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Synthesis and *in vitro* bioactivity evaluation of new glucose and xylitol ester derivatives of 5-aminosalicylic acid†

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New glucose and xylitol esters of 5-amino salicylic acid (**5-ASA**) were synthesized followed by evaluation of their *in vitro* antimicrobial, anti-cancer and anti-inflammatory activities. The results of the antimicrobial activity assessment revealed that the new final esters were more effective against Gram-negative as well as Gram-positive bacteria than the original drug. Furthermore, the new final products were confirmed by a cytotoxicity assay over HT-29 and 3T3 cell lines to be less toxic for normal cells compared to the initial drug. On the other hand, however, their suppressive effect against cancerous cells was somewhat lower. Meanwhile, the anti-inflammatory activity assay over a RAW264.7 macrophage cell line demonstrated that the NO inhibition activity of the conjugated drug to the previously mentioned saccharides, especially to glucose, has slightly improved compared to the non-conjugated drug. Finally, *in silico* screening was also performed in order to predict the potential interactions and binding energy of the novel products against cyclooxygenase (COX-1/COX-2) and lipoxygenase (5-LOX) proteins. Findings indicated that the new products had greater hydrogen bonds and binding affinities with the active sites of proteins towards **5-ASA**.

1. Introduction

Inflammatory bowel disease (IBD) is a high-incidence disease which is induced by a complex interplay among environmental, genetic and immunoregulatory factors.[1,2] IBD includes two major constituents, namely, ulcerative colitis and Crohn's disease, and if not cured properly, can lead to outbreak of collateral associated cancers.[3,4] Therefore, high priority should be accorded to the achievement of better therapeutic effects for the treatment of IBD. For more than half a century, 5-amino-salicylic acid (**5-ASA**) has been the most widely prescribed anti-inflammatory drug for the treatment of IBD. It reduces gastro-intestinal toxicity[5] and is tolerable for the majority of patients because it has limited side-effects.[4,6,7] In addition, its chemo-preventive activity, particularly against colitis-associated cancers,[8] and its antioxidant property against reactive oxygen and nitrogen species,[6] make it a multivalent drug. Hence, the derivatives of **5-ASA** with polymers,[9,10] nanoparticles[11] and amino acids[12] have been prepared in order to expand its applications. Its complexes with metals have shown greater antimicrobial activity than **5-ASA** alone.[13] For optimizing clinical use, chitosan[14] and azo-bond derivatives of **5-ASA**[15] have been produced to deliver the drug to targeted sites.

Despite the development of numerous beneficial **5-ASA** derivatives, patient life quality has so far not been improved. This is due to the fact that, when the drug is taken orally or used in the form of a suppository, the main portion of the drug is absorbed in the small intestine and hardly reaches the colon.[2] Its low stability in the gastrointestinal tract[16] and poor water solubility[15,17,18] are other problems which result in the necessary consumption of a higher dosage of the drug in order to be effective, the requirement of long-term treatment and a greater number of side-effects. In some cases, the disease relapses and surgery is required.[4,19,20]

On the other hand, the use of carbohydrate drug derivatives has been increasingly favoured in recent times. The versatile applications of carbohydrates in a variety of fields have attracted the attention of many researchers due to their biodegradability, nontoxicity,[21] as well as amphipathic and emulsifying characteristics.[22,23] A wide range of anticancer, antibiotic, antiviral or fungicidal active compounds relied on conjugating with carbohydrates for development of diverse medicinal applications.[22,24] Furthermore, it has been found that saccharides increase penetration into cell membranes and decrease toxicity.[22] In addition,

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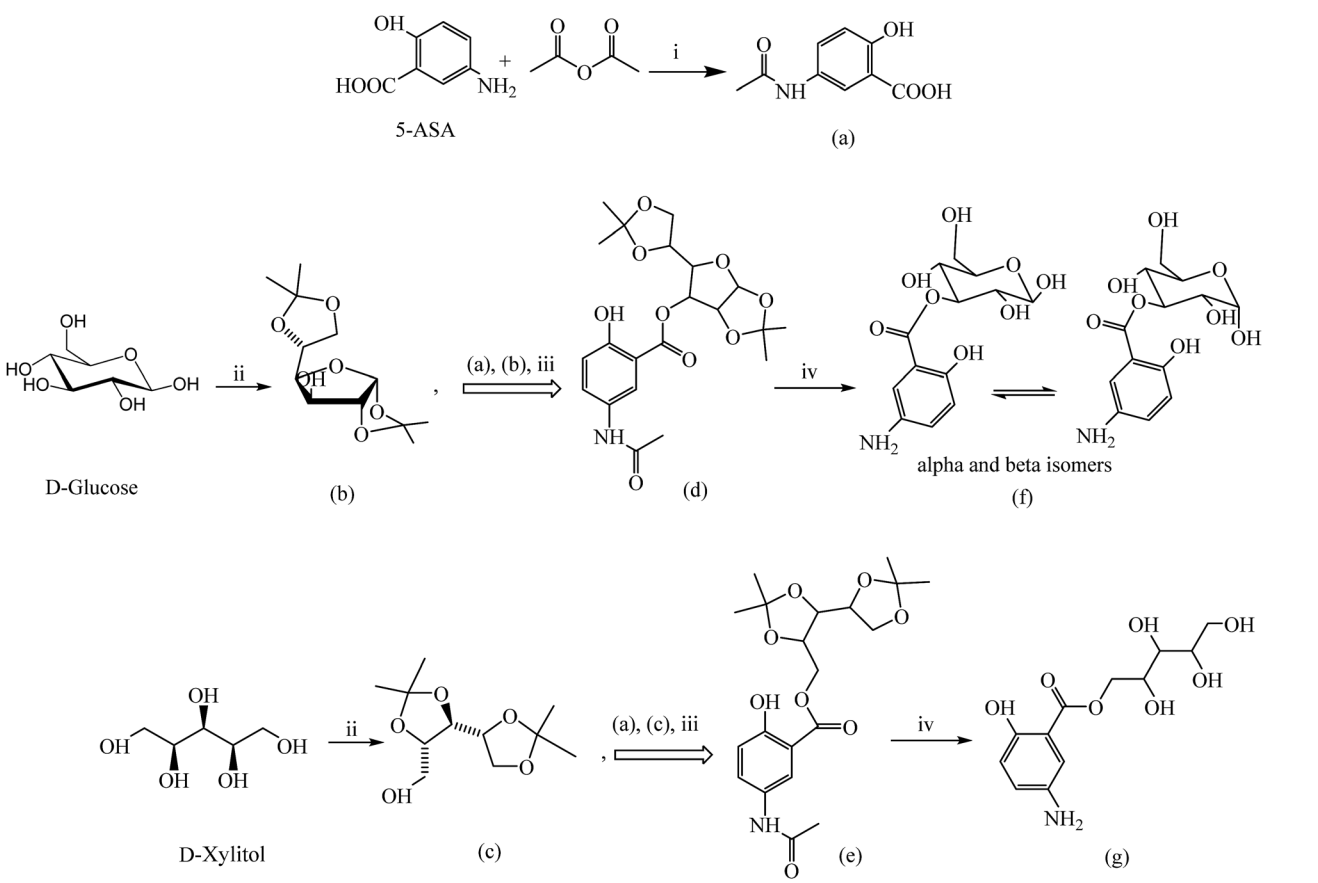
the anti-inflammatory properties of some synthetic compounds possessing glycosidic linkages have been recognized.[25] By comparison to ibuprofen, ibuprofen fructose ester exhibited fewer side-effects, longer stability in aqueous solution and better uptake of glycosylated drugs into tissue.[26] Meanwhile, compared to the parent drug, glucose-conjugated aspirin improved its solubility and anti-cancer activities.[27] By comparison with non-saccharide derivatives, porphyrin–saccharide incapacitated human breast cancer due to greater binding to cancerous cells.[28,29] Moreover, glycotargeting has demonstrated that carbohydrate ligands can be feasibly used to target protein receptors at sites of localization.[30] Additionally, carbohydrate fatty acid derivatives showed significant potential for improving antibacterial agents.[23] Sugar alcohols also have been proven to possess biological activities. Sugar alcohols selectively reduced the acute lethal toxicity of 1-(2-chloroethyl)-3-(methyl alpha-D-glucopyranos-6-yl)-1-nitrosourea without reducing its anti-tumour activity.[31] Anti-tumoural and antimicrobial specifications of xylitol esters have been documented as well.[21,32] Xylitol has been advocated as an optimal choice for linkage to drugs, due to the fact that its anti-biofilm activity has been proven to have efficiency in suppressing severe yeast condition and other harmful bacteria, as well as ulcers and stomach cancers.[33,34] Moreover, *in vitro* pharmacological investigations using xylitol butyric derivatives have shown potential in the treatment of sickle cell anaemia and cancer.[35] Furthermore, 3-*O*-perfluoroalkylated D-glucose and *O*-perfluoroalkylated D-xylitol amphiphiles have been prepared and biological assessment displayed stabilisation effects of sugars on perfluorocarbon emulsions.[36]

Thus, considering that conjugation of drugs with saccharides enriches the bioavailability and pharmaceutical properties of drug,[28,29] glucose and xylitol esters of **5-ASA** were prepared as models of sugar and sugar alcohol derivatives of **5-ASA**, followed by the evaluation of the *in vitro* bioactivity of new products for antibacterial, anti-inflammatory and anticancer activities and their comparison with the parent drug (**5-ASA**).

2. Results and discussion

2.1. Synthesis

In this work, esterification was purposed for preparing **5-ASA** derivatives, since the carbohydrate esters have been shown to be involved in varied biological processes such as antineoplastic, antidiabetic, anti-inflammatory, *etc.*[37,38] Furthermore, it has been found that ester bond is fairly fixed in the acidic medium and readily ruptured in basic environment which is compatible with gut nature[39] and may enable the drug glycoside on colonic tract releases the active drug through enzymatic action. Moreover, protection of hydroxyl functions by *O*-isopropylidenation has been performed as it is a well-known method due to its



Scheme 1 Reagents and conditions: (i) H_3PO_4, 80 °C; (ii) acetone, $ZnCl_2$, H_3PO_4, 5 h; (iii) DCC, DMAP, DCM, 24 h, r.t; (iv) acetic acid 85%, reflux under N_2 gas, 5 h.

convenient application in synthetic studies.[40] The di-and/or mono-*O*-isopropylidene derivatives have been utilized widely in total syntheses of some bioactive macromolecules and various natural products since their low toxicity and biological activities such as anti-inflammatory and antipyretic activities have been documented.[40,41] In addition, C-3 derivatives of D-glucose were introduced be to useful for biochemical studies as a key starting material.[42] For instance 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose has been used as a starting material for synthesis of glucose aspirin.[27] A series of 3-*O*-alkyl and 3-*O*-haloalkyl-D-glucoses and likewise in another work a series of fatty acid D-glucose derivatives were prepared from 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose and several of them showed antimicrobial efficacy comparable with commercially available antimicrobials.[21,43]

A chemical route designed to obtain **5-ASA** monosaccharide esters is shown in Scheme 1. The synthesis was commenced with the selective protection of the hydroxyl groups of sugars and protection of amine group of **5-ASA** to avoid becoming involved in the reaction. A highly efficient, one step and regioselective method for the direct *O*-isopropylidenation of D-glucose and D-xylitol is using anhydrous acetone in the presence of an acid catalyst.[41] Subsequently, esterification was carried out through a facile and one-step method which eludes harsh reaction conditions by using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as catalysts in dichloromethane (DCM) solvent.[44–46] As regards, the hydrolysis of the isopropylidene group occurs under mildly acidic conditions,[47] hence, finally, deprotection of compounds was achieved by refluxing under nitrogen gas in presence of acetic acid. The structures of the synthesized products were confirmed by ^{1}H NMR, ^{13}C NMR, FT-IR and mass spectroscopies.

2.2. Antibacterial evaluation

The antibacterial activity evaluation of the new monosaccharide esters of **5-ASA** (**f** and **g**) against both Gram-positive bacteria and

Table 1 *In vitro* antibacterial activity of samples, the results were duplicated and averages were recorded[a]

		Microbes			
		Diameter of inhibition zone in millimeter (±1 mm)			
Entry	Sample name	*Staphylococcus aureus* (+)	*Bacillus subtilis* (+)	*Escherichia coli* (−)	*Salmonella choleraesuis* (−)
1	**5-ASA**	NA	NA	NA	NA
2	Streptomycin	35	25	27	25
3	Ethanol	NA	NA	NA	NA
4	**f**	14	NA	10	NA
5	**g**	14	NA	12	NA

[a] NA: no activity found at the tested concentration. (+) Gram-positive and (−) Gram-negative bacteria.

Table 2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of samples[a]

		MIC/MBC (mg mL^{-1})			
Entry	Microbes	**5-ASA**	**f**	**g**	Streptomycin*
1	*Escherichia coli* (−)	NA/NA	1.92/>3.33	1.60/3.33	24.11/28.93
2	*Salmonella choleraesuis* (−)	NA/NA	NT/NT	NT/NT	NT/NT
3	*Staphylococcus aureus* (+)	3.33/>3.33	1.60/3.33	1.33/2.77	16.74/24.11
4	*Bacillus subtilis* (+)	NA/NA	NT/NT	NT/NT	NT/NT

[a] NA: no activity found at the tested concentration. (+) Gram-positive and (−) Gram-negative bacteria. NT: not tested. Streptomycin*: concentration unit μg mL^{-1}.

Table 3 *In vitro* nitric oxide (NO) suppression activity and cytotoxicity of samples on RAW 264.7 cells at 50 μM, S.E.M: standard error of the mean[a]

Entry	Sample name	NO inhibition (%) ± S.E.M	NO inhibition IC_{50} (μM) ± S.E.M	Cytotoxicity IC_{50} (μM)
1	Curcumin	99.3 ± 0.2	14.7 ± 0.2	>100
2	**5-ASA**	9.0 ± 1.1	ND	>100
3	**f**	13.2 ± 1.6	ND	>100
4	**g**	9.2 ± 3.0	ND	>100

[a] ND: not detected.

Gram-negative bacteria exhibited, both new compounds were more effective against *Escherichia coli* and *Staphylococcus aureus* bacteria than the initial drug which presented insignificant activity against all tested organisms. However, the antibacterial properties of the new products were not comparable with antimicrobial agents (streptomycin). Based on Table 1, there was no great difference between the antibacterial activities of **f** and **g**. Both new products demonstrated no outstanding inhibitions against *Salmonella choleraesuis* (Gram-negative) and *Bacillus subtilis* (Gram-positive) bacteria. On the other hand, both **f** and **g** showed an average inhibition zone of 14 ± 1 mm against *Staphylococcus aureus*. Furthermore, the average inhibition zones of 10 ± 1 mm and 12 ± 1 mm were observed against *Escherichia coli* for **f** and **g**, respectively. Accordingly, the inhibition activities of both **f** and **g** were found to be slightly greater against *Staphylococcus aureus* bacteria than against *Escherichia coli* ones (Table 1). Hence, the MIC and MBC tests were proceeded for the bacteria which showed sensitivity against samples in disc diffusion assay. Based on Table 2, minimum inhibition concentration of **g** against both *E. coli* and *S. aureus* was slightly lower than **f**, moreover, 3.33 mg mL^{-1} MIC was found for **5-ASA** against *Staphylococcus aureus* (Table 2).

2.3. Anti-inflammatory evaluation

Inflammation is a defensive response of the immune system against infections, tissue damage or harmful foreign stimuli and is accompanied by the production of pro-inflammatory mediators. Nitric oxide (NO) is a key inflammatory mediator which is secreted by inducible nitric oxide synthase (iNOS) from activated macrophages during the inflammatory response. When it occurs in excess, this factor contributes to a variety of conditions and diseases, including swelling, pain, asthma, cardiovascular disorders, cancer and other diseases.[48–50] Hereupon, the NO inhibition activity of the final esters (**f** and **g**) in IFN-γ/LPS-stimulated RAW264.7 macrophages (at 50 μM concentration) was screened and compared with the parent drug. Despite the NO inhibition activity of the new synthesized compounds and **5-ASA** was not as effective as positive control (curcumin), with less than 50% NO inhibition being achieved, conjugation of **5-ASA** to glucose increased the NO inhibition (%) activity of **5-ASA** by almost 1.5 fold, from 9.0 μM to 13.2 μM. Meanwhile, conjugating to xylitol was revealed to be less effective with the NO inhibition (%) activity of 9.2 μM. These results may contribute to the improvement of anti-inflammatory properties in the future. Additionally, the cell viability test results confirmed that NO inhibition at 50 μM was not due to the toxicity of the samples (Table 3).

Table 4 *In vitro* anticancer activity of samples against HT-29 and 3T3 cell lines ± SD (standard deviation)

Entry	Sample name	IC_{50} (μg mL^{-1}) for HT-29 cell line ± SD	IC_{50} (μg mL^{-1}) for 3T3 cell line ± SD
1	**5-ASA**	5.1 ± 0.01	6.1 ± 0.008
2	**f**	8.1 ± 0.01	2918.5 ± 0.008
3	**g**	9.9 ± 0.03	9254.0 ± 0.01

2.4. Anti-cancer evaluation

One of the predominant problems during the chemotherapy period is the side effects of drugs over normal cells, therefore, diminishing the toxicity of drugs against normal cells with maintaining anti-cancer properties of them is the priority. According to the evaluation of cytotoxicity against cancerous cells (HT-29 cell line), the inhibition activity of the new products, **f** (IC_{50}: 8.1 μg mL^{-1}) and **g** (IC_{50}: 9.9 μg mL^{-1}), was slightly reduced in comparison with **5-ASA** (IC_{50}: 5.1 μg mL^{-1}) (Table 4 and Fig. 1). On the other hand, the cytotoxicity test displayed that both **f** and **g** were much less harmful than the parent drug for normal cells (3T3 cell lines), since 50% of 3T3 cells were inhibited by approximately 2918.5 μg mL^{-1} of **f** and 9254.0 μg mL^{-1} of **g**, while only 6.1 μg mL^{-1} of **5-ASA** inhibited 50% of normal cells (Table 4 and Fig. 1). Consequently, although the anticancer activity of the new compounds was slightly less potent than that of **5-ASA**, their significant reduced toxicity to

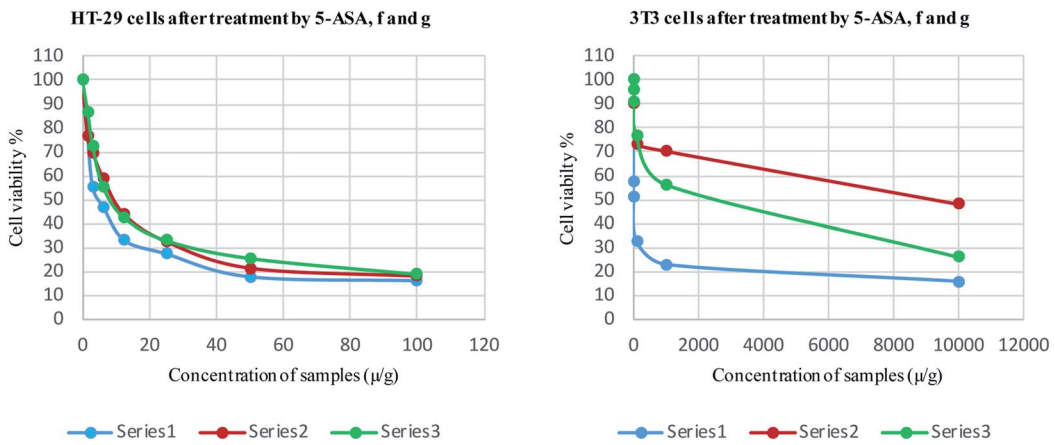


Fig. 1 Graphs were plotted for HT-29 and 3T3 cell lines with the percentage of cell viability against their respective concentration. HT-29 and 3T3 cell lines after treatment by: Series1: **5-ASA**, Series2: **g**, Series3: **f**.

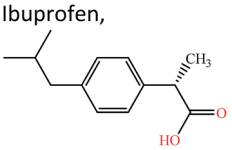
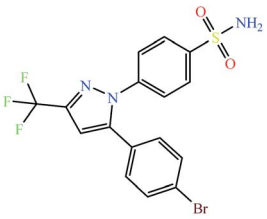
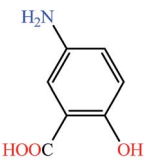
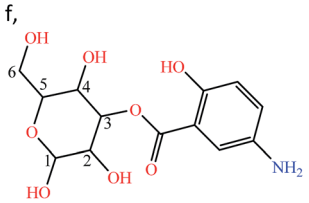
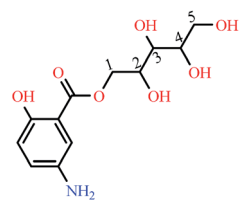
normal cells makes them promising options for future drug development.

2.5. Molecular docking analysis

Recently, the computer-based analysis are utilizing as a key tool to drug discovery in the field of molecular modelling. Indeed, molecular docking is a computer-assisted drug design with the aim of characterizing the predominant mode(s) of the small molecules (ligand) into the binding pocket of target proteins (three-dimensional structure) as well as estimating the binding affinity.[51–53] This prediction is of particular practical importance and is a prerequisite for successful structure-based drug design because it is used to screen virtual libraries of drug-like molecules to obtain leads for further drug development,[54] and anticipation of the biological activity of molecules[55] before experimental work which can be time-consuming and costly.[56]

Despite many evidence that proved the inhibition effect of **5-ASA** against cyclooxygenase protein (COX) and lipoxygenase, its mechanism of action remains controversial.[2,57,58] Cyclooxygenase has two main isoforms: COX-1 and COX-2. COX-1 causes cytoprotection in the gastrointestinal tract, whereas COX-2 mediates inflammation. On the other hand, lipoxygenase

Table 5 The docking results (AutoDock 4.2), regarding the binding free energy: [ΔG (kcal mol^{-1})], distances or lengths of hydrogen bonds: [D (Å)] and hydrogen bonds between compounds and amino acids involved in COX-1, COX-2 and 5-LOX

Compound name & structure	Hydrogen bonds between atoms of compounds and amino acids into related protein											
	COX-1				COX-2				5-LOX			
	ΔG	Atom of compound	Amino acid	D	ΔG	Atom of compound	Amino acid	D	ΔG	Atom of compound	Amino acid	D
Ibuprofen, 	−8.42	COO COO	Ty355 (*p*-OH) Arg120 (NH_2, NH)	2.59 2.61, 2.69	—	—	—	—	—	—	—	—
S58, 	—	—	—	—	−10.85	F S=O NH_2 NH_2	Arg120 (NH_2) His90 (N) Leu352 (COO) Gln192(COO)	3.20 2.79 2.76 2.89	—	—	—	—
5-ASA, 	−4.33	COO COO	Ty355 (*p*-OH) Arg120 (NH_2, NH)	2.59 2.61, 3.03	−3.94	NH_2 COO	Ala527 (COO) Tyr355 (*p*-OH)	2.95 2.93	−3.68	NH_2	Ala606 (COO)	2.48
f, 	−5.78	*p*-OH C_6-OH C_4-OH C_4-OH	Ser530 (COO) Arg120 (NH) Val349 (COO) Ser353 (NH_2)	2.62 3.12 3.02 2.90	−5.78	*p*-OH NH_2 C_1-OH C_6-OH C–O–C	Ty385 (*p*-OH) Met522 (COO) Tyr355 (*p*-OH) Arg120 (NH) Arg120 (NH)	2.80 2.93 2.62 3.11 3.01	−5.40	*p*-OH C_1-OH C_2-OH C_5-OH C_6-OH	Ile673 (COO) Gln363 (COO) His367 (N) Tyr181 (*p*-OH) Tyr181 (*p*-OH)	2.71 2.72 2.83 2.97 2.63
g, 	−4.22	NH_2 C_5-OH *p*-OH	Met522 (COO) Arg120 (NH) Ser530 (COO)	2.71 2.94 2.90	−4.07	*p*-OH NH_2 C_4-OH C_3-OH C_3-OH	Val523 (COO) Ty385 (*p*-OH) Arg120 (NH) Arg120 (NH) Ty355 (*p*-OH)	2.58 2.77 2.96 2.71 2.93	−4.55	NH_2 NH_2 C_3-OH C_4-OH C_5-OH	Ile406 (COO) Asn407 (H_2N–C=O) Gln363 (COO) Gln363 (COO) Thr364 (HC-OH)	3.00 3.03 2.53 2.74 2.64

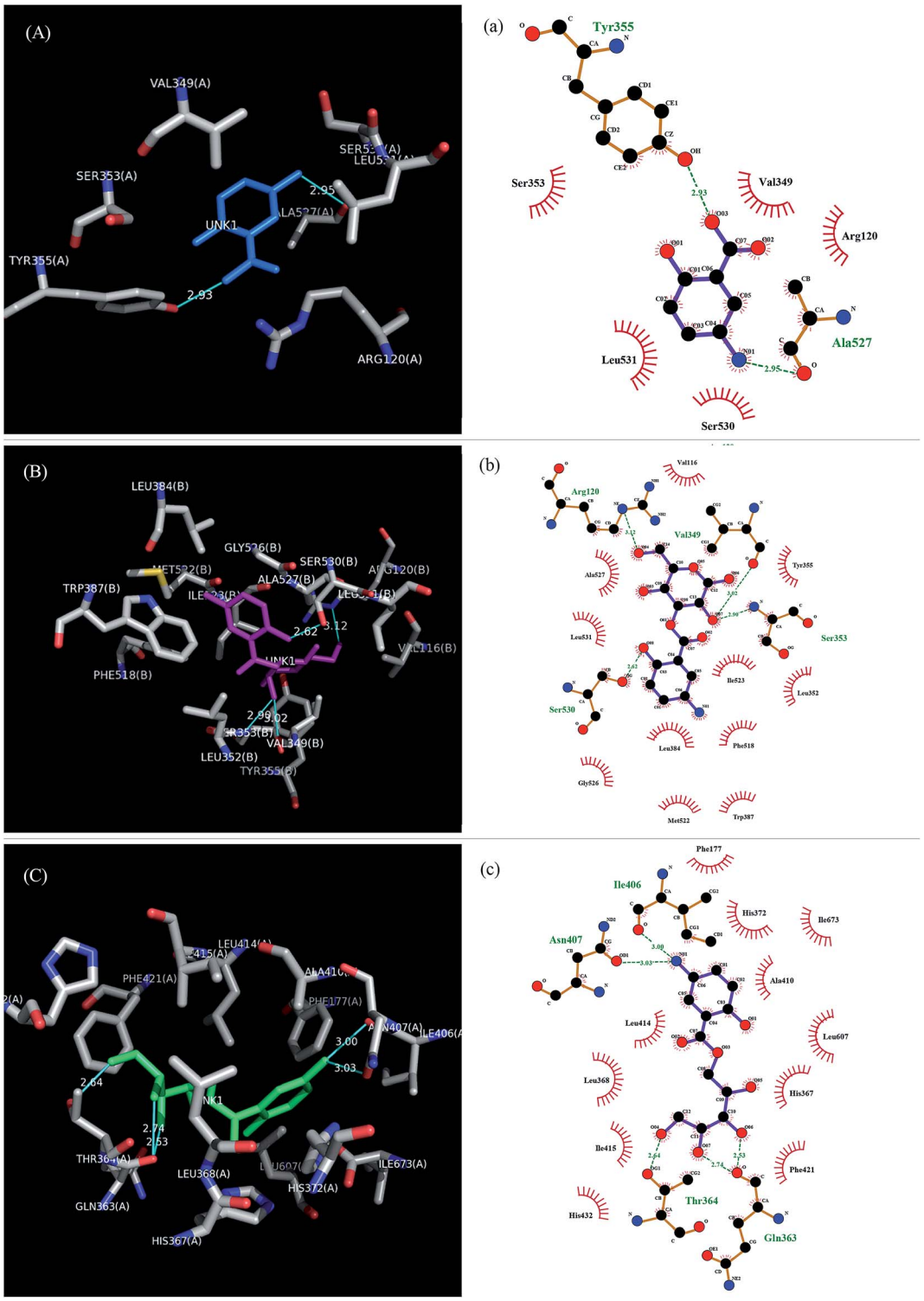


Fig. 2 Showing the examples of samples docked into the binding site of related protein in the best of their conformation. Images were rendered from Pymol (capital letter) and Ligplot softwares (small letter): images (A) and (a) illustrating **5-ASA** (blue) into COX-2, images (B) and (b) illustrating the best mode of **f** (purple) into COX-1 and images (C) and (c) illustrating the best pose of **g** (green) into 5-LOX. The meaning of the items for images taken by ligplot are as follows:

(LOX) is known for its essential role in leading to pro-inflammatory mediators especially in human body.[59] In the family of lipoxygenase, 5-lipoxygenase (5-LOX) has received attention due to its therapeutic potential. For treatment of inflammation, some dual COX/5-LOX non-steroidal anti-inflammatory drugs (NSAIDs) have been designed and achieved great successes.[59] Therefore, to understand possible interactions and predict binding ability of new synthesized compounds with the relevant amino acids in active site of the protein docking study has been performed into COX-1, COX-2 and 5-LOX enzymes and compared with parent drug.

In docking study toward COX-1 protein the co-crystallized ibuprofen was utilized as a positive control ligand. The key amino acids of COX-1 consist: His90, Arg120, Val349, Tyr355, Arg53, Met522 and Glu524 (ref. 57 and 59) where carboxylic group of ibuprofen formed two hydrogen bonds between NH and NH_2 groups of Arg120 and one hydrogen bond between phenolic OH of Ty355. The co-crystallized S58, was used to parameterize molecular docking study against COX-2. The main amino acids in this interaction include: Val349, Ser530, Leu352, Tyr385, Tyr348, Trp387, Gly526, Ala527, Met522, Leu384, His90, Arg120, Tyr355, Arg53, Phe518 and Gly526, where S58 formed four hydrogen bonds with His90, Arg120, Leu352 and Gln192 (ref. 57 and 59). It has been found that the active site of 5-LOX is around catalyst non-hem iron atom. The iron is coordinated by three conserved histidines (histidines 367, 372, and 550), as well as the main-chain carboxylate of the C terminus (Ile673). The other predominantly involved amino acids contain: Tyr181, Ala603, Ala606, His600 and Thr364.[60] As regards, there is no reference co-crystallized ligand into 5-LOX, the same parameters applied in docking against COX-1 and COX-2 were utilized. Additionally, hydrogen bond interactions between the samples and the amino acids in active site confirmed that docking carried out in the targeted site. As cited in the literatures,[59,61] if the RMSD (root mean square deviation) of the best docked conformation of co-crystal ligand is $\leq$2 Å from the experimental one the used scoring function is reliable. The RMSD value of 0.98 Å for ibuprofen and 1.57 Å for docked S58 validated the accuracy of the AutoDock4.2 performance.

In docking against COX-1, **5-ASA** exhibited two hydrogen bonds with NH and NH_2 groups of Arg120 and one hydrogen bond with phenolic OH of Ty355 from its carboxyl group. Moreover, four hydrogen bonds were observed between functional groups of **f** with Val349, Ser353, Arg120, and Ser530. **g** conserved three hydrogen bonds between its functional groups with Arg120, Met522 and Ser530. In case of molecular docking into COX-2, **5-ASA** conserved two hydrogen bonds between carboxyl of Ala527 and phenolic OH of Ty355 with its amine and carboxylic acid groups, respectively. Additionally, both new compounds became involved in three more hydrogen bonds with amino acids in active site of COX-2 protein than **5-ASA**. **f** exhibited five hydrogen bonds with Met522, Try385, Arg120 and Try355. Similarly, **g** formed five hydrogen bonds with Tyr385, Val523, Arg120, and Try355. In docking study toward 5-LOX, **5-ASA** formed one hydrogen bond from its amine group with carboxyl group of Ala606. Whiles, both new compounds formed five hydrogen bonds with amino acids in active site of 5-LOX protein.

In terms of the binding energy, the binding affinities of the new products into COX-1 observed to be moderate in comparison with ibuprofen (ΔG: -8.42 kcal mol^{-1}), however, compounds **f** (ΔG: -5.78 kcal mol^{-1}) revealed greater binding energy toward both **g** (ΔG: -4.22 kcal mol^{-1}) and **5-ASA** (ΔG: -4.33 kcal mol^{-1}) which exhibited almost the equal binding energies towards each other. Although the binding energies of the new derivatives into COX-2 were not comparable with the co-crystallized S58 (ΔG: -10.85 kcal mol^{-1}), but based on binding affinities **f** (ΔG: -5.78 kcal mol^{-1}) showed almost one and half fold greater binding energy than the parent drug (ΔG: -3.49 kcal mol^{-1}) and seems to be a more reasonable candidate than **g** (ΔG: -4.12 kcal mol^{-1}). Also, studying of binding energy of the new derivatives against 5-LOX predicted both **f** (ΔG: -5.40 kcal mol^{-1}) and **g** (ΔG: -4.55 kcal mol^{-1}) have stronger binding affinities than **5-ASA** (ΔG: -3.68 kcal mol^{-1}), (Table 5 and Fig. 2).

3. Conclusion

Glucose and xylitol esters of 5-amino salicylic acid were synthesized successfully in average yields with the approximately moderate to superior bioactivities toward **5-ASA** which offering a larger therapeutic window for preparation of a library of different **5-ASA**'s monosaccharide derivatives. Comparing bioactivities between sugar and sugar alcohol showed there was no significant difference between antibacterial activity of them also both derivatives exhibited slightly greater inhibition activity against *Staphylococcus aureus* (Gram-positive) than against *Escherichia coli* (Gram-negative) ones which is in accordance with previous studies on carbohydrate derivatives.[21,62] Also it can be noted that **5-ASA**'s glucose ester was better candidate for suppressing nitroxide over macrophage cells than xyilitol ester. Moreover, cytotoxicity assay for inhibiting HT-29 cells, revealed that **f** had greater potential than **g**, whereas **g** was less harmful than **f** against 3T3 cell lines. On the other hand, *in silico* docking study anticipated that although against all proteins **f** showed better binding energies, by comparison to the parent drug, both new ester derivatives of **5-ASA** (**f** and **g**) formed more hydrogen bonds with active site residues of the aforementioned proteins as well as had greater binding energy which is encouraging to further experimental investigation over chosen proteins and *in vivo* medicinal chemistry exploration.

4. Experimental

4.1. Materials

All materials in this project were purchased in analytical grade and used without further purification, otherwise stated. Merck silica gel 60 (70–230 mesh) was used for flash chromatography. Analytical thin layer chromatography (TLC) was implemented by using Merck 60 F254 precoated silica gel plate (0.2 mm thickness). Functional groups were detected by Fourier transform infrared spectroscopy (FT-IR); (Perkin Elmer Spectrum 100). NMR data were obtained by 500 MHz for ^{1}H NMR and 125.8 MHz for ^{13}C NMR (JEOL JNM ECA) spectrometer. Direct

infuse mass spectrometry (DIMS) was applied for mass characterization using ACQUITY® SQD with Single Quadrupole Detector (Waters Corporation, Milford, MA USA). Mass of compound (a) has been recorded by GC-MS QP2010 Plus SHIMADZU. Optical rotations of products were measured on a JASCO P-2000 Polarimeter.

All solvents prepared from J.T. Baker. **5-ASA**, DCC, DMAP, Molecular sieves 3 Å (8 to 12 mesh) were from ACROS Company. D-Glucose and D-xylitol were bought from Fisher Scientific Company. Nutrient agar and nutrient broth have been supplied from Sigma Aldrich. Bacterial strain subcultures were prepared in department of Microbiology, Faculty of Medicine and Health Sciences, UPM University Malaysia. Melting points were measured by Barnstead Electrothermal instrument. RAW 264.7 murine macrophages cells obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

4.2. Protection of 5-ASA and preparing (a)

The synthesis of 5-acetamido-2-hydroxy benzoic acid was conducted in accordance with previously described procedures.[63,64] Under the fume hood, **5-ASA** (3 g, 19.6 mmol) in an Erlenmeyer placed over warm water bath (80 °C) was dissolved in acetic anhydride (20 mL) and 5 to 10 drops of phosphoric acid (85%) were added to the mixture, which was then stirred for about 15 to 30 min. When the mixture was dissolved completely, it was removed from the warm water bath and 10 to 20 drops of distilled water were cautiously added to the warm mixture. Subsequently, over an ice bath 50 mL of cool distilled water was added and crystallization was induced by scratching the container's walls. Afterwards, the obtained crystals were filtered using the Buchner funnel and were washed several times with the cold distilled water to remove the rest of the acid. Ethanol (95%) was used to recrystallize the dried crystals and thus obtain pure (**a**). The product was identified initially with TLC (mobile phase 8 : 2, ethyl acetate/methanol) and visualized under the UV lamp, Scheme 1.

4.3. General procedure for protection of monosaccharides and preparation of (b) and (c)

Glucose and xylitol were selectively protected based on previous works[35,65,66] with a slightly different method. In brief, in the presence of 5 to 10 drops of H_3PO_4 and molecular sieves (2% w/w), monosaccharide (1 g, D-glucose: 5.5 mmol, D-xylitol: 6.5 mmol), freshly fused anhydrous $ZnCl_2$ (0.77 g, 5.7 mmol) and acetone (100 mL) were immediately mixed together in a flask fitted with an anhydrous calcium chloride guard tube. The mixture was stirred vigorously for 5 h at room temperature. The primary detection was performed by TLC (mobile phase 9 : 1 ethyl acetate/hexane). The mixture was filtered and neutralized gradually with $NaHCO_3$ (10%), then dried with $MgSO_4$ and freed from acetone under the reduced pressure. Afterwards, the product was extracted by hexane (5 × 50 mL) and hexane was collected and separated from precipitations by decanting. Once hexane evaporated under the diminished pressure, the precipitations were collected. Further purification was carried out through flash chromatography with the same mobile phase applied for TLC to obtain (**b**) and (**c**), Scheme 1.

4.4. General procedure for esterification and preparation of (d) and (e)

Over an ice bath, **a** (0.1 g, 0.5 mmol), **b** or **c** (0.2 g, **b**: 0.7 mmol, **c**: 0.8 mmol) and DMAP (0.09 g, 0.7 mmol) were dissolved in DCM. After that, DCC (0.15 g, 0.7 mmol) was added batch-wise (over 15–20 minutes) to the mixture. Subsequently, it was stirred for 24 h at room temperature. After initial identification by TLC (eluent solvent 8 : 2 ethyl acetate/hexane) dicyclohexylurea was separated by filtration and the solvent was removed under high vacuum. Next, the mixture was dissolved in the cold hexane (5 × 50 mL) to detach unreacted DCC. Later, precipitations were collected by decanting hexane. The surplus DMAP was extracted by NH_4Cl (20%) and DCM. Subsequently, the organic layer was dried with $MgSO_4$ and evaporated. Further purification was fulfilled through column chromatography (mobile phase 8 : 2, ethyl acetate/hexane) to obtain (**d**) or (**e**), Scheme 1.

4.5. General procedure for deprotection and preparation of (f) and (g)

In a round-bottomed flask, **d** or **e** (0.1 g, 0.2 mmol) and acetic acid (85%, 10 mL) were combined together and refluxed under nitrogen gas for 5–7 h to form a clear solution. The mixture was then concentrated under the reduced pressure and neutralized by $NaHCO_3$ (5%). Purification was performed with column chromatography (9 : 1 ethyl acetate/methanol) to acquire (**f**) or (**g**), Scheme 1.

4.6. Characterization

4.6.1. *N*-Acetyl-5-aminosalicylate or 5-acetamido-2-hydroxy benzoic acid (a). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.08 (3H, CH3), 6.83 (1H, d, J = 7.7 Hz, Ar-CH), 7.55 (1H, d, J = 7.9 Hz, Ar-CH), 8.05 (1H, s, Ar-CH), 10.41 (1H, s, COOH). ^{13}C NMR (125.8 MHz, DMSO-d_6) δ = 24.2 (C-1), 113.8, 114.4, 120.9, 129.7; (C-2, C-3, C-4, C-5), 130.4 (C-6), 156.7 (C-7), 165.9 (C-8), 169.8 (C-9). FT-IR (ν): 3561, 3239, 3064, 2818, 2699, 2573, 2490, 1796, 1674, 1618, 1537, 1486, 1208, 1018. Ms: GC-mass, $(M)^+$ m/z 195.0, [calcd; 195.053, formula ($C_9H_9NO_4$)]. Yield: 67.5%. M.p: 195 °C. White, needle-shape crystals.

4.6.2. 1,2:5,6-Di-*O*-isopropylidene-D-glucofuranose or diacetone-glucose (b). ^{1}H NMR (500 MHz, acetone-d_6): δ (ppm) = 1.22 (3H, s, CH_3), 1.29 (3H, s, CH_3), 1.33 (3H, s, CH_3), 1.45 (3H, s, CH_3), 2.22 (1H, s, OH), 3.95 (2H, dd, J = 6.3 Hz, J = 1.8 Hz, H-a, H-b), 4.12 (1H, t, J = 5.3 Hz, H-c), 4.28 (1H, t, J = 4.8 Hz, H-d), 4.41–4.50 (1H, m, H-e), 5.42 (1H, t, J = 5.1 Hz, H-f), 5.78 (1H, d, J = 3.6 Hz, H-g). ^{13}C NMR (125.8 MHz, acetone-d_6) δ = 23.8, 24.3, 25.7, 26.2; (C-1, C-2, C-3, C-4), 66.4 (C-5), 73.8 (C-6), 75.9 (C-7), 80.1 (C-8), 84.9 (C-9), 105.2 (C-10), 110.0 (C-11), 113.2 (C-12). FT-IR (ν): 3421, 2984, 1457, 1373, 1321, 1218, 1162, 1061, 1003. MS: HRMS $(M + Na)^+$, m/z 283.282, [calcd; 260.126, formula ($C_{12}H_{20}O_6$)]. Yield: 61%. M.p: 109–110 °C. White crystals. [α] 24/D −0.12, (c = 0.4 in acetone).

4.6.3. 2,3,4,5-Di-isopropylidene-D-xylitol or diacetone-xylitol (c). ^{1}H NMR (500 MHz, chloroform-d_1): δ (ppm) = 1.29

(6H, s, 3 each, 2 × CH_3), 1.34 (6H, s, 3 each, 2 × CH_3), 2.14 (1H, s, OH), 3.49 (2H, dd, J = 6.3 Hz, J = 2.5 Hz, H-a, H-b), 3.72–3.78 (3H, m, H-c, H-d, H-e), 3.90–3.99 (1H, m, H-f), 4.12–4.15 (1H, m, H-g). ^{13}C NMR (125.8 MHz, chloroform-d_1) δ = 25.5, 26.2, 27.0, 27.1; (C-1, C-2, C-3, C-4), 62.2 (C-5), 65.6 (C-6), 75.1 (C-7, C-8), 77.8 (C-9), 109.7 (C-10), 110.05 (C-11). FT-IR (ν): 3445, 2962, 2858, 1465, 1460, 1383, 1375, 1215, 1125, 1003. MS: HRMS (M + Na)$^+$, m/z 255.267 [calcd; 232.131, formula, ($C_{11}H_{20}O_5$)]. Yield: 57%. M.p: 30–33 °C. White crystals. [α] 24/D −0.02, (c = 0.4 in acetone).

4.6.4. 3-*O*-*N*-Acetyl-5-aminosalicylate-1,2:5,6-di-*O*-isopropylidene-D-glucofuranose (d). ^{1}H NMR (500 MHz, methanol-d_4): δ (ppm) = 1.28 (6H, bs, 3 each, 2 × CH_3), 1.49 (6H, s, 3 each, 2 × CH_3), 2.08 (3H, s, O=C–CH_3), 3.59 (2H, dd, J = 7.2 Hz, J = 3.3 Hz, H-a, H-b), 3.73 (2H, dd, J = 10.5 Hz, J = 5.7 Hz, H-c, H-d), 3.90 (1H, t, J = 6.3 Hz, H-e), 4.28 (1H, t, J = 7.6 Hz, H-f), 5.47 (1H, s, Ar-OH), 5.95 (1H, d, J = 3.4 Hz, H-g), 7.46 (1H, d, J = 7.8 Hz, Ar-CH), 7.63 (1H, d, J = 7.9 Hz, 1H, Ar-CH), 7.91 (1H, s, NH), 8.05 (s,1H, Ar-CH). ^{13}C NMR (125.8 MHz, methanol-d_4) δ = 23.8 (C-1), 26.4, 26.7; (C-2, C-3, C-4, C-5), 72.1 (C-6), 75.9 (C-7), 80.0 (C-8), 83.4 (C-9), 85.1 (C-10), 109.4 (C-11), 110.3 (C-12), 115.9 (C-13), 116.0 (C-14), 121.1 (C-15), 121.9 (C-16), 127.1 (C-17), 130.3 (C-18), 157.8 (C-19), 167.9 (C-20), 168.0 (C-21). FT-IR (ν): 3477, 2987, 2928, 1734.20, 1455, 1378, 1214, 1165, 1070, 1008. MS: HRMS (M + Na)$^+$, m/z 460.439 [calcd; 437.169, formula ($C_{21}H_{27}NO_9$)]. Yield: 57%. M.p: 182–185 °C. Light gray crystals.

4.6.5. 1-*O*-*N*-Acetyl-5-aminosalicylate-2,3,4,5-di-isopropylidene-D-xylitol (e). ^{1}H NMR (500 MHz, methanol-d_4): δ (ppm) = 1.15 (3H, brs, CH_3), 1.35 (9H, s, 3 each, 3 × CH_3), 1.76 (3H, s, O=C–CH_3), 3.40 (1H, t, J = 5.4 Hz, H-a), 3.56–3.61 (2H, m, H-b, H-c), 3.85–3.93 (1H, m, H-d), 3.99–4.18 (3H, m, H-e, H-f, H-g), 5.21 (1H, s, OH), 6.83 (1H, d, J = 8.5 Hz), 7.43 (1H, d, J = 7.8 Hz), 8.24 (1H, s, NH), 8.52 (1H, s). ^{13}C NMR (125.8 MHz, methanol-d_4) δ = 22.7 (C-1), 24.3, 26.1, 26.5; (C-2, C-3, C-4, C-5) 61.7 (C-6), 63.8 (C-7), 72.1 (C-8), 72.5 (C-9), 78.3 (C-10), 110.5 (C-11), 111.1 (C-12), 117.9 (C-13), 118.0 (C-14), 122.0 (C-15), 127.8 (C-16), 130.8 (C-17), 153.6 (C-18), 169.8 (C-19), 170.0 (C-20). FT-IR (ν): 3496, 3224, 3145, 2987, 2810, 1778, 1688, 1648, 1570, 1465, 1437, 1384, 1377, 1268, 1195, 1010, 1000. MS: HRMS (M + Na)$^+$, m/z 432.423 [calcd; 409.174, formula ($C_{20}H_{27}NO_8$)]. Yield: 47%. M.p: 151–153 °C. White crystals.

4.6.6. 3-*O*-5-Aminosalicylate-D-glucopyranoside (f). ^{1}H NMR (500 MHz, methanol-d_4): δ(ppm) = 2.08 (4H, s, 4 × OH), 3.61 (1H, dd, J = 5.8 Hz, J = 2.2 Hz, H-a), 3.75–3.77 (2H, m, H-b, H-c), 3.89 (2H, s, NH_2), 3.99 (1H, t, J = 4.2 Hz, H-d), 4.03 (1H, t, J = 4.9 Hz, H-e), 4.20 (1H, t, J = 5.2 Hz, H-f), 5.47 (1H, s, Ar-OH), 5.95 (1H, d, J = 1.8 Hz, H-g), 7.45 (1H, d, J = 8.8 Hz, Ar-CH), 7.64 (1H, d, J = 7.9 Hz, Ar-CH), 8.05 (1H, s, Ar-CH). ^{13}C NMR (125.8 MHz, methanol-d_4) δ (ppm) = 62.2 (C-1), 69.9 (C-2), 70.2 (C-3), 74.2 (C-4), 76.9 (C-5), 94.6 (C-6), 116.0 (C-7), 116.2 (C-8), 118.6 (C-9), 122.0 (C-10), 129.9 (C-11), 151.9 (C-12), 165.9 (C-13). FT-IR (ν): 3575, 3475, 3327, 3154, 2971, 2833, 2689, 1768, 1768, 1662, 1624, 1556, 1475, 1269, 1220, 1122. MS: HRMS (M + Na)$^+$, m/z 338.263 [calcd; 315.095, formula ($C_{13}H_{17}NO_8$)]. Yield: 41%. M.p: 191–193 °C. White crystals.

4.6.7. 1-*O*-5-Aminosalicylate-D-xylitol (g). ^{1}H NMR (500 MHz, methanol-d_4): δ = 2.09 (4H, s, 4 × OH), 3.40–3.46 (2H, m, H-a, H-b), 3.57–3.59 (1H, m, H-c), 3.65–3.70 (2H, m, H-d, H-e), 4.08 (2H, s, NH_2), 4.33–4.41(2H, m, H-f, H-g), 5.28 (1H, s, Ar-OH), 6.97 (1H, d, J = 8.8 Hz, Ar-CH), 7.19 (1H, d, J = 9.0 Hz, Ar-CH), 7.58 (1H, s, Ar-CH). ^{13}C NMR (125.8 MHz, methanol-d_4) δ = 63.9 (C-1), 64.0 (C-2), 69.9 (C-3), 71.9 (C-4), 73.8 (C-5), 114.4 (C-6), 116.4 (C-7), 116.6 (C-8), 120.1 (C-9), 149.6 (C-10), 156.4 (C-11), 168.0 (C-12). FT-IR (ν): 3601, 3536, 3446, 3121, 2986, 2977, 1719, 1601, 1549, 1475, 1310, 1282, 1000. MS: HRMS (M − H)$^-$, m/z 286.259 [calcd; 287.101, formula ($C_{12}H_{17}NO_7$)]. Yield: 38%. M.p: 164–166 °C. White crystals.

4.7. Anti-bacterial assay

4.7.1. Disc diffusion method. *In vitro* antibacterial test was carried out in keeping with the recommended standards of the National Committee for Clinical Laboratory Standards based on determination of inhibition zone in millimetres (mm) in nutrient agar (NA). In addition, the microbe cultures were standardized to 0.5McFarland standard which is approximately 10^8 cells.

The antibacterial activity of (**f**) and (**g**) (2 mg mL^{-1} concentration) was evaluated against pathogenic Gram-negative bacteria, (*Escherichia coli* (*E. coli*) and *Salmonella choleraesuis*) and Gram-positive bacteria, (*Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis*) *via* the disc diffusion method and compared with the initial drug (2 mg mL^{-1} concentration). Pure ethanol (which was employed to dissolve samples) and streptomycin (100 μg mL^{-1} concentration) were applied as a negative control and positive control, respectively.

In brief, the samples, which had been previously sterilised under UV, were inoculated onto 5 mm diameter paper discs which were then positioned on the NA surface of the plates on which microbial growth took place. The plates were inverted and incubated at 30–37 °C for 18–24 hours until sufficient growth occurred. After incubation, by ruler from the back of inverted Petri plates, the diameters of the zones around the samples which indicated amount of inhibition were measured to millimetre (Table 1).

4.7.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC/MBC assay was performed through the macro broth serial dilution technique as previously described methods by (Nakamura *et al.*, 1999, Karaman *et al.*, 2003, Singariya *et al.*, 2011, Fabry, *et al.*, 1998) with a slight modification.[67–70]

MICs were also investigated for the microbial strains which were determined to be sensitive to the compounds in the disc diffusion assay.

Samples initially prepared at the concentration of 4 mg mL^{-1} in ethanol, after that, they were diluted serially into eight consecutive tubes to obtain concentration range, 0.93–3.33 mg mL^{-1} (0.2 mL of samples into 1 mL of nutrient broth which pre-inoculated with bacteria adjusted to the 0.5 Mcfarland standard concentration, 10^8 cfu mL^{-1}). The final volume in each tube was 1.2 mL. Similar preparation was carried out for streptomycin from initial concentration of 50 μg mL^{-1} to obtain concentration range, 11.62–41.66 μg mL^{-1} as a positive control. Two control tubes also were set-up for each test batch. These include tube containing sample without inoculum and the tube containing the growth medium and inoculum. The tubes were

covered with a sterile sealer. Contents of each tube were mixed and incubated at 37 °C. After 24 h, MIC was recorded as the lowest concentration of the test compound that demonstrated no turbidity or visible growth of organism. Each sample tested in this study was screened two times against each organism.

The MBC was determined by sub-culturing the test dilution on nutrient agar and further incubated for 18–24 h. The MBC is defined as the minimum level of compound concentration produces no growth of microbial colonies on NA medium (Table 2).

4.8. Anti-inflammatory assay

The NO suppression of **f** and **g** in IFN-γ/LPS-stimulated RAW264.7 macrophages at 50 μM test concentration was examined, and curcumin was used as a positive control. Subsequently, the results were compared with the parent drug. Furthermore, an evaluation of cytotoxicity was carried out to support that the NO inhibition was not caused by sample toxicity.

4.8.1. NO investigation. According to Leong *et al.*'s method,[48] NO suppression was investigated. Briefly, into a 96-well culture plate with 50 μL of DMEM (Dulbecco's Modified Eagle's Medium) including 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 95% air and 5% CO_2 atmosphere RAW 264.7 murine macrophages cells were seeded (50 000 cells per well) and grown for 24 h at 37 °C. Thereafter, cells in 5 mg mL^{-1} of LPS (*Escherichia coli*, serotype 0111:B4) and 1 ng mL^{-1} of interferon-gamma (IFN-γ) were provoked in the presence or absence of test compounds for 17 h. Nitrite concentration was measured by reacting 50 μL of cell culture supernatant with 50 μL of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid) at room temperature. After 5 min of incubation at room temperature, the optical density was determined with a microplate reader, (Table 3).

4.8.2. Cell cytotoxicity determination for macrophage cell line. 100 μL DMEM was added in each well to remove supernatant, following that 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg mL^{-1}) was added, then the plate was incubated in a 95% air and 5% CO_2 atmosphere at 37 °C for 4 h. Subsequently, the mixture of culture media and MTT in all wells were detached and the purplish formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) and incubation was carried out at room temperature for 15 min. The color intensity was then calculated at 570 nm at room temperature, (Table 3).

4.9. Anti-cancer assay

In vitro MTT test was carried out for cell viability assessment against HT-29 and 3T3 cell lines. The HT-29 cell line which obtained from cultured human colon cancer cells was used in the study of intestinal cells. The 3T3 cell line which is the standard fibroblast cell line and was originally acquired from mouse embryo tissue was applied as a model for normal cells. This method was performed following the formerly published methods of Mosmann,[71] Boncler *et al.*,[72] and Vareed *et al.*[73] In brief, samples were put in the wells at concentrations of 0, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 μg mL^{-1} for HT-29 cell lines. For 3T3 cell lines, the concentrations used were 0, 0.1, 1, 100, 1000 and 10 000 μg mL^{-1}. For both cell lines, a concentration of zero was used for untreated control cells and **5-ASA** was applied as a reference drug as well. The incubation was carried out over 24 h and 48 h periods. When reduction of yellow MTT to purple formazan crystals was completed, the optical density (OD) of samples was calculated by ELISA reader at a wavelength of 570 nm (Table 4). IC_{50} value (the concentration of a drug that is required for 50% inhibition) was recorded using the following equation:

$$\text{Cell viability (\%)} = (\text{OD of sample/OD of control}) \times 100. \quad (1)$$

After triplication and validation, graphs were plotted with the percentage of cell viability against their respective concentrations (Fig. 1).

4.10. Docking study

4.10.1. Preparation of proteins and ligands. The crystal structures of proteins were retrieved from Protein Data Bank (http://www.rscb.org/pdb).[59,61] Both COX-1, COX-2 (PDB code: 1EQG, 1CX2, X-ray resolutions 2.61 Å, 3 Å, respectively)[57,59] exist as a homodimeric assembly, none of binding sites of them come in the interface of two domains, thus, only a subunit of the homodimeric COX-1 and COX-2 were utilized. 5-LOX (PDB code: 3O8Y, X-ray resolution 2.39 Å)[61] exists as a monomer. The water was removed from crystal structures to avoid masking the surface of protein from ligand. AutoDockTools-1.5.6 program[61] was utilized to add hydrogen and Kollman charges to protein. 3D structures of ligands were constructed using Pymol version 1.3 (ref. 57 and 74) and were converted to PDB.

4.10.2. Preparing grid box. Rigid protein and flexible ligand docking was fulfilled with 0.375 grid spacing. Grid of 50 × 50 × 50 Å size was set for COX-1 and COX-2 which centered at (x y z) 68.328, 22.231 and 189.725 for COX-1, 23.947, 21.582, 15.436 for COX-2. Grid size for 5-LOX was created 60 × 60 × 60 Å and (x y z) centered at 4.41, 20.412 and −0.648 for 5-LOX where surrounded the active side residues.

4.10.3. Preparing AutoDock. 100 runs of Lamarckian Genetics Algorithm[61] were carried out using AutoDock4.0 (ref. 61) (http://autodock.scripps.edu). All docking parameters were set to their default values. Eventually, the lowest binding energy was obtained for highest cluster members. All images were rendered using Pymol version 1.3 and binding interactions were analyzed using Ligplot software version v.1.4.5.

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