overexpression of Bax protein by 82.8%, 74.7% and 84.6%, respectively compared to control. Nitroso-Solamargine-induced overexpression of Bax was significantly (P < 0.01) higher than that of Solamargine by 39.2%, whereas Solanine overexpression of Bax was significantly (P <0.05) higher than that of Solamargine by 32% (Figure 22).
Figure 22a. Effect of Solanine, Solamargine and Nitroso-Solamargine on Bax protein expression in HepG2 cells. Data were expressed as Bax/β-tubulin ratio ± SEM. * Significantly different at P <0.05, ** significantly different at P <0.01 and ***significantly different at P <0.001.

Figure 22b. Western blot for Bax. Representative bands of experiment that is performed in triplicate. Bands are normalized using β-tubulin as loading control.
5.6 ELISA

The protein expression of intrinsic apoptosis pathway markers’ (Bax, Bcl-2, caspase-9 and caspase-3) was quantified via ELISA. Bax protein was induced significantly (P <0.001) by 78.4%, 65.2% and 69.8% after treatment with Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Nitroso-Solamargine exhibited significant (P <0.01) increase in Bax protein expression by 13% compared to Solamargine. Solanine exhibited significantly (P <0.001) higher Bax protein overexpression by 38% and 28.7% compared to Solamargine and Nitroso-Solamargine, respectively (Figure 23a).

The protein expression of the antiapoptotic marker Bcl-2 was also evaluated. Opposite to what is speculated, Bcl-2 protein levels were significantly (P < 0.001) upregulated by 12.6%, 6.6% and 12.2% after exposure to Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Nitroso-Solamargine significantly (P < 0.001) induced Bcl-2 expression levels by 5.9% compared to Solamargine. Solanine resulted in significantly (P <0.001) higher Bcl-2 expression levels by 6.4% compared to Solamargine (Figure 23b).

Bax/Bcl-2 ratio was estimated as means of evaluation of the responsiveness of the cells to apoptosis. Bax/Bcl-2 ratio was significantly (P <0.001) higher by 75.2% (4 folds), 62.4% (2.7 folds) and 65.4% (2.9 folds) for Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Nitroso-Solamargine exhibited significantly (P <0.001) higher Bax/Bcl-2 ratio by 7.98% (1 fold) compared to Solamargine. Solanine exhibited significant (P <0.001) increase in Bax/Bcl-2 ratio by 34% (1.5 folds) and 28.2% (1.4 folds) compared to Solamargine and Nitroso-Solamargine, respectively (Figure 23c).

Caspase-9 exhibited significant (P < 0.001) upregulated protein levels by 67% (3 folds), 55.7% (2.3 folds) and 60% (2.5 folds) after treatment with Solanine, Solamargine and Nitroso-Solamargine, respectively compared to control. Nitroso-Solamargine produced significant (P < 0.001) increase in caspase-9 protein levels by 9.97% (1 fold) compared to Solamargine. Solanine resulted in significant (P < 0.001) increase in caspase-9 protein
levels by 25.4% (1.3 folds) and 17.1% (1.2 folds) compared to Solamargine and Nitroso-Solamargine, respectively (Figure 24).

Similarly, caspase-3 protein levels exhibited significant (P < 0.001) induction by 28.4% (1.4 folds), 18.3% (1.2 folds) and 33.3% (1.5 folds) after treatment with Solanine, Solamargine and Nitroso-Solamargine compared to control, respectively. Nitroso-Solamargine has exhibited significant (P < 0.001) increase in caspase-3 protein levels by 18.4% (1.2 folds) and 6.9% (1.1 folds) compared to Solamargine and Solanine respectively. Solanine produced significant (P < 0.001) enhancement in caspase-3 protein levels by 12.3% (1 fold) compared to Solamargine (Figure 25).
Figure 23a. Effect of Solanine, Solamargine and Nitroso-Solamargine on Bax protein levels at 72 h treatment in HepG2 cells. Protein levels were measured using specific ELISA kit. Data are expressed as average of Bax pg/mg protein (mean ± SD) of three independent determinations (n=3). ** Significantly different at P <0.01 and *** Significantly different at P <0.001.
Figure 23b. Effect of Solanine, Solamargine and Nitroso-Solamargine on Bcl-2 protein levels at 72 h treatment in HepG2 cells. Protein levels were measured using specific ELISA kit. Data are expressed as average of Bcl-2 pg/mg protein (mean ± SD) of three independent determinations (n=3). *** Significantly different at P <0.001.
Figure 23c. Effect of Solanine, Solamargine and Nitroso-Solamargine on Bax/Bcl-2 ratio at 72 h of treatment in HepG2 cells. The protein levels of Bax and Bcl-2 was assessed via ELISA kits. Data are expressed as (mean ± SD) of three independent determinations (n=3). *** Significantly different at P <0.001.
Figure 24. Effect of Solanine, Solamargine and Nitroso-Solamargine on caspase-9 protein levels at 72 h treatment in HepG2 cells. Protein levels were measured using specific ELISA kit. Data are expressed as average of caspase-9 pg/mg protein (mean ± SD) of three independent determinations (n=3). *** Significantly different at P <0.001.
Figure 25. Effect of Solanine, Solamargine and Nitroso-Solamargine on caspase-3 protein levels at 72 h treatment in HepG2 cells. Protein levels were measured using specific ELISA kit. Data are expressed as average of caspase-3 pg/mg protein (mean ± SD) of three independent determinations (n=3). *** Significantly different at P <0.001.
6. Discussion

6.1 In vitro cytotoxic activity and structure activity relationship

*Solanum macrocarpon* and *Solanum seaforthianum* extracts have demonstrated promising cytotoxic activity on HepG2 cells. The in-vitro testing of the cytotoxic potential shown in (table 2, figures 6 and 7) of the ethanol extracts and both TGA and n-butanol fractions of the aerial parts of both species on HepG2 cell line revealed the highest potency for those of TGA fraction of *S. seaforthianum* Andr. with IC50 = 1.5 µg/ml followed by n-butanol fraction where the ethanol extract bottomed the cytotoxic potentiality with IC50 53.4 µg/ml. The results shed light on the cytotoxic potentiality of glycoalkaloids which are found in higher concentration in TGA fraction (Lee et al., 2004). The same order of cytotoxic activity was observed with different extract and fraction of *S. macrocarpon* L. but with higher IC50s compared to that of *S. seaforthianum* Andr. extracts and fractions. These results indicate that the glycoalkaloid content of *S. seaforthianum* is more effective against HepG2 cell line. Since TGASS has demonstrated the lowest IC50 value among the six main extracts and hence the most potent cytotoxic action, its fractions were investigated further.

The pure compounds; Solasonine (Figure 26), Solasodine (Figure 27) and Solamargine (Figure 28) were isolated from TGASS. Addition of sugar moiety seemed to enhance antitumor potency of glycosides, since Solasonine (solasodine + solatriose) and Solamargine (solasodine + chacotriose) (Table 4) demonstrated lower IC50 values than that of Solasodine. Usually glycoalkaloids show higher potency compared to their aglycones due to better pharmacokinetics rather than pharmacodynamics as sugar moieties increased solubility, absorption and distribution to the site of action. These results were consistent with findings reported by Lee et al. (2004), which explains the high IC50 values of the aglycones; Solanidine (Figure 29), tomatidine (Figure 30) and solasodine (Figure 27). Solanidine IC50 value was reduced greatly, upon incorporation of the solatriose sugar that yielded solanine glycoalkaloid (Figure 31). It also offers an explanation for the higher potency of solamargine (Figure 28) and solasonine (Figure 26), when compared to that of solasodine (Figure 27), suggesting that higher potency of
solamargine (Figure 28) is attributed to the trisaccharide “Chacotriose” sugar bonded within rather than the monosaccharide sugar moiety bonded within both of solasodine glucoside (Figure 32) and solasodine galactoside (Figure 33). Galactose (as a C-4 epimer of glucose) seemed to enhance cytotoxic potency more than glucose, which may be attributed to better H-bond interactions in case of galactose through OH at C4 (Table 4). These results were consistent with that of Ji et al. (2008) reporting a cytotoxic action of solanine on HepG2, with an IC$_{50}$ value of 14.47 µg/ml, via MTT assay.

Solamargine’s cytotoxic activity on HepG2 was consistent with the previous study of Munari et al. (2014) findings that reported cytotoxic potential of Solamargine on HepG2 cells with an IC$_{50}$ value of 4.58 µg/ml, via XTT assay. This, in addition to the work of Ding et al. (2012) who reported IC$_{50}$ value of solamargine on HepG2 of 19.88 µg/ml, via MTT assay. Moreover, Spirosolane glycoalkaloid (with Chacotriose sugar moiety) as solamargine (Figure 28) showed higher potency than Solasonine (Figure 26) (with solatriose sugar moiety) (Table 4). These results were consistent with other previous report of Ikeda et al. (2003). Solanine glycoalkaloid (Figure 31) (with solatriose sugar moiety), exhibited higher potency than that of solamargine (Figure 28) (with Chacotriose sugar moiety), which could be attributed to the indolizidine ring of solanine. This also explains its higher cytotoxic effect compared to the spirosolanes alkaloid (with oxazaspirodecan nucleus) of solamargine (Lee et al., 2004). Tomatidine (3β,5a,25S)-Spirosolan-3-ol) (Figure 30) is less cytotoxic than Solasodine (3β,22α,25R)-Spirosol-5-en-3-ol) (Figure 27) on HepG2 cell line which emphasizes the importance of configuration at 25 as 25S and that the absence of double bond at C5 decreases the cytotoxic activity against HepG2 Cell line. This result was contradictory to the work of Koduru et al. (2007). It is possible that the contradictory results were obtained because different cell lines were tested and that these compounds exhibit selective cytotoxic potential against HepG2 cell line.

Solasodine (Figure 27) was found to be more potent than N,O diacetylated derivative (Figure 34), which is less polar and less bioavailable and has less Hydrogen bond interaction with the receptor site as hydrophilic N and O are blocked by acetyl group. N-
The nitroso derivative of solamargine (Figure 35) had more potential cytotoxicity than solamargine itself due to alkylating potentiality of nitroso group like other alkylating anticancer agents. This finding was in agreement with the work of Wang et al. (1998).

Solanine, Solamargine and Nitroso-Solamargine demonstrated the most potent cytotoxic activity among all compounds in both of HepG2 and SCaBER cell lines. Solanine demonstrated the highest potency on both HepG2 and SCaBER cell lines, whereas, the addition of the Nitroso moiety to solamargine seemed to enhance cytotoxic activity of Solamargine lowering its IC$_{50}$ value to a comparable level to that of Solanine. This has encouraged for further scrutinization and characterization of the underlying mechanisms by which this potent cytotoxicity is achieved.

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>sugar</th>
<th>glycoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanidine</td>
<td>Solatriose</td>
<td>Solanine</td>
</tr>
<tr>
<td>Solasodine</td>
<td>solatriose</td>
<td>Solasonine</td>
</tr>
<tr>
<td></td>
<td>Chacotriose</td>
<td>solamargine</td>
</tr>
<tr>
<td></td>
<td>glucose</td>
<td>Solasodine-3-O-glucoside</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>Solasodine-3-O-galactoside</td>
</tr>
</tbody>
</table>

Table 4. Composition of alkaloids examined for their cytotoxic activity, demonstrating aglycone and sugar portions as well as whole glycosides formed.
activity against both cell lines at the 100
respectively; and (b) the corresponding inhibition values of the
levels and 12.8 and 19.0% at the 10 and 100
γ-chaconine results in the formation of the monosaccharide
activity against both cell lines at the 100
γ-chaconine.
Hydrolytic removal of two carbohydrate residues from
against both cell lines, and (c)
(b) the activity at the lower concentrations was much lower
activity against both cell lines at the 100
γ-chaconine of the colon cells was 0% at both the 0.1 and 1
γ-chaconine.

Table 2 shows that (a) the inhibition by
µ-c- and
V-o,
µ-o,
o,
u,
m,
V-o,
5,
µ-o,
o,
u,
s,
a,
a,
µ-o,
o,
u,
R=CH2OH
D-galactose
OH
CH2OH
β
O
HO
D-glucose
α
HO
O
OH
CH3
L-rhamnose

α-solasonine
Solatriose

Figure 26. Chemical structure of α-Solasonine. Image modified from Lee et al. (2004).

Figure 27. Chemical structure of Solasodine. Image modified from Lee et al. (2004).
Table 2 shows that compared to solamargin, the activity of solanine and chaconine results in the formation of the monosaccharide solanidine. Both compounds share the same aglycon but differ in the nature of the carbohydrate side chains attached to the aglycon.

- Figures 28 and 29 depict the chemical structures of α-Solamargine and Solanidine, respectively. Image modified from Lee et al. (2004).
Figure 30. Chemical structure of Tomatidine. Image modified from Lee et al. (2004).

Figure 31. Chemical structure of α-Solanine. Image modified from Lee et al. (2004).
Figure 32. Chemical structure of Solasodine glucoside. Image modified from Lee et al. (2004).

Figure 33. Chemical structure of Solasodine galactoside. Image modified from Lee et al. (2004).
Figure 34. Chemical structure of O,N-diacetyl Solasodine. Image modified from Lee et al. (2004).

Figure 35. Chemical structure of N-Nitroso- Solamargine. Image modified from Lee et al. (2004).
6.2 Cell cycle analysis and correlation with, proliferation index as well as apoptosis

Apoptosis hallmarks include chromatin condensation, formation of apoptotic bodies, emergence of the Pre-G1 peak displayed in the flow cytometry histogram, and the characteristic ladder pattern of DNA displayed after gel electrophoresis of fragmented DNA of oligonucelosomes (Brown & Attardi, 2005). All of the three compounds; Solanine, Solamargine and Nitroso-Solamargine have induced Pre-G peak (indicative of dead cells) on HepG2 cells, with Nitroso-Solamargine inducing the largest pre-G peak suggesting a strong contribution of the nitroso- group to the enhancement of apoptosis. Moreover, the peak induced by solamargine was higher than that of solanine, suggesting a more apoptotic induction ability of solamargine. These results were consistent with the findings reported by (Chang et al., 1998; Kuo et al., 2000), on the Solamargine’s pre-G inducing ability in Hep3B cells, as well as breast cancer cells (Shiu et al., 2007), K562 leukemic cells (Sun et al., 2011) and SMMC-7721 hepatoma cell line (Ding et al., 2012). Solanine’s ability to induce pre-G was also consistent with previous findings in HepG2 (Ji et al., 2008), and HT-29 colon cancer cells (S.-A. Yang et al., 2006).

All of the three compounds have also demonstrated reduction in the G0/G1 phase, with no significant difference in the inhibitions of the G0/G1 phase, between the three compounds. Regarding the S-phase; the findings were remarkably different, as Solanine has not shown significant reduction of the S-phase, compared to control, this was slightly comparable with findings reported by Ji et al. (2008), that has demonstrated an increase in the S-phase in HepG2, treated by solanine, rather than an anticipated reduction. Solamargine and Nitroso-Solamargine has differently demonstrated inhibition of the S-phase, indicating the ability of both compounds to inhibit one of the main proliferation phases, with Nitroso-Solamargine demonstrating a higher inhibition effect. This suggests an important role of the Nitroso moiety to enhance the cytotoxic action of Solamargine through enhancing inhibition of the S-phase.

The G2/M phase was demonstrated to be greatly inhibited by the three compounds; Solanine, Solamargine and Nitroso-Solamargine, with no significant difference in the extent of inhibition observed between the three compounds. This was consistent with the
previous studies reporting inhibition of G2/M phase by Solamargine in breast cancer cells (Shiu et al., 2007), in Hep3B hepatoma cells (Kuo et al., 2000) and SMMC-7721 hepatoma cells (Ding et al., 2012), as well as, inhibition of G2/M phase by Solanine in HepG2 cells (Ji et al., 2008). In conclusion, the three compounds inhibited G0/G1, S-phase and G2/M, yet only the S-phase, seemed to exhibit greater inhibition as a result of Nitroso-Solamargine. The latter has also induced the greatest Pre-G peak. These findings suggest a key role of the Nitroso moiety in enhancing inhibition of proliferation, mainly through enhancing suppression of the proliferative S-phase, and enhancing Pre-G peak indicative of dead cells.

The latter findings were further confirmed by estimating the proliferation index, which was reduced by the three compounds, with Nitroso-Solamargine possessing the lowest proliferation index, suggesting a promising role for the Nitroso-moiety in enhancing cytotoxic effect of solamargine through enhancing inhibition of proliferation. Interestingly, although Solanine has demonstrated lower IC\textsubscript{50} than that of Solamargine in both of HepG2 and SCaBER cell lines, yet Solamargine has demonstrated higher Pre-G and lower S-phase and lower proliferation index than that of Solanine, revealing an overall higher apoptosis-inducing potential of solamargine than that of solanine, mainly to cells in the S-phase.

To characterize molecules contributing in underlying pathways involved in glycoalkaloids mode of action, the expression of several proliferation and apoptotic markers expression were investigated as follows;

6.3 Impact on proliferation markers; Cyclin D1, ki67 and on IAP; Survivin.

6.3.1 CyclinD1
Cyclin D1 expression is maintained in the G1 phase and plays an important role for the initiation of the subsequent S phase, during which it declines rapidly once the S-phase has initiated and all over the S-phase consistently low cyclin D1 is detected, before rising again at the G2/M phases. This reveals an important role of cyclin D1 in the G1/S phase transition, where at the end of the S-phase the cell takes the decision of committing to the
next cycle of cell division or perhaps halt cell cycle at this point (Hitomi & Stacey, 1999). Only Solamargine and Nitroso-Solamargine have caused significant inhibition of cyclin D1 expression, which could be attributed at least partly to the G0/G1 phase and G2/M phase inhibition, during which cyclin D1 is highly expressed.

6.3.2 Ki67
In cells with progressing cell cycle, Ki67 expression is maintained through G1, S, and G2/M active cell cycle phases (Manoir et al., 1991). Moreover, (Tanaka et al., 2011) evaluated the proliferative action of the cell through estimating the S-phase as an indication of cells with progressing cell cycle. This was accompanied by Ki67 antibodies, that indicate actively proliferating cells, whereas, the S-phase is usually a direct indication of the magnitude of this proliferation. Hence correlation of both of the Ki67 and the S-phase markers as means of cell proliferation estimation, gives a more accurate indication of the cells’ proliferating state and magnitude, which is a very efficient tool during evaluation of tumor growth and progression. The three compounds; Solanine, Solamargine and Nitroso-Solamargine significantly suppressed Ki67 expression, with Nitroso-Solamargine demonstrating the highest suppression. This again reveals an important function of the nitroso group in enhancing proliferation inhibition action of Solamargine, to even a greater extent than that of Solanine. The ki67 inhibition could be partly attributed to the inhibition of the S-phase (Tanaka et al., 2011), caused by the three compounds. Nitroso-Solamargine produced the highest inhibition of the S-phase, which is concurrent with the highest ki67 suppression of Nitroso-Solamargine, again revealing the essential function played by the Nitroso moiety in enhancing the cytotoxicity action of Solamargine. Suppression of Ki67 could also be explained to be a result of the simultaneous inhibition of both of the G0/G1 and G2/M phases caused by the three compounds, during which Ki67 upregulation was previously reported to be maintained (Manoir et al., 1991).
6.3.3 Survivin

Survivin is expressed in all phases of cell cycle to ensure cell cycle progression, with the maximal expression during mitosis (Li & Altieri, 1999). It has an important function during mitosis as it represents an essential component of the chromosomal passenger complex stabilizing the centromere during chromosomal segregation during the G2/M phase of mitosis, where it peaks and has maximal overexpression at that phase (Dubrez-Daloz et al., 2008). In vivo knock-out of survivin in mouse model has resulted in deformation of microtubules functioning during mitosis (Tarnawski & Szabo, 2001). Lack of survivin leads to deformed cell cycle, as a result of defective chromosomes, impaired alignment and impaired cytokinesis. Hence abrogation of such defective cells is essential, through the absence of the second function of survivin as an antiapoptotic, and thus leading to stimulation of apoptosis and the eventual eradication of cell cycle-deformed cells (Li et al., 2005).

Our findings demonstrate a significant inhibition of survivin expression by the three compounds, with Nitroso-Solamargine exhibiting the greatest inhibition, again confirming the essential contribution of the nitroso group in enhancing proliferation inhibition action of Solamargine, to even a greater extent than that of Solanine, and thus revealing the importance of the Nitroso moiety and its role in enhancement of survivin inhibition, as a contributor to proliferation inhibition.

These findings were consistent with that of Liu et al. (2013) who have reported reduction in cells count percentage at the S-phase and the G2/M phases, upon knocking down of survivin in HepG2 and SMMC-721 hepatocellular carcinoma cells. Ambrosini et al., (1998) also reported an increase of the SubG1 peak and a reduction in the G2/M phase, upon knocking down survivin in HeLa cells. Hence, survivin inhibition post treatment with; Solanine, Solamargine and Nitroso-Solamargine could be attributed to the simultaneous reduction of cells in both of the S and G2/M phases, as well as the simultaneous increase of the pre-G peak, with Nitroso-Solamargine exhibiting the greatest inhibition at the S-phase, and the highest upregulation of the Pre-G peak. Dai et al. (2012) reported a reduction in G0/G1 phase cells upon knocking down of survivin in
HepG2 cells. Thus survivin downregulation by the three compounds (Solanine, Solamargine and Nitroso-Solamargine) could also be partially correlated with the G0/G1 phase inhibition triggered by the three compounds.

Furthermore, chemotherapy with survivin targeting approach, provides an important advantage of being selective to highly proliferating cells as cancer cells, where survivin is highly expressed, rather to the low survivin-expressing normal cells. Hence survivin targeting drugs would be expected to exhibit limited toxicity to normal cells versus cancer cells (Andersen, 2002). Indeed, Survivin antagonists did not exhibit toxic effects on bone marrow and hematopoietic cells (Ryan et al., 2009). This might suggest a selective advantage of the three compounds investigated here to cancer cells. Nitroso-Solamargine is suggested to possess the highest selectivity, since it exhibits the highest survivin inhibition. This could imply that the Nitroso moiety has enhanced Solamargine’s selectivity towards cancer cells.

Survivin upregulation in HepG2 and other hepatocellular carcinoma cell lines as; Huh-7 and sk-Hep1, was correlated with resistance to chemotherapy (as in taxol treatment for instance) and thus inefficiency of the anti-tumor therapy (Altieri, 2003; Chau et al., 2007). For example; Taxol (known for its stabilizing action on microtubules and thus cell cycle arrest) treatment on HeLa cells that are co-transfected with survivin, has exhibited reduced induction of apoptosis (Giodini et al., 2002). Our results indicate targeting and downregulation of survivin by the action of solanine, solamargine and nitroso-solamargine hence indicates a promising ability of these compounds to overcome resistance of other currently used chemotherapies. The highest effect of Nitroso-Solamargine on survivin inhibition, suggests a role of the Nitroso-group in enhancing the resistance-overcoming advantageous property of solamargine. This finding warrants further investigations through assessing the synergistic potential of these glycoalkaloids with conventional antitumor agents.
6.4 Impact on the levels apoptotic markers caspase-9, caspase-3, Bax and Bcl-2

Apoptosis is a central regulatory process, for maintaining the balance between proliferation and growth. During apoptosis upregulation of the intrinsic pathway apoptotic markers; Bax, caspase-9, caspase-3, as well as downregulation of Bcl-2 takes place (Kerr et al., 1994; Zimmermann & Green, 2001). In this study, the three compounds; Solanine, Solamargine and Nitroso-Solamargine have demonstrated upregulation of the intrinsic apoptotic markers; Bax, caspase-9 and caspase-3 in HepG2 cells. On the transcriptional level, caspase-9 demonstrated significant upregulation by the three compounds, whereas, caspase-3 -although being upregulated by the three compounds- was only significantly overexpressed by Solanine treatment. On the translational level, both of caspase-9 and caspase-3 demonstrated significant upregulation by the three compounds. This could be attributed to the experimental settings that estimated mRNA upregulation post 48 h of treatment, whereas the protein levels were assayed post 72 h of treatment. Caspase-9 is upstream of caspase-3 in the intrinsic apoptotic pathway and hence is expressed earlier (Zimmermann & Green, 2001), thus is expected to demonstrate higher expression levels than that of caspase-3 post 48 of treatment, and accordingly exhibit more significant upregulation. Indeed, this postulation was confirmed by comparing the caspase-3 and caspase-9 levels on the protein level, post 72 hours of treatment, which showed that even though the three compounds significantly overexpressed both of caspase-3 and caspase-9, yet caspase-9 has also demonstrated higher expression levels than that of caspase-3 by all of the three compounds.

Bax was found to be significantly upregulated as assessed by western blot and that was further confirmed by ELISA assay. Nitroso-Solamargine has demonstrated higher significant expression of Bax, caspase-9 and caspase-3, than that of Solamargine and hence revealing the essential role of the Nitroso moiety in enhancing Solamargine’s function of inducing expression of intrinsic apoptotic markers. The expression of antiapoptotic Bcl-2, was opposite to what is speculated to be down regulated, according to previous studies that demonstrated downregulation of Bcl-2, with simultaneous overexpression of Bax, caspase-9 and caspase-3 by solamargine treatment in osteosarcoma cell line (U2OS) (Li et al., 2011), lung cancer cells (Liang et al., 2004; Liu
et al., 2004), breast cancer cells (Shiu et al., 2007) and leukemic K562 cells (Sun et al., 2010). Solanine had similar reports of downregulating Bcl-2, as well as upregulation of Bax and caspase-3 in HepG2 cells (Ji & Gao, 2012), and in pancreatic cancer cells (Sun et al., 2014). Despite this unexpected upregulation of Bcl-2 that would suggest development of some resistance to the treatments on hand as consistent with Cho et al. (2006), yet the Bax/Bcl-2 ratio was still significantly higher than control revealing active capability of Bax to form the apoptotic dimer Bax/Bak (Willis et al., 2003) and hence inducing mitochondrial intrinsic apoptotic pathway. Nitroso-Solamargine has demonstrated higher Bax/Bcl-2 ratio than that of Solamargine. Addition of the Nitroso moiety to solamargine seemed to enhance its apoptotic inducing function (through enhancing higher expression of Bax, caspase-3 and caspase-9), yet not to the extent of surpassing that of Solanine’s that has demonstrated higher expression of caspase-3, caspase-9 and Bax than those of Solamargine.

This could be substantially correlated with survivin downregulation. In HeLa cells (Ambrosini et al., 1998) and neural cancer cells (Shankar et al., 2001); knocking down survivin was reported to stimulate apoptosis. Upregulation of caspase-3, DNA fragmentation and chromatin condensation, upon knocking down of survivin in HepG2 was also reported (Dai et al., 2012; W. Liu et al., 2013). Moreover, Survivin overexpression in human embryonic kidney (HEK) 293 cells has led to inhibition of both of Bax (intrinsic pathway) and Fas (extrinsic pathway) mediated apoptosis (Tamm et al., 1998), with a more pronounced inhibition of the intrinsic apoptotic pathway (Grossman et al., 2001). Survivin was reported to suppress activity of the cytochrome c cleaving function, suggesting its protective function against apoptotic stimulation (Tamm et al., 1998). Marusawa et al. (2003) reported that survivin forms a complex with hepatitis B X-inter-acting protein (HBXIP), which interacts with the initiator procaspase-9 leading to hindering of Apaf-1 binding to the apoptosome complex and thus impeding the progression of apoptosis. Survivin also decreased apoptosis, when overexpressed in keratinocytes, post UV-exposure (Grossman et al., 2001). Survivin was also reported to interact with and sequester Smac/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl), and hence alleviating
the latter’s suppressing action on the rest of IAPs, thus relieving other IAPs and allowing their suppressing action on caspases (Tarnawski & Szabo, 2001). Our findings demonstrate a concurrent upregulation of Bax, caspase-9 and caspase-3, upon survivin downregulation induced by the three compounds; Solanine, Solamargine and Nitroso-Solamargine. Moreover, Nitroso-Solamargine had demonstrated the highest caspase-3 upregulation with a simultaneous lowest expression of survivin, confirming the role of Nitroso moiety in enhancing induction of apoptosis of Solamargine.
7. Conclusion

Glycoalkaloids extracted from the *Solanaceae* family have demonstrated promising antitumor potential, with Solanine, Solamargine and Nitroso-Solamargine exhibiting the most potent cytotoxic activity. Incorporation of the Nitroso moiety within Solamargine, resulted in enhanced cytotoxicity of Solamargine, and reduced the IC$_{50}$ values of Solamargine to levels that are comparable to the low IC$_{50}$ values of Solanine, in both of hepatocellular carcinoma and urinary bladder cancer cell lines. This has driven further interest for exploring the underlying molecular mechanisms by which these molecules achieve their cytotoxic action. Further investigations have indicated a remarkable capability of the three compounds to activate the intrinsic apoptotic pathway through upregulation of its markers; Bax, caspase-9 and caspase-3. The anti-apoptotic marker; Bcl-2 was not down-regulated as expected, yet Bax/Bcl-2 ratio was significantly higher after treatment with the three compounds indicating their ability to induce the mitochondrial intrinsic apoptotic pathway. Nitroso-Solamargine has exhibited a stronger effect than that of Solamargine, revealing the importance of the Nitroso moiety in enhancing the cytotoxic action of Solamargine through stimulating the intrinsic apoptotic pathway, yet not reaching that of Solanine. Proliferation and cell cycle markers as; cyclin D1, Ki67 and survivin were also greatly inhibited by the three compounds in HepG2 cells, with the Nitroso-Solamargine exhibiting the greatest inhibition, and hence confirming the importance of the Nitroso moiety in enhancing the cytotoxic activity through enhancing proliferation-inhibition action of Solamargine to a level even lower than that induced by Solanine.

In conclusion, glycoalkaloids extracted from *Solanaceae* family exhibit promising cytotoxic activity. Solanine, Solamargine and Nitroso-Solamargine exhibited the most potent activity. Nitroso moiety incorporation to Solamargine significantly improved the cytotoxic activity of Solamargine, as examined and confirmed by the several approaches mentioned above. The only other potent competitor to Solamargine’s antitumor potency, among all of the screened alkaloids; is Solanine.
8. Future Prospective
Investigating the antitumor activity of the three promising glycoalkaloids (Solanine, Solamargine, and Nitroso-Solamargine) on primary tissue cultures of hepatocellular carcinoma and urinary bladder cancer isolated from Egyptian patients, would reveal a lot about the efficiency of these treatments specifically for Egyptian cancerous patients. This in addition to, in vivo experiments using animal xenograft models that would determine if they possess adequate bioavailability and can exhibit the same potency in vivo or not. Another very important aspect to be taken into consideration while evaluating and comparing the potency of each of these compounds is examining and comparing their cytotoxic effect on normal cells, in order to determine which one exhibits the least toxicity to normal cells, while having the highest toxicity to cancer cells. Furthermore, exploring the underlying molecular mechanisms for their cytotoxicity in urinary bladder cancer cells would reveal whether these compounds follow similar or different molecular mechanisms on different cancer cell types.
9. References


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