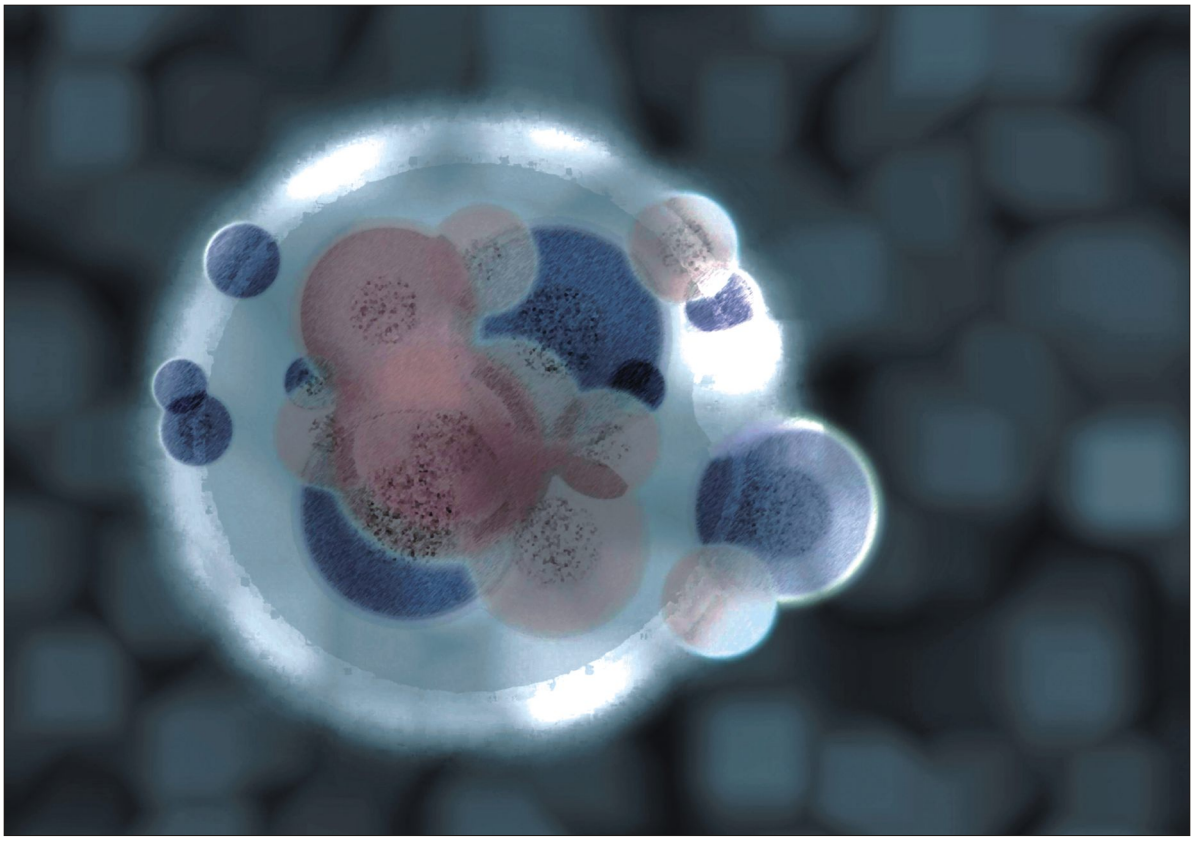

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2021.59.69

Research Article
In silico and *in vitro* Analysis of Alkylated Salicylic Acid Inhibition Activity on Cervical HeLa Cancer Cell Line

[1]Priscilla Ardianto, [2]Ade Arsianti, [3]Khaerunissa Anbar Istiadi and [2,3]Fadilah Fadilah

[1]Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
[2]Department of Medical Chemistry, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
[3]Bioinformatics Core Facilities IMERI Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Abstract

Background and Objective: Cervical cancer is one of the most frequently found cancers in women. Studies have demonstrated the correlation between inflammation mediated by COX-2 and cancer proliferation. This research was conducted to observe and compare the effects of different alkylated salicylic acid derivatives on the proliferation of cervical cancer HeLa cell. **Materials and Methods:** Alkylated salicylic acid was synthesized by reacting salicylic acid with n-alcohol through Steglich esterification. Its products' physical and chemical properties were measured by using apparatus and by observation. The products of the reaction were analyzed by thin-layer chromatography. Alkylated derivatives of salicylic acid were tested using an MTT assay to determine their percentage inhibition and IC_{50} value against HeLa cell. The IC_{50} values were compared with the IC_{50} value of salicylic acid. **Results:** The thin layer chromatography profile for alkylated salicylic acids using non-polar eluent (n-hexane:ethyl acetate = 4:1) are methyl salicylate (Rf =0.75), ethyl salicylate (Rf = 0.78), butyl salicylate (Rf = 0.90), isoamyl salicylate (Rf = 0.95) and octyl salicylate (Rf = 0.81). The MTT result shows that both salicylic acid and its alkylated derivatives showed cytotoxic activity. **Conclusion:** All alkylated derivatives of salicylic acid have better anti-proliferative activity compared to salicylic acid. The length of the alkyl chain was not related to the anti-proliferative activity. Out of all alkylated salicylic acid derivatives, butyl salicylate had the best anti-proliferative activity.

Key words: Alkylated, COX-2, HeLa cell, IC_{50}, salicylic acid

Citation: Ardianto, P., A. Arsianti, K.A. Istiadi and F. Fadilah, 2021. *In silico* and *in vitro* analysis of alkylated salicylic acid inhibition activity on cervical HeLa cancer cell line. J. Biol. Sci., 21: 59-69.

Corresponding Author: Fadilah Fadilah, Department of Medical Chemistry, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is undoubtedly a major health concern in both developed and developing countries. It is currently the second leading cause of mortality worldwide and is responsible for 9.6 million deaths in 2018[1]. Among the different types of cancer, cervical cancer is the fourth most frequently found cancer in women. There are approximately 570,000 new cases of cervical cancer worldwide in 2018, making up 6.6% of all female cancers. Furthermore, cervical cancer ranks fourth as the cause of cancer-related deaths in women[2,3]. In Indonesia, cervical cancer placed second as the most common cancer with an incidence of 8.7 per 100.000 women[4].

Although prevention by vaccination is one of the best tools in reducing the incidence, diagnosis of cervical cancer in advanced stages is still frequently found especially in developing countries[5]. Hence continued pursuit for better treatment option is still a necessity. In the past years, there have been an increasing number of pieces of evidence that support the relation between inflammation which is mainly maintained by cyclooxygenase-2 (COX-2) enzyme activity and cancer cells proliferation, progression and dissemination[5,6]. The HPV oncogene can regulate the Cyclooxygenase (COX)-Prostaglandin (PG) axis in cervical cancer cells to bring about inflammation, persistent infection as well as tumorigenesis.

Inflammation has been indicated to have a role in the modulation of cancer progression. Among the modulators of inflammation, cyclooxygenase seems to be implicated in cancer[7]. The COX enzymes act in a coupled reaction which the first reaction involves COX activity which mediates the conversion of arachidonic acid into prostaglandin H2 (PGG2). The second reaction converts the PGG2 from the first reaction into prostaglandin H2 (PGH2) via peroxidase activity. These two reactions will then be followed by another reaction mediated by isomerases to convert PGH2 into thromboxane A2 and other prostaglandins such as PGD2, PGF2α, PGE2 and PGI2[7].

An example of possible treatment options that target COX-2 is anti-inflammatory agents such as salicylic acid and its derivatives. Research is done by Soriano-Hernandez AD *et al*. in 2015[5] demonstrate the antineoplastic effect of anti-inflammatory agents such as sulindac, meclofenamic acid, flufenamic acid, mefenamic acid and celecoxib against HeLa cell. The research also concludes that meclofenamic acid displays the highest efficacy. Another research in 2014[8] demonstrates that salicylic acid and its derivative, acetylsalicylic acid (aspirin) induce apoptosis on the cervical cancer cell. The same research also showed that salicylic acid has more effect in reducing the viability of cervical cancer cell line compared to aspirin due to differences in chemical properties and structure of the drugs.

Although there have been researches that demonstrate the antineoplastic capability of anti-inflammatory agents, research development can still be made especially in modifying the chemical structure of the active substance. As demonstrated in the research above, the chemical structure of the drug may impact its efficacy[8]. Hence, this study aimed to analyze the effect of methyl salicylate, ethyl salicylate, butyl salicylate, isoamyl salicylate and octyl salicylate as anti-inflammatory agents in the growth of HeLa cervical cancer cell line and determine the interaction between those salicylic acid derivatives towards COX2 molecule by molecular simulation.

MATERIALS AND METHODS

Study area: This research was done at the Faculty of Medicine Universitas Indonesia, at the Department of Medical Chemistry laboratory in February and March, 2020.

Molecular simulation and docking

Preparation of COX2 molecule and ligand: The 3D structure of the COX2 molecule (6BL3) was downloaded from PDB (http://www.pdb.org/). The series of salicylic acid derivatives as a ligand was made in 2D using Marvin Sketch 15.1.19 software and saved in 3D structure.

Prediction of the active site: The active site was predicted by using the site finder option of using MOE 20 (Molecular Operating Environment) software licensed from Universitas Indonesia (valid through December 7th, 2021). Calculations were made to determine potential sites for ligand binding and docking and restriction sets for rendering partial molecular surfaces.

Molecular docking: Molecules and the ligands being optimized using MOE software to fix the charge, added the hydrogen and minimizing energy. Both molecules and ligands stored in molecular format for later use. Docking calculations executed by the Lamarckian algorithm in MOE software. Docking interactions had been clustered to decide the Gibbs energy (ΔG) and optimum docking energy conformation was considered as the fine docked pose. The generated conformations had a related value of the Gibbs energy (ΔG). An estimated inhibition concentration (Ki) was used for the determination of binding energies of various docking

conformations, ranking according to their binding rankings. Contact residue of the COX2-ligand complex from the docking results identified using MOE software.

Physical and chemical properties measurement: Alkylated salicylic acid was synthesized before the experiment. The synthesis products were examined for their physical and chemical properties. The molecular weight was obtained by calculation. The physical properties tested were appearance, odour, melting point and solubility. The appearance and odour of the sample were tested by the observation method. The melting point was measured by using Innotech DMP 100 melting point apparatus. Solubility was physically observed by dissolving the synthesis products in water.

Thin layer chromatography: Thin layer chromatography was a method to separate different mixtures of substance into its components. In this experiment, thin-layer chromatography was conducted for each synthesis products that was the alkylated salicylic acids. This method was used to determine the purity of the products. Thin-layer chromatography had two phases: a stationary phase and a mobile phase. The stationary phase used here was alumina coated plate while the mobile phase (eluent) was n-hexane and ethyl acetate with the ratio of 4:1 (n-hexane: ethyl acetate).

First, a pencil line was drawn at the bottom of the alumina coated plate which was called the starting line and another line was drawn 1 cm from the top border of the plate. Then, 4 spots were created at the starting line by dropping 4 different solutions: salicylic acid, alcohol (i.e., methanol, ethanol, butanol, etc.), products of synthesis and a mixture of reagents and products.

When the eluent has reached the drawn line at the top of the plate, then the thin layer chromatography process was stopped. Afterwards, the plate was stained with iodine and read under UV light with wavelength 254 nm. The distance travelled by the stain from the starting line was measured to find the retention factor (Rf value).

Purification of products: The thin layer chromatography done previously was able to elucidate the purity of the products of synthesis. If the TLC result of the products of synthesis showed more than 1 stain, a purification process must be conducted to the synthesis products. The purification process was done by using column chromatography with silica gel as the stationary phase and the n-hexane: ethyl acetate eluent as the mobile phase. The ratio for the eluent varies from one alkylated salicylic acid samples to another. This is due to the different polarity of the alkylated salicylic acid samples. The eluent ratio for each compound is described in Table 1.

Table 1: Eluent ratio for purification of the process of alkylated salicylic acids

Compounds	Mobile phase
Methyl salicylate	n-hexane: Ethyl acetate ratio 20:1-10:1
Ethyl salicylate	n-hexane: Ethyl acetate ratio 20:1-10:1
Butyl salicylate	n-hexane: Ethyl acetate ratio 10:1-5:1
Isoamyl salicylate	n-hexane: Ethyl acetate ratio 10:1-5:1
Octyl salicylate	n-hexane: Ethyl acetate ratio 10:1-1:1

MTT cytotoxicity assay: The100 µL HeLa cell suspension with a cell density of 2.62×10^6 mL^{-1} medium was taken using a micropipette and placed on each well of the 96-well plate. The cell suspension was then incubated for 24 hrs at 37°C with 5% CO_2 and 95% O_2. Following incubation, 100 µL of the prepared alkylated salicylic acid solution (methyl salicylate, ethyl salicylate, butyl salicylate, isoamyl salicylate and octyl salicylate) with different concentration was placed inside the wells which contain HeLa cells suspension. Besides, some of the wells were also given 100 µL of culture medium (RPMI) as control, 100 µL of DMSO as solvent control and another number of wells were administered with 100 µL of doxorubicin which acts as the positive control.

The 96-well plate was further incubated for 24 hrs at 37°C with 5% CO_2 and 95% O_2. After incubation, the culture medium was disposed and 100 µL 10% MTT solution will be added. Another incubation was performed at the same condition for 3-4 hrs. Live cells react with MTT, forming formazan crystals that give purple colour. The absorbance of the sample will then be read using an ELISA reader at wavelength 492 nm.

The absorbance result obtained from the ELISA reader was converted to find out the percentage inhibition of the sample against the proliferation of HeLa cell. The conversion was done using the following formula[9]:

$$\text{Inhibition } (\%) = \frac{A_T}{A_C} \times 100$$

Where:

n = Percentage of cell death after treatment

A_T = Absorbance of HeLa cell suspension treated with negative control (RPMI 1640 medium)

A_C = Absorbance of HeLa cell suspension that had been treated with alkylated salicylic acid samples or doxorubicin samples

Data analysis: Microsoft Excel software was used to find the IC_{50} value which is the sample concentration that was able to inhibit 50% of HeLa cell growth. The Inhibition data (%)

obtained previously were plotted on a scatter plot where the X-axis was the log concentration of the sample and the Y-axis was the Inhibition (%). Afterwards, the most suitable linear regression was plotted using the trendline function of Microsoft Excel. After a linear function is obtained, the IC_{50} value could be calculated.

The Inhibition data (%) and the IC_{50} value was further analyzed statistically using the IBM SPSS statistics software. The purpose was to prove whether the findings were able to confirm the hypothesis. The statistical analysis performed on the %Inhibition data was done to prove the significance of differing concentrations of a sample in inhibiting HeLa cell growth. Meanwhile, the IC_{50} between samples was analyzed statistically to show the difference of the five alkylated salicylic acid samples tested in inhibiting HeLa cell growth.

RESULTS AND DISCUSSION

Prediction of active site COX2 (6BL3) with site finder: Protein receptors consist of the active side and the all-electric side. The substrate will bind to protein receptors on the active side. The active site of the enzyme can only bind to the corresponding substrate. Meanwhile, the active side of protein receptors will adjust to the shape of the substrate that will bind to the enzyme. So that in molecular simulations are carried out predictions of the active side of receptors. Ligands or active compounds are substances that can increase or decrease the activity of enzymes. Inhibitors are compounds that inhibit the activity of enzymes. The bond between non-competitive inhibitors and the allometric side of the enzyme results in the active site of the enzyme changing.

Active site of COX2 (6BL3) with Site finder predictor in MOE shown in Fig. 1. Prediction active site of receptor COX2 (6BL3) used site finder in MOE 20 software have 41 sites. Active site number 1 has size 475 TPSA and residue Thr76, Lys79, Leu80, Leu81, Lys83, Pro84, Thr85, Pro86, Asn87, Thr88, Val89, His90, Leu93, Ile111, Lys114, Tyr115, Val116, Ser119, Arg120, Tyr122, Leu123, Val349, Gln350, Ser353, Gly354, Tyr355, Leu359, Arg467, Lys468, Arg469, Phe470, Ser471, Leu472, Lys473, Pro474, Tyr475, Glu480, Glu510, Lys511, Pro512, Arg513, Gly519, Glu520, Thr521, Val523, Glu524, Ala527 Pro528, Leu531.

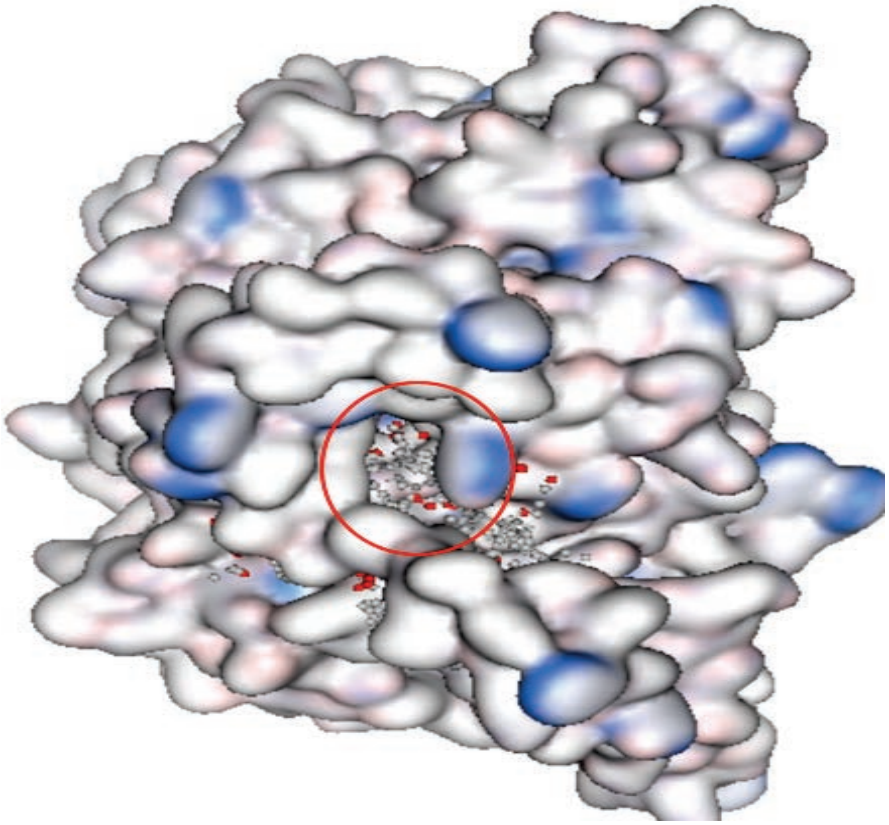

Fig. 1: Active site prediction (red circle) of COX2 receptor (used MOE 20)

Table 2: Docking Result of alkylated salicylic acid derivatives with COX2

	Salicylate							
	Methyl	Ethyl	Propyl	Butyl	Isoamyl	Pentyl	Hexyl	Octyl
Molecular weight (g mol^{-1})	152.15	166.17	180.201	194.23	208.25	208.254	222.280	250.33
Gibbs energy (Kcal mol^{-1})	-6.6231	-8.9231	-8.3412	-10.9035	-10.3823	-9.3520	-10.0282	-10.2337
Inhibition concentrate-ion (μM)	5.673	4.893	5.972	7.734	7.562	6.925	7.013	7.542
Hydrogen interaction	Ala527, Val116, Val89	Ser119, Tyr115, Val116	Val116, Val89, Leu93	Tyr365, Ala527, Val116, Val89, Leu93, Val523	Val89, Leu93, Tyr115, Val116, Ser119, Arg120	Leu93, Ser119, Tyr115, Val116	Val116, Ser119, Arg120	Ser119, Tyr115, Val116, Tyr365, Ala527

In COX2 receptors the predicted results showed the presence of 41 active sides with different sizes. Figure 1 showed that the assets side number 1 has a lot in common with the active site of the COX2 database in NCBI. According to the database, www.ncbi.nlm.nih.gov/protein/6BL3_A the protein sequence region 58..545 are region prostaglandin endoperoxide synthase,and substrate-binding site or chemical binding in the region site are 86, 174, 314, 317..318, 322, 324, 350, 354, 356, 487, 491, 495..496, 499..500, 503). The homodimer interface or polypeptide binding in the site are region 94, 96..98, 105..109, 111, 198, 288..292, 295..296, 299, 302..303, 306, 336..343, 506..507, 510..514, 516..518, 520..521.

If this salicylic derivative compound interacts with the residues on the active site in hydrogen bonds, Hydrogen bonds that occur are formed by oxygen atoms (O) in salicylic acid compounds that have a partial charge with hydrogen atoms (H) in amino acid residues according to the active site then able to inhibit the study of COX2.

Molecular docking: Docking is performed on the active side of COX2 where there are 51 amino acids on the active site of the cavity. The outerness of the molecular docking is in the form of poses and scores. The pose is the position and conformation of ligands on the active side of COX2. The pose will determine the interaction of ligands with the surrounding COX2 amino acid residues. The docking results between the COX2 and salicylic acid derivatives showed in Table 2.

The interaction of butyl salicylic compounds with amino acids on the active side showed in Fig. 2a is a guide mechanism for the interaction of other COX2 inhibitor compounds. Butyl salicylate has Gibbs energy-10.9035 Kcal moL^{-1} and the interaction of butyl salicylate with amino acids Glu524 and Arg120 become the basis of constraint enforcement by giving more value to the pose of active compounds in salicylic acid derivatives that interact with COX2. The enforcement of constraint in the scoring function is expected to lead to better exploration of poses. Besides, butyl salicylic compounds have hydrophobic interactions against Tyr365, Ala527, Val116, Val89, Leu93, Val523.

Isoamyl salicylic compounds interact with Arg120 residues given in Fig. 2b. Isoamyl salicylate has Gibbs energy-10.3823 Kcal moL^{-1}. The interaction between isoamyl salicylate and Arg120 residue is hydrogen bonding and hydrophobic interaction through aromatic rings on isoamyl salicylate with rings on Arg120 residues. In addition to interacting hydrophobically, isoamyl salicylic aromatic rings have phi electrons capable of binding electropositive atoms.

Octyl salicylic compounds by bonding hydrogen with Arg120 residues and interacting hydrophobic with residues of Ser119, Tyr115, Val116, Tyr365, Ala527 showed in Fig. 2c. Octyl salicylic have Gibbs energy-10.2337 Kcal moL^{-1} Hydrogen bonds form between the O atom in the salicylic octyl and the H atom in the Arg120 residue.

The score is usually stated as an energy-based score where the lower the value indicates the more stable the ligand-receptor interaction so that the ligament affinity against the receptor is stronger. From docking result that energy-based score from low energy to high energy Butyl salicylate >Isoamyl salicylate >Octyl Salicylate >Hexyl salicylate >pentyl salicylate >ethyl salicylate > propyl salicylate >methyl salicylate. Butyl salicylate has the best energy binding than other derivatives.

Physical and chemical properties of sample: Methyl salicylate ($C_8H_8O_3$) has a molecular weight of 152.15 g moL^{-1} with melting temperature-8.6°C and a colourless yellowish appearance. Ethyl salicylate ($C_9H_{10}O_3$) has a molecular weight of 166.17 g moL^{-1} with a melting temperature of 1°C. Butyl salicylate ($C_{11}H_{14}O_3$) has a molecular weight of 194.23 g moL^{-1} with a melting temperature of-5.9°C. Isoamyl salicylate ($C_{12}H_{16}O_3$) has a molecular weight of 208.25 g moL^{-1} with melting temperature-75°C and Sweet herbaceous green, slightly floral odour. Octyl salicylate ($C_{15}H_{22}O_3$) has a molecular weight of 250.33 g moL^{-1} with a melting point at 25°C and a slightly floral odour. Methyl Salicylate, Ethyl Salicylate are slightly soluble in water meanwhile Butyl Salicylate, Isoamyl Salicylate and Octyl Salicylate are insoluble in water. In-room temperature, ethyl and Butyl Salicylate have a colourless liquid

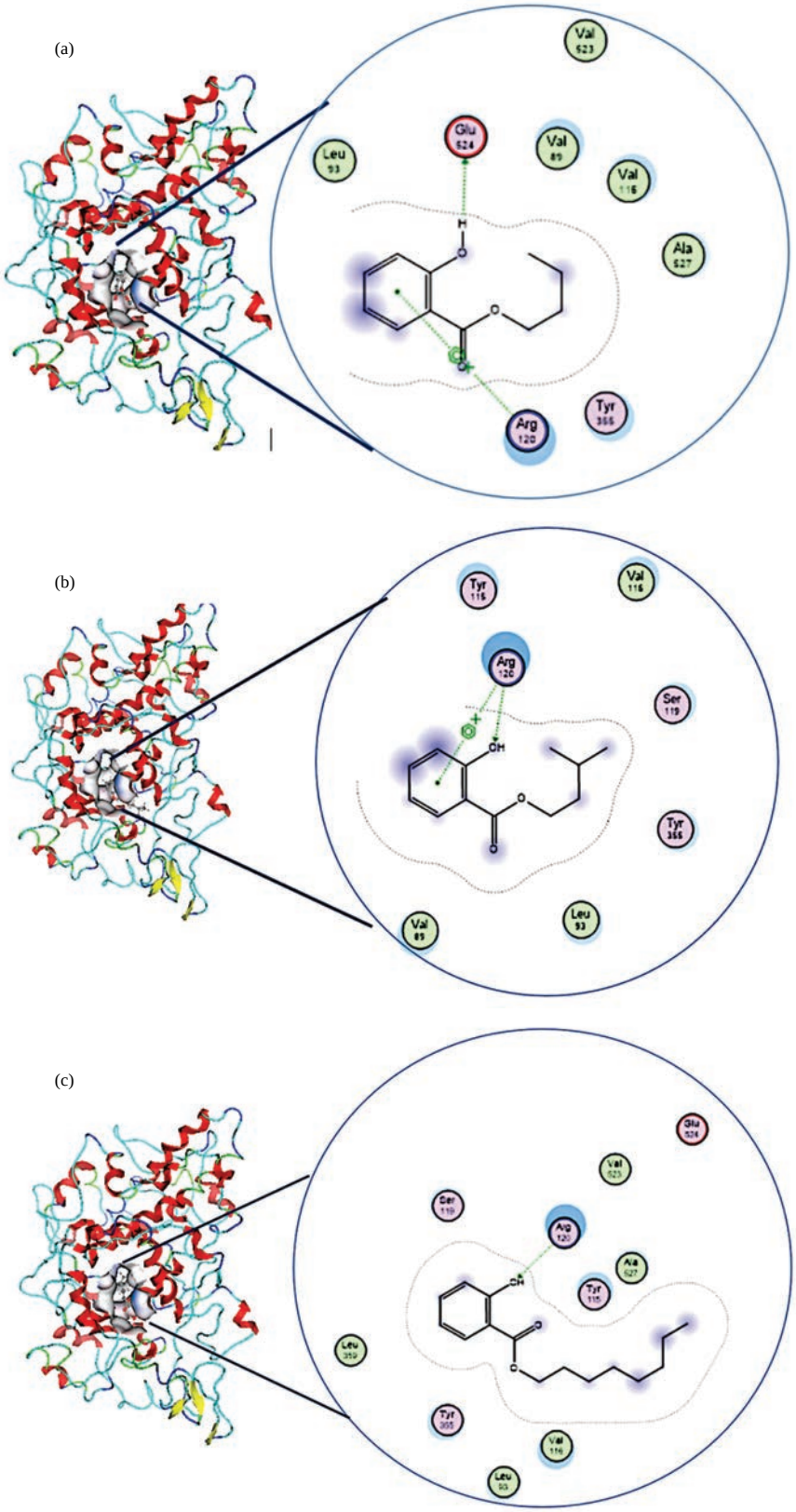

Fig. 2(a-c): Complexes of alkyl salicylate with COX2, (a) Butyl salicylate-COX2, (b) Isoamyl salicylate-COX2 and (c) Octyl salicylate-COX2

Table 3: Physical and chemical properties of alkylated salicylic acid derivatives

	Salicylate				
	Methyl	Ethyl	Butyl	Isoamyl	Octyl
Formula	$C_8H_8O_3$	$C_9H_{10}O_3$	$C_{11}H_{14}O_3$	$C_{12}H_{16}O_3$	$C_{15}H_{22}O_3$
Molecular weight (g mol^{-1})	152.15	166.17	194.23	208.25	250.33
Melting point	-8.6°C	1.0°C	-5.9°C	-75°C	25°C
Solubility in water	Slightly soluble	Slightly soluble	Insoluble	Insoluble	Insoluble
Physical description at room temperature	Colourless yellowish liquid	Colourless liquid	Colourless liquid	Colourless oily liquid	Colourless oily liquid
Odour	Wintergreen odour	Wintergreen odour	Wintergreen odour	Sweet herbaceous green, slightly floral odour	Slight floral odour

Table 4: Thin layer chromatography profile

	Rf values				
	Methyl salicylate	Ethyl salicylate	Butyl salicylate	Isoamyl salicylate	Octyl salicylate
Salicylic acid	0.22	0.21	0.34	0.30	0.20
Alcohol	0.43	0.46	0.71	0.76	0.60
Mix	0.22	0.21	0.32	0.28	0.18
	0.43	0.45	0.71	0.76	0.60
	0.62	0.65	0.90	0.95	0.81
	0.75	0.78	-	-	-
Product	0.43	0.46	0.40	0.30	0.60
	0.63	0.65	0.71	0.74	0.81
	0.75	0.78	0.90	0.91	-
	-	-	-	0.95	-

appearance. Isoamyl and octyl salicylate liquid has a colourless oily appearance. Methyl, ethyl and butyl salicylate has wintergreen odour given in Table 3.

The length of the alkyl chain in the synthesised alkylated salicylic acid derivatives affects both its physical and chemical properties. From the samples tested, methyl salicylate has the shortest alkyl chain, followed by ethyl salicylate, butyl salicylate and isoamyl salicylate and octyl salicylate has the longest alkyl chain. Hence as the length of the chain is increased from methyl to octyl salicylate, its molecular weight will also increase. This is in line with the result of the experiment where out of all samples, methyl salicylate has the lowest molecular weight and octyl salicylate has the highest molecular weight.

The next property observed is the melting point of the substance. Melting and boiling processes occur when intermolecular forces between identical molecules are disrupted. As the intermolecular bond between molecules is stronger, the higher the energy required to disrupt those bonds, resulting in higher melting and boiling point. In general, molecules that have longer chain should have a higher boiling point as a longer chain allows the molecules to have more surface area for intermolecular forces, such as the Van der Waals forces[10].

As all the melting point of the alkylated derivatives of salicylic acid tested is below the room temperature, all samples existed in the liquid state. From the result of the experiment illustrated, the melting point of samples observed generally increases as the length of the chain increases, which is in line with the theory. The molecule with the shortest chain, i.e., methyl salicylate does have the lowest melting point at -8.6°C, ethyl salicylate with a slightly longer chain has a higher melting point than methyl salicylate at 1°C, while octyl salicylate, the molecule with the longest chain has the highest melting point at 25°C. However, there are some exceptions for the melting point of butyl salicylate and isoamyl salicylate which was lower compared to ethyl salicylate, despite having a longer chain.

The reason for this phenomenon may be related to the fact that aside from the size of the molecule and the strength of intermolecular forces, the melting point is also affected by the molecule's shape and how well they pack tightly together[10]. For example, isoamyl salicylate has an additional $-CH_3$ attached at the end of the carbon chain (making it branched). This disrupted the ability of isoamyl molecules to pack together tightly, hence resulting in a much lower melting point compared to the other samples. Another reason is that the melting point is related to the balance between entropy and enthalpy of fusion. In a longer alkyl chain, enthalpy is the deciding factor as there are enhanced Van der Waals forces of attraction, hence the melting point will increase as the chain length increases. However, in a shorter alkyl chain (methyl,

ethyl and butyl) the entropy plays a larger factor so that those molecules with higher liquid phase entropy will have a lower melting point, e.g., butyl salicylate.

Another observed physical property is solubility. The samples tested have different solubility in water. Methyl salicylate and ethyl salicylate were slightly soluble in water, while butyl, isoamyl and octyl salicylate were insoluble. These differences can be attributed to the length of the carbon chain. The longer the carbon chain, the more insoluble it becomes as derivatives with longer chain would have larger hydrophobic non-polar region, causing them to be less polar and less soluble[10].

Thin Layer Chromatography (TLC) analysis of alkyl salicylate: Thin layer chromatography is a technique that is used to separate the components of a mixture. The principle of thin layer chromatography is that different compounds will be separated based on their polarity properties. The more polar the tested compound is more likely it to interact strongly with the polar stationary phase, i.e., the alumina plate. This results in a lower Rf value. On the other hand, if a compound is less polar, it will have a higher Rf value as it is more soluble in the non-polar mobile phase, i.e., the n-hexane: ethyl acetate eluent following the like-dissolves-like principle[11,12]. Thin layer chromatography was done in this experiment to separate the products of synthesis and compare their Rf value to its reagent.

In this experiment, the thin layer chromatography profile of products of synthesis given in Table 4: for methyl salicylate, there are 3 stains with Rf: 0.43, 0.63 and 0.75. For ethyl salicylate, there are 3 stains with an Rf value of 0.46, 0.65 and 0.78. For butyl salicylate, there are 3 stains with Rf: 0.40, 0.71 and 0.90. For isoamyl salicylate, there are 4 stains with Rf value 0.30, 0.74, 0.91, 0.95. Lastly, for octyl salicylate, there are 2 stains with an Rf value of 0.60 and 0.81.

The products of synthesis was a mixture of different compounds as the TLC profile of the products have more than 1 stain with different Rf values, meaning that the products of the reaction were not pure, hence required purification. The Rf values that are inside the square are the Rf values of the products. Hence: methyl salicylate (Rf = 0.75), ethyl salicylate (Rf = 0.78), butyl salicylate (Rf = 0.90), isoamyl salicylate (Rf = 0.95) and octyl salicylate (Rf = 0.81).

By comparing the Rf values of the products with the Rf value of the reagents, some of the compounds in the products of synthesis are identified as leftover reagents of the synthesis, i.e. salicylic acid and n-alcohol as the Rf value was the same as the Rf value of the reagents. For example, for octyl salicylate, there are 2 stains in the products of synthesis with Rf value: 0.60 and 0.81. One of the compounds has the same Rf value as octanol which is 0.60. Hence, it can be assumed that one of the compounds in the products of synthesis is octanol.

As the products of synthesis still contain the reagent, it can be assumed that the esterification reaction is not optimal. For all the reagents to be fully used up in the reaction, further optimisation is needed. Examples of optimisation that can be done include optimisation of reaction time and temperature and optimisation by doing molarity calculation for the reagents.

Aside from the alkylated salicylic acids and the reagents, some of the products of reaction also have an additional stain with a different Rf value from the reagents. This can be observed at the products of synthesis of methyl salicylate, ethyl salicylate and isoamyl salicylate. This additional strain may indicate the presence of secondary products of the reaction. The structure of the secondary products needs to be further examined by using FTIR, mass spectroscopy and NMR.

Anti-proliferative activity of samples against HeLa cell: Based on the graphic shown in Fig. 3, all samples tested (methyl salicylate, ethyl salicylate, isoamyl salicylate, butyl salicylate, octyl salicylate, doxorubicin and salicylic acid) had a positive value for percentage inhibition in all groups of concentration tested. In other words, all samples were able to inhibit the growth of cervical cancer HeLa cell within the concentration range tested. The general trend showed that samples' ability to inhibit the growth of HeLa cell was positively correlated with the samples' concentration. The data also showed that there was a significant difference in percentage inhibition value between groups of concentration. Salicylic acid and all its alkylated derivatives tested have shown cytotoxic activity against cervical cancer HeLa cell. Meaning that these compounds have potential anti-cancer activity as they can inhibit the growth of HeLa cell.

From the results in Fig. 3, the percentage inhibition of salicylic acid alkylated salicylic acid derivatives and positive control doxorubicin increases as the concentration of samples increases. The percentage inhibition value between most groups of concentration has also been shown to be significant. This implies that the cytotoxic activity of all samples tested is concentration-dependent. The concentration-dependent properties of salicylic acid and doxorubicin are also supported by previous studies.

A study by Mohammed I, *et al*., [13] demonstrated that cell viability inhibition of salicylic acid in HeLa cell was more

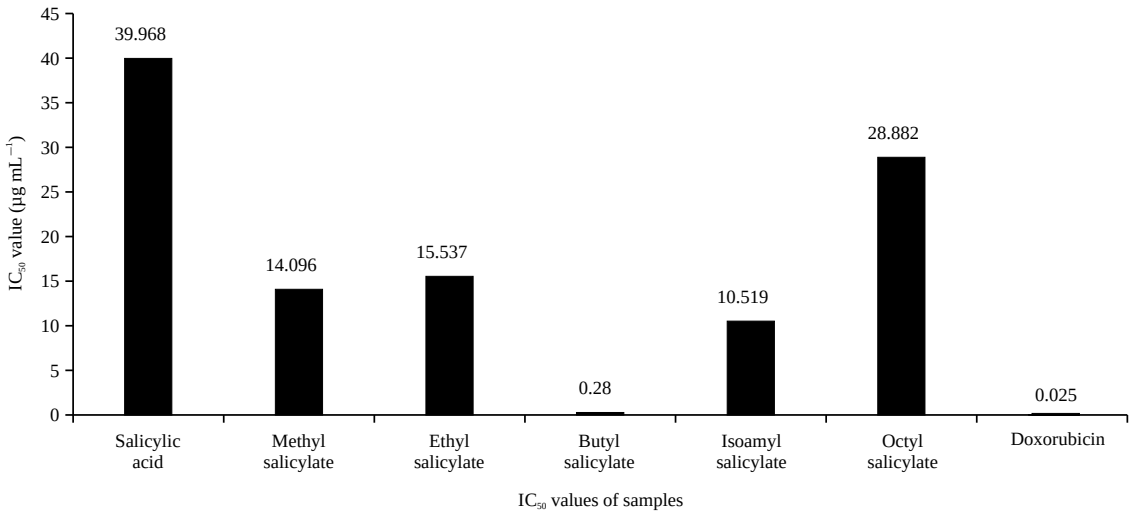

Fig. 3: IC_{50} values of alkylated salicylic acid, salicylic acid and doxorubicin

effective in higher concentrations. The dose-dependent properties of doxorubicin against HeLa cell was supported by previous studies such as the one by Kibria *et al.*[14] used the WST-8 assay, an alternative to MTT Assay to measure the cytotoxicity of doxorubicin against HeLa cell and found that the activity was dose-dependent.

For the other alkylated salicylic derivatives e.g. methyl salicylate, ethyl salicylate, butyl salicylate, isoamyl salicylate and octyl salicylate, no previous studies have been conducted. However, from the result of this experiment, the positive slope in the graph of percentage inhibition against concentration indicated a positive correlation between concentration and cytotoxic activity of the aforementioned samples.

IC_{50} value could be used to determine the activity of samples tested as a potential anti-cancer substance. Doxorubicin has the lowest IC_{50} value (0.025 µg mL^{-1}), while salicylic acid has the highest IC_{50} value (39.968 µg mL^{-1}). All the alkylated salicylic acid derivatives tested had lower IC_{50} value compared to salicylic acid. According to Shapiro-Wilk Normality Test, the IC_{50} value of samples had a p-value of <0.05, meaning that they were not normally distributed. Following the normality test, the Kruskal-Wallis test was performed and the p-value was 0.007 (<0.05), indicating a significant difference between the IC_{50} values of some samples. Post-Hoc Mann Whitney U test was then performed. Most of the IC_{50} value comparison between samples gave out a significant difference (p-value <0.05), except comparison between methyl salicylate, ethyl salicylate and isoamyl salicylate, between ethyl salicylate and octyl salicylate and between octyl salicylate and salicylic acid. According to Atjanasuppat *et al.*,[15] the activity can be classified as high activity, moderate activity, low activity and inactive. The IC_{50} range values for those classifications are: <20, 20-100, 100-1000 and >1000 µg mL^{-1} respectively. Hence, methyl salicylate (IC_{50}: 14.096 µg mL^{-1}), ethyl salicylate (IC_{50}: 15.537 µg mL^{-1}), butyl salicylate (IC_{50}: 0.280 µg mL^{-1}), isoamyl salicylate (IC_{50}: 10.519), doxorubicin (IC 50: 0.025 µg mL^{-1}) have high anticancer activity against HeLa cell. Meanwhile, salicylic acid (IC_{50}: 39.968 µg mL^{-1}) and octyl salicylate (IC_{50}: 28.882 µg mL^{-1}) have moderate activity. Even though some of the samples tested have high activity against HeLa cell (i.e., methyl salicylate, ethyl salicylate, butyl salicylate and isoamyl salicylate) and has the potential to become an anti-cancer substance, their anti-proliferative activity is still significantly lower in comparison to the already established anti-cancer drug, doxorubicin.

Both salicylic acid and its alkylated derivatives displayed anti-proliferative activity against cervical cancer HeLa cell. However, the experiment result showed that the alkylated derivatives had significantly lower IC_{50} value compared to salicylic acid (IC_{50}: 39.968 µg mL^{-1}), except for octyl salicylate whose difference was not statistically significant. Hence, it can be inferred that the addition of the alkyl chain to salicylic acid increases its anti-proliferative activity.

Various studies have demonstrated that the ability of salicylic acid in inhibiting the growth of HeLa cell was related to various mechanisms such as the COX-dependent mechanisms and the COX-independent mechanisms. The COX-2 dependent mechanism is related to salicylic acid's ability in downregulating the COX2/PGE2 pathway. While the

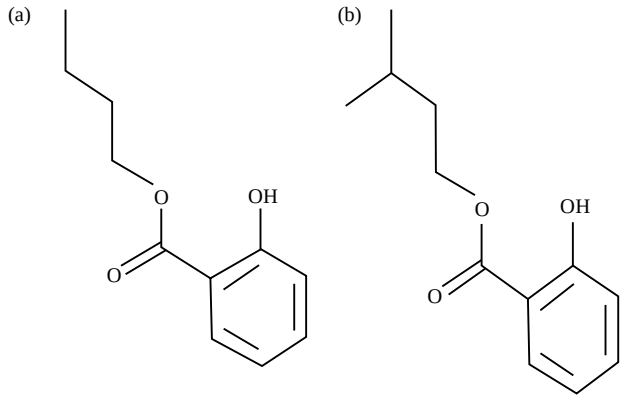

Fig. 4(a-b): (a) Structure of Butyl Salicylate and (b) Structure of Isoamyl Salicylate

COX-independent pathway is related to the inhibitory activity of salicylic acid towards NF-B. As both pathways have been proven to promote cell's survival by increasing its resistance to apoptosis, inhibiting it would result in decreased cancer cell proliferation[7,16-20].

As there are no previous studies that examine the anti-proliferative activity of alkylated derivatives of salicylic acid, it is still unknown whether the alkylated derivatives share the same mechanism with the parent structure, salicylic acid. Further research is required to find the mechanisms of the anti-proliferative activity of the alkylated derivatives as this may explain the reason behind their better performance compared to salicylic acid in inhibiting the growth of HeLa cell. Amongst the alkylated salicylate, butyl salicylate has the lowest IC_{50} value of 0.280 μg mL^{-1}, while octyl salicylate has the highest IC_{50} value of 28.882 μg mL^{-1}. Statistically, the IC_{50} value of octyl salicylate (8 carbon chain in length) is significantly higher compared to the other alkylated salicylic acid samples. Meanwhile, the value of butyl salicylate (4 carbon chain in length) is significantly lower compared to the other alkylated salicylic acid samples. The difference in IC_{50} values between methyl salicylate (1 carbon chain), ethyl salicylate (2 carbon chain) and isoamyl salicylate (4 carbon chain) is not significant. Hence, the length of the carbon chain does not seem to be correlated with the anti-proliferative activity of the substance tested.

The reason that butyl salicylate has the best activity can be attributed to its polarity and size. Butyl salicylate may have the optimum polarity to interact with the binding site of COX-2. Aside from that butyl salicylate also may have the optimum polarity to cross both the hydrophilic and the hydrophobic part of the phospholipid bilayer membrane, allowing it to reach the COX-enzymes inside the cell. Besides, the butyl salicylate molecule is also smaller compared to long-chained octyl salicylate which allows for easier transport.

Both butyl salicylate and isoamyl salicylate are 4 carbon chain in length. The difference between the two is that for isoamyl salicylate, there is an additional methyl branch at the third carbon position of the chain, making the structure branched showed in Fig. 4a and b[4].

The addition of the methyl group in isoamyl salicylate compared to butyl salicylate decreases isoamyl salicylate's anti-proliferative activity significantly. Based on the result of this experiment, the anti-proliferative activity of branched chain salicylic acid derivatives may be lower than its straight-chained counterpart. However, further studies are required to validate this observation.

The result of *in silico* and *in vitro* that butyl salicylate has the best energy binding and *in vitro* test have IC_{50} better than other derivatives. Butyl salicylate has the potential to become an anti-cancer substance, their anti-proliferative activity is still significantly lower in comparison to the already established anti-cancer drug, doxorubicin.

CONCLUSION

All alkylated salicylic acids have positive percentage inhibition on HeLa cell, meaning that they were able to inhibit the proliferation of HeLa cell. The concentration needed to inhibition 50% growth of HeLa cells are: salicylic acid (IC_{50}: 39.968 μg mL^{-1}), methyl salicylate (IC_{50}: 14.096 μg mL^{-1}), ethyl salicylate (IC_{50}: 15.537 μg mL^{-1}), butyl salicylate (IC_{50}: 0.280 μg mL^{-1}), isoamyl salicylate (IC_{50}: 10.519 μg mL^{-1}) and octyl salicylate (IC_{50}: 28.882 μg mL^{-1}). All alkylated derivatives of salicylic acid have significantly better anti-proliferative activity compared to salicylic acid, except for octyl salicylate whose difference is not statistically significant.

SIGNIFICANCE STATEMENT

This study modified the chemical structure of salicylic acid into several derivatives: ethyl salicylate, ethyl salicylate, butyl salicylate, isoamyl salicylate and octyl salicylate to discover its potential as anti-inflammatory agents towards HeLa cervical cancer cell line. This study discovered the alkylated salicylic acid derivatives that can inhibit proliferation of HeLa cell and has potential as anti-cancer substance. Its potential capabilities can be improved further by other researchers by modification of the chemical structure.

ACKNOWLEDGMENT

We thank Universitas Indonesia who had given financial support for this research, particularly for PUTI Prosiding Grant2020No. NKB-3523/UN2.RST/HKP.05.00/2020. Molecular analysis executed by MOE 20 software with license number AC1F6BD22BCF 7788, licensed from Universitas Indonesia (valid through December 7th, 2021).

REFERENCES

1. Bray, F., J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre and A. Jemal, 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: Cancer J. Clin., 68: 394-424.
2. Ferlay, J., M. Ervik, F. Lam, M. Colombet and L.P.M. Meryl, 2018. Global cancer observatory: Cancer today. Lyon: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed 01/02/2021
3. Hull, R., M. Mbele, T. Makhafola, C. Hicks and S.M. Wang *et al*., 2020. Cervical cancer in low and middle-income countries (review). Oncol. Lett., 20: 2058-2074.
4. Belsito, D., D. Bickers, M. Bruze, P. Calow and H. Greim *et al*., 2007. A toxicologic and dermatologic assessment of salicylates when used as fragrance ingredients. Food Chem. Toxicol., 45: S318-S361.
5. Soriano-Hernandez, A.D., D. Madrigal-Pérez, H.R. Galvan-Salazar, M.L. Martinez-Fierro and L.L. Valdez-Velazquez *et al*., 2015. Anti-inflammatory drugs and uterine cervical cancer cells: Antineoplastic effect of meclofenamic acid. Oncol. Lett., 10: 2574-2578.
6. Hold, G.L. and M.E. El-Omar, 2008. Genetic aspects of inflammation and cancer. Biochem J., 410: 225-235.
7. Sobolewski, C., C. Cerella, M. Dicato, L. Ghibelli and M. Diederich, 2010. The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. Int. J. Cell Biol., Vol. 2010. 10.1155/2010/215158.
8. Mohammed, I., A.M. Tangaza and R.I. Tsamiya, 2014. Effects of acetylsalicylic acid and salicylic acid on the growth of HT3 cervical cancer line. Am. J. Pharm. Pharmacol., 1: 32-44.
9. Blumenthal, R.D., 2005. Chemosensitivity. Humana Press, New Jersey, pp: 72–78.
10. Soderberg, T., 2010. Organic Chemistry with a Biological Emphasis. Vol. 1. Chemistry Publishers, University of Minnesota Morris Digital Well, pp: 300–302.
11. Bele, A.A. and A. Khale, 2011. An overview on thin layer chromatography. Int. J. Pharm. Sci. Res., 2: 256-267.
12. Santiago, M. and S. Strobel, 2013. Thin layer chromatography. Methods Enzymol., 533: 303-324.
13. Mohammed, I., M.O. Musa and A. Umar, 2016. Effects of acetylsalicylic acid and salicylic acid on the growth of HeLa cervical cancer cells line. J. Adv. Med. Pharm. Sci., Vol. 10. 10.9734/JAMPS/2016/29034.
14. Kibria, G., H. Hatakeyama, K. Akiyama, K. Hida and H. Harashima, 2014. Comparative study of the sensitivities of cancer cells to doxorubicin and relationships between the effect of the drug-efflux pump P-gp. Biol. Pharm. Bull., 37: 1926-1935.
15. Atjanasuppat, K., W. Wongkham, P. Meepowpan, P. Kittakoop, P. Sobhon, A. Bartlett and P.J. Whitfield, 2009. *In vitro* screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. J. Ethnopharmacol., 123: 475-482.
16. Tilborghs, S., J. Corthouts, Y. Verhoeven, D. Arias, C. Rolfo, X.B. Trinh and P.A. van Dam, 2017. The role of nuclear factor-kappa B signaling in human cervical cancer. Crit. Rev. Oncol. Hematol., 120: 141-150.
17. Xie, T.X., Z. Xia, N.U. Zhang, W. Gong and S. Huang, 2010. Constitutive NF-κB activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma. Oncol. Rep., 23: 725-732.
18. Zhang, Z., F. Chen and L. Shang, 2018. Advances in antitumor effects of NSAIDs. Cancer Manag. Res., 10: 4631-4640.
19. Čeponytė, U., M. Paškevičiūtė and V. Petrikaitė, 2018. Comparison of NSAIDs activity in COX-2 expressing and non-expressing 2D and 3D pancreatic cancer cell cultures. Cancer Manag. Res., 10: 1543-1551.
20. Giardiello, F.M., R.A. Casero, S.R. Hamilton, L.M. Hylind and J.D. Trimbath *et al*., 2004. Prostanoids, ornithine decarboxylase and polyamines in primary chemoprevention of familial adenomatous polyposis. Gastroenterology, 126: 425-431.