COLORANTS WITH MIXT NAPHTHOQUINONE-AZOMETHINE CROMOPHORE-SYNTHESIS, CHARACTERIZATION AND IN VITRO TOXICITY ANALYSIS

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A fost studiată sinteza unor noi compuşi de concepţie originală, bazată pe condensarea unor aldehide heterociclice compact condensate obţinute prin reacţia 2,3 dicloro -1,4-naftochinonei şi a derivaţilor săi substituiţi cu salicilaldehida, urmată de condensarea cu diverse amine aromatice în vederea obţinerii bazelor Schiff corespunzatoare. În vederea utilizării în produse cosmetice, coloranţii au fost supuşi testării "in vitro" a toxicităţii pe culturi celulare specifice.

The paper presents the synthesis of some new original compounds based on a compact condensed heterocyclic aldehyde obtained by reacting 2,3-dichloro-1,4-naphthoquinone and its 5- substituted derivatives with salicylaldehyde, followed by the condensation of the resulting products with different aromatic amines, leading to the corresponding Schiff bases. In order to use thyese compounds in cosmetic products an "in vitro" toxicity analysis on specified cell cultures was performed.

Keywords: azomethine colorants, mixed naphthoquinone-azomethine chromophore, 2,3-dichloro-1,4-naphthoquinone, "in vitro" toxicity

1. Introduction

The later studies performed and presented elsewhere **[1, 2]** showed that the naphthoquinone-furane chromophore is characterized by a high luminescence, compounds in this class being characterized by high shifting of the emission band towards the absorption bands and by relatively high intensities. As the absorption

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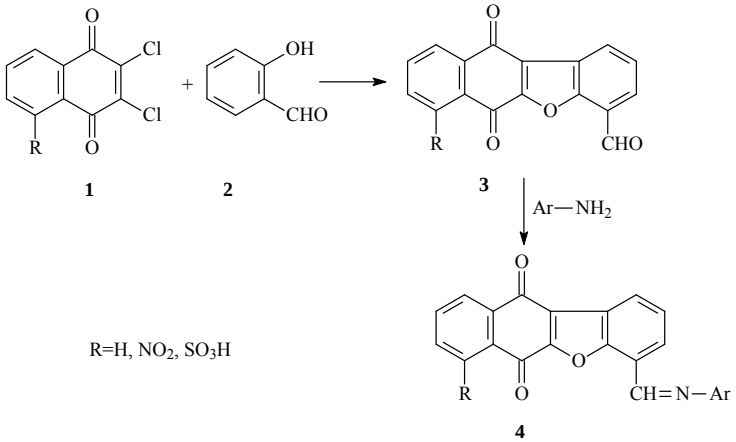
extinction coefficients are rather low, these compounds could be used especially as shade colorants and not as pigments.

Due to this observation, the supplementation of the chromophore system was made by linking an azomethine structure to the chromophore, thus increasing the tinctorial properties of the studied compounds.

The azomethine colorants **[3]** are compounds which contain as a chromophore, the C=N double bond. The properties of these colorants combine those of the azo and the polymethine dyes, having the tinctorial properties of the first and the luminescent properties of the last. Most of the luminophore commercial products can be found in this category, due to their great stability and high quantic yields.

The compounds synthesized in this paper are new compact condensed heterocyclic azomethines obtained by the reaction of 2,3-dichloro-1,4-naphthoquinone **1** and its 5- substituted derivatives with salicylaldehyde **2** leading to compounds **3**, followed by the condensation of the resulting products with different aromatic amines, leading to the corresponding Schiff bases **4.** The reaction steps are given in scheme **1**.

The "*in vitro*" toxicity was evaluated by the cells viability upon addition of the colorants. This type of test was chosen in order to determine the best concentrations to be used in cosmetic products. Progressive concentrations of **CND** – azomethine-naphtoquinone derivative – 4 – formyl – benzo(b) – naphto[2,3-d]furan – 6,11 – dione (orange coloring agent) and **CNA** – azomethine naphtoquinone derivative – 4N phenyl imino-benzo (b) – naphto[2,3 – d]furan-6,11-dione (shining yellow coloring agent), were tested starting with a dilution of 1/100 in order to determinate the cytotoxicity limit for each substance depending on the studied cell type.



Scheme 1**.** The Synthesis of 4-Formyl-Benzo(b)-Naphto[2,3-d]Furane-6,11-Dione and its 7-Substituted Derivatives

2. Experimental

2.1. Reagents

All reagents used for synthesis were of the highest purity, commercially available. 2,3-dichloro-1,4-naphthoquinone and salicylaldehyde were purchased from Merck. Also, cell culture 3T3, BALB 3T3 and Jurkat were purchased from Cell Bank - Interlab Cell Line Collection (ICLC).

2.2. Apparatus

The synthesized compounds were tested on Shimadzu spectrophotometer UNICAM UV3-100. The IR spectra were recorded on a FT-IR Perkin Elmer Spectrometer with Pike Miracle ATR reflection Zones device. Refrigerated incubator Binder KB 400 is needed for incubating the cells suspensions at 35°C and the hemocytometer Burger-Türck for counting the viable cells.

2.3. Procedure

A. Synthesis of 4-formyl-benzo(b)naphto [2,3 d]furan-6,11-dione (3a)

10 g (0,044 moles) of 2,3-dichloro-1,4-naphthoquinone, 90 ml pyridine and 6 g (0,049 moles, 5,5 ml) of salicylic aldehyde are introduced into a reaction flask. The mixture is refluxed for 3 hours, then cooled and the compound 4-formyl-benzo (b) [2,3-d]-furan-6,11-dione is precipitated and filtered.
10,3 g product is obtained, yield = 85%.

Elemental analysis: Calculated : %C=73,91; %H=2,89;
Found: %C=73,96; %H=2,86;

Melting point: 297^0C (recrystallized from ethanol)

IR (cm^{-1}) : ν_{CHO} = 1720, 2725, $\nu_{COquinone}$= 1679, 1703
other spectrum bands :733, 785, 866, 879, 924, 975, 1237, 1312, 1426, 1460, 1526, 1569.

^{1}H-RMN spectrum - $CDCl_3$ - δ (ppm): $H^{1,2,3}$ - 7.7(m, 3H), H^4 - aldehyde -8.8 (s, 1H), $H^{7,10}$- 8.2 (t, 2H), $H^{8,9}$ - 7.8(t,2H)

B. Synthesis of 7-nitro-4-formyl benzo(b)naphto [2,3 d]furan-6,11-dione (3b)

12 g (0,044 moles) of 5-nitro-2,3-dichloro-1,4-naphthoquinone, 90 ml of pyridine and 6 g (0,049 moles, 5,5 ml) of salicylic aldehyde are introduced into a reaction flask. The mixture is refluxed for 3 hours, then cooled. The precipitate is separated by filtration and 11,5 g product are obtained, yield 81%.

Elemental analysis: Calculated : %C=63,55; %H=2,18; %N=4.36

Found: %C=63,56; %H=2,16; %N=4.40

Melting point: 247^0C (recrystallized from ethanol)

IR (cm^{-1}) : ν_{CHO}=1728, 2726, $\nu_{COquinone}$= 1671, 1693
other spectrum bands:733, 785, 866, 879, 924, 975, 1237, 1312, 1426, 1460, 1526, 1569.

C. Synthesis of 4-formyl benzo(b)naphto [2,3 d]-furan-6,11-dione-7-sulphonic acid (3c)

15 g (0,044 moles) of 2,3-dichloro-1,4-naphthoquinone-5-sulphonic acid, 90 ml of pyridine and 6 g (0,049 moles, 5,5 ml) salicylic aldehyde are introduced into a reaction flask. The mixture is refluxed for 3 hours, cooled and the precipitated product is separated by filtration. 14,6 g of 4-formyl benzo(b)naphto [2,3 d]-furan-6,11-dione-7-sulphonic acid is obtained, yield = 84%.

Elemental analysis: Calculated : %C=57,30; %H=2,24; %S=8.98
Found: %C=57,25; %H=2,26; %S=8.92

Melting point: 247^0C (recrystallizated from ethanol)

IR (cm^{-1}) : ν_{CHO}=1730, 2729, $\nu_{COchinona}$ = 1684, 1701
other spectrum bands :733, 785, 866, 879, 924, 975, 1237, 1312,1426, 1460, 1526, 1569.

D. The synthesis of componds with mixed naphtoquinone azomethine chromophore

In a reaction flask 0.01 moles derivate of 4-formyl -benzo(b)naphto [2,3 d]-furan-6,11-dione are dissolved in 20 ml ethyl alcohol then 0.012 moles aromatic amine and 0.5 ml HCl 35% are added. The mixture is refluxed for 2 hours. After cooling the mixture the colorant precipitates,is then filtered and dried.

3. Results and discussions

A. The synthesis and characterization of colorants with mixt naphthoquinone-azomethine cromophore

The synthesized colorants are new compact condensed heterocyclic azomethines obtained by reacting 2,3-dichloro-1,4-naphthoquinone **1** and its 5-substituted derivatives with salicylaldehyde **2** followed by the condensation of the resulting products with different aromatic amines.

The synthesis of these products starts from 2,3-dichloro-1,4-naphthoquinone or its 5- substituted derivatives (5-nitro-2,3-dichloro-1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone-5-sulfonic acid) which are

condensed in pyridine, by means of two nucleophile substitutions, with salicylaldehyde, when the compact condensed 4-formyl derivatives are obtained.

After boiling, the reaction mixture is cooled and the 4-formyl-benzo(b)-naphtho[2,3-d] furane-6,11-dione derivatives are precipitated and separated by filtration.

The obtained products are recrystallized from ethanol and characterized by boiling point, elemental analysis and IR spectra.

The insolubility of the compounds in usual solvents hindered the obtaining of the ^{1}H-NMR spectra for the compound **3c** (R=H)**.**

The presented data confirm the theoretically proposed structures. The IR spectral analysis allows the attribution of the bands of the characteristic frequencies for the aldehyde C=O group [**4, 5**] at higher values (1720-1730 cm^{-1}) than those of the quinone C=O groups (1670 - 1703).

Other characteristic peaks for the aldehyde group appear in the range 2720-2730 cm^{-1}, corresponding to a combining band vibration or to an aromatic valence vibration of the C-H bond.

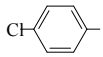
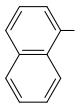
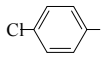
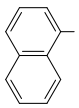
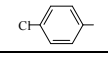
The ^{1}H-NMR spectrum performed in $CDCl_3$, at 300MHz, indicates a chemical shift of 8.8 ppm for the aldehyde proton. Its desecranation is due to the H bonds, which can be formed with the oxygen atom of the furan cycle. The protons in positions 7 and 10 are also strongly shifted (8.2 ppm) due to the quinone-neighboring group. The protons in positions 8 and 9 have signals at 7.8 ppm. The influence of the formyl group together with the compact condensed molecular system makes the protons in positions 1, 2 and 3 to have higher chemical shifts than normally for a benzene ring.

The synthesis of some azomethine colorants by the reaction of 4-formyl-benzo(b)naphto[2,3-d]-furane-6,11-dione and various substituted aromatic amine and the characterization of these compounds is performed in ethyl alcohol, at a molar ratio of aldehyde: amine = 1 : 1.1. The colorant precipitates by cooling and is then filtered and dried. The reaction yields are high, in the range 90-98%, but they decrease in the case of 7-sulfonic derivative due to the solubility of the product. The reaction time varies for every compound.

The synthesized colorants were recrystallized from ethanol and their melting points, IR and UV-VIS spectra are presented in Table 1.

Table 1

Spectral behavior of colorants with mixed naphthoquinone-azometinic chromophore

Compound	R	Ar	M.p.	IR Spectra	UV-VIS Spectrum (EtOH)				
				KBr pellet	Absorption spectra		Emission spectra		
			°C	$\nu_{CH=N}$ (cm^{-1})	λ_{max} (nm)	$lg\varepsilon_{max}$	λ_F (nm)	I_F (div)	η_F
4a	H		296	1631	418	2,5	480	220	0.78
^{1}H-RMN Spectra - DMSO - δ(ppm)									
$H^{1,2,}$ - 7.8(d, 2H), H^3 8.6(t, 1H), H^4 - azomethine - 8.88 (s, 1H), $H^{7,10}$- 8.13 (t, 2H), $H^{8,9}$ - 8.0 (d,2H), H_{phenyl}- 7.4 (m, 5H)									
4b	H		227	1631	436	3,02	525	395	0.89
4c	H		>330	1657	426	2,31	505	250	0.81
4d	H		214	1644	453	1,6	530	201	0.76
4e	NO_2		316	1618	423	1,78	520	415	0.80
^{1}H-RMN Spectra - DMSO - δ(ppm)									
$H^{1,2,}$ - 7.9(d, 2H), H^3 -8.6 (t, 1H), H^4 - azomethine - 8.9 (s, 1H), $H^{9,10}$- 8.15 (m, 2H), H^8 - 8.81 (d,1H), H_{phenyl} - 7.4 (m, 5H)									
4g	NO_2		320	1618	418	3,75	535	530	0.94
4h	NO_2		175	1618	426	1,6	515	320	0.86
4i	NO_2		232	1618	463	1,6	544	250	0.81
4j	SO_3H		>330	1611	475	1,236	563	310	0.86
^{1}H-RMN Spectra - DMSO - δ(ppm)									
$H^{1,2,}$ - 7.3(d, 2H), H^3 -8.4 (t, 1H), H^4 - azomethine - 8.9 (s, 1H), H^8 - 8.7 (d,1H), H^9 - 7.9(d,1H), H^{10}- 8.13 (d, 1H), $H_{sulphonic\ group}$ - 9.2 (s, 1H), H_{phenyl} - 7.0 (m, 5H)									
4k	SO_3H		>330	1605	430	3,5	530	320	0.86

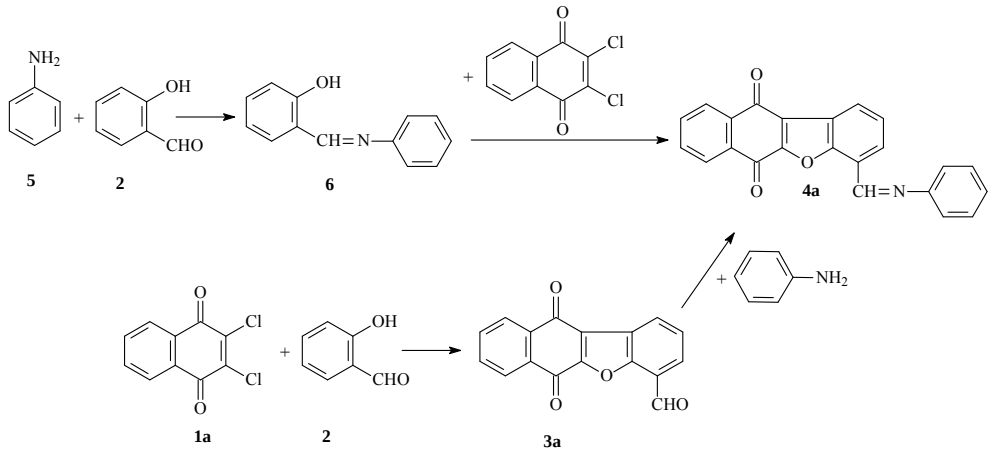
The analysis of the IR spectra shows frequency values of the azomethine group vibration at values between 1605 and 1657 cm^{-1}. Lower values are

encountered for the sulfonic derivatives. The frequencies of the carbonylic groups in the quinone system do not show important changes with respect to the raw materials.

The ^{1}H-NMR spectrum for the compound **4a** shows important differences among the protons of the compact condensed system. If the proton of the azomethine group has values close to those of compound **3a,** its nitrogen is shifted by the H bonds formed with the proton in position 3 of the benzene nucleus.

The linkage of substituents into the naphthalene residues leads to shiftings of the neighboring proton (position 8) and differences in the signals of protons in position 9 and 10 (compound **4j**).

In order to ensure the correctness of the proposed structures of the synthesized compounds, an intersection synthesis for compound **8** was performed, as presented in scheme 2.



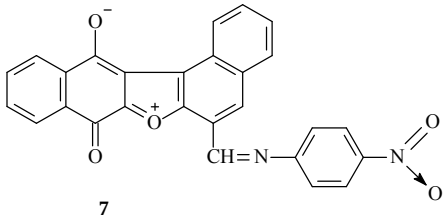
Scheme 2**.** The intersection synthesis

The reaction between salicylic aldehyde and aniline was performed in ethanol at reflux for 1 hour, using a molar ratio aldehyde : amine of 1 : 1.1. The intermediary product was separated by cooling the reaction mixture and filtration. Then it was condensed with 2,3-dichloro-1,4-naphthoquinone in pyridine, at reflux temperature for 3 hours. The final compound was separated by cooling and filtration.

The performed analysis (IR and melting point) showed that the reaction products obtained by the two ways are identical.

The results of the electronic spectra show an important bathochrome effect when electron-accepting substituents are introduced into the aldehyde component. The shiftings are higher than 50 nm in the case of a sulfonic group.

The introduction of a NO_2 group in the amine component produces a bathochromic shifting in the absorption spectrum of the component **9** of 18 nm. The effect on the compound fluorescence is very important as a shifting towards red of the emission bands of 89 nm has been observed. This behavior can be explained by the appearance of some polar structures of type **7** or **8** induced by the withdrawing effect of the carbonyl groups or the nitro group respectively.



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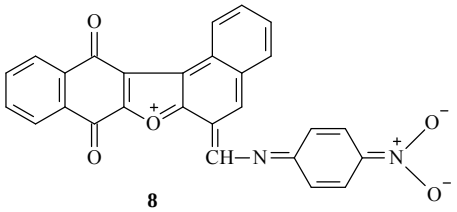

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These statements are supported by the fact that, when introducing electron-accepting groups with -I, -E effects in the α-position of the naphthoquinone unit (nitro or sulfonic groups in the case of **4h**, **4i** and **4k** compounds), there is a higher shifting of the emission bands (more than 100 nm) and also an important increase of the fluorescence intensity.

If halogen type substituents are introduced into the aminic component, a weak bathochromic effect and a strong decrease of the extinction coefficients appears.

The use of polycyclic amine components (α-naphthylamine) leads to important bathochromic shiftings both in the absorption and emission spectra and to a decrease of the extinction coefficients and fluorescence intensity.

It is also important to notice that by coupling the naphthoquinonic and the azometine chromophores, their spectral properties are improved.

B. "In vitro" toxicity tests

"In vitro" toxicity was evaluated by cells viability analysis. This type of test was chosen to establish the concentrations of colorant to be used for the colored cosmetic formulations. Progressive concentrations of **CND** – azomethine-naphtoquinone colouring agent - 4–formyl – benzo(b)naphto [2,3-d]furan–6,11 – dione (orange coloring agent) and **CNA** – azomethine naphtoquinone dye – 4N-phenylimino-benzo(b)naphto [2,3-d]furan-6,11-dione (shining yellow dye) -were tested, starting with a dilution of 1/100 (equivalent molar concentrations are

shown in Tables 3, 4, 5, 6) in order to determinate the cytotoxicity limit for each substance depending on the studied cell type.

An aliquot from the existing cellular suspension is centrifuged five minutes at 100 rot/min. for viability testing. and the supernatant liquid is removed. The size of the aliquot depends on the approximate number of cells. The aliquot must contain a suitable number of cells for countings when suspended in 1 ml of PBS and than it is diluted by the addition of 0.4 % trypan blue solution (e.g., 5 × 105 cells/ml).

The diluted solution of the colorant is mixed with one fraction of cells suspension (from diluted cells). The mixture is incubated for 3-5 minutes at 35°C and the cells are counted using a counting chamber (hemocytometer Burger-Türck).

The cells must be numbered in the first 3 to 5 minutes from the mixture preparation, a longer incubation period leading to the death of the cells and the decrease of the number of viable cells.

The mixture can be easier prepared in a bucket plate or in a small plastic vial, using between 10 and 20 µl of each coloring agent solution cellular suspension.

The non-colored (viable cells) cells and the colored cells (dead cells) are counted separately.

The counting of viable cells (%) is shown below:

$$\textbf{Viable cells \%} = \frac{total\ number\ of\ viable\ cells}{total\ number\ of\ cells} \times 100 \qquad (1)$$

Exclusion of marker is an ordinary and fast technique for counting the cells viability, but it is sensible to several causes: the viability is defined indirectly by the integrity of the cell membrane. In this way, the cellular viability can be compromised (counting the growth capacity or the activity) though integrity of the membrane is maintained. Also, the integrity of cellular membrane can be abnormal, but the cells can be capable to auto-repair and in this way the cells become completely viable. Another possible problem could be related to the amount of assimilated substance (absorbed by the cell) is subjectively evaluated and small concentrations of fluorescent indicator assimilated by destroyed cell can pass unnoticed.

There coloring agent solutions were used in dilution: 1/100, 1/500, 1/1000, 1/1500, 1/2000; in all this situations, as blank assay, the same cellular lines in the presence of eosin were used. Blank assay evaluation depends on the eosin characteristics to penetrate in dead cells to color them red (pink), making possible the differentiation between viable cells and the dead cells. 20µl cellular suspension is transferred in a tube and 80µl 0,1%eosin solution is added. The

mixture is read in 5 minute. The lamella is attached to the hemocytometer. In the hemocytometer chamber 10µl eosin cellular suspension is transferred with a pipette. All cells will be counted (viable- non-colored and dead - colored) from a central 1 mm^2 and from the four perimeters in the corners. Viable cells and dead cells are counted separately

The results are centralized in tables below:

Table 2

3T3 Cellular Line

Tested substance	%Viability	Tested substance	% Viability
Blank assay	99.1		
CND 1/100	0	CAN 1/100	42
CND 1/500	28	CAN 1/500	72
CND 1/1000	42	CNA 1/1000	89
CND 1/1500	78	CNA 1/1500	92
CND 1/2000	87	CNA 1/2000	98

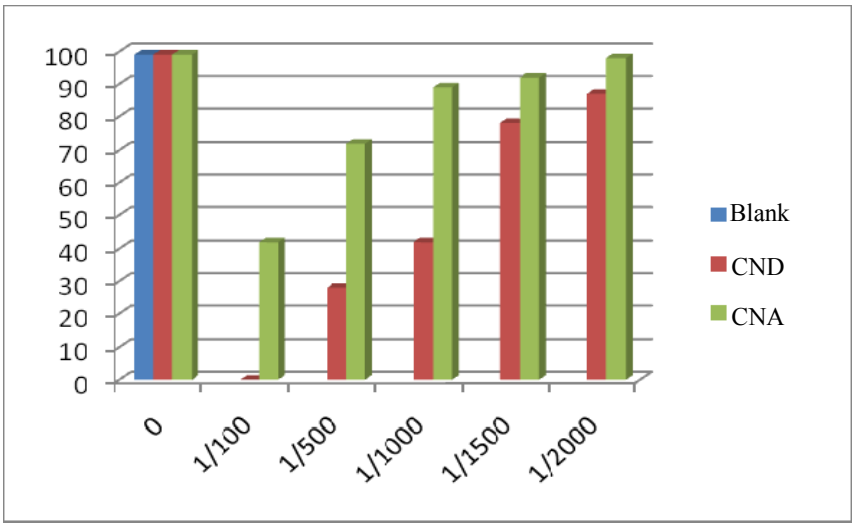

Fig. 1. Cells viability 3T3

CND is cytotoxic for cellular line 3T3 at dilutions between 1/100 and 1/1000, but CNA is well tolerated beginning with 1/500.

Table 3

BALB/3T3 Cellular Line

Tested substance	% Viability	Tested substance	% Viability
Blank assay	98		
CND 1/100	0	CNA 1/100	58
CND 1/500	12	CNA 1/500	63
CND 1/1000	72.3	CNA 1/1000	70
CND 1/1500	83.8	CNA 1/1500	78
CND 1/2000	89.4	CNA 1/2000	92

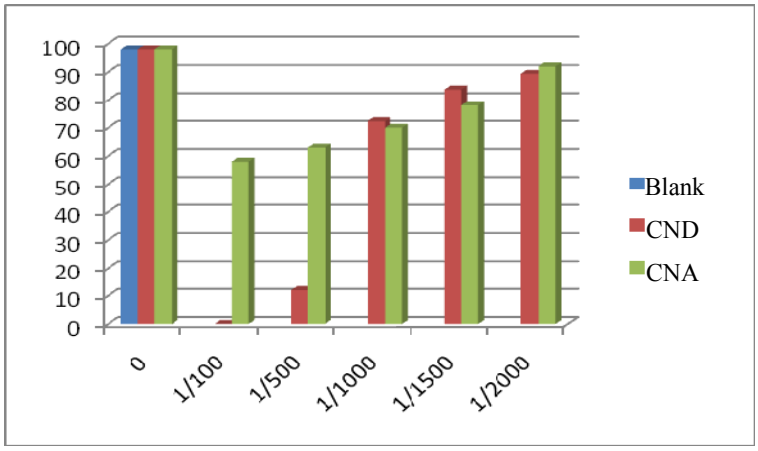

Fig. 2. BALB/3T3 cells viability

BALB/3T3 fibroblasts cellular line is more resistant at the action of CND and CNA, subsequently viabilities tests show over 70%, for 1/1000 dilution for CND and CNA. At lower dilutions (1/100, 1/500) CND is more toxic than CNA.

Table 4

Jurkat cellular line

Tested substance	% Viability	Tested substance	% Viability
Blank assay	98.2		
CND1/100	15	CNA 1/100	61
CND1/500	35	CNA 1/500	80
CND1/1000	63	CNA 1/1000	91
CND1/1500	65	CNA 1/1500	92
CND1/2000	68.3	CNA 1/2000	93

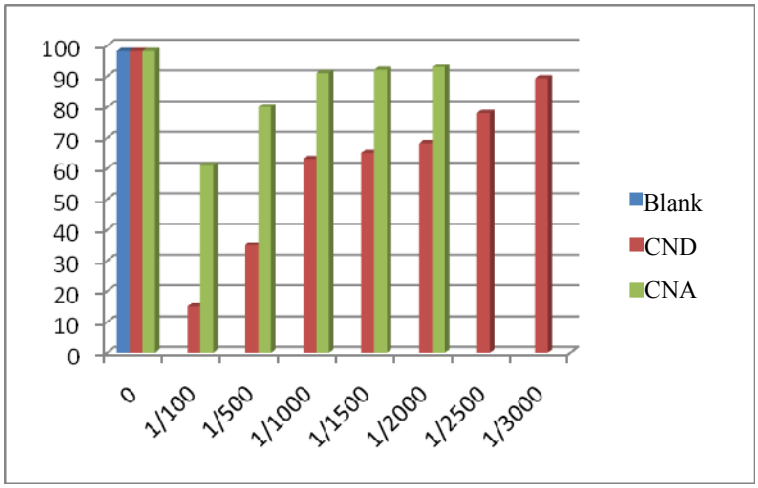

Fig. 3. Jurkat cellular viability

CND is more toxic than CNA, when the tested concentration is over 1/2000. In cultures, CNA keeps a well viability starting with 1/500 dilution.

Table 5

Normal human lymphocytes separated from peripheral blood

Tested substance	% Viability	Tested substance	% Viability
Blank assay	99.3		
CND1/100	0	CNA 1/100	54
CND1/500	72	CNA 1/500	83
CND1/1000	91	CNA 1/1000	94
CND1/1500	96	CNA 1/1500	96
CND1/2000	98	CNA 1/2000	98.3

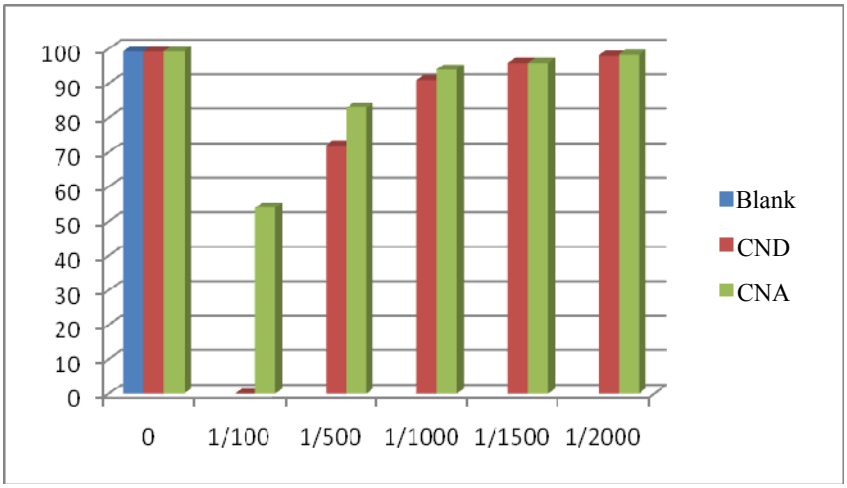

Fig. 4. Lymphocytes- cells viability

At small dilutions (1/100), CND is very toxic by comparison with CNA, but starting with 1/500 for CNA and 1/1000 for CND, the substances are not cytotoxic for the lymphocytes in the culture.

4. Conclusions

A series of new carbonyl compounds (4-formyl-benzo(b)naphtho[2,3-d]furane-6,11-dione and its 5-nitro and 5-sulfo derivatives) were synthesized and characterized (3 of them). Their synthetic procedure is based on the condensation of 2,3-dichloro-1,4-naphthoquinone and of some of its derivatives with salicylic aldehyde.

The synthesized aldehydes were used for the obtaining of 10 new azomethin colorants not cited in the literature. These colorants were analyzed by IR, ^{1}H-NMR and UV-VIS spectra. Their structure was also proved by two intersection synthesis. By coupling the naphtoquinone and azometine chromophores, the spectral properties of the colorants are greatly improved.

An important bathochrome shifting was observed when electron-accepting groups are linked to the naphthalene ring or when polycyclic amine components are used. The amine components that contain halogen lead to the obtaining of some colorants with high extinction coefficients.

“In vitro” toxicity is determined for azomethine colorants to indicate the possibility to their use as coloring agents in cosmetic preparations.

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