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Effects of Acetylsalicylic Acid and Salicylic Acid on the Growth of HeLa Cervical Cancer Cells Line

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Authors' contributions

This work was carried out in collaboration among all authors. Author IM designed the study, wrote the protocol and carried out the experimentation. Author AU collected all data, performed the statistical analysis and wrote the first draft of the manuscript. Author MOM did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Cervical cancer is the fourth most common cancer affecting women in the world, 527,000 new cases were reported and over 250,000 deaths were reported each year. The major risk factor of cervical cancer is infection with HPV. The aim of our research is to determine the effect of Aspirin (Acetylsalicylic Acid) and Salicylic Acid on cervical cancer cells in vitro using HeLa cervical cancer cells line, also to determine the mechanism by which these drugs can kill cervical cancer cells through apoptosis, also to find the possibility of using ASA and SA as cervical cancer drugs.
Materials and Methods: Cell viability was determined using cell titre blue from 1,000,000 to 31,250 cells per ml on HeLa. Dose response for the drugs was carried out at concentrations from 0-20 mM incubated at time intervals 24, 48 and 72 hrs incubation. A 10 mM concentration of ASA and SA were used to determine the caspase activity using caspaseglo for the period of 0-24 hrs incubations. Western blot was carried out using active anti- caspase3 antibody for caspase3 proteins.
Results and Conclusion: The cell viability shows the absorbance increases as the number of cells

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increases. There is effectiveness of viability inhibition from 15 mM to 20 mM concentration for 24, 48 and 72 hrs incubation on dose response. There is much higher increase in caspase activities from 8 hrs to 16 hrs on both drugs, with much more effect with SA than ASA. Western blot shows no expression of protein for caspase3, but shows expression using β actin as a housekeeping gene. ASA and SA can induce apoptosis and bring about cell death on cervical cancer; therefore these drugs can be good drugs for the treatment of cervical cancer.

Keywords: HeLa cervical cancer; acetylsalicylic acid; salicylic acid; caspaseglo; prostaglandin; angiogenesis.

1. INTRODUCTION

1.1 Cervical Cancer

Cervical cancer is the fourth most common cancer worldwide for females, and the seventh most common cancer overall, with more than 527,000 new cases diagnosed in 2012 (8% of female cases and 4% of the total). Cervical cancer incidence rates are higher in Eastern Africa and lowest in Western Asia, but this partly reflects varying data quality worldwide [1]. The major risk factor of cervical cancer is transmissible infection with Human Papilloma Virus (HPV), which is necessary for the growth of cervical cancer, as HPV DNA sequences are identified in greater than 99% of cervical cancers [2,3]. Infection with "high risk" HPV subtypes brings about tumor progression through abrogating cell cycle control as well as apoptosis checkpoints via viral oncoprotein E6 as well as E7, which inactivate the p53 and RB tumor suppressor pathways respectively [2]. This results in the development of noninvasive (in situ) cervical dysplasia referred to as High-grade Squamous Intraepithelial Lesions (HSILs) [3,4,5]. Nevertheless, these HPV induced dysplasia are asymptomatic and most regress, showing that HPV is not enough to develop cervical cancer [2]. The development of cervical dysplasia to invasive, lethal cervical cancers has been suggested to other risk factors, which include immune, hormonal, nutritional status and co-infection with other sexually transmitted diseases [2]. Insertional mutagenesis by HPV is one of the suggested tumors promoting mechanism, but most of new studies did not agree with this hypothesis [6].

In Sub-Saharan African countries, cervical cancer is the leading gynecological cancer [7,8]. The main risk factor associated with the development of cervical cancer is HPV [9]. HPV oncogene has the ability to regulate the Cyclooxygenase (COX) - Prostaglandin (PG) axis in cervical cancer cells to bring about inflammation, persistent infection as well as tumorigenesis [10,11]. Many studies revealed that the inflammatory COX-PG axis is elevated in cervical cancers [12]. COX is an enzyme in human body, which is of two isoforms COX-1 and COX-2, which brings about the rate limiting conversion of arachidonic acid to PG [12]. After their biosynthesis, PG is actively conveyed from the cell, where they functioned locally in an autocrine/paracrine manner through PG receptors [12]. Evidences have shown that PG biosynthesized following induction of COX-1 as well as COX-2 in cervical cancer cells, elevates the expression of potent pro-angiogenic factors like basic fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF) as we all as angiopoitins [12,13]. After biosynthesis and release from neoplastic cervical cells, angiogenic factors can then trigger a paracrine activity on endothelial cells to promote blood circulation through angiogenesis to enhance tumor development [12]. These evidences guide us to propose that suppression of the inflammatory COX-PG axis with non-steroidal anti-inflammatory drugs (NSAIDs) such as Salicylic acid and Acetylsalicylic acid could be important drugs for preventing cervical cancer progression in poor countries in Africa, through suppressing potent inflammatory and angiogenic pathways that can support cancer progression.

1.2 HeLa Cervical Cancer Cell Line

HeLa cell line was the first human cell line produced in culture [14] and has since been used worldwide in biological research laboratory. The application of this cell line has contributed more to the scientist toward the characterization of important biological processes and more than 70,000 publications. The origin of this cells line was from a cervical cancer tumor from a patient called Henrietta Lacks, who died as a result of the cancer in 1951, from which the name of the cell line originates (HeLa) [15]. HeLa cells line was first used for the processing of vaccine against the polio Virus [16]. Later, HeLa cell was

discovered to play a role in link between human papilloma virus and cervical cancer as well as the role in telomerase in preventing chromosome degradation.

1.3 Acetylsalicylic Acid

Acetylsalicylic acid (ASA), known as Aspirin, was one of the drugs used by public every day for the treatment of different types of diseases and some condition for decades. This drug was first known as an analgesic, anti-pyretic and anti-inflammatory. For more than 25 years, some researches revealed that aspirin can be used to suppress the occurrence as well as growth of epithelial cell cancers, specifically colorectal cancer [17,18,19,20]. The application of aspirin for long period of time may possibly reduce the incidence of many cancers that are fatal to human, such as breast cancer, bladder cancer, and gastric cancer by 40-50% [21].

The molecular processes involved in aspirin's action on tumor cells are well known. Some factors have to be put into consideration when using aspirin as anti-cancer drug, such as concentration of the drugs, duration, stage of the cancer and type of cancer to be cured.

Aspirin as COX inhibitor; its physiological actions are based on its ability to inhibit COX-2 enzymes as shown in Fig. 1 [22]. The inhibition of COX-2 in tumor cells brings about reduction in the production of prostaglandin, which at the end initiates a decrease cell growth and cell death occur through apoptosis, also the inhibition of COX-2 by aspirin will as well increase immune surveillance and result to cell death as shown in Fig. 1 [23]. There are many non-steroidal anti-inflammatory drugs (NSAIDs), but aspirin varies from others due to its ability to inhibit irreversibly both COX-1 and COX-2, but much especially COX-1 [18]. Evidences have shown that, COX-1 is over-expressed in cervical cancer; therefore, the effect of aspirin may be a dual COX inhibitor.

Aspirin has the ability to acetylate proteins associated with tumor cells, the effect of COX and prostaglandins may not be due to chemopreventive action of aspirin, because similar action has been seen in COX deficient cell model and prostaglandin deficient animal model studies [18]. Aspirin contained an acetyl and salicylate group and all the groups were recognized to have a clear difference in their molecular ways of actions. The ability of aspirin to acetylate is one of the reasons that it varies from other NSAIDs.

Many researches have shown that aspirin has the capacity to attach and modulate multiple proteins in cancer cells through acetylation of lysine and serine residues [24]. For example, action of aspirin as a COX inhibitor is due to the binding of its acetyl group with serine residue on cyclooxygenase, this brings about an irreversible inhibition of its function as shown in Fig. 1 [25]. At physiological concentration, aspirin has the capacity to acetylate majority of cellular constituents relevant to cervical cancer, such as tumor suppressor gene p53, that has the ability to control over growth or cell death [26].

Apoptosis, one of the ways by which cell dies (known as programmed cell death) due to inflammation, cell damage, cell stress or mutation; Caspases which initiates and control apoptosis, can respond to extracellular inducers like tumor necrosis factor (TNF) of Fas ligand or to intracellular inducers such as mitogen activated kinases. More importantly, this process is associated with tumor suppressor gene p53 and mitochondria where cytochrome C is released to initiate apoptosis regulated by Bcl_2. Cervical cancer cells are known to inhibit apoptosis through inhibition of many different types of pro-apoptotic proteins such as p53 and up regulation of anti-apoptotic proteins [23,27].

From the previous studies it has been revealed that aspirin has the ability to promote and brings about caspase-dependent apoptosis in various cancer cell types, this occurs mostly through up regulation of p53 [28]. Aspirin can modulate calpain gene expression, as a result of that it will activate caspase-3 and bring about cell death through apoptosis [29]. However, the inhibition of COX by aspirin results in aggregation of ceramide, which also initiates and induces caspase-dependent apoptosis through cellular stress signaling [18].

Apoptosis is a programmed cell death, usually occurs in two pathways; extrinsic and intrinsic. An extrinsic apoptotic pathway occurs through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, this is a cell membrane-bound protein that initiates tumor-specific apoptosis. The activation of TRAIL receptor pathway is selective mechanism specifically for removing cancer cells completely, although, in some circumstances there are most of the cancers that are known to be resistant to TRAIL therapy. With the advent of multiple therapies through combining TNF and aspirin, the treatment has shown a good improvement in

caspase-induced cell death in cancer cell rather than TRAIL therapy alone [30]. This is why in this current research we want to combine acetylsalicylic acid and salicylic acid to see if there is effect on the growth of cervical cancer using cell line. Aspirin also reduces certain amount of protein surviving and sensitizes cells to TNF/TRAIL-induced cell death [31].

Aspirin has the ability to stop angiogenesis in tumor cell (see Fig. 1), angiogenesis is very important for the growth and development of cancer cell; cancer cell has the ability to produce a new blood capillaries within its vicinity so as to supply nutrients and oxygen for the survival and growth. Previous research has shown that COX has the ability to control angiogenesis in colon cancer via promoting endothelial activity [32]. It has been also documented that there is possibility for aspirin to inhibit vascular remodeling as well to inhibit pro-angiogenic factors such as metalloproteinases (MMPs) or vascular endothelial growth factor (VEGF) [33]. In cervical cancer especially cervical neoplasia, COX-1 expression plays a vital role in improving and maintaining angiogenesis [12]. Aspirin as an effective inhibitor of COX-1, has the ability to inhibit and stop angiogenesis through COX-1 dependent pathways in cervical cancer as shown in Fig. 1.

An epidemiological research was conducted on ASA as anti-tumor drugs on colorectal cancer, the result shows effectiveness of ASA on colorectal cancer [17,34]. The anti-proliferative and chemopreventive characteristic of ASA in cell cultures and tumor cells was proved to be as a result of induction of apoptosis [35,36]. There are different molecular pathways involved in apoptotic effect of ASA recognized, these include; p38 MAP Kinase (Mitogen-activated protein kinases) activation in human fibroblast [37], effect on human telomerase reverse transcriptase in colorectal cancer cells [38], caspase activation [39,40], down regulation of the anti-apoptotic Bcl-2 protein [41,42], beside that it triggers alteration of the signaling pathway of the transcription factor NF-*κ*B in human gastric cells [43]. ASA induce tumor apoptotic cell death through promoting the onset of the mitochondrial permeability transition [44,45].

1.4 Salicylic Acid

Salicylic acid (SA) as a COX inhibitor performed its action by COX-independent mechanisms, from Fig. 1. SA plays a vital role in COX-2 inhibition than acetylation, as acetylation of serine on COX-2 possibly may not bring about activation [18]. A research has shown that SA might possibly be a weak competitor for COX with its target arachidonic acid [46]. Evidence from a previous research has shown that SA is a weak inhibitor of COX directly, but is still seen to reduce the amount of COX metabolites as well as prostaglandins [47]. It has also been shown that SA might modulate COX indirectly via nuclear transcription factors, which results in the equality between cell life and cell death as shown in Fig. 1. The inhibition of nuclear factor kappa B (NFκB) transcriptional activation might be the major target of SA on cancer cells, as NFκB plays a vital role in controlling COX expression [48,49]. However, SA can also degrade NFκB inhibitory co-factor genes in cervical cancer models [50,51]. The mechanism in which SA succeeded in this process was considered to be independent of COX [52]. Other nuclear transcription factors associated with COX in cervical cancers may possibly be due to pharmacological action of aspirin, such as AP-1 and PPAR receptor [47,53,54].

Mitochondrion is one of the most important components of the cell, plays a vital role in cell death through apoptosis, apoptosis occurs in cell by the action of Bcl_2 proteins and the release of cytochrome C [55]. ASA and SA have the ability of altering these proteins. It has also been found that SA can down-regulate the anti-apoptotic Bcl_2 family group MCL-1 [56]. Evidences from previous research has shown that ASA has the ability to initiate apoptosis through mitochondrial cytochrome-C release by inhibition of the ubiquitin proteasome pathway, which can brings about stopping protein signaling of the key transcription factors [57]. Mitochondrial calcium uptake inhibition is another mechanism by which ASA brings about cell death as shown in Fig. 1 [58].

Previous studies have shown that ASA and SA are found to be inhibitor of COX enzymes, these drugs have been in use for many treatments such as anti-oxidative, anti-microbial and anti-inflammatory [59,60,61,62,63,64]. Evidences from many studies have shown that ASA and SA could be used for the assessment for anti-cancer drugs in many cancer cell lines [65,66,67,68].

ASA and SA have been used as chemopreventive agents of cancers to induce apoptosis and bring about cell death; it has also been shown to reduce the possibility of cancer formation and development in many organs,

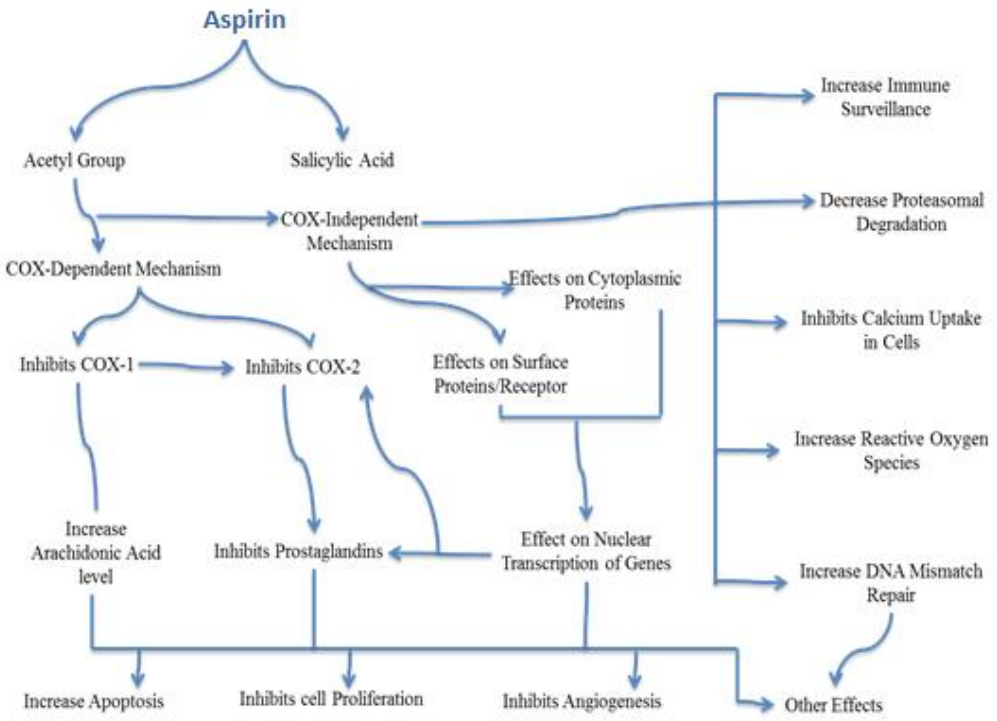

Fig. 1. Molecular pathway showing multiple anticancer effects of Aspirin in epithelial cancer

such as colorectal cancer [35], lung cancer [69], and stomach cancer [70]. ASA initiates and promotes apoptotic cell death by mitochondrial permeability transition and result to death of cell by TRAIL [45,71].

In conducting a research on the effects of ASA and SA on cervical cancer growth, there are certain factors that need to be put into consideration, such as concentration of the drugs, and in oral administration there are chances that most of its effects would not be observed due to clearance and excretion limitations. However, what is seen in one epithelial cancer might not be possibly the same to other epithelial cancer cells. Most of the previous studies conducted on the effects of aspirin are on colorectal cancer; there must be some variations from the results conducted on cervical cancer, due to differences in mutations and some genes associated with these tumors. Other factors to be considered are the adverse side effects of the drugs (ASA and SA), such as ulceration, hypersensitivity reactions, gastrointestinal bleeding [72], renal damage as well as macular degenerative disease [73].

Cervical cancer is the fourth most common cancer affecting women in the world with high rate of mortality. Awareness, screening and treatments are being in application every day, but still the mortality rate is increasing. The aim of our research is to determine the effect of Aspirin (Acetylsalicylic Acid) and Salicylic Acid on cervical cancer cells in vitro using HeLa cervical cancer cells line, also to determine the mechanism by which these drugs can kill cervical cancer cells through apoptosis, also to find the possibility of using ASA and SA as cervical cancer drugs. We used ASA and SA as drugs for this research; because these drugs are COX inhibitors in cancer cells, also COX is over expressed in cervical cancer. These drugs are also available everywhere, very cheap affordable for both rich and poor people.

2. MATERIALS AND METHODS

2.1 Determination of Cell Viability of Adherent Cells Using Cell Titer Blue™

This experiment was carried out in order to determine the viability of the cell line HeLa before assessing the viability with the drugs. HeLa cervical cancer cell line was grown to 80% maximum cell as recommended by the provider (American Collection of Cell Cultures). Briefly,

the cells were washed with dulbecco's PBS once, the cells were trypsinized using Cardiff Metropolitan University (CMU) tissue culture guidelines, and re-suspended in 3 ml of fresh supplement media. Cells were counted using haemocytometer and re-suspended to a density of 1 million cells per ml. Doubling dilutions was performed of the cell numbers of 1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250 and 0 cell per ml as media only (negative control). The microplates were incubated for overnight. The media from the wells were aspirated out and a fresh media containing 20 µL Cell titer Blue per 100 µL media was added into each well and incubated in the dark for 1 hour. The microplates were inserted into the Tecan M200 Infinite multi detection reader and the fluorescence of each well was detected using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain).

2.2 Dose Response for Acetylsalicylic Acid and Salicylic Acid Using Cell Titer Blue™

HeLa cervical cancer cells line was processed as mentioned earlier. 100 µL at a density of 200000 cells per ml of cells were pipetted into 96 well plate. The plate was incubated for overnight. The media in the plate was aspirated out, 100 µL of different concentrations of ASA (acetylsalicylic acid), and SA (Salicylic acid) from 0, 1, 5, 7.5, 10, 15 and 20 mM was pipetted in to each well, one row left without the drugs media only as control and incubated for 24 hours. The media was aspirated again from the wells; it was washed once with 300 µL DPBS (Dulbecco's Phosphate-Buffered Saline). Prepared pre-warmed media containing 20µL Cell titer Blue per 100 µL media was added into the wells. The plate was incubated for 1 hour in dark. The micro plate was inserted into the Tecan M200 Infinite multi detection reader and the fluorescence of each well was detected using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). The experiment was repeated for viability after 48 hours incubation as well as after 72 hours incubation, and the fluorescence of each was measured using the Tecan M200 Infinite multi detection reader.

2.3 Determination of Caspase Activity in Adherent Cells Using Caspase 3/7 GLO™

HeLa cervical cancer cell line was processed as mentioned earlier. 50 µL at a density of 100000 cells per ml of HeLa cells were pipetted into 96 wells plate. The plate was incubated for overnight. The media in the plate was aspirated out and 10 mM of ASA, 10 mM of SA solution were prepared using phenol red, 50 µL of ASA solution was pipetted into three rows of the plate, 50 µL of SA solution into another three rows of same plate, one row was left without the drugs, media only as control, the plate was incubated for 24 hours. Caspaseglo reagent from refrigerator was thawed beforehand to equilibrate to room temperature. One vial of caspaseglo buffer was mixed with one vial of caspaseglolyophilised substrate. 50 µL of mixed caspaseglo was added into each well of the plate, it was wrapped with aluminium foil to protect it from light (Caspaseglo is light sensitive), and the plate was incubated at room temperature for 1 hour. The plate was inserted into the Tecan M200 Infinite multi detection reader and the luminescence of each well was determined using i-control software. Caspase activity was measured through the following incubation period 0, 2, 4, 6, 8, 16, 20 and 24 hours respectively.

2.4 Western Blotting Detection of Active Anti Caspase 3

2.4.1 Method

20 µL of the samples HeLa cells with the drugs were pipetted into the wells of electrophoretic gel, about 12 samples together with a ladder, it was filled up with buffer solution and an electric current passed through for 50 minutes in dark room. The gel was removed; iBlot machine was used to blot membrane paper for 5 minutes. 5% non-fat dried milk in TBST (Tris- Buffered Saline with Tween) was used as blocking buffer to block the antibody on blotting paper; the blotting paper was placed on shaking machine for 1 hour. The blotting paper was washed with buffer TBST solution 3 times 5 minutes interval each on shaking machine. A primary antibody Active anti caspase (abcam NO: 32042) 1:600 dilution, 25 µL in 15 ml (5% TBST) was applied and incubated at 4°C on shaking machine overnight. The blotting paper was washed with buffer TBST solution 3 times 5 minutes interval each on shaking machine. A secondary antibody goat FAB2 (abcam NO: ab6013) 1:5000 dilution, 3 µL in 15 ml (5% blocking buffer) was added and incubated for 1 hour at room temperature. The blotting paper was washed again with buffer TBST solution 3 times 5 minutes interval each on shaking machine. The membrane paper was

incubated in West Pico Dura Luminol substrate (2.5 ml luminol and 2.5 ml peroxide substrate) for 5 minutes at room temperature, excess solution was blotted using tissue paper, air bubble was removed using roller. The membrane was placed on protected membrane in a film cassette with the protein side facing up, it was exposed to KODAK X-Ray film for 1 minutes. The film was developed using 20% (v/v) developer and fixing agent (100 ml in 400 ml deionised water), various bands were produced on the film, all the processes for developing carried out in dark room.

The result from western blot analysis did not show any expression of proteins from the samples. The above processes were repeated using β-actin as primary antibody (Housekeeping gene) in order to confirm the western blot analysis was done correctly. The membrane paper was washed in buffer TBST solution 3 times 5 minutes interval each on shaking machine in order to remove active anti-caspase antibody and apply β-actin antibody as housekeeping gene, because no expression shown from the results of western blot for active anti-caspase, the process was repeated on the membrane paper, but in this case β-actin was used as primary antibody. It was exposed for KODAK X-Ray film for 1 second.

3. RESULTS

3.1 HeLa Cervical Cancer Cells Viability and Growth

From the figure below; the graph from the result (absorbance) of HeLa cervical cancer cell line using cells titre blue. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). A doubling dilution was carried out from 1000000 cells per ml to 7800 cells per ml from the cell line (HeLa), from the graph of Fig. 2 it shows that the absorbance increases as the number of cells increases. The relationship between cells density and absorbance on HeLa cervical cancer cells line shown as the mean ± SEM (n=3).

3.2 Dose Response for Acetylsalicylic Acid (ASA) and Salicylic Acid (SA) on HeLa Cervical Cancer Cell Line

Figs. 3 and 4 shows the graphs from the results (absorbance) of the effects of ASA and SA on viability of HeLa cervical cancer cell line using cell titre blue. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). Fig. 3, shows the effectiveness of viability inhibition of Acetylsalicylic acid on HeLa cells line from the concentration of 10 mM to 20 mM and from 48 to 72 hrs incubations period. But there is much effective of viability inhibition from 15 mM to 20 mM concentration for 24, 48 and 72 hrs incubation. The effectiveness of viability inhibition on 42 hrs incubation at 10 mM is very low. 10000 cells per ml were chosen because in the relationship between cells density and absorbance results, there is much increase in absorbance which started above 10000 cells per ml. Effect of cells viability on HeLa cervical cancer cells line with ASA shown as the mean ± SEM (n=3). VC- Vehicle control, this serves as negative control for the experiment and it did not show any effect of inhibition on the viability of HeLa cells line after 24, 48 and 72 hrs incubations.

Graph from Fig. 4, shows the effectiveness for inhibition of viability of salicylic acid on HeLa cells line from 10 mM to 20 mM concentrations at 48 and 72 hrs incubations. At 15 mM and 20 mM concentrations, the effectiveness is from 24 to 72 hrs incubations with more in 48 and 72 hrs incubations. 10000 cells per ml were chosen because in the relationship between cells density and absorbance results, there is much increase in absorbance, which started above 10000 cells per ml. The effects of cells viability on HeLa cervical cancer cell line with SA shown as the mean ± SEM (n=3). VC- Vehicle control, this serves as negative control for the experiment and it did not show any effect of inhibition on the viability of HeLa cells line after 24, 48 and 72 hrs incubations.

3.3 Caspase Activity on HeLa Cervical Cencer Cell Line

Graph in Fig. 5 shows the result (absorbance) of differential effects of ASA and SA on caspase activity on HeLa cervical cancer cells line using caspaseglo. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). The result shows the differential effects of ASA and SA on caspase activity on HeLa cervical cancer cells line, it shows a much higher increase in caspase activities from 8 hrs to

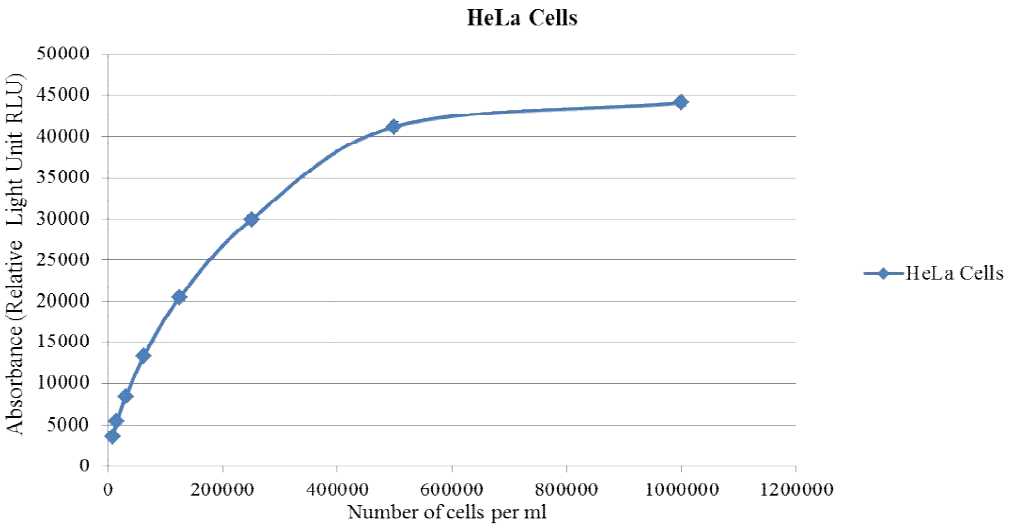

Fig. 2. Relationship between cell density and absorbance on HeLa cell using cell titre blue

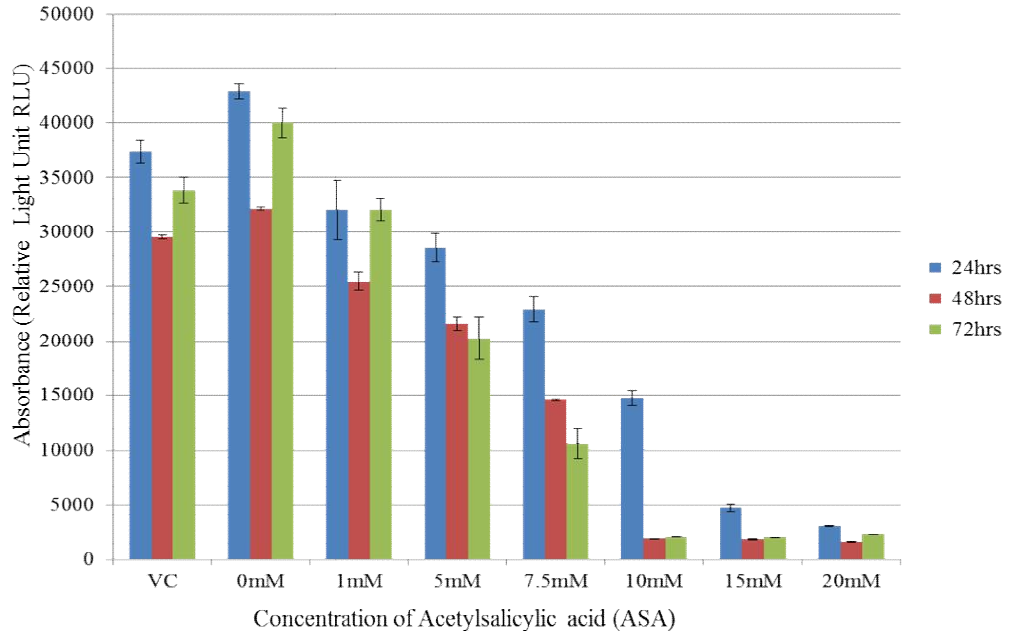

Fig. 3. Effects of acetylsalicylic acid on viability of HeLa cervical cancer cell line

16 hrs on both drugs, with much more effect with SA than ASA, and the activities fall after 16 hrs to 24 hrs. Differential effects of ASA and SA on caspase activity on HeLa cervical cancer cells line shown as the mean ± SEM (n=3).

3.4 Western Blot Analysis for Active Anti Caspase 3 [74]

Fig. 6 shows the results of western blot analysis of the samples using active anti-caspase 3 antibody for the expression of proteins in the samples. From the results no any expression is shown by active anti-caspase 3. β-actin as housekeeping gene was used in order to confirm western blot by active anti-caspase 3 was done correctly, also to confirm the presence of protein in the samples. A ladder was used as a molecular weight size marker with a known concentration and size, as a standard that are used to identify the approximate size, base pair, different bands were produced by the samples

on the gel [74]. The ladder shows different bands of molecular weight ranging from 20 to 220kDa. Active anti-caspase 3 expected to show a band around 17kDa, but no any expression is shown from this result.

Fig. 7 shows the expression of protein for β-actin as a housekeeping gene at molecular weight of 43kDa after 1-second exposure of the film; lane 8, 9 and 10 shows more expression of protein than others, while lane 3 shows very small expression of protein. A ladder was used as a molecular weight size marker with a known concentration and size, as a standard that are used to identify the approximate size, base pair, different bands were produced by the samples on the gel [74]. The ladder shows different bands of molecular weight ranging from 20 to 220kDa. From this result it shows that western blot analysis was done correctly, it also shows the presence of protein for active anti-caspase 3 in the samples, it might be as result of small amount of sample used in western blot analysis or the protein is in small quantity in the sample that is why it did not show any expression.

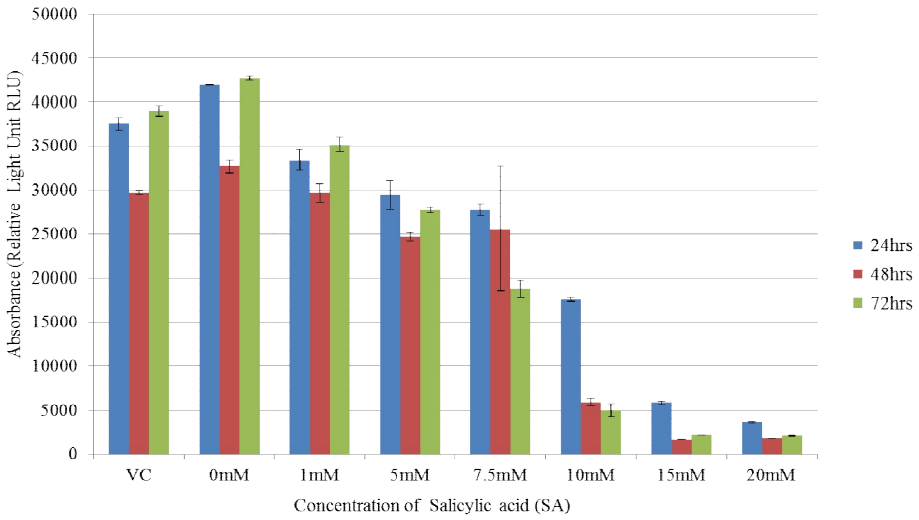

Fig. 4. Effects of salicylic acid on viability of HeLa cervical cancer cell line

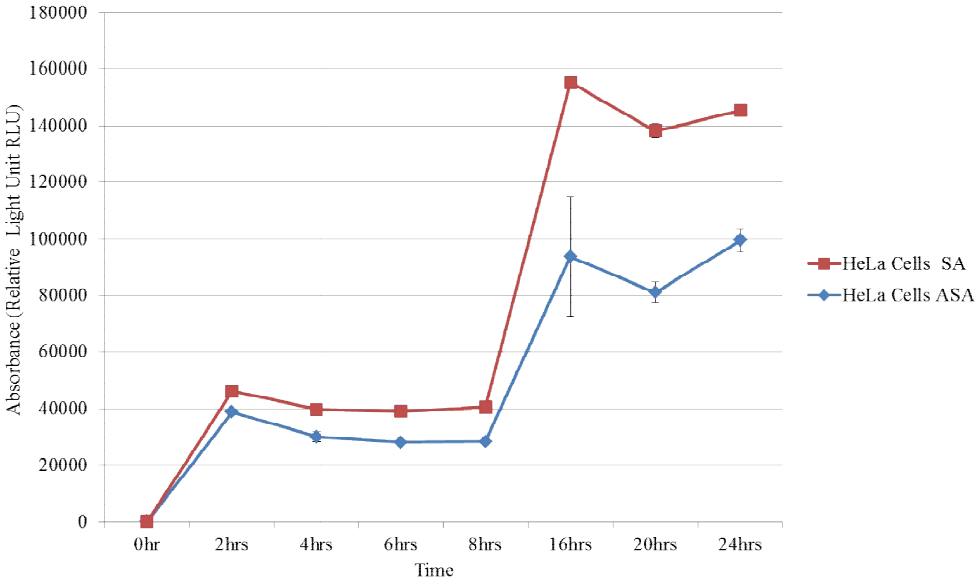

Fig. 5. Differential effect of ASA and SA on caspase activity on HeLa cervical cancer cell line

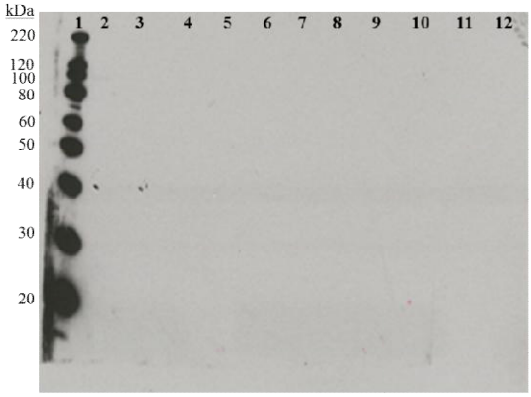

Fig. 6. Electrophoretogram illustration migration of active anti-caspase, 1 minute exposure

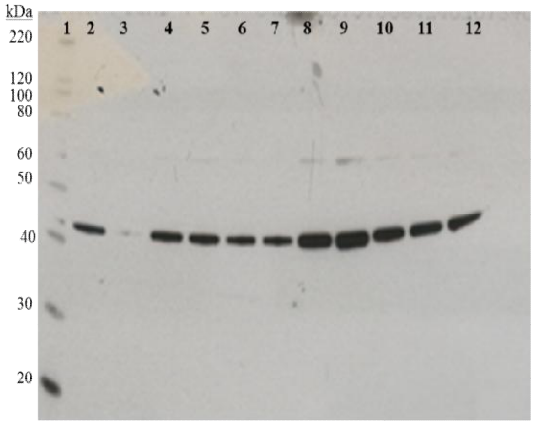

Fig. 7. Electrophoretogram illustrating migration of β-action, 1 second exposure

4. DISCUSSION

We found out that the relationship between cells density and absorbance on HeLa cervical cancer cells line using cell titre blue by doubling dilution from 1000000 to 7800 increases as the number of cells increases from 0 cell per ml to 1000000 cells per ml. In Fig. 2 above, it shows that the relationship between cells density and absorbance continues as the number of cells increases the absorbance of the cells increases too from 0 cells per ml to 1000000 no break up. From these results we found out that HeLa cells show more relationship between cells density and absorbance; this could be as result of HeLa cells being as HPV-16 positive. Cervical cancer cells that are HPV-16 or -18 positive are known to express small amount of p53 transcript as a result of E6-activated ubiquitin-dependent protease digestion [75]. It was also discovered that there is possibility for cervical cancer with smaller HPV copy number to be more susceptible to growth inhibition by adeno-associated virus [76].

The results from Fig. 3 shows the effect of Acetylsalicylic acid (ASA) on viability of HeLa cervical cancer cells line at concentrations from 0 to 20 mM for 24 hrs, 48 hrs and 72 hrs incubations. The result shows no inhibition of viability by the drug on the cells from 0 to 10 mM concentrations after 24 hrs incubation, but it shows an inhibition of cells viability from 15 to 20 mM after 24 hrs with much more inhibition of cells viability at 20 mM concentration. There is also no inhibition of cells viability by the drug from 0 to 7.5 mM concentrations after 48 hrs incubation, but it shows a much inhibition of cells viability from 10 to 15 mM concentrations after 48 hrs. At 72 hrs incubation there are no effects on cells viability from 0 to 7.5 mM concentrations of the drug, but from 10 to 20 mM concentrations the drug has the effect on the cell by inhibiting the viability of the cells much more. From our results we found out that ASA has effect on viability of HeLa cervical cancer cells line from 15 to 20 mM concentrations after 24 hrs incubation and 10 to 20 mM after 48 and 72 hrs incubation. Aspirin as a COX inhibitor, the physiological actions of aspirin are related specifically to its ability to inhibit COX-2 enzymes [22]. The inhibition of COX-2 in cancer cells brings about a reduction in the production of prostaglandin, which eventually results in decrease cell viability, initiates apoptosis as well as increased immune surveillance [23].

Fig. 4 shows the effect Salicylic acid (SA) on viability of HeLa cervical cancer cells line from 0 to 20 mM concentrations of SA after 24, 48 and 72 hrs incubations. Our results show that there are no effects on the viability of the cells by the drug from 0 to 10 mM after 24 hrs incubation, but it shows effectiveness on cells viability at the concentration of 15 and 20 mM, with much more inhibition of cells viability at 20 than 15 mM. No inhibition observed by the drugs from 0 to 7.5 mM concentrations after 48hrs, but the drugs inhibit the growth of the cells from the concentrations of 10 to 20 mM, with much more inhibition at the concentration of 15 and 20 mM. After 72 hrs incubation, the drug shows no inhibition on cells viability at 0 to 7.5 mM, but it inhibits the cells viability from 10 to 20 mM concentrations with much more inhibition of cells viability at 15 and 20 mM concentrations. The results of our research show that, SA inhibits the

viability of HeLa cervical cancer cells line from 15 to 20 mM concentrations at 48 to 72 hrs incubations, this shows that SA can inhibit the growth of cervical cancer at high dose. SA acts through COX-independent pathways, SA plays a vital role in COX-2 inhibition than acetylation, as acetylation of serine on COX-2 may not result to inactivation [18]. Some studies documented that SA might possibly be a weak competitor for COX with its target arachidonic acid [46]. Salicylic acid is a weak inhibitor of COX directly, so far is still seen to decrease amount of COX metabolites, the prostaglandins that will result in inhibition of cells viability [47].

The graph in Fig. 5 shows the results of differential effect of SA and ASA on caspase activities on HeLa cervical cancer cells line seeded with 10 mM concentration of the drugs with caspaseglo after 0 to 24 hrs incubations. The result shows differential increase in caspase activity from 8 to 16 hrs incubation on HeLa cervical cancer cells line with both drugs SA and ASA, but there is no increase in caspase activity from 0 to 6 hrs incubations for both drugs, and the caspase activity reduces from 16 to 24 hrs incubations in both, but SA shows much higher in caspase activity. HeLa cervical cancer cells line induces caspase activity; this might be as a result of HeLa cervical cancer cell line was recommended as HPV positive because of the up-regulation of COX by viral oncoproteins in Hela cells [10]. From our findings, we observed that SA induced more caspase activity than ASA; this might be due to the differences in chemical properties and chemical functions of the two drugs [77,78].

Non-steroidal anti-inflammatory drugs; that is Acetylsalicylic acid and Salicylic acid were applied as an anti-inflammatory and analgesic drugs for decades [61,63,64,79], previous studies have revealed that these drugs are applicable in order to decrease the risk of some cancers in clinical observations [59,60,62,68], it has also been documented that these drugs can induce apoptosis in different types of cancers [65,66,67]. Another study also reported that Acetylsalicylic acid might be applicable as an anti-tumor drug in different cancer cell lines [65,66,67]. In our study non-steroidal anti-inflammatory drugs Acetylsalicylic acid and Salicylic acid were used on cervical cancer cells line (HeLa), from our findings it shows that these drugs inhibit the growth of cervical cancer cells line (HeLa), it also induces apoptosis through caspase 3 activation.

Previous researches revealed that Acetylsalicylic acid and Salicylic acid have anti-neoplastic effects on colorectal cancer [17,34]. The anti-tumor effect of Acetylsalicylic acid has been observed through apoptosis [35,36]. Mechanisms associated with apoptotic effect of Acetylsalicylic acid seen in human fibroblast are; p38 MAP kinase activation [37], inhibition of human telomerase transcriptase in colon cancer cells [38] and activation of caspases [39,40]. In this study we found out that Acetylsalicylic acid and Salicylic acid inhibit the viability of cervical cancer cells line (HeLa), it also shows a differential effect of the drugs on the cells and increase in caspase activity by the drugs on the cells line.

Acetylsalicylic acid and Salicylic acid have been used as chemopreventive drugs of cancers to induce apoptosis and reduce the risk of cancer development in many organs of the body such as colon [35], lung [69] and stomach [70]. ASA brings about apoptotic cell death by mitochondrial permeability transition [45,71]. In our study we used ASA and SA to determine the effectiveness on the viability of cervical cancer using HeLa cells line, our result shows inhibition of cells line viability, it also shows a differential effect on caspase activity using caspase 3 glo which brings about apoptosis and cells death.

Many studies were carried out on the effect of Salicylic acid on colorectal cancer, and the results concluded that SA can decrease precancerous adenomas through blocking COX-2 [80,81,82,83], the current study was carried out on the effect of Salicylic acid and acetyl salicylic acid on cervical cancer cells line viability, we found out this drug can possibly decrease the growth of cervical cancer.

Previous research also recommended that Acetylsalicylic acid has the ability to attach and modulate multiple cellular proteins in cancer cells by acetylation of lysine and serine residues [29]. Acetylsalicylic acid as COX inhibitor; its functions are due to the attachment of its acetyl group with serine residues on cyclooxygenase, which results to an irreversible inhibition of its function in cancer cells [25]. At physiological amount of acetylsalicylic acid, it has the capacity to acetylate different components that are associated to cervical cancer, such as tumor suppressor gene p53, responsible for regulation of cell development and result to cell death [26]. From our results we found out that at certain concentrations 10 to 20 mM of the drugs ASA

and SA, inhibit the viability of cervical cancer cells line (HeLa).

Apoptosis is a programmed cell death as a result of cell stress, cell damage, inflammation or mutations. Caspases control and initiate apoptosis that will result to cell death, can as well respond to extracellular inducers and intracellular inducers. Caspases are also associated to tumor suppressor gene p53 and mitochondria that initiates apoptosis and result to cell death. Cervical cancer cells are seen to inhibit apoptosis by inhibition of pro-apoptotic proteins and up regulation of anti-apoptotic proteins [23,27]. From the previous researches it was revealed that Acetylsalicylic acid could influence and promote caspase-dependent apoptosis in differents cancer cells by regulation of p53 [28]. Lee et al. [29] recommended that Acetylsalicylic acid modulate calpain gene expression and results to activation of caspase-3. However, inhibition of COX by Acetylsalicylic acid initiates caspase-dependent apoptosis by cellular stress signaling [84]. This previous studies corroborate with our findings in which we used caspaseglo to show the inhibition of cells viability, and from our results, we found out that there is differential effects of the drugs on caspase activity and inhibition of the viability of the cervical cancer cells line. A previous study also revealed that cell death occurs by apoptosis, extrinsic apoptotic pathway that occurred by tumor necrosis factor-related apoptosis-inducing ligand receptor, which results to tumor apoptosis. Through combination with acetylsalicylic acid and tumor necrosis factor for treatment of cancer has revealed a successful capacity to promote caspase-induced cell death in cancer [30]. This study also corroborate with our work in which we used a caspaseglo in combination with Acetylsalicylic acid and Salicylic acid on cervical cancer cells line (HeLa), from study results we observed a differential effects of the drugs on caspase activity and observed the inhibition of cervical cancer cell viability by the drugs.

From the results of our study we found out that Acetylsalicylic acid and salicylic acid inhibit the viability of cervical cancer cells line (HeLa). It was reported that Acetylsalicylic acid being an effective inhibitor of COX-1 has the ability to inhibit angiogenesis by COX-1 depended pathways in cervical cancer [12]. We also found out that salicylic acid inhibits the viability of cervical cancer cells line (HeLa) more than the acetylsalicylic acid. It was recommended that Salicylic acid might possibly be a weak competitor for COX inhibition [46]. Salicylic acid is a weak inhibitor of COX directly, but still observed to reduce amount of COX metabolites [47]. It was also revealed that Salicylic could degrade NFκB's inhibitory co-factor genes in cervical cancer [50,51].

According to results of our research, we found out that caspase activity occurred in HeLa cervical cancer cells line with Acetylsalicylic acid and salicylic acid that results to initiation of apoptosis and cell death. It was recommended that mitochondria are one of the initiators for cell death through apoptosis as execution of apoptosis through action of Bcl_2 proteins and release of cytochrome C [55]. It was also revealed that Salicylic acid and Acetylsalicylic acid are capable of changing these proteins and results to the release of cytochrome C and initiate apoptosis and cell death [56]. There is also possibility that Acetylsalicylic acid to initiate apoptosis through the release of mitochondrial cytochrome C [57], mitochondrial calcium uptake inhibition is another mechanism by which Acetylsalicylic acid brings about cell death [58].

From the results of this study we found out that SA induces greater caspase activity than ASA this might be as a result of differences in chemical properties between AS and ASA and result to differences in their mode of actions, SA has a chemical structure C_6H_4 (OH) COOH while ASA has $C_9H_8O_4$ in which hydroxyl group in SA was replaced with acetyl group in ASA [76,77], the hydroxyl group in SA attaches itself with serine residue in COX enzymes and inhibits the action of COX, while the acetyl group in ASA attaches itself with serine residue in COX and inhibits the action of COX. The hydroxyl group in SA is soluble in water, it will make the bond so strong; also it can directly penetrate the cell and start its action immediately when compared with ASA. However, the acetyl group in ASA is slightly insoluble in water, the bond will be very weak and hence cannot penetrate the cell immediately and start its function. Similarly, SA has been revealed to activate adenosine monophosphate-activated protein kinase (AMPK), and might be the reason for its anti-tumor effects more than ASA [85].

ASA and SA decreases inflammation by inhibition of the production of prostaglandins (PGs) as well as induce apoptosis in many types of tumor cells [57,86,87]. Cancer cells are found to undergo resistant to some drugs treatment and irradiation because of the inhibition of stimuli

in the tumor cell. ASA and SA have been recognized to induce apoptosis in resistance tumor cells [71]. However, the main basic molecular mechanisms by which ASA and SA induce apoptosis and results to anti-cancer effect are not well understood. But the most important role these drugs played is the molecular inhibition of COX. However, it was revealed from previous researches that there are some other mechanisms apart from COX inhibition, that may possibly results to anti-tumor and anti-inflammatory effects of these drugs [72,88,89]. These mechanisms include; increased reactive oxygen species (ROS) production, alterations in mitochondrial function and cell signaling [36,86,90,91,92].

In our study we used ASA and SA to see the effects on HeLa cervical cancer cells line, the reason we used this cell line (HeLa) was that, HeLa cells are HPV positive. HPV is the greatest causative agent of almost all cervical cancer infections [93,94], and this is achieved by up-regulation of COX enzymes [10]. It was found that over-expression of COX enzymes was recognized in significant numbers of cervical cancers at every significant phases of growth, including precancerous lesions [95,96]. In our study, we used ASA and SA as our drugs to find their effects on cervical cancer cells line (HeLa), this is because, these drugs were shown to inhibit COX in cancer cells, and COX over-expressed in cervical cancers. These drugs are found to induce apoptosis in cancer cells. In the growth of cervical cancer, COX has the capacity to promote the growth, chronic inflammation, carcinogen production, angiogenesis, and tumor invasiveness, inhibits immune recognition and apoptosis [10,18,22,97]. These drugs; ASA and SA can block COX and stop all these processes in cancer growth and development.

Western blotting analysis is a technique usually applied for the identification and analysis of proteins based on their ability to bind to a certain antibody. Western blotting is a simple procedure that needs simple equipment and inexpensive reagents, rapid technique. The specificity of the antibody-antigen reaction makes the target protein to be determined in the midst of complex protein mixture [98]. Fig. 6 shows the results of western blot of active anti-caspase, from our results we found out no any expression of proteins, which is contrary to some literatures in which an active anti-caspase-3 Ab (for pro-caspase and activated caspase peptide) shows an expression around 17kDa large unit and 12kDa small subunits [99]. We used β-actin again as a housekeeping gene, we found out an expression of the proteins with different bands of the samples around 43kDa shown in Fig. 7 above. We finally concluded that the Western blot analysis for active anti-caspase was done correctly; we are suggesting that it's as a result of using small amount of sample in an electrophoretic gel, or that the protein for active anti-caspase3 was not enough in the sample to show the expression.

5. CONCLUSIONS

In conclusion, this study that determined the effect of Acetylsalicylic acid (ASA) and Salicylic acid (SA) on the growth of HeLa cervical cancer cells line, indicating compliance with the finding of the above-described studies. Based on our results we found out that Acetylsalicylic acid and Salicylic acid at 10 mM to 20 mM concentrations inhibit the viability of cervical cancer cells line (HeLa) after 48 and 72 hrs incubations. We also found out a differential increase in caspase activities by the drugs on the cells line from 8 to 16 hrs incubations. This shows that Acetylsalicylic acid and Salicylic acid can induce apoptosis and bring about cell death on cervical cancer, and these drugs could be good drugs for the treatment of cervical cancer. Based on Western blot analysis we concluded that the active anti-caspase protein is in very small amount that is why it did not show any expression from the Western blot.

6. LIMITATIONS

Cost factor is a major limitation, establishment of infrastructure, equipment and other facilities are expensive for the tissue culture. Another difficulty with some cultured cells is their tendency to change their morphology, functions, or the range of genes they express.

Western blots are more expensive (antibody costs, plus membrane, reagents and visualization equipment), Western blots are much more likely to fail as there are many more steps than just staining a gel. A non-intended protein has a slight chance of reacting with the secondary anti-body, resulting in the labeling of an incorrect protein. Western blots are manually intensive and time-consuming, generally requiring 4 to 24 hour when taking into account gel preparation, separation, electro-blotting, and multiple incubations.

7. RECOMMENDATION

We are recommending further studies using animal model to induce cervical cancer and the use of these drugs for treatment before application to human.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0. Cancer Incidence *and* Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013.
2. Steben M, Duarte-Franco E. Human papillomavirus infection: Epidemiology and pathophysiology. Gynecology Oncology. 2007;107:S2–5.
3. Ellenson LH, Wu TC. Focus on endometrial and cervical cancer. Cancer Cell. 2004;5:533–538.
4. Crum CP, Lee KR. Diagnostic gynecologic and obstetric pathology. Carlsbad, CA: Saunders; 2006.
5. Arbyn M, Bergeron C, Klinkhamer P, Martin-Hirsch P, Siebers AG, Bulten J. Liquid compared with conventional cervical cytology: A systematic review and meta-analysis. Obstetric Gynecology. 2008;111: 167–177.
6. Dall KL, Scarpini CG, Roberts I, Winder DM, Stanley MA, Muralidhar B, Herdman MT, Pett MR, Coleman N. Characterization of naturally occurring HPV16 integration sites isolated from cervical keratinocytes under noncompetitive conditions. Cancer Research. 2008;68:8249–8259.
7. Sitas F, Madhoo J, Wessie J. Incidence of histologically diagnosed cancer in South Africa, 1993–1995. National Cancer Registry of South Africa, South African Institute for Medical Research, Johannesburg; 1998.
8. Arbyn M, Castellsague X, de Sanjose S, Bruni L, Saraiya M, Bray F, Ferlay J. Worldwide burden of cervical cancer. Annals of Oncology. 2011;22(2011):2675–2686.
9. ZurHausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009;384:260–265.
10. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase-2 transcription is regulated by human papillomavirus 16 E6 and E7 oncoproteins: Evidence of a corepressor/coactivator exchange. Cancer Research. 2007;67:3976–3985.
11. Oh JM, Kim SH, Lee YI, Seo M, Kim SY, Song YS, Kim WH, Juhnn YS. Human papillomavirus E5 protein induces expression of the EP4 subtype of prostaglandin E2 receptor in cyclic AMP response element-dependent pathways in cervical cancer cells. Carcinogenesis. 2009;30:141–149.
12. Sales KJ, Jabbour HN. Cyclooxygenase enzymes and prostaglandins in the pathology of the endometrium. Reproduction. 2003;5(126):559-67.
13. Sales KJ, Katz AA, Howard B, Soeters RP, Millar RP, Jabbour HN. Cyclooxy-genase-1 is up-regulated in cervical carcinomas: Autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin e receptors, and angiogenic factors by cyclooxygenase-1. Cancer Research. 2002;62:424–432.
14. Gey GO, Coffman WD, Kubicek MT. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Research. 1952;12: 264–265.
15. Skloot R. The immortal life of Henrietta lacks. MacMillan, New York; 2010.
16. Scherer WF, Syverton JT, Gey GO. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix.

Journal of Experimental Medicine. 1953;97:695–710.
17. Kune GA, Kune S, Watson LF. Colorectal cancer risk, chronic illnesses, operations, and medications: Case control results from the Melbourne colorectal cancer study. Cancer Research. 1988;48(1):4399–4404.
18. Simmons DL, Botting RM, Hla T. Cyclooxygenase isoenzymes: The biology of prostaglandin synthesis and inhibition. Pharmacological Reviews. 2004;3(56):387-437.
19. Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, Jankowski J, La Vecchia C, Meyskens F, JörgSenn H, Thun M. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: An international consensus statement. Lancet Oncology. 2009;5(10):501-7.
20. Manzano A, Perez-Segura P. Colorectal cancer chemoprevention: Is this the future of colorectal cancer prevention? Scientific World Journal. 2012;2012:327341.
21. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic and clinical issues. Journal of National Cancer Institute. 2002;4(94):252-66.
22. Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: Promise, perils and pharmacogenetics. Nature Reviews Cancer. 2006;2(6):130-40.
23. Dannenburg AJ, Dubois RN. COX-2: A new target for cancer prevention and treatment. Progress in Experimental Tumour Research. Basel: Karger. 2003;37.
24. Alfonso LF, Srivenugopal KS, Bhag G. Does aspirin acetylate multiple cellular proteins? (Review). Molecular Medicine Reports. 2009a;4(2):533-7.
25. Vane JR, Botting RM. The mechanism of action of aspirin. Thrombosis Research. 2003;5-6(110):255-8.
26. Alfonso LF, Srivenugopal KS, Arumugam TV, Abbruscato TJ, Weidanz JA, Bhat GJ. Aspirin inhibits comptothecin-induced p21CIP1 levels and potentiates apoptosis in human breast cancer cells. International Journal of Oncology. 2009b;3(34):597-608.
27. Brown JR, DuBois RN. COX-2: A molecular target for colorectal cancer prevention. Journal of Clinical Oncology. 2005;12(23):2840-55.
28. Ranganathan S, Joseph J, Mehta JL. Aspirin inhibits human coronary artery endothelial cell proliferation by upregulation of p53. Biochemical and Biophysical Research Communications. 2003;1(301):143-6.
29. Lee SK, Park MS, Nam MJ. Aspirin has anti-tumour effects via expression of calpain gene in cervical cancer cells. Journal of Oncology. 2008;2008:285374.
30. Im SR, Jang YJ. Aspirin enhances TRAIL-induced apoptosis via regulation of ERK1/2 activation in human cervical cancer cells. Biochemical and Biophysical Research Communications. 2012;1(424):65-70.
31. Lu M, Strohecker A, Chen F, Kwan T, Bosman J, Jordan VC, Cryns VL. Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing surviving levels. Clinical Cancer Research. 2008;10(14): 3168-76.
32. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell. 1998;5(93):705-16.
33. Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R, Arthur HM. Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a COX-independed mechanism. Federation of American Societies for Experimental Biology. 2006;12(20):2009-16.
34. Chan AT, Giovannucci EL, Schernhammer ES, Colditz GA, Hunter DJ, Willett WC, Fuchs CS. A prospective study of aspirin use and the risk for colorectal adenoma. Annals of Internal Medicine. 2004;3(140): 157–166.
35. Qiao L, Hanif R, Sphicas E, Shiff SJ, Rigas B. Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. Biochemical Pharmacology. 1998;55(1):53-64.
36. Piqué M, Barragán M, Dalmau M, Bellosillo B, Pons G, Gil J. Aspirin induces apoptosis through mitochondrial cytochrome c release. Federation of European Biochemical Societies. 2000;480(2):193–196.
37. Schwenger P, Alpert D, Skolnik EY, Vilček J. Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumour necrosis factor-induced IκBα phosphorylation and degradation. Molecular Cell Biology. 1998;18(1):78–84.

38. He H, Xia HH, Wang JD, Gu Q, Lin MC, Zou B, Lam SK, Chan AO, Yuen MF, Kung HF, Wong BC. Inhibition of human telomerase reverse transcriptase by non-steroidal anti-inflammatory drugs in colon carcinoma. Cancer. 2006;106(6):1243–1249.
39. Castaño E, Dalmau M, Barragán M, Pueyo G, Bartrons R, Gil J. Aspirin induces cell death and caspase-dependent phosphatidylserine externalization in HT-29 human colon adenocarcinoma cells. British Journal of Cancer. 1999;81:294–299.
40. Power JJ, Dennis MS, Redlak MJ, Miller TA. Aspirin-induced mucosal cell death in human gastric cells: Evidence supporting an apoptotic mechanism. Digestive Diseases and Sciences. 2004;49(9):1518–1525.
41. Gao J, Niwa K, Sun W, Takemura M, Lian Z, Onogi K, Seishima M, Mori H, Tamaya T. Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and up-regulate cyclooxygenase-2 protein expression in endometrial cancer cells. Cancer Science. 2004;95(11):901–907.
42. Redlak MJ, Power JJ, Miller TA. Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2005;289:G731–G738.
43. Stark LA, Dunlop MG. Nucleolar sequestration of RelA (p65) regulates NF-κB-driven transcription and apoptosis. Molecular Cell Biology. 2005;25(14):5985–6004.
44. Uyemura SA, Santos AC, Mingatto FE, Jordani MC, Curti C. Diclofenac sodium and mefenamic acid: Potent inducers of the membrane permeability transition in renal cortex mitochondria. Archives of Biochemistry and Biophysics. 1997;342(2): 231–235.
45. Oh KW, Qian T, Brenner DA, Lemasters JJ. Salicylate enhances necrosis and apoptosis mediated by the mitochondrial permeability transition. Toxicological Sciences. 2003;73(1):44–52.
46. Mitchell JA, Saunders M, Barnes PJ, Newton R, Belvisi MG. Sodium salicylate inhibits cyclo-oxygenase-2 activity independently of transcription factor (nuclear factor kappaB) activation: Role arachidonic acid. Molecular Pharmacology. 1997;51(6):907-12.
47. Franz B, O'Neil EA. The effect of sodium salicylate and aspirin on NF-kappa B. Science. 1995;5244(270):2017-9.
48. Kopp E, Gosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science. 1994;5174(265):956-9.
49. Wu KK. Aspirin and salicylate: An old remedy with a new twist. Circulation. 2000;17(102):2022-3.
50. Xu XM, Sansores-Garcia L, Chen XM, Matijivic-Aleksic N, Du M, Wu KK. Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. Proceedings of the National Academy of Sciences USA. 1999;9(96): 5292-7.
51. Kutuk O, Basaga H. Aspirin inhibits TNF-alpha and IL-1- induced NF kappa B activation and sensitizes HeLa cells to apoptosis. Cytokine. 2004;5(25):229-37.
52. Rigas B, Shiff SJ. Is inhibition of cyclooxygenase required for the chemopreventive effect of NSAIDs in colon cancer? A model reconciling the current contradiction. Medical Hypotheses. 2000; 2(54):210-5.
53. Dong Z, Huang C, Brown RE, Ma WY. Inhibition of activator protein 1 activity and neoplastic transformation by aspirin. Journal of Biological Chemistry. 1997;15(272):9962-70.
54. Han S, Inoue H, Flowers LC, Sidell N. Control of COX-2 gene expression through peroxisome proliferator-activated receptor gamma in human cervical cancer cells. Clinical Cancer Research. 2003;12(9): 4627-35.
55. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annual Review of Biochemistry. 2004;73:87-106.
56. Klampfer L, Cammenga J, Wisniewski HG, Nimer SD. Sodium salicylate activates caspases and induces apoptosis in myeloid leukemia cell lines. Blood. 1999;7(93):2386-94.
57. Dikshit P, Chatterjee M, Goswami A, Mishra A, Jana NR. Aspirin induces apoptosis through the inhibition of proteasome function. Journal of Biological Chemistry. 2006;39(281):29228-35.
58. Núñez L, Valero RA, Senovilla L, Sanz-Blasco S, García-Sancho J, Villalobos C. Cell proliferation depends on mitochondrial Ca2+ uptake: Inhibition by salicylate.

Journal of Physiology. 2006;Pt 1(571):57-73.

59. Hsieh CC, Hernández-Ledesma B, de Lumen BO. Lunasin, a novel seed peptide, sensitizes human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induced apoptosis. Chemico-Biological Interactions. 2010;186:127-34.
60. DeLuna-Bertos E, Ramos Torrecillas J, García-Martínez O, Díaz-Rodríguez L, Ruiz C. Effect of aspirin on cell growth of human MG-63 osteosarcoma line. Scientific World Journal. 2012;2012: 834246.
61. Dhillon PK, Kenfield SA, Stampfer MJ, Giovannucci LE, Chan MJ. Aspirin use after a prostate cancer diagnosis and cancer survival in a prospective cohort. Cancer prevention research (Philadelphia, Pa.). 2012;5:1223-8.
62. Pazhang Y, Ahmadian S, Javadifar N, Shafiezadeh M. COX-2 and surviving reduction may play a role in berberine-induced apoptosis in human ductal breast epithelial tumour cell line. Tumor Biology. 2012;33:207-14.
63. Kodela R, Chattopadhyay M, Goswami S, Gan ZY, Rao PN, Nia KV, Velázquez-Martínez CA, Kashfi K. Positional isomers of aspirin are equally potent in inhibiting colon cancer cell growth: Differences in mode of cyclooxygenase inhibition. Journal of Pharmacology and Experimental Therapeutics. 2013;345:85-94.
64. Yan F, He Q, Hu X, Li W, Wei K, Li L, Zhong Y, Ding X, Xiang S, Zhang J. Direct regulation of caspase-3 by the transcription factor AP-2α is involved in aspirin-induced apoptosis in MDA-MB-453 breast cancer cells. Molecular Medicine Reports. 2013;7: 909-14.
65. Xiaoxin M, Yingnan J, Yanxia L, Shu L, Yuanqi H, Hongwei L. Experimental research on the depressant effect of aspirin on Ishikawa adenocarcinoma endometrium cell growth. International Journal of Gynaecological Cancer. 2009;19:1182-5.
66. Ou YQ, Zhu Wb, Li Y, Qiu PX, Huang YJ, Xie J, He SM, Zheng XK, Leng TD, Xu D, Yan GM. Aspirin inhibits proliferation of gemcitabine-resistant human pancreatic cancer cells and augments gemcitabine-induced cytotoxicity. Acta Pharmacologica Sinica. 2010;31:73-80.
67. Park IS, Jo JR, Hong H, Nam KY, Kim JB, Hwang SH, Choi MS, Ryu NH, Jang HJ, Lee SH, Kim CS, Kwon TG, Park GY, Park JW, Janng BC. Aspirin induces apoptosis in YD-8 human oral squamous carcinoma cells through activation of caspases, down-regulation of Mcl-1, and inactivation of ERK-1/2 and AKT. Toxicology *in vitro.* 2010;24:713-20.
68. Singh A, Rathaur S. Combination of DEC plus aspirin induced mitochondrial mediated apoptosis in filarial parasite *Setariacervi.* Biochimie. 2010;92:894-900.
69. Hosomi Y, Yokose T, Hirose Y, Nakajima R, Nagai K, Nishiwaki Y, Ochiai A. Increased cyclooxygenase 2 (COX-2) expressions occur frequently in precursor lesions of human adenocarcinoma of the lung. Lung Cancer. 2000;30(2):73-81.
70. Wong BC, Zhu GH, Lam SK. Aspirin induced apoptosis in gastric cancer cells. Biomedicine & Pharmacotherapy. 1999; 53(7):315-318.
71. Kim KM, Song JJ, An JY, Kwon YT, Lee YJ. Pre-treatment of acetylsalicylic acid promotes tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis by down regulating BCL-2 gene expression. Journal of Biological Chemistry. 2005;280:41047-41056.
72. Langley RE, Burdett S, Tierney JF, Cafferty F, Parmar MK, Venning G. Aspirin and cancer: Has aspirin been overlooked as an adjuvant therapy? British Journal of Cancer. 2011;8(105):1107-13.
73. Liew G1, Mitchell P, Wong TY, Rochtchina E, Wang JJ. The association of aspirin use with age-related macular degeneration. Journal of American Medical Association Internal Medicine. 2013;4(173):258-64.
74. Mohammed I, Tangaza AM, Tsamiya RI. Effects of acetylsalicylic acid and salicylic acid on the growth of HT3 cervical cancer cell line. American Journal of Pharmacy and Pharmacology. 2014;1(4):32-44.
75. Brinton LA. Epidemiology of cervical cancer: Overview. In: Munoz KV, Bosch KV, Meheus A, editors. The Epidemiology of Human Papilloma Virus and Cervical Cancer. Oxford University Press. 1992;23–33.
76. Su PF, Wu FY. Differential suppression of the tumorigenicity of HeLa and SiHa cells by adeno-associated virus. British Journal Cancer. 1996;73:1533–1537.

77. Salicilyc acid.
Available:www.Inchem.org
(Retrieved 2008-10-13)
(Access on 16/11/2013)
78. Aspirin.
Available:http://www.wiley.com/legacy/college/boyer/0471661791/cutting_edge/aspirin/aspirin.htm
(Access on 13/11/2013)
79. El Kebir D, Filep JG. Modulation of neutrophil apoptosis and the resolution of inflammation through β2 integrins. Frontiers in Immunology. 2013;4:60.
80. Flossmann E, Rothwell PM. On behalf of the British doctors aspirin trial and the UK-TIA aspirin trial. Effect of aspirin on long-term risk of colorectal cancer: Consistent evidence from randomised and observational studies. Lancet. 2007;369:
81. Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, Meade TW. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomized trials. Lancet. 2010;376:1741–50.
82. Cole BF, Logan RF, Halabi S, Benamouzig R, Sandler RS, Grainge MJ, Chaussade S, Baron JA. Aspirin for chemoprevention of colorectal adenomas: Meta-analysis of the randomised trials. Journal of National Cancer Institute. 2009;101:256–66.
83. Chan AT, Ogino S, Fuchs CS. Aspirin and risk of colorectal cancer in relation to expression of COX-2. New England Journal of Medicine. 2007;356:2131–42.
84. Elwood PC, Gallagher AM, Duthie GG, Mur LA, Morgan G. Aspirin, salicylates, and cancer. Lancet. 2009;9671(373):1301-9.
85. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ, Peggie MW, Zibrova D, Green KA, Mustard KJ, Kemp BE, Sakamoto K, Steinberg GR, Hardie DG. The ancient drug salicylate directly activates AMP-activated protein kinase. Science. 2012;6083(336):918–22.
86. Baron JA. Epidemiology of non-steroidal anti-inflammatory drugs and cancer. Progress in Experimental Tumour Research. 2003;37:1–24.
87. Adachi M, Sakamoto H, Kawamura H, Wang W, Imai K, Shinomura Y. Non-steroidal anti-inflammatory drugs and oxidative stress in cancer cells. Histology and Histopathology. 2007;22:437–442.
88. Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. Science. 2000;290:989–992.
89. Gao J, Liu X, Rigas B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proceedings of the National Academy USA. 2005;102: 17207–17212.
90. Sun Y, Huang L, Mackenzie GG, Rigas B. Oxidative stress mediates through apoptosis the anticancer effect of phosphor-nonsteroidal anti-inflammatory drugs: implications for the role of oxidative stress in the action of anticancer agents. Journal of Pharmacology and Experimental Therapeutics. 2011;338(3):775–783.
91. Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumour necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proceedings of the National Academy USA. 1997;94:2869–2873.
92. Husain SS, Szabo IL, Tamawski AS. NSAID inhibition of GI cancer growth: Clinical implications and molecular mechanisms of action. American Journal of Gastroenterology. 2002;97:542–553.
93. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ. International Agency for Research on Cancer Multicentre Cervical Cancer Study Group. Epidemiological classification of human papillomavirus types associated with cervical cancer. New England Journal of Medicine. 2003;6(348):518-27.
94. Muñoz N, Bosch FX, Castellsagué X, Díaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. International Journal of Cancer. 2004;2(111):278-85.
95. Kulkarni S, Rader JS, Zhang F, Liapis H, Koki AT, Masferrer JL, Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2 is overexpressed in cervical cancer. Clinical Cancer Research. 2001;2(7):429-34.
96. Dursun P, Yuce K, Usubutun A, Ayhan A. Cyclooxygenase-2 expression in cervical intraepithelial neoplasia III and squamous cell cervical carcinoma, and its correlation

with clinicopathologic variables. International Journal of Gynaecological Cancer. 2007;1:164-73.

97. Grösch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anti-carcinogenic effects of selective COX-2 inhibitors. Journal of the National Cancer Institute. 2006;11(98): 736-47.

98. Western blotting (Theory); 2013.
Available:http://amrita.vlab.co.in/?sub=3&brch=187&sim=1331&cnt=1
(Accessed on 12/9/2013)

99. Anti-active caspase 3 antibody; 2013.
Available:http://www.abcam.com/active-caspase-3-antibody-e83-77-ab32042.html
(Accessed on 26/10/2013)

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