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Coumarins from Creston Apple Seeds: Isolation, Chemical Modification, and Cytotoxicity Study

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ABSTRACT

In the present work, preliminary phytochemical screening tests were performed on extracts of Creston apple seeds. Extraction was performed using two methods, which are serial Soxhlet extraction and kinetic maceration utilizing water, methanol, chloroform, and n-hexane as extraction solvents. Three coumarin derivatives acquired from chloroform extract in the order of increasing polarity were isolated via column chromatography and one of them was chemically modified by esterification, Fries rearrangement, and methylation afforded three semisynthetic derivatives. Detection of physicochemical properties and analysis of FTIR, ^{1}H-NMR and ^{13}C-NMR spectra of the natural and semisynthetic coumarins were used to identify their structures. *In vitro* cytotoxic activity of the aforementioned coumarin derivatives was studied on three cancer cell lines, which are MCF-7, AMN3, and HeLa using MTT assay. The results indicated that compounds N3, S4, S5 and S6 have IC_{50} values lower than that of 5- fluorouracil against MCF-7 cell line and all compounds have higher IC_{50} values than that of 5-fluorouracil against AMN3 and HeLa cell lines.

INTRODUCTION

Cancer is a major public health problem and is the second leading cause of death worldwide. According to the American Cancer Society, there were 1.7 million new cancer cases and 0.6 million cancer deaths throughout the United States nationally in 2018 (Siegel *et al.*, 2018).

During the last decades, natural products and their derivatives played an influential role in drug discovery and development programs because of their enormous biological properties (Crane and Gademann, 2016) and their effectiveness in treating several human diseases, including cancer (Newman and Cragg, 2016). Although the molecular mechanisms of action for most of these compounds are yet uncertain, recent evidence arises from molecular biology, high throughput screening, proteomic and genomic approaches can enable many researchers to collect some pieces of the fact (Kok *et al.*, 2012; Li-Weber, 2015; Tsuchiya, 2015).

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Coumarin (1,2-benzopyrone, 2*H*-1-benzopyran-2-one, or phenylpropanoids, Fig. 2) and its derivatives are a family of oxygen-containing fused heterocycles and they are widely distributed throughout nature (Swayam *et al.*, 2012). Until 2013, more than 1,300 coumarins have been identified as secondary metabolites in different plants, bacteria and fungi (Venugopala *et al.*, 2013). In the plant kingdom, coumarins are found in over 30 different families as *Umbelliferae, Rutaceae and Clusiaceae* (Stefanachi *et al.*, 2018) and they are distributed in various parts of these plants especially seeds, roots and leaves (Rosselli *et al.*, 2009).

Creston apple (*Malus domestica, Pomoideae* subfamily, *Rosaceae* family) is a name of both the tree and fruit. As a tree, it is cultivated worldwide and is originated in Central Asia where its wild ancestor, *Malus sieversii*, is still found today. Also, this tree has been grown for thousands of years in Asia and Europe and was brought to North America by European colonists (Quamme *et al.*, 1999).

The seeds of Creston apple are mildly poisonous due to the presence of a small amount of cyanogenic glycoside called amygdalin; the amount of which (0.6 mg/g of seeds) is relatively low and is unlikely to cause harmful effect to humans, but it is

possible to cause that effect when extremely high amount of seeds has been consumed (Nelson *et al.*, 2007).

According to our survey conducted in September 2017, the available reports regarding the phytochemicals of Creston apple studied them in whole fruit without seeds (Jelodarian *et al.*, 2012; Anbu Jeba Sunilson *et al.,* 2016), peel (Konarska, 2013; Vineetha *et al.*, 2014) or leaves (Liaudanskas *et al.*, 2014; Sowa *et al.*, 2016). We failed to find any data on the chemical composition of the Creston apple seeds, which provides a motive to initiate this work.

The aim of this work is to carry out phytochemical screening tests on four different extracts (water, methanol, chloroform and n-hexane) obtained from Creston apple seeds (using serial Soxhlet extraction and kinetic maceration), to isolate any coumarins found in the extracts **(**compounds **N1, N2** and **N3)** and then chemically modifying **N3** to afford new semisynthetic derivatives **(**compounds **S4, S5** and **S6)**, and finally to screen the cytotoxic activity of the isolated coumarins and their semisynthetic derivatives on three cancer cell lines, which are: MCF-7, AMN3 and HeLa using MTT assay.

MATERIAL AND METHODS

The chemicals and solvents used in the extraction and synthesis were purchased from Sigma-Aldrich and Tokyo Chemical Industry (TCI), and they were utilized without further purification. The chemicals and reagents used in a cytotoxic study included the following: Trypsin/EDTA (T4049), Roswell Park Memorial Institute (RPMI) 1640 medium (R8758) and fetal bovine serum (F2442) were purchased from Sigma-Aldrich while MTT stain (42000092-1) was obtained from Bio-World. The fruit was purchased from the local market and the taxonomic identity for 30 plant specimens was confirmed by the College of Agriculture and Forestry/University of Mosul.

Solvent Extractor SER 148 Series (VELP Scientifica) was used for serial Soxhlet extraction, while for kinetic maceration, SWBR17 SHEL LAB shaking water bath was used. Bruker-Alpha ATR was used to scan IR spectra, and Varian UV/Visible spectrophotometer was used to identify the IR spectra of the products. Among other UV absorption bands, the wavelength of maximum absorption (λ_{max}) was utilized in this work. Proton-nuclear magnetic resonance (^{1}H-NMR) and carbon-nuclear magnetic resonance (^{13}C-NMR) spectra of products were scanned on Bruker Avance 300 and 400 MHz, respectively.

EXTRACTION

Preparation of sample

After authentication, Creston apples (100 kg) were washed properly with running water, then with distilled water and sliced manually into small pieces. The obtained seeds were shade dried at room temperature for 2 weeks, pulverized in a blender, and sieved affording a fine powder (138.4 g) which was placed in amber tightly-closed containers in the refrigerator for further use (Mahlo *et al.*, 2016).

Serial Soxhlet extraction

Powdered material (20 g) was extracted successively in Soxhlet with 200 mL of each of the following solvents: water, methanol, chloroform, and n-hexane; once in order of decreasing polarity and secondly in the order of increasing polarity. Each time before extracting with the next solvent, the powdered material was dried. The extracts were filtered using Whitman No.1 filter paper and the resulted solutions were concentrated under reduced pressure, stored in amber tightly-closed containers apparently labeled and kept in the refrigerator until used for phytochemical screening tests (Apu *et al.*, 2012).

Extraction by kinetic maceration

Powdered material (2.5 g) was added to a beaker containing 25 mL of water, methanol, chloroform or n-hexane placed in a shaker water bath adjusted at 37°C for 24 hours. The extracts were filtered using Whitman No. 1 filter paper and the resulted solutions were concentrated under reduced pressure, stored in amber tightly-closed containers apparently labeled and kept in the refrigerator until used for phytochemical screening tests (Naviglio *et al.*, 2016).

QUALITATIVE PHYTOCHEMICAL SCREENING TESTS

Extracts obtained from the previously mentioned extraction methods were subjected separately to the phytochemical screening tests reported by Harborne (Harborne, 1998).

ISOLATION OF COUMARINS

The chloroform extract was dried under reduced pressure. The solid was treated with 50 mL of 1N NaOH and stirred using a condenser apparatus at 50°C for 3 hours. The mixture was filtered and the resulted yellow filtrate was acidified with 1N HCl; the crystals were collected via filtration after cooling the mixture for 24 hours in the refrigerator.

The TLC plate eluted with $CHCl_3$: acetone (4:1) indicated the presence of three spots; accordingly, the separation was conducted via column chromatography using silica gel as a stationary phase and mixture of ethyl acetate: ether as an eluent in gradient ratio ranging from 1:9 to 9:1.

Three compounds (**N1**, **N2** and **N3**) (N for natural) were identified respectively, each one as a single spot, in the following fractions: (7:3), (5:5) and (9:1) (Ghanbari *et al.*, 2011).

5,6-dihydropyrone(4,3-g)coumarin **(N1)** white powder from a mixture of ether: $CHCl_3$ (1:3); m.p 176-178°C; λ_{max} (EtOH) 323 nm; R_f 0.846; IR (ν, cm^{-1}): 3056 (=C-H str.), 2980 (C-H str., alkyl), 1724 (C=O str., ester), 1670 (C=C str.), 1585 (C=C str., aromatic), 1267 (C-O str., ester); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 8.4 (s, 1H, C5-**H**), 7.8 (d, 1H, *J* = 9.6 Hz, C4-**H**), 7.1 (s, 1H, C8-**H**), 6.5 (d, 1H, *J* = 9.6 Hz, C3-**H**), 4.7 (t, 2H, *J* = 6.0 Hz, O-C**H**$_2$-CH$_2$), 3.0 (t, 2H, *J* = 6.0 Hz, O-CH$_2$-C**H**$_2$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 168.3 (C6-**C**=O), 161.5 (C2), 156.7 (C9), 146.0 (C4), 140.3 (C7), 128.1 (C5), 127.7 (C6), 127.2 (C10), 120.1 (C8), 117.2 (C3), 72.3 (O-**C**H$_2$-CH$_2$), 30.8 (O-CH$_2$-**C**H$_2$).

5-Ethyl-6,7-dimethylcoumarin **(N2)** white powder from a mixture of EtOH: $CHCl_3$ (1:2); m.p 121-123°C; λ_{max} (EtOH) 326 nm; R_f 0.830; IR (ν, cm^{-1}): 3035 (=C-H str.), 2950, 2883 (C-H str., alkyl), 1700 (C=O str., ester), 1666 (C=C str.), 1588 (C=C str., aromatic), 1270 (C-O str., ester); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 7.9 (d, 1H, *J* = 9.6 Hz, C4-**H**), 6.8 (s, 1H, C8-**H**), 6.4 (d, 1H, *J* = 9.6 Hz, C3-**H**), 2.6 (m, 2H, *J* = 6.3 Hz, C5-C**H**$_2$-CH$_3$), 2.4 (s, 3H, C6-C**H**$_3$), 2.2 (s, 3H, C7-C**H**$_3$), 1.2 (t, 3H, *J* = 6.3 Hz,

C5-CH_2-C**H**$_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 163.1 (C2), 147.9 (C9), 147.3 (C4), 137.9 (C5), 137.5 (C7), 134.3 (C6), 124.9 (C10), 120.0 (C8), 116.1 (C3), 17.0 (C5-**C**H_2-CH_3), 16.2 (C5-CH_2-**C**H_3), 14.5 (C7-**C**H_3), 9.0 (C6-**C**H_3).

7-Hydroxy-4-methoxycoumarin **(N3)** white powder from a mixture of EtOH: $CHCl_3$ (1:1); m.p 164-166°C; λ_{max} (EtOH) 322 nm; R_f 0.727; IR (ν, cm^{-1}): 3300 (O-H str., phenol), 3076 (=C-H str.), 2898 (C-H str., alkyl), 1708 (C=O str., ester), 1678 (C=C str.), 1573 (C=C str., aromatic), 1302 (asymmetrical C-O-C str., ether), 1169 (C-O str., ester), 1064 (symmetrical C-O-C str., ether); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 10.1 (s, 1H, O-**H**), 7.6 (d, 1H, *J* = 9 Hz, C5-**H**), 6.7 (d, 1H, *J* = 9 Hz, C6-**H**), 6.6 (s, 1H, C8-**H**), 5.5 (s, 1H, C3-**H**), 3.6 (s, 3H, O-C**H**$_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 176.6 (C4), 162.7 (C2), 157.2 (C7), 150.6 (C9), 128.8 (C5), 121.7 (C10), 113.4 (C6), 105.5 (C8), 88.2 (C3), 50.6 (O-**C**H_3).

CHEMICAL SYNTHESIS OF COMPOUND (N3) DERIVATIVES (SCHEME 1)

Synthesis of 7-Acetoxy-4-methoxycoumarin (S4)

A mixture of **N3** (1.92 g, 10 mmol) and anhydrous K_2CO_3 (2.76 g, 20 mmol) in 30 mL ethanol was stirred for 30 minutes at room temperature and then concentrated under reduced pressure. The solid was filtered, re-dissolved in 40 mL acetone and filtered. The filtrate was evaporated to afford a sodium salt of **N3**.

The solution of the resultant salt in 30 mL acetone was dropwise added to a solution consisting of acetic anhydride (1.5 mL, 0.014 M) and glacial acetic acid (1 mL, 0.016 M). The reaction solution was heated with constant stirring for 1 hour in a water bath and then poured into a mixture of water and crushed ice. The solid was collected by filtration, washed with water and recrystallized from the EtOH to afford **S4 (S for semisynthesized).** The method used here is a modified method to that employed by (Sandhya *et al.*, 2010).

(**S4**): Pale yellow powder; (1.92 g, 82% yield); m.p 140-143°C; λ_{max} (EtOH) 492 nm; R_f 0.803; IR (ν, cm^{-1}): 3078 (=C-H str.), 2897 (C-H str., alkyl), 1706, 1690 (C=O str., ester), 1677 (C=C str.), 1577 (C=C str., aromatic), 1298 (asymmetrical C-O-C str., ether), 1170 (C-O str., ester), 1063 (symmetrical C-O-C str., ether); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 7.8 (d, 1H, *J* = 9 Hz, C5-**H**), 6.9 (d, 1H, *J* = 9 Hz, C6-**H**), 6.85 (s, 1H, C8-**H**), 5.6 (s, 1H, C3-**H**), 3.5 (s, 3H, O-C**H**$_3$), 2.0 (s, 3H, C7-OCO-C**H**$_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 176.5 (C4), 169.0 (C7-O**C**O-CH_3), 164.2 (C2), 155.3 (C7), 150.0 (C9), 126.1 (C5), 120.3 (C10), 118.5 (C6), 114.3 (C8), 90.1 (C3), 52.2 (O-**C**H_3), 15.5 (C7-OCO-**C**H_3).

Synthesis of 8-Acetyl-7-hydroxy-4-methoxycoumarin (S5)

A mixture of **S4** (1.17 g, 5 mmol) and anhydrous $AlCl_3$ (2.2 g, 16.5 mmol) in 40 mL dry ACN was reflexed (85°C) for 4 hours. The cloudy solution was slowly and carefully poured into 20 mL of 1M HCl at 0°C to destroy the excess of $AlCl_3$. The mixture was extracted with ethyl acetate (2 × 30 mL) that was sequentially washed with 20 mL of 5% $NaHCO_3$ solution, 20 mL of saturated NaCl solution and 20 mL of distilled H_2O. The ethyl acetate layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. **S5** was recrystallized from a mixture of EtOH: $CHCl_3$ (1:2). This synthetic method is a modified one to that reported by (Traven *et al.*, 2000).

(**S5**): Yellowish powder; (0.73 g, 63% yield); m.p 171-173°C; λ_{max} (EtOH) 543 nm; R_f 0.746; IR (ν, cm^{-1}): 3316 (O-H str., phenol), 3058 (=C-H str.), 2858 (C-H str., alkyl), 1712 (C=O str., ester), 1668 (C=O str., ketone), 1570 (C=C str., aromatic), 1303 (asymmetrical C-O-C str., ether), 1172 (C-O str., ester), 1063 (symmetrical C-O-C str., ether); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 11.5 (s, 1H, O-**H**), 7.9 (d, 1H, *J* = 9 Hz, C5-**H**), 6.8 (d, 1H, *J* = 9 Hz, C6-**H**), 5.2 (s, 1H, C3-**H**), 3.5 (s, 3H, O-C**H**$_3$), 2.8 (s, 3H, C8-CO-C**H**$_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 196.1 (C8-**C**O-CH_3), 176.1 (C4), 162.6 (C2), 157.9 (C7), 157.4 (C9), 130.2 (C5), 122.5 (C8), 121.4 (C10), 110.1 (C6), 90.1 (C3), 51.2 (O-**C**H_3), 24.2 (C8-CO-**C**H_3).

Synthesis of 4,7-Dimethoxycoumarin (S6)

To a stirred mixture of **N3** (1.92 g, 10 mmol) and anhydrous K_2CO_3 (2.76 g, 20 mmol) in 90 mL dry acetone, a solution of dimethyl sulfate (DMS) (1.15 mL, 12 mmol) in 10 mL dry acetone was dropwise added. The reaction mixture was refluxed for 3 hours under anhydrous conditions and filtered; the inorganic salts were washed several times with hot acetone (10 mL). The combined acetone solution was concentrated under reduced pressure; heated to 50°C and directly poured into a mixture of water and crushed ice. The precipitate was filtered, washed with water and recrystallized from a mixture of methanol: ether (3:1) to afford **S6**. This is a modified method to that reported by (Karteek *et al.*, 2015).

(S6): White powder; (1.44 g, 70 % yield); m.p 149-152°C; λ_{max} (EtOH) 320 nm; R_f 0.824; IR (ν, cm^{-1}): 3063 (=C-H str.), 2892, 2838 (C-H str., alkyl), 1700 (C=O str., ester), 1596 (C=C str., aromatic), 1310 (asymmetrical C-O-C str., ether), 1148 (C-O str., ester), 1042 (symmetrical C-O-C str., ether); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 7.4 (d, 1H, *J* = 9 Hz, C5-**H**), 6.6 (d, 1H, *J* = 9 Hz, C6-**H**), 6.5 (s, 1H, C8-**H**), 5.4 (s, 1H, C3-**H**) 3.7 (s, 3H, C7-O-C**H**$_3$), 3.3 (s, 3H, C4-O-C**H**$_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm: 173.5 (C4), 162.6 (C2), 160.1 (C7), 150.1 (C9), 130.0 (C5), 119.0 (C10), 111.9 (C6), 107.5 (C8), 94.3 (C3), 58.0 (C7-O-**C**H_3), 50.6 (C4-O-**C**H_3).

CYTOTOXICITY STUDY

The cancer cell lines on which the cytotoxic effects of the studied compounds were tested were MCF-7, AMN3, and HeLa. They were obtained from Iraq Biotech Cell Bank Unit and maintained in a medium composed of RPMI 1640 supplemented with fetal bovine serum (heat-inactivated, 10%), 100 units/mL penicillin, and 100 µg/mL streptomycin. The harvest cells were passaged via Trypsin-EDTA, reseeded at 50% confluence twice weekly, and incubated in 5% CO_2 environment at 37°C (Attar *et al.*, 2017).

MTT cell viability test was performed to determine the cytotoxic effect of the isolated and semisynthetic coumarins using 96-well plates. The cell line was seeded at 1×10^4 cells/well and after 24 hours it was treated separately with each of the tested compounds. Later than 72 hours of treatment, cell viability was measured by removing the medium, adding 28 µl of 2 mg/mL solution of MTT and incubating the cells for 1.5 hours at 37°C. As MTT solution was removed, the crystals remaining in the wells were solubilized by the addition of 130 µl DMSO followed by

incubation at 37°C for 15 minutes with shaking (Al-Shammari *et al.*, 2016).

The absorbance was determined on a microplate reader at 492 nm and the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated according to the following equation: Inhibition rate = (A − B)/A × 100, where A is the mean optical density of untreated wells, and B is the optical density of treated wells (Shahneh *et al.*, 2013).

RESULTS AND DISCUSSION

In the recent years, natural products and their semisynthetic derivatives are gaining more and more popularity as potential drugs for the treatment of various illnesses affecting human beings (Atanasov *et al.*, 2015; Sanjeewa *et al.*, 2016).

Because apple is the fourth most consumed fruit in the world, and because of several lines of evidence which indicate that apple and its constituents have a wide range of pharmacological properties (White *et al.*, 2013) and ratified to the ancient saying "an apple a day keeps the doctor away", our team targeted the isolation and identification of coumarins present in Creston apple seeds.

Table 1: Phytochemical analysis of extracts obtained from serial Soxhlet extraction in the order of decreasing polarity. The solvent columns are arranged according to polarity starting from the most polar to the left.

Phytoconstituents	Water extract	Methanol extract	Chloroform extract	n-hexane extract
Flavonoids	+	+	-	-
Coumarins	-	+	+	-
Tannins	-	-	-	-
Terpenoid	-	+	+	-
Carbohydrates	+	+	-	-
Alkaloids	+	+	-	-
Fatty acids	-	-	+	+
Emodins	-	-	-	-
Anthraquinones	-	-	-	-
Betacyanine	+	+	-	-
Anthocyanine	-	-	-	-
Phenol	+	+	+	-
Protein	+	+	-	-
Amino acid	+	+	-	-
Steroids	-	+	+	-
Saponine	+	+	-	-
Glycosides	-	-	-	-

PHYTOCHEMICAL ANALYSIS

Qualitative phytochemical screening tests were carried out on three types of extracts; the first one was obtained from serial Soxhlet extraction in the order of decreasing polarity, the second was acquired using the same extraction method but in the order of increasing polarity, and the last type was attained from kinetic maceration method of extraction. Based on the results of the phytochemical analysis as showed in (Tables 1, 2 and 3), coumarins could be detected in methanol and chloroform extracts.

Table 2: Phytochemical analysis of extracts obtained from serial Soxhlet extraction in the order of increasing polarity. The solvent columns are arranged according to polarity starting from the most polar to the left.

Phytoconstituents	Water extract	Methanol extract	Chloroform extract	n-hexane extract
Flavonoids	-	+	-	-
Coumarins	-	-	+	-
Tannins	-	-	-	-
Terpenoid	-	+	+	-
Carbohydrates	+	+	-	-
Alkaloids	+	+	-	-
Fatty acids	-	-	-	+
Emodins	-	-	-	-
Anthraquinones	-	-	-	-
Betacyanine	-	+	-	-
Anthocyanine	-	-	-	-
Phenol	-	+	+	-
Protein	-	+	+	-
Amino acid	-	+	+	-
Steroids	-	+	+	-
Saponine	+	+	-	-
Glycosides	-	-	-	-

Table 3: Phytochemical analysis of extracts obtained from kinetic maceration method of extraction. The solvent columns are arranged according to polarity starting from the most polar to the left.

Phytoconstituents	Water extract	Methanol extract	Chloroform extract	n-hexane extract
Flavonoids	+	+	-	-
Coumarins	-	+	+	-
Tannins	-	-	-	-
Terpenoid	-	+	+	-
Carbohydrates	+	+	-	-
Alkaloids	+	+	-	-
Fatty acids	-	-	+	+
Emodins	-	-	-	-
Anthraquinones	-	-	-	-
Betacyanine	+	+	-	-
Anthocyanine	-	-	-	-
Phenol	+	+	+	-
Protein	+	+	+	-
Amino acid	+	+	+	-
Steroids	-	+	+	-
Saponine	+	+	-	-
Glycosides	-	-	-	-

Coumarins can be present in both polar and nonpolar solvents. This can be due to more than one reason. For example, the types of substituents present on the isolated coumarin can make it soluble in more than one type of solvents. The amount of solvent used, which can be considered huge relative to the amount of the isolated coumarin could be another reason, where the coumarin will dissolve because of a large amount of solvent present (Hrdlovic *et al.*, 2010).

ISOLATION OF COUMARINS

One of the most important chemical characteristics of coumarins is the presence of cyclic ester that opens when attacked by a strong base like NaOH to give yellow solutions of the salts of the corresponding *cis*-cinnamic acids. These salts are recycled to original coumarins when acidified with a sufficient amount of strong acid. This chemical property as displayed in Scheme 2 was employed in this work to isolate coumarins from other phytoconstituents (Lopez-Castillo *et al.*, 2013). According to the selected method of isolation and the presence or absence of flavonoids, the chloroform extract from serial Soxhlet extraction in the order of increasing polarity was chosen to identify its coumarin components.

IDENTIFICATION OF ISOLATED COUMARINS

Chemical structures of **N1, N2** and **N3** were identified by analyzing their FTIR, ^{1}H NMR and ^{13}C NMR spectra. For **N1**, the identification data showed the presence of characteristic chemical shifts of coumarin nucleus (Shults *et al.*, 2003) and of the dihydropyran-2-one ring (Bonvin *et al.*, 2005). For **N2**, the identification data reported the presence of characteristic chemical shifts of coumarin nucleus substituted at positions 6 and 7 (Perel'son *et al.*, 1970) and of coumarin nucleus substituted at position 5 with ethyl group (Appendino *et al.*, 1999). For **N3**, the identification data informed the presence of characteristic chemical shifts of 7-hydroxycoumarin substituted at position 4 (Ramesh *et al.*, 2008). Accordingly, the chemical structures of **N1**, **N2,** and **N3** were elucidated as displayed in Fig. 1.

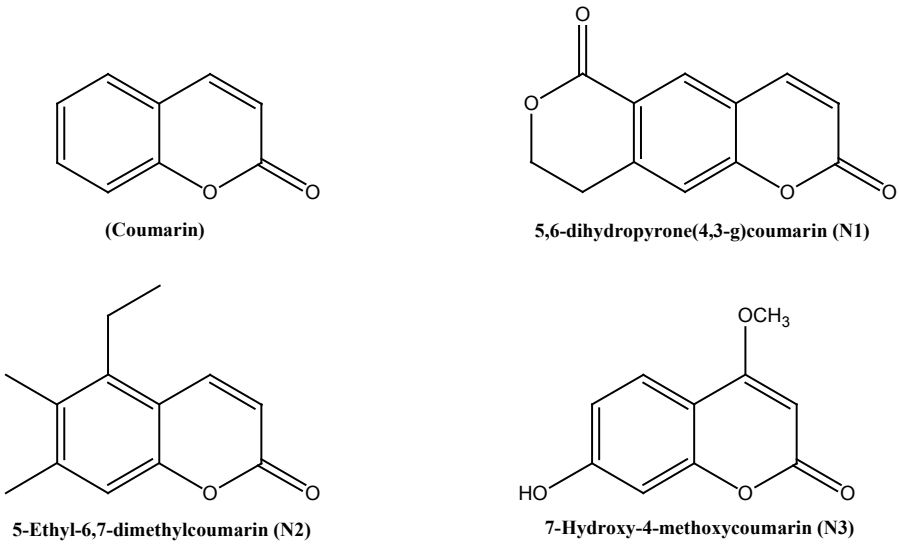


Fig. 1: Chemical structures of coumarin and of coumarins isolated from Creston apple seeds.

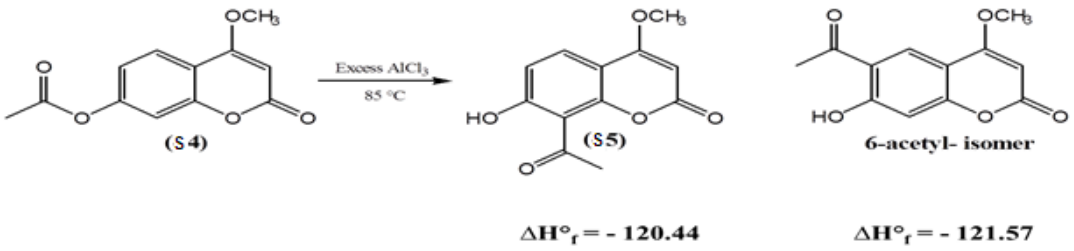


Fig. 2: Heat of formation of each of **S5** and its 6-acetyl-isomer expressed in kcal/mol.

CHEMICAL SYNTHESIS OF COMPOUND (N3) DERIVATIVES

7-Acetoxy-4-methoxycoumarin (S4)

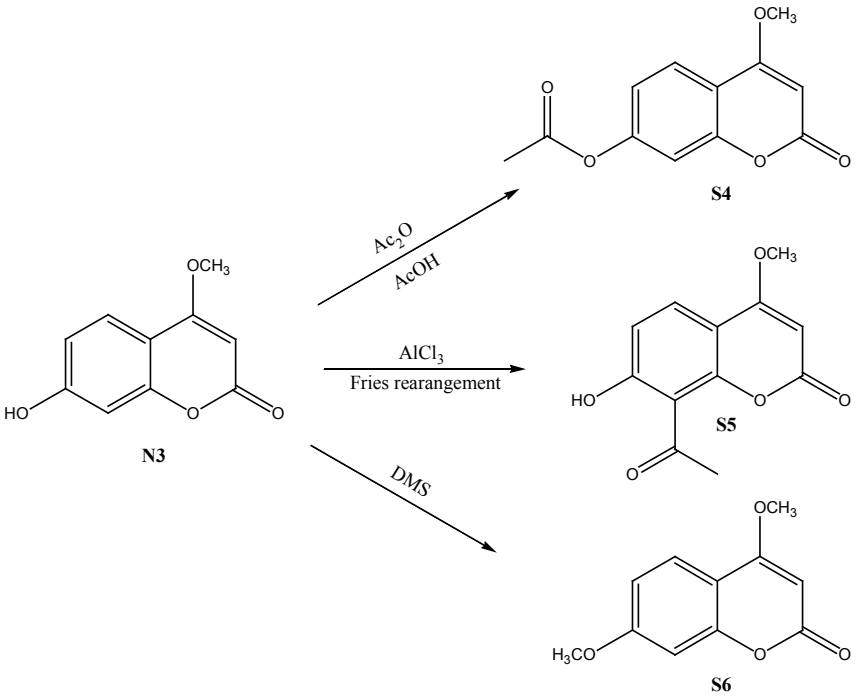
It was synthesized by condensing **N3** with acetic anhydride; both reactants have been activated prior to their condensation by enhancing the nucleophilicity of phenolic hydroxyl group as it is converted to metal phenoxide and by improving the electrophilicity of carbonyl carbon of acetic anhydride when it is protonated via glacial acetic acid (Habibi *et al.*, 2013).

Identification data of **S4** reported the absence of the chemical shift of phenolic hydroxyl group resonated at 10.1 ppm found in the ^{1}H-NMR spectrum of **N3** and the appearance of the chemical shift of acetate methyl group resonated at 2.0 ppm. This confirmed the formation of acetate ester linkage at position 7 of **S4**.

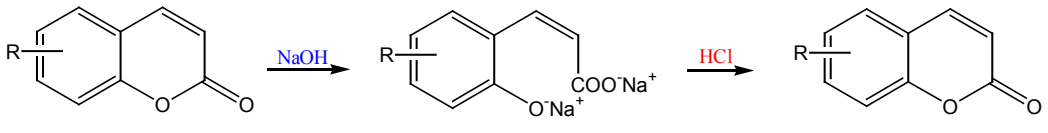
8-Acetyl-7-hydroxy-4-methoxycoumarin (S5)

It was synthesized by heating **S4** with an excess of $AlCl_3$ at 85°C in a Fries rearrangement reaction. At that temperature,

S4 is the predominant product comparing with its 6-acetyl-isomer (Traven, 2004), this is mainly due to kinetic factors since both, **S5** and its 6-acetyl- isomer, have approximately the same thermodynamic stability (Fig. 2) according to AM1 calculation that was performed using Scigress Explorer software (Fujitsu, Japan).



Scheme 1: Synthetic pathways of **N3** derivatives.



Scheme 2: base-catalyzed ring opening of coumarin derivatives and their recycling by strong acid.

Identification data of **S5** reported the presence of the chemical shifts of phenolic hydroxyl group resonated at 11.5 and the chemical shift of acyl methyl group resonated at 2.8 ppm. This confirmed the rearrangement of phenolic ester found in **S4** into O-hydroxy aryl ketone present in the chemical structure of **S5**.

4,7-Dimethoxycoumarin (S6)

DMS is one of the most commonly used reagents for methylation of O-, N- and S-functional groups, and it is miscible with many solvents such as ether, acetone, and dioxane that must be dry since it is hydrolyzed by water (Selva and Perosa, 2008).

The synthesis of **S6** is similar in scope to Haworth methylation (DMS with 30% aqueous NaOH) (Lamoureux and Agüero, 2009) but it is different in the type of base. K_2CO_3 was used instead of NaOH, which can open the coumarin lactone ring of **S6**, to initiate the deprotonation of phenolic hydroxyl group enhancing its nucleophilicity and to neutralize the acidity of methyl hydrogen sulfate byproduct (Bernini *et al.*, 2011).

Identification data of **S6** reported the absence of the chemical shift of phenolic hydroxyl group resonated at 10.1 ppm found in the ^{1}H-NMR spectrum of **N3** and the appearance of the chemical shift of methoxy group resonated at 3.7 ppm. This confirmed the formation of methyl ether bond at position 7 of **S6**.

CYTOTOXICITY STUDY

Tables 4 indicated that **N1** and **N2** have IC_{50} values higher than that of positive control against MCF-7 cancer cell line as calculated by using the MTT assay. The results also showed that **N3, S4, S5,** and **S6** have IC_{50} values lower than that of the positive control, with the best cytotoxic activity attributed to **S6**. Accordingly, these compounds may be considered as potential cytotoxic agents for the treatment of breast cancer.

The results also showed that the isolated and semisynthetic compounds have IC_{50} values higher than that of positive control against AMN3 cancer cell line with the best cytotoxic activity attributed to **S6**. Accordingly, this compound may be considered as a candidate cytotoxic agent for the treatment of mammary gland cancer.

In addition, the results presented in (Table 4) revealed that the isolated and semisynthetic compounds have IC_{50} values higher than that of positive control against HeLa cancer cell line

with the best cytotoxic activity attributed to **S5**. Accordingly, this compound may be considered as a candidate cytotoxic agent for the treatment of cervical cancer.

Table 4: Mean ± SD IC_{50} values of positive control, natural and semisynthetic coumarin derivatives against MCF-7, AMN3 and HeLa cancer cell lines.

Compound Name	MCF-7	AMN3	HeLa
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
5- Fluorouracil	12.50 ± 1.477	15.20 ± 2.621	11.90 ± 0.737
N1	36.80 ± 2.788	75.20 ± 5.834	37.50 ± 3.722
N2	26.20 ± 1.324	49.20 ± 4.475	39.22 ± 2.576
N3	9.83 ± 1.135	35.60 ± 2.763	37.83 ± 3.682
S4	8.30 ± 0.449	41.30 ± 3.286	42.30 ± 6.772
S5	12.48 ± 0.572	32.50 ± 1.406	21.50 ± 1.52
S6	8.22 ± 0.321	21.22 ± 1.804	23.52 ± 1.372

Mean ± SD IC_{50} values were determined by MTT assays following 72 h exposure of cells to test compounds (n = 3) and expressed as a mean and standard deviation of 3 independent trials.

CONCLUSION

Two extraction methods (Soxhlet extraction and kinetic maceration) performed on Creston apple seeds revealed the presence of coumarin derivatives in the chloroform extract. These natural coumarin derivatives were isolated, purified and characterized; one of them was chemically modified affording three semisynthetic coumarin derivatives. The *in vitro* cytotoxic activity of the isolated and semisynthetic coumarins using MTT assay on three different cancer cell lines indicated that **N3, S4, S5,** and **S6** have a significant cytotoxic activity against MCF-7 cancer cell line. Also, **S6** showed an encouraging cytotoxic activity against AMN3 while **S5** showed the same activity against HeLa cancer cell line.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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