New Bioactive Metabolites from a Crown Gall Induced on an *Eucalyptus tereticornis* Sm. Tree

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Applying a bioactivity-guided isolation strategy for the ethanolic extract of crown gall tumours induced on an *Eucalyptus tereticornis* tree, two new compounds in addition to a known one were isolated. The new compounds were identified as an amino acid derivative named 1-ethyl-6-(1'-methyl-1'-phenylethyl) piperidin-2-one (**1**) and a lanostane tetracyclic triterpene named 3*β*-hydroxy-24-methyllanosta-8,17(20),24(28)-trien-22-oic acid (**2**), together with stigmasterol-3-*O*-glucoside (**3**). The three compounds exhibited significant cytotoxic activity against two human cell lines, breast (MCF7) and colon (HCT116), with IC_{50} values of 1.01, 1.54, and 2.15 μg/ml, respectively, against MCF7 and 3.49, 3.83, and 3.39 μg/ml, respectively, against HCT116. Furthermore, in rats elevated levels of blood cholesterol, triglycerides, and low-density lipoprotein (LDLc) were significantly reduced, while the level of high-density lipoprotein (HDLc) was significantly increased by administration of the ethanolic extract as well as of **3**. These results support a correlation between the reduction of blood cholesterol levels and improvement of colorectal cancer.

Key words: Crown Gall, New Amino Acid Derivatives, Colorectal Cancer

Introduction

Microbial secondary metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities. Novel antibiotics and other bioactive secondary metabolites can still be discovered from microbial sources. In the last few decades, these products have been obtained through intensive screening involving millions of microorganisms. However, not all microorganisms are equally capable of producing secondary metabolites. In fact, this capability is restricted to a few groups of bacterial or eukaryotic microbes (Donadio *et al.*, 2002).

Agrobacterium tumefaciens (revised scientific name: *Rhizobium radiobacter*) (Young *et al.*, 2001) is the causal agent of crown gall disease defined as the formation of tumours in over 140 species of Dicotyledons. It is a Gram-negative, soil-born bacterium occurring in different strains, some of which are virulent, while others are avirulent. To be virulent, the strain must contain a tumour-inducing (Ti) plasmid which is responsible for crown gall formation (Escudero *et al.*, 1995). Symptoms are caused by the insertion of a small segment of DNA (known as T-DNA, for transfer DNA) into the plant cell which is incorporated at a semi-random location into the plant genome (Chilton *et al.*, 1977). The infection usually occurs during the rainy seasons and/or after cutting trees in relatively humid environment.

In the last decades, crown gall tumour tissues have attracted great interest related to the study and isolation of bioactive metabolites, as well as their biosynthesis and metabolism (Morris *et al.*, 1982; Palni and Horgan, 1983; Hanold, 1993; Stahlhut, 1994). Some unusual amino acid derivatives not ordinarily detected in normal cells have been found in crown gall tumours. These compounds were identified as N^2-(1-carboxyethyl)-L-amino acids, N^2-(1,3-dicarboxypropyl)-L-amino acids, N^2-(1'-deoxy-D-mannitol-1'-yl)-L-glutamine-1,2'-lactone (agropine) (Biemann *et al.*, 1960; Ménage and Morel, 1964; Chilton *et al.*, 1977; Kemp, 1977; Coxon *et al.*, 1980; Tate *et al.*, 1982; Chang *et al.*, 1983), and N^5-(L-1-carboxyethyl)-L-ornith-

ine (Firmin and Fenwick, 1977; Thompson, 1989). Furthermore, indole alkaloids (Wang *et al.*, 1994), cardenolides (Luckner *et al.*, 1990; Pinkwart *et al.*, 1993), and diterpenes (Ghosh and Mukerjee, 1997) have also been isolated from crown gall tumours.

Cancer will remain a major cause of worldwide deaths in the 21st century. More than 50% of the new cancer cases in the world occur in developing countries. Nearly 80% of the people living in developing countries still depend on plant-based traditional medicine for their primary health care, and almost three-fourths of the herbal drugs used worldwide are derived from medicinal plants (Verma and Singh, 2008). Natural products from plants used in traditional medicines are currently one of the main sources in cancer chemo-preventive drug discovery (Abdullaev, 2001). Considering the almost unlimited genetic variety of microorganisms and the multitude of secondary compounds formed by them, it is to be expected that compounds useful in cancer treatment will be found increasingly among microbial secondary metabolites (Umezawa, 1978).

Breast carcinoma is the most prevalent cancer among Egyptian women and constitutes 29% (Omar *et al.*, 2003), while colorectal cancer affects younger population with an incidence of 2 to 6% (Khafagy *et al.*, 2000).

Globalization and the wide-spread of Western diet to the developing world have produced an explosive increase in the rates of obesity and hypercholesterolemia in those regions. Therefore, hypercholesterolemia and its associated cardiovascular diseases represent one of the greatest worldwide economic, social, and medical challenges that we are facing now (AHA, 2005; Olshansky *et al.*, 2005).

Crown galls are a source of bioactive metabolites, especially of such that possess both antihypercholesterolemic (Sato *et al.*, 1996) and antineoplastic activities (Ghosh and Mukerjee, 1997). Such compounds are attractive drug candidates, because recent research has uncovered a relationship between factors causing hypercholesterolemia and factors leading to some types of cancer, such as colorectal cancer (CRC) (Herbey *et al.*, 2005).

For this reason, we have investigated the phytoconstituents of a crown gall of an *Eucalyptus tereticornis* Sm. tree with the aim of isolating new secondary metabolites and studying their cytotoxic activity against two human cancer cell lines, breast (MCF7) and colon (HCT116). We wished to examine the ability of these metabolites to reduce risk factors and thus prevent hypercholesterolemia and CRC.

Material and Methods

Plant material

The host tree, a specimen of *Eucalyptus tereticornis* Sm. (Mysore Gum, Forest Red Gum), family Myrtaceae, was growing in the garden of the campus of the School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia. The tree (23 m in height, 6 years old) was identified by Dr. Wondo Genet, College of Forestry and Natural Resources (WGCF), Addis Ababa, Ethiopia. The crown gall tissues were obtained from the tumour induced on this tree.

Induction of crown gall tumour

The tumour was induced during the rainy season by incision of the stem of the tree with an axe infected with both *Agrobacterium tumefaciens* strain C58 [carrying a tumour-inducing plasmid (Ti plasmid)] and *Agrobacterium rhizogenes* (strain A4) [carrying a root-inducing plasmid (Ri plasmid)]. The tumour, 25–27 cm in diameter and weighing about 350 g, was formed within two months, cut off and dried in an oven at 60 °C, and stored in a dry room. Both plasmids were identified in the tumour tissue after infection using restriction analysis in the Microbiology Department, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia. The tumour was easily powdered due to its spongy nature, kept in completely filled, tightly closed glass containers, and brought to Egypt by one of us (A. M. E.).

General procedures and materials

Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70–230 mesh ASTM; Fluka, Steinheim, Germany) for column chromatography (CC), and Sephadex LH 20 (Pharmacia, Stockholm, Sweden) were used. Thin-layer chromatography (TLC) was performed on silica gel GF254 precoated plates (Fluka). The chromatograms were visualized after spraying with *p*-anisaldehyde/sulfuric acid, ninhydrin, and Dragendorff's spray reagents (Wagner and Balt, 2001). ^{1}H and ^{13}C NMR spec-

tra were recorded in $CDCl_3$ with a Jeol JNA-LAA 400WB-FT (^1H, 400 MHz; ^{13}C, 100 MHz; Jeol, Tokyo, Japan) spectrometer with tetramethylsilane (TMS) as an internal standard. High-resolution ESI-MS was performed on a TQ-FT-ICR (liquid transfer quest-fourier transfer-ion cyclotron resonance) instrument. A Perkin Elmer (Santa Clara, CA, USA) Analyst 300 atomic absorption spectrometer was used for elemental analysis. Kits from Biomérieux (Craponne, France) were used for the determination of cholesterol, triglycerides, LDLc (low-density lipoprotein) and HDLc (high-density lipoprotein). Simvastatin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Bioactivity-guided extraction and isolation

The powdered crown gall (1 kg) was extracted by percolation with 95% ethanol (4 x 2 l) at room temperature, followed by evaporation of the solvent under reduced pressure to yield 57 g of dried ethanolic extract. Forty g of the ethanolic residue were chromatographed over a VLC column (silica gel H, 200 g, 7 cm x 20 cm). Elution was carried out using successively *n*-hexane, $CHCl_3$, EtOAc, and 50% (v/v) methanol in ethyl acetate. Fractions of 200 ml each were collected, subjected to TLC screening, and similar fractions were pooled to yield four major fractions (A–D) which were screened for anticancer and antihypercholesterolemic activities. The two biologically active fractions A and C were further purified on silica gel columns. Fraction A (eluted with 100% *n*-hexane) was purified over a silica gel 60 column using *n*-hexane and *n*-hexane/EtOAc (90:10, v/v). Seventy fractions each of 10 ml were collected and subjected to TLC on silica gel GF254 precoated plates, using *n*-hexane/EtOAc (80:20, v/v) as a solvent system. Fractions 18–32 were pooled to yield compound **1** (33 mg, yellowish oil) after evaporation of the solvent, while the pooled fractions 43–62 yielded compound **2** (243 mg, white amorphous powder). Fraction C (eluted with 100% ethyl acetate) was re-chromatographed over a silica gel 60 column using chloroform/methanol (96:4, v/v) as eluent. Thirty five fractions each of 10 ml were collected and subjected to TLC on silica gel GF254 precoated plates, using chloroform/methanol (90:10, v/v) as a solvent system. Fractions 20–30 were pooled to yield eventually compound **3** (110 mg, white microcrystalline powder).

Animals

Adult male Sprague-Dawley rats [130–150 g body weight (BW)] were obtained from the animal house of the National Research Centre, Giza, Egypt. They were kept under hygienic conditions and were fed the basal diet recommended by the American Institute of Nutrition (AIN, 1977), a semi-purified diet that contained (g/kg BW): casein (200), sucrose (555), cellulose (100), fat blends (100) (cholesterol and lard, 1:5), vitamin mix (35), and mineral mix (35), for one week before the experiment. Water was supplied *ad libitum*. The temperature was maintained at (22 ± 2) °C in a well ventilated room, with a relative humidity of 50–60%, and with a 12-h cycle of light and dark. All rats were individually housed in metal cages. Care was taken to avoid stressful conditions. After adaptation, the rats were divided into seven groups of six animals each on the basis of body weight. All experimental procedures were performed between 8–10 a.m. They were conducted in accordance with internationally accepted principles of laboratory animal use and care, and had been approved by the Ethics Committee of the National Research Centre (No. 9-031) in accordance with recommendations for the proper care and use of laboratory animals (NIH Publication No. 80-23; revised 1978).

Determination of LD_{50} values

One hundred and twenty adult male Sprague-Dawley rats were used to test the ethanolic extract of the crown gall as well as of fractions A–D. The animals of each group were divided into five subgroups ($n = 6$) which received increasing doses of 100, 250, 500, and 1000 mg/kg BW daily for a period of 3 weeks. The animals were observed during the first hour continuously, then every hour for 6 h, again after 12 and 24 h, and finally after every 24 h up to 3 weeks for any physical signs of toxicity such as writhing, gasping, palpitation, and decreased respiratory rate or mortality. The LD_{50} values of the ethanolic extract of the crown gall as well as of fractions A–D were calculated according to Karber (1931).

Assessment of cytotoxic activity

The cytotoxicity of the ethanolic extract, its four subfractions, and the isolated compounds was measured using the sulforhodamine B assay

(SRB) (Skehan *et al.*, 1990) against two human cancer cell lines: colon cancer cell line (HCT116) and breast cancer cell line (MCF7), in which the cells were placed in a 96-multi-well plate (10^4 cells/well) for 24 h before treatment with the test extracts to allow attachment of the cells to the wall of the plate. Different concentrations of each respective extract (0, 5, 12.5, 25, and 50 μg/ml) were added to the cell monolayer. Triplicate wells were incubated with the samples for 48 h at 37 °C in an atmosphere of 5% CO_2. The cells were then fixed, washed and stained with SRB stain. Excess stain was washed off with acetic acid and attached stain was recovered with Tris-EDTA buffer. The colour intensity was measured in an ELISA reader at 564 nm. The relation between surviving fraction and extract concentration was plotted to obtain the survival curve of each tumour cell line for the specified test compound. The curves were fitted using linear regression, and IC_{50} was calculated.

The assessment was performed in the National Cancer Institute (NCI) in Egypt. The IC_{50} (concentration which reduces the survival to 50%) and IC_{10} (concentration which reduces the survival to 10%) values for each test sample were calculated and compared to those of the reference drug doxorubicin®.

Assessment of antihyperlipidemic effect

The antihyperlipidemic effect of the ethanolic extract, its four subfractions, and compound **3** were evaluated by administration of an oral daily dose of 100 mg/kg BW of each of the test samples and compared to simvastatin as a standard drug (at the same dose level). Hyperlipidemia was induced according to the procedure of Neiy *et al.* (1988) and assessed by measuring serum cholesterol (Allain *et al.*, 1974), triglycerides (Bucolo and David, 1973), HDLc, and LDLc levels (Stein, 1986), using Biomérieux kits. The risk factor (LDLc/HDLc) was also calculated.

Statistical analysis

Results were expressed as mean ± standard error of the mean (S.E.M.) for six rats in each group. Data were analysed using one-way analysis of variance (ANOVA).

Results and Discussion

The ethanolic extract of the crown gall induced on an *Eucalyptus tereticornis* Sm. tree was tested for its anticancer and antihyperlipidemic activities, after it had proven to be safe for administration based on its high LD_{50} value (8.3 g/kg BW). The results (Tables I and II) revealed its potent anticancer action against MCF7 (IC_{50} 0.94 μg/ml) and HCT116 (IC_{50} 1.61 μg/ml) cells and also its significant antihyperlipidemic action, as it lowered the risk factor to 0.89 and caused a decrease of about 74% in the level of LDLc and an increase of about 80% in the level of HDLc after 8 weeks of administration of 100 mg/kg BW. Subsequently, these two effects served as the basis for the bioactivity-guided fractionation of the extract. Fractionation of the ethanolic extract yielded four major fractions, A–D, which were screened for the above mentioned activities. The two fractions A and C exhibited cytotoxic activ-

Table I. Cytotoxic effect of the ethanolic extract, fractions A–D, and the isolated compounds from the crown gall.

Tested sample	IC_{50}[a] [μg/ml]	
	MCF7[b]	HCT116[c]
Ethanolic extract	0.94 ± 0.10	1.61 ± 0.21
Faction A	0.60 ± 0.07	0.67 ± 0.22
Faction B	–	–
Faction C	0.67 ± 0.05	1.95 ± 0.25
Faction D	–	–
1	1.01 ± 0.50	3.49 ± 0.32
2	1.54 ± 0.49	3.83 ± 0.61
3	2.15 ± 0.41	3.39 ± 0.55
Doxorubicin®	0.7 ± 0.01	0.69 ± 0.01

[a] Concentration which reduces the survival to 50%, expressed as mean ± S.E.M.
[b] Breast cancer cell line.
[c] Colon cancer cell line.

Table II. Antihyperlipidemic effect of the ethanolic extract, fractions A–D, and compound **3** from the crown gall.

Treatment[a]		Antihyperlipidemic effect				
		Zero time	4 weeks	% of change	8 weeks	% of change
Ethanolic extract	Cholesterol	196.8 ± 7.2	148.2 ± 5.6*	24.69	96.3 ± 4.1*	51.06
	Triglycerides	151.7 ± 5.3	121.3 ± 4.9*	20.0	86.4 ± 3.5*	43.05
	HDLc	23.1 ± 0.8	31.2 ± 1.1*	35	41.6 ± 1.2*	80.10
	LDLc	143.4 ± 5.8	92.7 ± 3.7*	35.36	37.4 ± 1.1*	73.92
	Risk factor	6.2	2.97		0.89	
Fraction A	Cholesterol	205.1 ± 7.2	201.9 ± 2.3	1.56	198.0 ± 7.1	3.46
	Triglycerides	161.8 ± 5.0	160.9 ± 1.9	0.56	159.5 ± 6.2	1.73
	HDLc	20.7 ± 0.6	21.2 ± 0.4	2.42	21.9 ± 1.1	5.79
	LDLc	148.0 ± 1.2	146.9 ± 2.7	0.74	145.5 ± 0.8	1.69
	Risk factor	7.71	6.93		6.64	
Fraction B	Cholesterol	211.9 ± 5.1	208.3 ± 6.4	1.69	205.2 ± 8.1	3.16
	Triglycerides	171.6 ± 4.3	181.2 ± 5.1	1.39	167.5 ± 4.1	2.38
	HDLc	21.6 ± 0.9	22.1 ± 1.9	2.31	29.7 ± 2.6	37.5
	LDLc	166.7 ± 3.6	157.5 ± 5.2	5.52	156.3 ± 5.9	6.20
	Risk factor	7.71	7.12		5.26	
Fraction C	Cholesterol	191.6 ± 4.3	161.7 ± 5.6*	15.61	132.4 ± 3.4*	30.89
	Triglycerides	173.2 ± 4.1	126.8 ± 3.9*	26.79	104.6 ± 2.9*	39.61
	HDLc	18.7 ± 0.4	23.8 ± 0.6*	27.27	42.4 ± 0.8*	126.70
	LDLc	138.3 ± 5.4	112.5 ± 4.2*	18.66	69.1 ± 2.3*	50.0
	Risk factor	7.39	4.72		1.62	
3	Cholesterol	211.6 ± 5.2	156.2 ± 4.9*	26.18	113.8 ± 3.7*	46.22
	Triglycerides	159.4 ± 4.7	118.7 ± 3.6*	25.53	91.8 ± 2.3*	42.41
	HDLc	19.2 ± 0.8	28.6 ± 0.4*	48.95	45.3 ± 1.2*	135.9
	LDLc	160.5 ± 5.2	103.9 ± 3.7*	35.26	50.1 ± 1.6*	68.78
	Risk factor	8.35	3.6		1.10	
Fraction D	Cholesterol	216.9 ± 8.4	201.8 ± 9.5	6.96	243.6 ± 11.7	7.98
	Triglycerides	167.8 ± 6.2	164.2 ± 5.8	2.14	191.3 ± 6.1	9.80
	HDLc	19.6 ± 0.7	21.1 ± 0.9	7.65	18.3 ± 0.6	13.27
	LDLc	163.7 ± 4.6	153.9 ± 4.7	5.99	187 ± 5.2	10.14
	Risk factor	8.35	7.29		6.63	
Simvastatin	Cholesterol	208.3 ± 7.9	121.4 ± 5.2*	41.70	85.2 ± 3.1	59.10
	Triglycerides	161.3 ± 3.6	94.2 ± 2.4*	41.60	71.1 ± 2.9	55.92
	HDLc	21.3 ± 0.7	37.4 ± 1.1*	75.59	48.6 ± 1.3	128.17
	LDLc	154.7 ± 5.1	65.2 ± 2.3*	57.40	22.4 ± 0.6	85.52
	Risk factor	7.26	1.7		0.46	

[a] The extract, fractions, compound **3**, and simvastatin were given in a dose of 100 mg/kg BW.
* Statistically significant difference from zero time at $P < 0.01$.
– Percentage decrease. + Percentage increase.

ity against MCF7 (IC_{50} 0.60 and 0.67 μg/ml, respectively) and against HCT116 cells (IC_{50} 0.67 and 1.95 μg/ml, respectively) (Table I), but only fraction C significantly reduced the risk factor in the treated group to 1.62 and caused a 50% decrease in LDLc and an 127% increase in HDLc levels (Table II). Since the LD_{50} values of fractions A and C confirmed their safety up to 7.9 and 7.6 g/kg BW, respectively, these two fractions were selected for further purification. Repeated CC of fractions A and C resulted in the isolation of two new cytotoxic compounds, the amino acid derivative **1** (33 mg) and the lanostane triterpene **2** (243 mg) from fraction A, in addition to the known compound **3** (110 mg) from fraction C.

Compound **1** was isolated as yellowish oil; it responded positively to ninhydrin reagent which suggested its nitrogenous nature. The ESI mass spectrum of compound **1** in the positive mode gave a molecular ion peak $[M + H]^+$ at *m/z* 246.18, compatible with the molecular formula $C_{16}H_{23}NO$. The elemental analysis of compound **1** showed the presence of C (78.32%), H (9.45%), N (5.71%), and O (6.52%). The spectroscopic

analysis of compound **1** supported that it is a derivative of an aromatic amino acid. The ^{1}H NMR spectrum of compound **1** (Table III) showed the presence of two aromatic signals (m, each) at δ_H 7.70 and 7.52 ppm integrated to five protons which correspond to five aromatic protons present on a mono-substituted aromatic ring assigned to H-2", -3", -4", -5", and -6". The ^{13}C NMR spectroscopic data of **1** (Table III) showed 16 signals (two coincided at δ_C 128.7 and 130.8 ppm); six aromatic carbon atoms, one attached to the nitrogen atom, three methyl carbon atoms, one methine carbon atom, and one carbonyl carbon atom at δ_C 167.7 ppm indicating the presence of a carbonyl group involved in a cyclic amide linkage (Semak *et al.*, 2010). Direct correlations of protons to their respective carbon atoms were revealed in the HMQC spectrum. The structure of **1** was confirmed by the key HMBC [H-6/C-5, C-2, C-1', C-4, C-3; H-2", H-6"/C1", C-3", C-4", and C-5"; H-3", H-5"/C-2" and C-6"; H_3-2' and H_3-3'/C-1', C-6; H-3/C-2] correlations. On the basis of the above evidences, the structure of **1** was elucidated as 1-ethyl-6-(1'-methyl-1'-phenylethyl) piperidin-2-one (Fig. 1) which is a new natural product.

Compound **2** was isolated as a white amorphous powder. The ESI mass spectrum of compound **2** in the negative mode gave a molecular ion peak $[M-H]^+$ at *m/z* 481.38, compatible with the molecular formula $C_{32}H_{50}O_3$. The ^{1}H NMR spectrum (Table IV) displayed a total of eight methyl signals, *i.e.*, two secondary methyl groups at δ_H 0.92 ppm (d, J = 6.8 Hz) and six tertiary methyl groups at δ_H 0.68, 0.69, 0.78, 0.82, 0.86, and 1.43 ppm, an oxymethine group at δ_H 2.98 ppm (m), and two allylic protons at δ_H 4.71 and 4.63 ppm (each s). The ^{13}C NMR spectrum displayed 32 carbon signals, due to eight methyl carbon atoms, three methine carbon atoms (including one oxymethine group at δ_C 76.7 ppm), one quaternary carbon atom (a carbonyl at δ_C 176.5 ppm), and six olefinic carbon atoms at δ_C 106.7, 125.0, 133.3, 134.2 (two signals overlapped), and 156.0 ppm. The long-range ^{1}H-^{13}C NMR correlations indicated that **2** is a lanostane triterpenoidal derivative with the same A–D ring system as pisosterol (Gill *et al.*, 1989; Zamuner *et al.*, 2005). This was clear from the HMBC correlations between the protons of the allylic methyl group at δ_H 1.43 ppm (of C<u>H</u>$_3$-21)

Table III. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data of compound **1**.

Position	δ_H	δ_C
1	–	–
2	–	167.7
3	2.36, m	23.6
4	1.25, m	22.9
5	1.31, m	28.9
6	4.21, t (J = 2.4 Hz)	68.1
1'	–	38.7
2'	1.25	29.7
3'	1.25	29.7
1"	–	132.4
2"	7.70, m	128.7
3"	7.52, m	130.8
4"	7.70, m	128.7
5"	7.52, m	130.8
6"	7.70, m	128.4
N-<u>C</u>H$_2$CH$_3$	1.75, m	38.7
N-CH$_2$<u>C</u>H$_3$	0.87, m	14.0

Table IV. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data of compound **2**.

Position	δ_H	δ_C
1	1.38, m	35.2
2	1.56, m	26.4
3	2.98, m	76.7
4	–	38.5
5	0.94, m	50.0
6	1.95, m	21.5
7	1.87, m	27.5
8	–	134.2
9	–	134.2
10	–	36.5
11	1.48, m	18.9
12	1.92, m	28.1
13	–	46.5
14	–	48.9
15	1.32, m	28.3
16	1.65, m	33.2
17	–	133.3
18	0.69	15.6
19	0.82	17.4
20	–	125.0
21	1.43, s	18.9
22	1.62, m	26.4
23	1.84, m	33.2
24	–	156.0
25	1.86, m	24.0
26	0.92, d	22.0
27	0.92, d	22.0
28	4.71, 4.63 (each s)	106.7
29	0.68, s	17.4
30	0.86, s	29.9
31	0.78, s	25.9
<u>C</u>OOH	–	176.5

and the two olefinic carbon atoms C-20 and C-17 at δ_C 125.0 and 133.3 ppm, respectively, confirming the same skeleton as in pisosterol with an additional double bond between C-17 and C-20. The HMQC spectrum showed correlations between the signals at δ_H 4.71 and 4.63 ppm with the carbon signal at δ_C 106.7 ppm, indicating the presence of a double bond between C-24 and C-28. Additionally, the HMBC spectrum confirmed a correlation between the aforementioned protons ($C\underline{H}_2$-28) and two carbon signals at δ_C 33.2 and 24.0 ppm (C-23 and C-25, respectively). Also the proton signal at δ_H 1.86 ppm (H-25) correlated with δ_C 33.2 ppm (C-23). The HMBC spectrum showed a clear coupling between C-24 at δ_C 156.0 ppm and δ_H 1.84 ppm (H-23) and δ_H 1.86 ppm (H-25) which in turn correlated with C-26 and C-27 at δ_C 22.0 ppm (the two doublet methyl groups), respectively. The quaternary carbon atom at δ_C 176.5 ppm which correlated with δ_H 1.84 ppm (H-23) in the HMBC spectrum was assigned as a carbonyl group of a carboxylic acid group at C-22. Thus, compound **2** was identified as 3*β*-hydroxy-24-methyllanosta-8,17(20),24(28)-trien-22-oic acid (Fig. 1) which is a new natural product.

Compound **3** was identified as stigmasterol-3-*O*-glucoside (Fig. 1) by comparison of its spectroscopic data with reported values (Akisha *et al.*, 1988; Leitao *et al.*, 1992; Goad and Akihisa, 1997).

The three isolated compounds were consequently examined for their cytotoxic activity against the two tested human cancer cell lines, MCF7 and HCT116, while compound **3** only was tested for its antihyperlipidemic effect, as fraction C had proven to be the only active fraction with antihyperlipidemic activity. Compounds **1**, **2**, and **3** exerted significant cytotoxic activity against MCF7

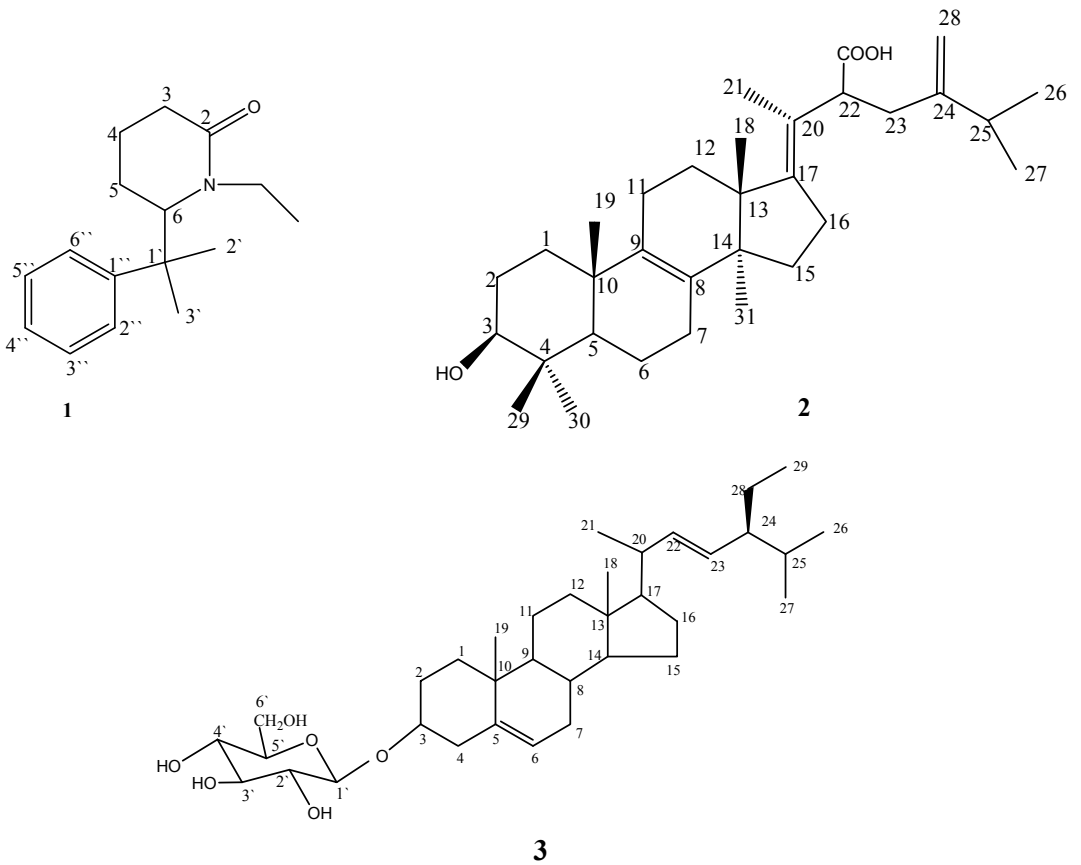

Fig. 1. Chemical structures of 1-ethyl-6-(1'-methyl-1'-phenylethyl) piperidin-2-one (**1**), 3*β*-hydroxy-24-methyllanosta-8,17(20),24(28)-trien-22-oic acid (**2**), and stigmasterol-3-*O*-glucoside (**3**) isolated from the crown gall.

cells with IC_{50} values of 1.01, 1.54, and 2.15 μg/ml, respectively, and 3.49, 3.83, and 3.39 μg/ml against HCT116 cells, respectively. Previous investigations reported cytotoxic activity of lanostane triterpenes too (Ukiya and Akihisa, 2002; Akihisa *et al*., 2007; Monténégro *et al*., 2008; Zhou *et al*., 2008; Arpha *et al*., 2012). As compound **2** was found to be cytotoxic against the human cancer cell lines studied here, our results provide further support for the cytotoxic activity of lanostane triterpenes. Ghosh *et al*. (2011) reported that stigmasterol decreased the tumour volume, packed cell volume, and viable cell count in Ehrlich ascites carcinoma (EAC)-bearing mice, and increased the mean survival time, thereby extending their life span. In our present study, stigmasterol-3-*O*-glucoside (**3**) exerted significant cytotoxic activity against the tested human cancer cell lines, which is in good agreement with previous studies.

Reduction of the blood cholesterol level by administration of drugs or by modification of the diet decreases the risk of coronary diseases (Daksha *et al*., 2010). Clinical trials revealed that a one percent reduction in total cholesterol causes a two percent reduction in heart attacks (NIH Reports, 1988). Compound **3** proved promising antihyperlipidemic activity, as it reduced the risk factor in the treated group to 1.10, as compared to the time before the onset of treatment, and caused an almost 69% decrease in LDLc and 136% increase in HDLc (Table II). Plant sterols have been reported to be effective in lowering plasma total cholesterol and LDL cholesterol by virtue of inhibiting the absorption of cholesterol from the small intestine (Sabir *et al.*, 2003). Phytosterols are cholesterol-like molecules absorbed only in trace amounts, but they inhibit the absorption of intestinal cholesterol including recirculating endogenous biliary cholesterol, and this is a key step in cholesterol elimination (Daksha *et al*., 2010).

Colorectal cancer (CRC) is one of the most common neoplasms of the digestive system and one of the most lethal causes of cancer death worldwide. The most studied risk factors causing CRC development are heredity, age, low-fiber and high-fat diet, alcohol, tobacco use, obesity, low physical activity, and environmental pollution. All of these factors could lead to hypercholesterolemia, which in turn increases the risk of CRC development (Herbey *et al.*, 2005). Hyperlipidemia is a relatively frequent complication in FAP [familial adenomatous polyposis that results from germline *adenomatous polyposis coli* (APC) gene mutations]; many affected patients die from CRC which arises from colorectal polyps. Moreover, there is a tendency for higher serum triglyceride (TG) levels in patients who developed CRC. This suggests the possible link of hyperlipidemia to CRC development. Consequently, improving a hyperlipidemic state might be of benefit for protection against neoplasia or other adverse outcomes (Mutoh *et al*., 2006). Administration of statins was associated with a relative reduction in the risk of CRC (Poynter *et al*., 2005; Broughton *et al*., 2012). In the present study, compound **3** reduced the risk factor, decreased the LDLc and increased the HDLc level (Table II). On the other hand, it also exhibited a significant cytotoxic activity against human colon cancer cells (HCT116) with an IC_{50} value of (3.39 ± 0.55) μg/ml. From these results we can presume a possible correlation between the reduction in hypercholesterolemia and the improvement in the incidence of CRC.

Conclusion

The ethanolic extract, fraction C, and stigmasterol-3-*O*-glucoside (**3**) of the crown gall tissues obtained from the tumour induced on an *Eucalyptus tereticornis* tree reduced the risk factors and thus prevented hypercholesterolemia. The two new compounds **1** and **2** showed a potent activity against both breast and colon cancer cell lines. Administration of **3** was associated with a lower incidence of CRC as well as a lower blood cholesterol level. Stigmasterol-3-*O*-glucoside may have a protective effect against the development of CRC, however, further studies examining the effects of stigmasterol-3-*O*-glucoside are required.

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