Novel Antisickling, Antioxidant and Cytotoxic Prenylated Flavonoids from the Bark of *Morus alba* L

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Abstract: Two novel prenylated flavonoids and quercetrin were isolated from extract of the bark of *Morus alba* L. The structures of these compounds were established based on physicochemical data, UV spectral data, 1H-NMR, 13CNMR, 1H-1H COSY, HMBC and EIMS. Extract and isolated compounds exhibited a significant antisickling activity, a powerful antioxidant activity and remarkable cytotoxic activity. Both antioxidant & cytotoxic activity were supported & evidenced by docking structure of isolated compounds in the receptor binding site and estimation of binding affinity into 17beta-hydroysteroid dehydrogenase type 1 (17beta-HSD1) (3HB5) and glutathione reductase (1XAN) as a standard docked model.

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1. Introduction

The White Mulberry (*Morus alba*) is a short-lived, fast-growing, small to medium sized tree to 10–20 m tall, native to northern China known as Tuta in Sanskrit and Tuti in Marathi, and widely cultivated elsewhere. It is also known as Tuta in Egypt (Bailey, 1953; Leung & Foster, 1977 & Taylor *et al.*, 2006). Bark is characterized by light tannish brown colour while stems are yellowish brown with sweet taste. White substance appears when stem is broken in warm conditions (Bailey, 1953). Trees are extensively grown (e.g. southern Europe, India) for their leaves as food for silkworms. Fruits may be eaten raw or cooked. Fruits are an ingredient of a particularly seductive drink known as Mulberry Wine. Stem bark is fibrous and used in China and Europe for paper making. Twigs are used as binding material and for making baskets. Trees often used as ornamentals, roadsides or boundary markers (C.S.I.R, 1948 & Reed, 1976)

In Traditional Chinese Medicine, the fruit is used to treat prematurely grey hair, to "tonify" the blood, and treat constipation and diabetes. The bark is used to treat cough, wheezing, edema, and to promote urination. It is also used to treat fever, headache, red dry and sore eyes, as well as cough (Taylor *et al.*, 2006).

Medicinally, fruits are laxative, refrigerant in fevers, and used locally as remedy for sore throat, dyspepsia. Roots and bark are purgative, anthelmintic, and astringent; leaves considered disphoretic and emollient; a decoction of leaves being used as a gargle for inflammation of throat (Reed, 1976).

Drug design is an important tool in the field of drug discovery where new compounds are synthesized by molecular or chemical manipulation of the lead moiety in order to produce highly active compounds with minimum side effect (Cavasotto & Abagyan, 2004). Search for new ligands and the assessment, improvement and extension of the lead is a very important step in identification of new chemical entities (Borges *et al.* , 2002). Nowadays, the use of computers to predict the binding of libraries of small molecules to known target structures is an increasingly important component in the drug discovery process (Schoichet, 2004 & Koppen, 2009). Docking of small molecules in the receptor- binding site and estimation of binding affinity of the complex is a vital part of structure based drug design (Seeliger & Groot, 2010).

Natural Drugs are usually discovered by means of high-throughput screening approaches that use *in vitro* experiments to evaluate the activity of a large number of compounds against a known target. This procedure is very costly, time-consuming and consume large amount of natural isolated compounds. If the crystallographic structure of the protein target is available, then molecular docking simulations can be a helpful computational approach in the drug-discovery process. This computer simulation process allows for faster and cheaper identification of promising drug candidates using structure-based virtual screening (Bellows & Floudas, 2010; De Azevedo, 2010; De Azevedo, 2010 & Kim *et al.*, 2010; Hernandes *et al.*,2010; Krystof & Uldrijan, 2010; Mitrasinovic, 2010 & Rizzolio *et al.* , 2010).

Subsequently, *in vitro* tests can be performed to further evaluate the drug candidates found by the virtual screening process (Thomsen & Christensen, 2006).

Reviewing the available current literature, different organs of *Morus alba* species have been

investigated previously (Deshpande, 1968; Anca Maier *et al.*,1997; Shin-Ichi & Setsuko, 1997; Jiang *et al.*,2003; Park *et al.*,2003; Sohn *et al.*,2004; Chen *et al.*,2005; Singab *et al.*,2005; Chen *et al.*,2006; El-Beshbishy *et al.*,2006; Katsube *et al.*,2006; Ercisli & Orhan, 2007; Kaushik *et al.*, 2008; & Zheng *et al*, 2008).

Few works were done on root bark of some Morus species (Jiang *et al.*,2003; Park *et al.*,2003 &Singab *et al.*,2005;). Little was reported concerning the study of flavonoids of *Morus alba* root bark in the available literature (Jiang *et al.*,2003).

Preliminary phytochemical screening of bark of the plant revealed the presence of different phenolics. Therefore, it was deemed of interest to isolate and identify these constituents; as well as to evaluate certain biological activities of the plant with evidence based approach by docking structure of isolated compounds in the receptor binding site and estimation of binding affinity.

We found two novels prenylated falvonoids 1-2, as well as known compound 3. In addition some biological activities of these compounds were carried out & these activities were proved by docking module. Now we report our results of this study in detail

2. Material and Methods

2.1. Plant material:

Plant material was collected from bark of *Morus alba* L. was collected from plants cultivated in the private farm, Zagazig, EL-Sharkya, Egypt. The identity of the plant was kindly confirmed by Prof. Dr. Mohamed Nabil Attia, Head of Cultivated Plants Department, Faculty of Agriculture, Zagazig University. The plant was air-dried, reduced to fine powder and kept in tightly closed amber coloured glass containers. Voucher specimens are kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

2.2. Chemicals:

2.2.1. Reference samples:

Flavonoids from Sigma (USA) and Aldrich (Germany)

2.2.2. Material for chromatography:

Silica gel G (60 mesh) for TLC, silica gel (70-230 mesh) for CC, precoated TLC plates (silica gel 60 GF254) from E. Merck (Darmstadt, Germany), sephadex LH-20 from Pharmacia (Uppsala, Sweden)

2.2.3. Solvent systems:

S1: Hexane-ethyl acetate-methanol (in different ratios v/v)

S2: Chloroform - Methanol (in different ratios v/v).

Spray reagents; Sulphuric acid (50%) and P-anisaldehyde were used.

2. 3. Material for biological evaluation:

2.3.1. Plant extracts:

The biological evaluation was performed on the three isolated compounds in addition to the Methanol (95 %) extract of the air-dried powdered bark. Bark of *Morus alba* L. were air-dried, milled and extracted with methanol for 24 hrs by maceration. The MeOH extract was evaporated in rota-vapor to yield a semisolid residue (650 g). The solvent-free residue was kept for investigation

2.3.2. Animals:

Male albino rats of Sprague Dawely Strain (120-150g) were used. Animals were obtained from the animal house, of the National Research Center, Dokki, Giza, Egypt. The animals were fed on a standard laboratory diet composed of vitamin mix (1 %), mineral mix (4 %), corn oil (10 %), sucrose (20 %), cellulose (0.2 %), casein (10.5 %) and starch (54.3 %)

2.3.3. Tumor cell lines:

Tumor cell lines (cervix, HELA), (liver, HEPG2) and (breast, MCF7) from National Cancer Institute of Egypt were used for cytotoxic screening

2.3.4. Reference drugs and kits:

Glutathione Kit (Wak, company – Germany), and Vitamin E (Pharco Pharmaceutical Co, Egypt) in addition to Testosterone propionate (4µg/ml TC199) were used in the pharmacological screening

2. 4. Apparatus:

UV-visible spectrophotometer, Shimadzu UV 240 (P/N 204-58000); Mass spectrometer: Varian 90 NMR spectrophotometer; NMR Jeol GLM, Jeol TMS Route instrument (^{1}H-NMR, 300 MHZ, ^{13}C, 75 MHZ, Japan); Koffler's heating stage microscope

2.5. Software for doking:

ChemDraw 3D structures were constructed using Chem 3D ultra 8.0 software (Molecular Modeling and Analysis; Cambbridge Soft Corporation, USA (2010)), and then they were energetically minimized by using OPAC (semi-empirical quantum mechanics), Jop Type with 100 iterations and minimum RS gradient of 0.01, and saved as DL MolFile (mol**)**

There is a wide range of software packages available for the conduct of molecular docking simulations like, **Molsoft** (Murcko & Stouten, 1997; Anderson & Weng, 1999; Halperin, 2002 & Wang *et al.*, 2003;) as flexible docking program enable us to predict favorable protein-ligand complex structures with reasonable accuracy and enhanced docking speed.

All docking studies were performed using "Internal Coordinate Mechanics (Molsoft IC 3.4-8C)". ICM docking is probably the most accurate predictive tool of binding geometry today (Murcko & Stouten, 1997; Anderson & Weng, 1999; Halperin, 2002 & Wang *et al.*, 2003).

2.6. Phytochemical study:

2.6.1. Investigation of flavonoidal content:

2.6.1.1. Extraction, isolation and Identification:

Bark of *Morus alba* L. were air-dried, milled and extracted with methanol for 24 hrs by maceration. The mark left was repeatedly extracted five times similarly, for complete extraction. The MeOH extract was evaporated in rota-vapor to yield a semisolid residue (650 g)

The methanolic residue (650g) was successively fractionated over silica gel VLC (silica for TLC packed on column; 10 cm i.d. × 20 cm) with *n*-hexane, ethyl acetate, methanol. The solvents were removed to give fractions of *n*-hexane (3.2 g), ethylacetate (2.6 g) and methanol (9.6 g).

The ethylacetate fraction (2.6 g) (was subjected to a silica gel column chromatography (SiO2 column; 20 mm i.d. × 25 cm) using solvent system of CHCl3-MeOH (9:1). The active fraction of CHCl3:MeOH (9:1) eluate (115 mg) was further separated & purified by Sephadex LH-20 column chromatography (30 mm i.d. × 50 cm, MeOH) to give compound **1**(23 mg) & compound **2**(21 mg).

The methanolic fraction of VLC (9.6 g) was column chromatographed over silica gel (SiO2 column; 40 mm i.d. × 20 cm) using chloroform and methanol, step gradient as eluents to yield compound **3** (15 mg) from fraction eluted by CHCl3-MeOH (8:2).

The structure elucidation of compounds (**1**-**3**) was established based on physico-chemical data, UV spectral data, ^{1}H-NMR, ^{13}C-NMR and EIMS

2.7. Biological study:

2.7.1. Antioxidant activity:

The antioxidant activity of the tested samples (Methanolic extract and isolated compounds 10 mg/kg) was assessed by measuring the glutathione level in blood samples collected from alloxan-induced diabetic rats as compared to Vitamin E (12 mg / kg b.wt., positive control) and adopting the procedure described by Beutler (Beutler *et al*., 1963). The restoration of blood glutathione levels (reduced due to induction of diabetes) was taken as a measure of antioxidant activity. The percentage change observed after dose administration was, in each case, calculated according to the following equation: % of change=$(G_c - G_t)\times 100/G_c$ The results are recorded in Table (1) and illustrated in Figures (2)

2.7.2. Antisickling activity:

Sickle cell anaemia (SCA) is a hereditary anaemia that results from an abnormal β-haemoglobin molecule (valine is substituted for glutamic acid), forming haemoglobin AS instead of normal one (haemoglobin AA). Sickle cell crisis occurs when an individual homozygous for the sickle cell gene is exposed to an adverse state (hypoxia, dehydration), causing the cell to take on a sickle shape, with subsequent occlusion of small vessels (Pousada *et al*., 1996).

Antisickling activity was determined as described (Sofowara & Isaac, 1971)**.** Tested samples were redissolved in tissue culture medium no. 199 (T.C. 199). The final suspensions were filtered, the volume made up to 180 ml with T.C. 199 and the resulting solutions autoclaved. Tested solutions were similar, in pH, to T.C. 199. Testosterone propionate was diluted with T.C. 199 to give tested sample concentrations a 4 µg/ml suspension which was used for comparison.

Blood sampling and preparation for the test samples of haemoglobin AA and haemoglobin AS blood were obtained by veinpuncture from males aged 25 and 36, respectively. Both donors were in good health and the blood was collected in acidic citrate-dextrose-saline. The blood was added either to a well, formed by sealing a drilled sterile microscope slide (diameter of hole =3 mm) with cover slips, or to a sterile Bijou bottle. To haemoglobin AA blood, an equal volume of tested samples or testosterone propionate suspension (4µg/mL) was added. To the haemoglobin AS sample, an equal volume of sterile 2% sodium metabisulphite was first added until sickling was observed in 95% of the red cells (usually after one hr incubation at 37°C) and the same volume of tested samples or testosterone propionate suspension was then added.

A control containing a volume of T.C. 199 in place of the tested samples. All solutions were sterilized before use and aseptic techniques were used throughout the experiments. The drilled slides were observed directly under the microscope, while samples from the Bijou bottles were put on slides and sealed over with cover slips just before examination. Observations consisted of estimates of the effect of control (TC199) and tests (methanolic extract and isolated compounds.) on haemoglobin AA cells which crenated and on haemoglobin AS which sickled after incubation at 37oC for 18 hrs. At this stage, maximum effect occurred and could be compared with the effect of testosterone propionate (concentration 4µg /mL) as standard. The results are recorded in Table (2) and illustrated in Figure (3)

2.7.3. Evaluation of Potential Cytotoxicity by SRB Assay:

Potential cytotoxicity of the tested samples (Compounds 1, 2 and 3) was tested at the National Cancer Institute of Egypt adopting the method of Skehan (Skehan & Strong, 1990). Cells were plated in a 96-wells plate (10^4cells/well) for 24hrs before treatment with the tested sample to allow attachment of the cells to the wall of the plate. Different concentrations of each of the tested samples under study (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and were incubated for 48hrs at 37°C in an atmosphere of 5% CO_2. After 48hrs, cells were fixed, washed and stained with Sulphorodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer. Colour intensity was measured in an ELISA reader. The survival curves of each of the tumor

cell lines (cervices, liver and breast) were plotted and IC50 was calculated for each of the tested samples table (3)

2.8. Docking of Isolated compounds in the receptor-binding site:

2.8.1. Generation of Ligand and Enzyme Structures:

The crystal structures of target protein compounds1, 2, 3 into 17beta-hydroysteroid dehydrogenase type 1 (17beta-HSD1) (3HB5) and glutathione reductase (1XAN) active site were retrieved from the Protein Data Bank (http//www.rcsb.org/pdb/welcome.do). All bound waters, ligands and cofactors were removed from the protein. The amino acids of the binding site where defined using data in pdbsum (http//www.ebi.ac.uk/thoronton-srv/databases /pdbsum.)

2.8.2. Docking using Molsoft IC 3.4-8C program:

2.8.2.1. Convert our PDB file into an IC object.

This conversion involves addition of hydrogen bonds, assignment of atoms types, and charges from the residue templates.

2.8.2.2. To perform IC small molecule docking

a) Setup Docking Project:
-Set Project Name
-Setup the Receptor
-Review and Adjust Binding Site
-Make Receptor Maps
b) Start docking simulation

2.8.2.3. Display the result:

IC stochastic global optimization algorithm attempts to find the global minimum of the energy function that include five grid potentials describing interaction of the flexible ligand with the receptor and internal conformational energy of the ligand, during this process a stack of alternative low energy conformations is saved (Tables 4& 5). The mode of interaction of compounds 1, 2, 3 into 17beta-hydroysteroid dehydrogenase type 1 (17beta-HSD1) (3HB5) and glutathione reductase (1XAN) was used as a standard docked model. All inhibitors were compared according to the best binding free energy (minimum) obtained among all the run.

3. Results

The structure elucidation of compounds (**1-3**) Figure 1, was established based on physico-chemical data, UV spectral data, ^{1}H-NMR, ^{13}C-NMR and EIMS.

Compound 1: 23 mg, yellow crystals

MP: 235-240 ºC.

Rf : 0.53 ($CHCl_3$-MeOH, 9:1).

UV/Vis λ_{max} (MeOH) nm (log ε): 228 sh, 293,339 + NaoMe, 248, 285, 333; + AlCl3, 221, 316, 392; + NaoAc, 287, 297,334 + H3BO3).

^{1}H NMR (300 MHz, DMSO-d6): 9.08 (1H, s, OH-3'), 8.86 (1 H, s, OH-4'), 6.73 (1 H, d, J= 1.5 Hz, H-2'), 6.70 (1 H, d, J= 1.6 Hz, H-6'), 5.88 (1 H, d, J=2.4 Hz, H-8), 5.70 (1 H, d, J=2.4 Hz, H-6), 5.2 (1H, m, H-2"), 5.1 (1H, m, H-2'"), 3.60 (1 H, dd, J= 7.1 Hz, J= 9.2 Hz H-βH-3), 2.8 (1 H, dd, J= 7.1 Hz, J= 9.2 Hz H-αH-3), 2.65(2H, d, J= 4.7 Hz, H-1"), 2.60(2H, d, J= 4.7 Hz, H-1'"), 1.25(6H, s, H-5", H-5'"), 1.06(6H, s, H-4", H-4'")

^{13}C NMR (75 MHz DMSO-d6): 197 (C-4), 157 (C-7), 156 (C-9), 145.6 (C-3$^{\backslash}$), 145.5 (C-4$^{\backslash}$),140.1 (C-3'"), 138.2 (C-3"), 131 (C-1$^{\backslash}$),126.5 (C-2"), 126.5 (C-2'"), 124.5 (C-5), 119 (C-6$^{\backslash}$),115.5 (C-5$^{\backslash}$),115 (C-2$^{\backslash}$),100 (C-10), 96 (C-6), 94.5 (C-8), 82 (C-2), 43 (C-3), 30.5 (C-1'"), 29.5 (C-1'"), 26.7 (C-5"), 26.7 (C-5'"), 13.1 (C-4"), 13.1 (C-4'").

MS (EI, 70 eV): m/z (%) = 408 (M^{+}, 88%), 324 (45%),.

Compound 2: 21 mg, yellowish white crystals

MP: 246-248 ºC.

Rf : 0.82 ($CHCl_3$-MeOH, 9:1).

UV/Vis λ_{max} (MeOH) nm (log ε): 229 sh, 298, 333 + NaoMe, 248, 276, 333; + AlCl3, 220, 392; + NaoAc, 284, 295,336 + H3BO3).

^{1}H NMR (300 MHz, DMSO-d6): 9.08 (1H, s, OH-3'), 8.86 (1 H, s, OH-4'), 6.73 (1 H, d, J= 1.5 Hz, H-2'), 6.70 (1 H, dd, J= 8 Hz, J= 1.6 Hz, H-6'), 6.62 (1 H, d, J=9.1 Hz, H-5'), 5.88 (1 H, d, J=2.4 Hz, H-8), 5.70 (1 H, d, J=2.4 Hz, H-6), 5.88 (1 H, d, J=2.4 Hz, H-8), 5.70 (1 H, d, J=2.4 Hz, H-6), 5.2 (1H, m, H-2"), 4.8(1H, d, J=7.5,H-2), 4.41(1H, m, H-3), 2.65(2H, d, J= 4.7 Hz, H-1"), 1.25(3H, s, H-5"), 1.06(3H, s, H-4")

^{13}C NMR (75 MHz DMSO-d6): 196.5 (C-4), 157.2 (C-7), 155.8 (C-9), 145.8 (C-3$^{\backslash}$), 145.7 (C-4$^{\backslash}$), 138.4 (C-3"), 131.3 (C-1$^{\backslash}$),126.5 (C-2"), 124.5 (C-5). 118.8 (C-6$^{\backslash}$), 115.6 (C-5$^{\backslash}$),115.1 (C-2$^{\backslash}$),99.7 (C-10), 95.8 (C-6), 94.6 (C-8), 81.7 (C-2), 67.1 (C-3), 29.5 (C-1"), 26.4 (C-5"), 13.4 (C-4").

MS (EI, 70 eV): m/z (%) = 356 (M^{+}, 10%), 326 (9%).

Compound 3:15 mg, yellow powder

MP: 181-183 ºC.

Rf : 0.95 ($CHCl_3$-MeOH, 9:1).

UV/Vis λ_{max} (MeOH) nm (log ε): UV/Vis λ_{max} (MeOH) nm (log ε): 259, 299sh, 358; + NaoMe, 272, 320sh, 415; + AlCl3 271, 300sh, 328sh, 430; + NaoAc, 275, 324sh, 395; + H3BO3 260, 324sh, 377

^{1}H NMR (300 MHz, DMSO-d6): Aglycone: 12.5-(S, OH), 7.4-(2H, d, J=8Hz, H-2',6'), 6.85-(1H, d, J=7.5Hz, H-5'), 6.4-(1H, d, J= 2.5Hz, H-8), 6.23-(1H, d, J=2.5Hz,H-6)

Sugar: 5.3-(1H, d, J=2.5Hz, H-1"), 3.1-3.7-(m, other protons of sugar), 1.1-(3H, d, J =6.5Hz, Me)

MS (EI, 70 eV): m/z (%) = 448 (M^{+}, 18%), 302((38%), 146 (18%).

The different biological activities evaluated (antioxidant, antisickling and cytotoxic) for the aforementioned extract and isolated compounds, represented in (Tables 1-3), revealed variable although significant efficacy & potency for all the samples when compared to standard and suggest their incorporation in herbal formulations after necessary clinical trials.

Concerning docking study results; It was reported that (Mazumdar *et al.*, 2009) Oestradiol is a well-characterized sex hormone that stimulates breast cancer and other oestrogen-related diseases. 17beta-hydroysteroid dehydrogenase type 1 (17beta-HSD1) catalyses the last step in the synthesis of oestradiol and androstenediol in breast tumour tissue. The enzyme's high expression and activity after simultaneous blockade of oestrogen receptors and inhibition of aromatase in the tumour shows the necessity for its inhibition as a requirement for breast cancer therapy.

On the other hand Glutathione reductase is an important glutathione reductases housekeeping enzyme for redox homeostasis both in human cells. It showed that inhibitor to bind noncovalently with some amino acid residues explains the reduction at the flavin of glutathione reductase (Savvides & Karplus, 1996).

In the present study, we have screened 3 flavonoides (Figure 1) for cytotoxic effect in (breast & cervix cancer) cell lines and the antioxidant activities. So, we docked the isolated compounds 1, 2 & 3 from plant under study into high affinity crystal Structure of 17beta-hydroysteroid dehydrogenase type 1 (17beta-HSD1) (3HB5) (Mazumdar *et al.*, 2009) in complex with a natural inhibitor E2B {3-(3',17'beta-dihydroyestra-1',3',5'(10')-trien-16'beta-methyl) benzamide} and into the crystal Structure of glutathione reductase (1XAN) (Savvides & Karplus, 1996) active sites in order to predict their binding modes, their binding affinities and orientation of these compounds at the active site of the enzymes. The ICM score values and hydrogen bonds formed with the surrounding amino acids obtained by docking our titled compounds shows good agreement and predicted binding affinities obtained by molecular docking studies on (3HB5) as verified by antiproliferative (Table 4, Fig. 4), and on 1XAN as verified by antioxidant screening (Table 5, Fig. 5).

Table 1. Antioxidant effect of methanol extract and isolated compounds obtained from the bark of *Morus alba* L. grown in Egypt.

Tested Samples	Blood glutathione (mg%)	
	Mean ± S.E	Potency[2]
Control (Saline)	35.2±0.5	-
Diabetic	23.9±0.1*	-
Methanol extract	32.2±0.4	92.2
Compound 1	34.3±0.9	98.1
Compound 2	31.1±1.3	89.1
Compound 3	33.9±1.1·	97.1
Diabetic + Vit E.	34.9±0.5	100

1: Blood glutathion level expressed in mg % as mean ± S.E. 2: Percentage of Potency as compared to standard.
* Significantly different from the control group at $p < 0.01$

Table 2. Antisickling effect of methanol extract and isolated compounds obtained from the bark of *Morus alba* L. grown in Egypt.

Tested Samples	Effect on RBCs	
	% of crenated cells after 18	% of sickled cells after 18 hrs
Control (T.C. 199)	74%	91%
Methanol extract	45%	65%
Compound 1	49%	59%
Compound 2	41%	52%
Compound 3	43%	54%
Testosterone propionate (4µg/ml TC199)	40%	51%

Table 3. Cytotoxic activity of compounds 1 & 2 isolated from the bark of *Morus alba* L. grown in Egypt.

Cell Line	IC_{50} (υg/ml)		
	Compound 1	Compound 2	Compound 3
Cervix (MCF7)	0.71	0.92	0.89
Breast (HELA)	0.56	1.41	0.97
Liver (HEPG2)	0.52	0.45	0.68

Table 4. ICM Scores of inhibitor ligand E2B, NADP, the compounds, and hydrogen bonds formed with amino acid residues and their lengths.

Tested Samples	ICM score	No. of hydroge bonds	Involved group of ami acid	Atom of lig involved	Length of hydrogen bo (A)
Ligand	-133.03	7	S142 hg -- m	o3	2.56 A
			V188 hn -- m	n1	2.78 A
			C185 o -- m	h04	2.26 A
			V188 o -- m	h11	2.52 A
			V188 o -- m	h12	1.47 A
			T190 og1 -- m	h02	1.88 A
			E282 oe1 -- m	h03	2.11 A
NADP	-303.59	41	S11 hn -- m	o9	2.58 A
			S11 hn -- m	o10	1.85 A
			S12 hn -- m	o9	1.29 A
			I14 hn -- m	o1	2.24 A
			G15 hn -- m	o1	1.56 A
			R37 hn -- m	o11	1.68 A
			R37 he -- m	o13	2.02 A
			R37 he -- m	n6	2.65 A
			R37 hh21 -- m	o13	1.61 A
			R37 hh21 -- m	o12	2.71 A
			R37 hh22 --m	o13	1.02 A
			T41 hg1 -- m	o12	2.59 A
			R67 he -- m	o14	2.38 A
			R67 hh22 -- m	o14	2.32 A
			N90 hn -- m	n3	2.54 A
			N90 hd22 -- m	o5	1.94 A
			A91 hn -- m	n3	1.87 A
			K159 hz1 -- m	o16	2.23 A
			K159 hz1 -- m	o15	2.79 A
			K159 hz2 -- m	o16	2.69 A
			K159 hz2 -- m	o15	1.81 A
			T8 og1 -- m	h06	2.72 A
			G9 o -- m	h09	1.92 A
			G9 o -- m	h10	1.21 A
			G9 o -- m	h13	2.43 A
			S11 og -- m	h14	2.04 A
			S12 og -- m	h11	2.73 A
			S12 o -- m	h11	1.89 A
			S12 o -- m	h01	1.53 A
			S12 o -- m	h13	1.75 A
			G13 o -- m	h01	2.74 A
			D65 od1 -- m	h16	1.65 A
			C89 o -- m	h07	1.39 A
			N90 od1 -- m	h10	2.15 A
			N90 o -- m	h32	1.43 A
			A91 o -- m	h06	2.61 A
			A91 o -- m	h32	2.45 A
			A91 o -- m	h05	1.25 A
			T140 o -- m	h08	2.34 A
			G141 o -- m	h19	2.79 A
			Y155 oh -- m	h20	1.56 A
Comp.1	-111.44	9	S11 hn -- m	o4	1.91 A
			S11 hn -- m	o3	2.5 A
			S12 hn -- m	o4	1.44 A

			I14 hn -- m	o2	2.36 A
			K159 hz1 -- m	o5	1.63 A
			K159 hz2 -- m	o5	2.45 A
			S11 og -- m	h10	1.99 A
			S12 o -- m	h11	2.09 A
			Y155 oh -- m	h12	2.22 A
Comp. 2	-91.71	9	I14 hn -- m	o5	1.99 A
			G15 hn -- m	o5	2.27 A
			T118 hg1 -- m	o3	2.48 A
			K159 hz1 -- m	o6	1.67 A
			K159 hz2 -- m	o6	1.58 A
			K159 hz3 -- m	o4	1.97 A
			N114 o -- m	h9	2.07 A
			T118 og1 -- m	h8	2.49 A
			Y155 oh -- m	h18	2.54 A
Comp. 3	-99.36	14	I14 hn --m	o8	2.51 A
			N90 hd21 –m	o11	1.56 A
			N90 hd22 --m	o11	1.23 A
			T140 hg1 --m	o11	2.26 A
			S142 hg --m	o6	1.79 A
			G9 o -- m	h19	1.57 A
			N90 od1 --m	h18	2.58 A
			N90 od1 --m	h20	2.72 A
			N90 o --m	h18	1.45 A
			N90 o --m	h20	1.74 A
			A91 o --m	h7	1.38 A
			T140 o --m	h8	1.31 A
			C185 o --m	h9	2.36 A
			G186 o --m	h9	2.70 A

Table 5. ICM Scores of FAD, the compounds, and hydrogen bonds formed with amino acid residues and their lengths.

Tested Samples	IC scores	No. of hydrogen bo	Involved group of amin acid	Atom of liga involved	Length of hydro bond (A)
FAD	-281.49	31	G29 hn -- m	o5	2.42 A
			G31 hn -- m	n5	1.93 A
			T57 hn -- m	o6	2.37 A
			T57 hg1 -- m	o3	2.12 A
			T57 hg1 -- m	o6	2.46 A
			T57 hg1 -- m	o7	2.57 A
			K66 hz3 -- m	n9	2.37 A
			G158 hn -- m	o1	2.33 A
			G158 hn -- m	o3	2.29 A
			S177 hg -- m	o10	1.76 A
			R291 hh11 -- m	o12	2.60 A
			R291 hh12 -- m	o9	1.84 A
			R291 hh12 -- m	o10	2.44 A
			R291 hh21 -- m	o7	2.02 A
			R291 hh21 -- m	o9	2.22 A
			N294 hd21 -- m	o1	2.18 A
			N294 hd21 -- m	o2	2.72 A
			D331 hn -- m	n3	2.75 A
			D331 hn -- m	n4	1.50 A
			E50 oe1 -- m	h41	2.56 A
			T57 og1 -- m	h22	1.77 A
			V61 o -- m	h15	1.56 A
			V61 o -- m	h18	2.36 A
			G62 o -- m	h07	1.54 A

			D331 od1 -- m	h51	1.74 A
			G158 o -- m	h11	1.31 A
			G158 o -- m	h01	2.28 A
			S177 og -- m	h16	1.40 A
			D178 od1 -- m	h14	1.90 A
			D178 od2 -- m	h13	2.30 A
			D331 od1 -- m	h07	2.28 A
Comp. 1	-112.05	6	T57 hn -- m	o2	1.96 A
			Y197 hh -- m	o3	2.13 A
			D331 hn -- m	o5	2.13 A
			T339 hn -- m	o4	2.41 A
			V329 o -- m	h12	2.08 A
			L337 o -- m	h11	1.53 A
Comp. 2	-92.09	8	T57 hn -- m	o6	2.68 A
			C58 hn -- m	o2	2.22 A
			G157 hn -- m	o4	2.46 A
			V332 hn -- m	o4	2.23 A
			L338 hn -- m	o5	1.76 A
			A155 o -- m	h8	1.46 A
			V329 o -- m	h9	1.67 A
			L337 o -- m	h10	1.39 A
Comp. 3	-101.46	18	G29 hn -- m	o11	2.57 A
			T57 hn -- m	o2	2.46 A
			T57 hn -- m	o8	1.57 A
			T57 hg1 -- m	o2	2.54 A
			T57 hg1 -- m	o8	2.31 A
			T156 hn -- m	o5	2.19 A
			T156 hn -- m	o4	1.91 A
			L338 o -- m	o5	1.22 A
			G157 hn -- m	o4	2.56 A
			D331 hn -- m	o1	2.07 A
			L338 hn -- m	o6	2.51 A
			T339 hn -- m	o6	2.58 A
			E50 oe1 -- m	h19	2.72 A
			I154 o -- m	h7	2.10 A
			A155 o -- m	h18	1.72 A
			T156 o -- m	h8	1.50 A
			V329 o -- m	h7	2.55 A
			G157 hn -- m	h9	1.79 A

Figure 1. Isolated compounds from methanol extract of *Morus alba* L. bark.

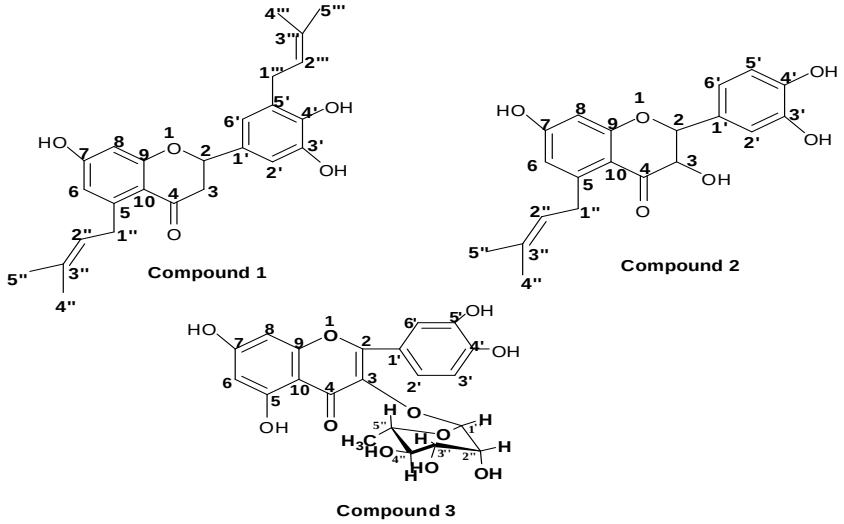


Figure 2. % potancy of methanol extract and isolated compounds obtained from the bark of *Morus alba* L. grown in Egypt.

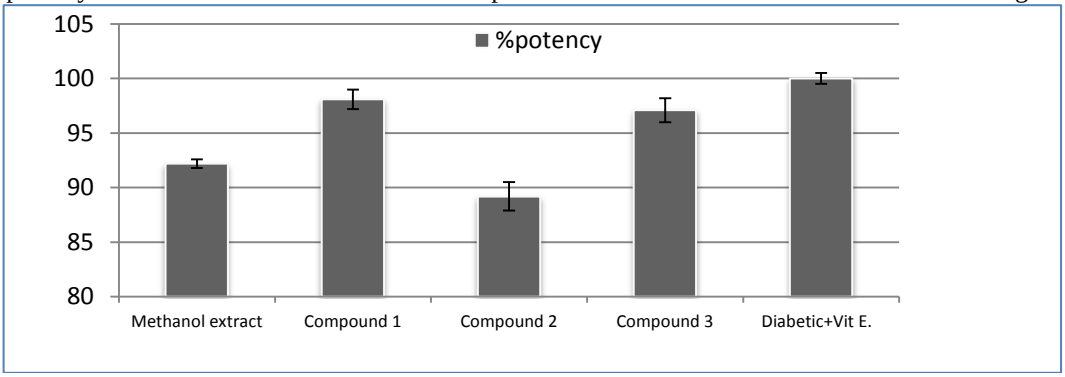


Figure 3. Antisickling effect of methanol extract and isolated compounds obtained from the bark of *Morus alba* L. grown in Egypt.

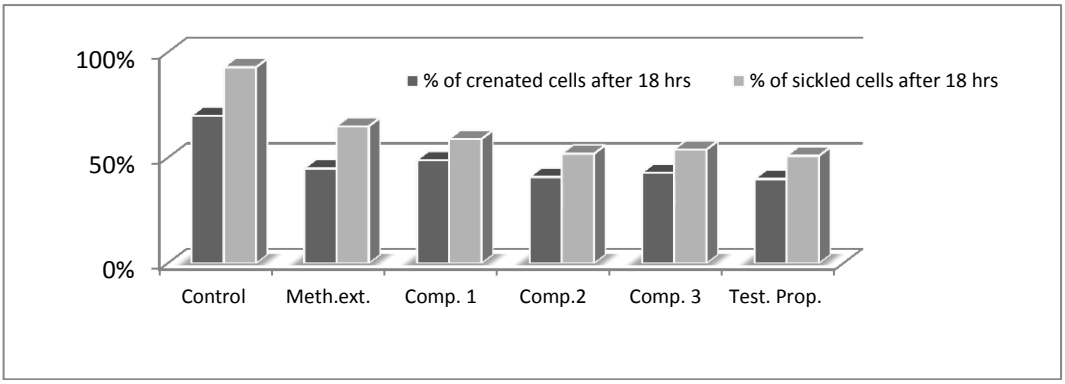


Figure 4. Orientation of active compounds 1, 2, 3 at 3HB5 active site.

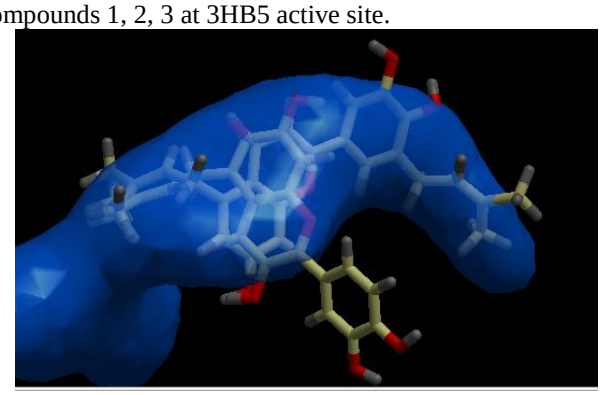
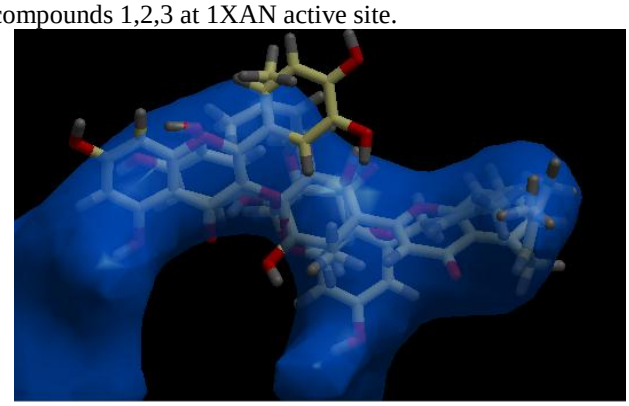


Figure 5. Orientation of active compounds 1,2,3 at 1XAN active site.



4. Discussion

Compound **1** designated as 5, 5'-diprenyl-7, 3', 4'-trihydroxy flavanone. Its molecular formula was established as C25H28O5 due to parent ion at m/z 408 (M) + and (M + 1) at 409. The characteristic U.V absorption bands (λmax MeOH nm = 228 sh, 293,339 + NaoMe, 248, 285, 333; + AlCl3, 221, 316, 392; + NaoAc, 287, 297,334 + H3BO3) suggested a flavanone structure (Ercisli & Orhan, 2007; Kaushik *et al.*, 2008 & Zheng *et al*., 2008). ^{1}HNMR spectrum showed the signals characteristic for flavanone (Geissman, 1962; Harborne *et al.*, 1975 & Mabry *et al.*, 1996). 5,7,3',4',5' substituted derivative (absence of 5,7,3',4'and 5'protons). The chemical shifts of aromatic carbon signals and the molecular weight of compound **1** showed that the A-ring and the B-ring were substituted by one hydroxyl group and by two ortho hydroxyl groups, respectively. The presence of a pair douplet of douplet of the two geminal protons at H-3 β at 2.8 & α at 3.2 ppm respectively with one proton integration, each due to geminal coupling & coupling with H-2. ^{1}HNMR showed a douplet of douplet at 4.8ppm assigned for β H-2, due to coupling with α & β protons at 3. The prenyl group was observed at δ1.06, 1.2 (3H, each, s, for 4", 4'" Me & 5", 5'"Me), 2.6 and 2.65 (2H, each d, for 1", 1'"CH2), 5.1, 5.2 (1H, each, m, 2", 2'" CH). ^{13}CNMR (ppm) showed 25 signals characteristic for flavan nucleus substituted by two isoprene units. 5 signals of oxygenated aromatic carbons at δ: 186.2(**C-4**) 158 (**C-7**), 157 (**C-9**), 146.8 (**C-3**\) and 146.5 (**C-4**\), 7 signals of nonoxygenated aromatic carbons at 131.3 (**C-1**\), 119.07 (**C-6**\),115.7 (**C-5**\),115.2 (**C-2**\), 99.7(**C-10**), 95.8 (**C-6**) and 94.5 (**C-8**) and 3 signals of central ring carbon at 81.7 (**C-2**) and 67.1 (**C-3).**

Prenyl carbons were recorded at δ: 139.2, 139 (**C-3''', 3''**; respectively), 125.6, 125.5 (**C-2'''**, **2''**; respectively), 30.2, 28.5 (**C-1'''**, **1''**, respectively), 24.1, 24.2(**C-5'''**, **5''**, respectively) and 12.4, 12.5(**C-4'''**, **4''**, respectively). The two prenyl groups were attached to C5 and C5' this was confirmed from ^{1}H-^{1}H COSY and HMBC correlation between methylene signals at δH 2.65(H-1") & δH 2.60(H-1'") and carbon signals at δ C 124.5 (C-5) & δ C 115.5 (C-5'); respectively. In addition 6, 8 and 2',6' meta coupling and geminal coupling of 2H on C3 were confirmed from 1H-1H COSY correlation between these protons. Mass spectrum showed a molecular peak at m/z = 408 ascribed structure formula C25H28O5.

Compound **2** designated as 5-prenyl-3, 7, 3', 4'-tetrahydroxy flavanonol.Spectral data of compound 2 is different from compound 1 in absence of prenyl moiety in ring B and this was confirmed by the presence 20 carbons only in^{13}CNMR. ^{1}HNMR spectrum revealed the presence of H5' proton at 6.60 (1H, d, *J*'=9.1 Hz, ortho coupled with 6') presence of 3-OH assigned for flavanonol nucleus and this was confirmed by the presence of one proton at 4.41 (1H, d, *J*'=7.2 Hz due to coupling with H-2) and absence of geminal coupling. The prenyl group was attached to C5 and this was confirmed from ^{1}H-^{1}H COSY and HMBC correlation between methylene signal at δH 2.65(H-1") and carbon signal at δ C 124.5 (C-5). In addition 6,8 and 2',6' meta coupling were confirmed from 1H-1H COSY correlation between these protons. Mass spectrum showed a molecular peak at m/z = **356** ascribed structure formula C20H20O6.

Compound **3** exhibited a purple colour in UV light changing to yellow on exposure to ammonia vapours and $AlCl_3$ reagent. Properties and spectra were identical to those reported earlier (24, 25) for Quercetin-3-O-α-L-rhamnoside (Quercetrin)

From the previously mentioned physicochemical and spectral data, as well as, comparison with the published data (26-28), compounds **C1-C3**(Figure 1) could be identified as 5, 5'-diprenyl-7, 3', 4'-trihydroxy flavanone, 5-prenyl-3, 7, 3', 4'-tetrahydroxy flavanonol and Quercetin-3-O-α-L-rhamnoside (Quercetrin). Compounds C1 and C2 were isolated for the first time from *M. alba* L.

The variation in biological activities of the tested samples may be attributed to the difference in chemical nature of tested sample. As a matter of fact, all tested samples exerted high potency as antioxidant (Table 1 & Figure 2); which may be due to synergetic effect of methanol extract content and phenolic nature of isolated compounds (Stephen & Duke, 1996).

The experiments tested with haemoglobin AA blood which crenated in acidic citrate-dextrose-saline (Table 2 & Figure 3) showed that the tested samples caused appreciable reversal of crenation (% of crenated cells decreased from 74% in control to about 41-49% in tested samples). Haemoglobin AS samples also exhibited reversal of sickling in the presence of the tested samples (% of sickled cells decreased from 91% in control to 52-65% in tested samples).

The results in all cases were more or less equal to testosterone propionate in the concentrations 4μg/mL (decrease % of crenated and sickled cells to 40% & 51%, respectively). Effect may be due to direct effect of phenolics on RBCs membrane (Gurib *et al.*, 1992).

Considering cytotoxic activity a high potency of compound 1 was noticed especially against cervices & breast tumor cell lines. On the other hand compound 2 recorded higher activities against liver tumor cell line than compound 1

To understand the biological data on structural basis, we evaluate the isolated compounds **1, 2 & 3** through docking techniques using Molsoft ICM 3.4-8C program, by docking our titled compounds on the crystal structures of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) in complex with its natural inhibitor E2B {3-(3',17'beta-dihydroxyestra-1',3',5'(10')-trien-16'beta-methyl)benzamide}available

through the RCSB Protein Data Bank (PDB entry 3HB5) and on another crystal structures of peroxiredoxin available through the RCSB Protein Data Bank (PDB entry 1XAN) (http//www.rcsb.org/pdb/welcome.do & http//www.ebi.ac.uk/thoronton-srv/databases /pdbsum).

Docking studies on 3HB5 reveals that E2B (the original ligand) has ICM score of -133.03 and form two hydrogen bonds with Ser-142, and C-185 and docking of NADP reveals ICM score of -303.59 and form three hydrogen bonds with Ser-11, and three bond with Ser-12, another four bonds with K-159, four bonds with N-90, four bonds with A-91, one bond with Thr-140, and another bond with Tyr-155 (Table 4, Fig. 4). Compound 2 exhibits relatively weak binding affinity with ICM score of -91.71 but form three hydrogen bonds with with K-159, another one bond with Tyr-155 (Table 4, Fig.4). Compound 3 possess ICM scores of -99.36 and form one bond with Ser-142, and another bond with C-185, and form six bonds with N-90, and one bond with A-91, and another bond with Thr-140 (Table 2, Fig. 4). Compound 1which is the most active compound as antiproliferative possesses ICM scores of -111.44 and form three hydrogen bonds with Ser-11, and two bond with Ser-12, two bonds with K-159, and another bond with Tyr-155 (Table 4, Fig. 4).

In addition, docking studies on 1XAN reveals that FAD has ICM score of -281.49, forms five hydrogen bonds with Thr-57, four bonds with Asp-331, and another bond with G-29 (Table 5, Fig. 5). Compound 2 exhibits relatively weak binding affinity with ICM score of -92.09 and form one hydrogen bond with Thr-57 (Table 5, Fig. 5). Compound 3 possess ICM scores of -101.46 and form four bonds with Thr-57, one bond with Asp-331, and another bond with G-29 (Table 5, Fig. 5).

Compound **1**which is the most active compound as antioxidant possesses ICM score of -112.05 and forms one hydrogen bond with Thr-57, and another bond with Asp-331(Table 5, Fig. 5), where compounds **1** is the most active as antitumor against tumor cell lines (breast, MCF7) and (cervix, HELA) of IC_{50} 0.56 υg/ml, and 0.71 υg/ml respectively, also, shows maximum potency as antioxidant of 98.1%, and shows good affinity with the receptor that reveals ICM score value of -111.44.

Compound **3** has less antioxidant activity than compound 1 with antioxidant potency of 97.1%, while compound **2** which reveal less activities as antiproliferative against tumor cell lines (breast, MCF7) and (cervix, HELA) of IC_{50} 1.41 υg, 0.92 υg respectively, and shows least potency as antioxidant of 89.1%, and shows least binding affinity with ICM score value of -91.71.

The data reported herein indicates that prenylated flavonoid represents a new interesting class of potentially useful compounds as antiproliferative and antioxidant. Di prenylated derivative compound **1** represents the most active than the monosubstituted analogue compound 2 of flavonoid systems and may enhances its binding affinity with enzymes, it was found that hydrogen bonds formation with Ser-142, C185, K-159, Ser-11, Ser-12, N-90 amino acid residues may be responsible for the antiproliferative activity as referred to natural inhibitor E2B, and NADP, while to be active as antioxidant it may need formation of hydrogen bonds with Thr-57, Asp-331.

Conclusions

According to this article, we can conclude that compound 1 appears to be the most interesting compound among the newly isolated and seem potentially attractive as antiproliferative and antioxidant candidates. (3HB5) and (1XAN) enzymes, which are potential, target of hormone dependent cancer & antioxidant activity; respectively were considered for docking and screening studies. This work finds application using these targets to design drugs for the treatment of hormone dependent cancer & act as preventive to risk factors of cancer. Consequently, the approach is useful in designing novel molecules for treatment & prevention of hormone dependent cancer

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