Study of Physico-chemical Characteristics and Pharmacological Effects of 1-Ethyl-Salicyldene-*bis*-Ethylene-Diamine and Its Complex with Mn(II)

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The paper presents the synthesis of a new complex combination of a Bis-Schiff base with Mn(II) ions with great potential for antimicrobial and anti-inflammatory activity. A new complex of the Salen-type ligand, 1-ethyl-salicylidene-bis-ethylene diamine was synthetized using Mn(II) ions. The chemical structure was confirmed through ^{1}H-NMR and IR spectroscopy. The antimicrobial activities of the Bis-Schiff base and its complex were tested in comparison with Ampicillin, Chloramphenicol, Tetracycline, Ofloxacin and Nystatin. Those compounds were found to be active against Gram-positive or Gram-negative bacteria, and had an anti-inflammatory effect comparable to that of Indomethacin.

Keywords: Bis-Schiff base, Mn(II) complex, anti-inflammatory effect, antimicrobial effect.

Schiff bases can be synthesized from an aliphatic or aromatic amine and a carbonyl compound by nucleophilic addition forming a hemiaminal, followed by dehydration to generate an imine. Schiff bases are common ligands in coordination chemistry. The imine nitrogen is basic and exhibits π-acceptor properties. The ligands are typically derived from alkyl diamines and aromatic aldehydes [1,2].

Schiff bases are well known as anticancer [3], antimicrobial [4,5], anti-inflammatory [6,7], antiviral [8], analgesic [9], and antioxidant [10] agents.

Schiff bases can be used to mass-produce nanoclusters of transition metals inside halloysite. That naturally abundant mineral has a structure of rolled nanosheets (nanotubes), which can be used for the synthesis of metal nanocluster products. Those nanoclusters can be made of metals such as Ag, Ru, Rh, Pt or Co, and may catalyze various chemical reactions [11]. The complex combinations of Bis-Schiff bases with metallic ions [12] represent a class of compounds with very interesting properties from the point of view of their chemical and biological behavior. Regarding their anti-inflammatory effects, it was reported that several Schiff bases with pyrazole, thiazole, thiazoline and benzothiazole moiety and their metal complexes with Mn(II), Cu(II), Ni(II), Zn(II) posses important anti-inflammatory effects [13-22]. Those compounds inhibit the activity of cyclooxygenase (COX) and 5-lipooxygenase enzymes [23], they can also scavenge free radicals, which are well-known for their implication in inflammatory diseases [24], and also reduce the synthesis of some chemical mediators of acute inflammation such as leukotrienes which are involved in the formation of free radicals [25].

Based on the above-mentioned applications of Schiff bases, the study presents the synthesis, physico-chemical characterization, toxicity degree, antimicrobial and anti-inflammatory effects of a new Bis-Schiff base and its Mn(22) complex [25].

Ligands derived from substituted salicylaldimine have played an important part in revealing the preferred coordination geometries of metal complexes. Of particular interest have been those involving Mn(II), since they reveal surprising molecular diversity not only in coordination geometry but also regarding more subtle changes in the ligands. Thus complexes with four, five or six donors or with marked tetrahedral *distortions* are accompanied by bond length changes and deviations from expected ligand geometry [26].

The new Bis-Schiff base [27] from figure 1 and its Mn(II) complex were evaluated for the antimicrobial activity against Gram-positive and Gram-negative bacteria. The antimicrobial activity of the complex was tested in comparison with the activity of the Bis-Schiff base on the following strains: *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633, *Kiebsiella spp. Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, and it was compared to the antimicrobial activity of Ampicillin, Chloramphenicol Tetracycline, Ofloxacin and Nystatin.

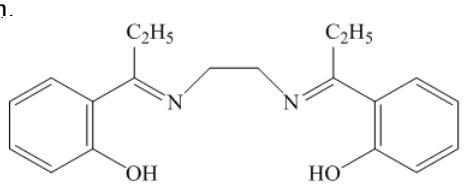

Fig. 1. Structure of 1-ethyl-salicylidene-bis-ethylene diamine (BSB)

The DL50 values of new Bis-Schiff bases and their complexes with metallic ions, have also been established.

Experimental part

The following reagents were used: $MnSO_4 . H_2O$, dimethyl sulfoxide (DMSO) sodium carboxymethyl cellulose (Na-CMC), and methanol. They were produced by Merck Germany or Chimopar Romania.

The melting points were determined using a Boetius apparatus. Elemental analysis was carried out using an Elemental Vario El Analyzer. The quantitative determination of Mn(II) ions from the synthesized complex was performed using the spectrometer AAS-IN Carl-Zeiss-Jena. The ^{1}H-NMR spectra of the Bis-Schiff base (BSB) and Mn(II) complex were obtained using a Brucker AM250 apparatus operating at 250 MHz. The spectra were obtained in $CDCl_3$ for BSB, and in DMSO for the complex, while the chemical shifts were calculated in ppm with respect to TMS ($\delta = 0$). The FT-IR spectra were recorded on a FTS-135 BIO-RAD apparatus in KBr pellets (4000-400 cm^{-1} range). The UV-

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Vis spectra have been obtained on a Hewlett-Packard 8453 spectrophotometer.

The manganese (II) complex ($Mn(BSB)_2$) was synthesized according to the general method from the scientific literature [1, 25, 28]. Firstly, 25 mL of 0.0592M $MnSO_4 . H_2O$ solution prepared in 10^{-3}M HCl was added to 50 mL of 0.014238M BSB solution prepared in anhydrous methanol while stirring at 40°C. The brown complex precipitated immediately. After cooling at room temperature, the precipitate was filtered and then washed with distilled water at first, and then with a methanol-water mixture and finally with ether. After drying in vacuum, a fine brown crystalline powder - $Mn(BSB)_2$ - was obtained and then it was analyzed.

The melting point of the $Mn(BSB)_2$ was 358-359°C. The crystalline powders proved to be stable at room temperature, insoluble in water, ethanol, benzene, and $CHCl_3$, but soluble in DMSO, methanol and dimethyl-formamide (DMF).

The experimental versus calculated results of the elemental analysis of $Mn(BSB)_2$ were: 67.20% C (68.47); 5.78% H (6.56); 8.06% N (7.98) and 7.76% Mn (7.82).

Based on the characteristic absorbance of $Mn(BSB)_2$ [29], the formular weight was determined according to the following equation:

$$\mathbf{F} = \frac{a \cdot \varepsilon}{\mathbf{V} \cdot \mathbf{A}} \qquad (1)$$

where: F = formular weight of the complex, a = the amount of complex obtained; ε = the molar absorption of the complex (for λ = 275 nm), A = absorbance determined experimentally, and V = volume of the solution.

The acute toxicity of the Bis-Schiff base and $Mn(BSB)_2$ was estimated by orally administrating their 0.1% suspensions in Na-CMC to groups of 6-10 Swiss male mice, each weighing between 20 and 25 g, according to the classical laboratory methodology [30]. The animals received food and water *ad libidum.* Three hours before testing their access to water was discontinued.

Acute toxicity was evaluated using geometrically progressing doses in single administrations. The death of the animals and their behavioral reactions has been followed for 10 days. The testing was made in accordance with the international legislation and the internal regulations of the University of Medicine and Pharmacy concerning experiments using lab animals [14, 15].

Interpretation of the results was made by analyzing the regression lines and the data were submitted to ANOVA testing.

The qualitative antimicrobial assay of the compounds was performed by the agar diffusion method according to standard accepted disk sensitivity criteria of National Committee for Clinical Laboratory Standards [31, 32] using 10^{-2}M methanolic solution of BSB, 10^{-2}M methanolic solution of $MnSO4 . H_2O$, 1000 µg/mL BSB solution in DMSO and 1000 µg/mL $Mn(BSB)_2$ solution in DMSO. The bacterial strains used on Sabouraeud medium were: *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579 *(Bc), Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Kiebsiella spp.* The reference substances were used in very small quantities: 30 µg *Chloramphenicol* and *Tetracycline*, 10 µg *Ampicillin*, 5 µg *Ofloxacin*, and 100 µg *Nystatin* dissolved in DMSO and impregnated in discs of sterile paper.

For the qualitative assay, suspensions of the compounds, prepared in sterile peptone water from 24 h cultures of microorganisms were adjusted to 0.5 McFarland. Muller-Hinton Petri dishes of 90 mm were inoculated using those suspensions. The tested compounds were dissolved in DMSO and brought to 1000 µg/mL concentration levels. *Chloramphenicol*, *Tetracycline, Ampicillin*, *Ofloxacin* and *Nystatin* dissolved in DMSO were used as reference substances. The 6 mm discs impregnated with 10 µL solution of each compound were used as negative controls and placed in a circular pattern in each inoculated plate. Incubation of the plates was done at 37°C for 24 h. Evaluating the results was done by measuring the diameters of the inhibition zones generated by the tested substances. Toxicity tests of the DMSO solvent showed that the concentrations used in antibacterial activity assays did not interfere with the growth of the microorganisms [33, 34].

The anti-inflammatory activity was determined using male Wistar rats, weighting 180-200 g using carrageenan induced rat paw edema method [35-37]. The animals were randomly divided into groups of six. The standard drug (Indomethacin) and test compounds: BSB and $Mn(BSB)_2$, were administered *p.o.* as a suspension in 0.5% Na-CMC solution, one hour before the carrageenan injection. The control group received only 0.5% Na-CMC solution. The right hind paw edema was induced by sub-plantar injection of 0.2 mL of 2% carrageenan solution in saline (0.9%). The volume of paw edema (mL) was determined using plethysmometric method before and after 1, 2, 4, 6, 8 and 24 hours of carrageenan injection. The anti-inflammatory activity was evaluated as the variation of the volume of inflammation paw edema (mL).

The results were analyzed using one-way analysis of variance (ANOVA) and expressed as mean ± standard error of mean (S.E.M.).

Results and discussions

The complex combination was characterized from the physic-chemical and chemical point of view by elemental analysis, ^{1}H-NMR, UV-Vis, FT-IR techniques, which confirmed the structure and radio of metal/ BSB combination.

The elemental analysis of the complexes indicated the formation of the complexes in a 1:2 metal/ligand molar ratio. The formular weight of the complex was calculated using equation (1) based on the experimental data: a = 0.2100 mg; ε = = 10702.34 $mol^{-1} . L . cm^{-1}$, A = 0.320, and V = 10 mL. By calculating the formular weight (F) of the $Mn(BSB)_2$ complex, the theoretical (700.94) and the experimental values (702.34) obtained, confirmed the 1:2 molar ratio.

The ^{1}H-NMR spectra of BSB in $CDCl_3$ exhibited three singlets (δ_{OH} = 12.80 ppm, $\delta_{=CH(aromatic)}$ = 6.89 - 7.62 ppm, δ_{-CH2C} = 2.15 ppm) and two doublets ($\delta_{CH=N}$ = 3.82 ppm). The ^{1}H-NMR spectra of the complex presented significant changes when compared to that of BSB, due to the coordination process. The -OH proton signal of the BSB (12.80 ppm) disappeared upon complexation with Mn(II). The aromatic protons were shifted, while the methyl proton did not seem to presents a significant change because of the coordination.

The UV-Vis spectra of BSB in DMF showed two strong absorption bands in the 200-450 nm region, attributed to π-π* and n-π* transitions. The spectra of the complex presented modifications in the position and intensity of the bands characteristic to the free BSB, as well as the occurrence of new bands which were attributed to d-d or d-π* transition. The UV spectrum of BSB showed two maxima at 255(3.16) nm and 320(3.27) nm, but the latter suffers a batochromic shifting at 275(2.99) nm and 460(5.50) nm in the spectrum of the complex which

suggested the involvement of the C=N group in the coordination reaction with Mn(II). In the spectrum of the complex, a small shoulder appeared at 425 nm, probably due to the coordination with the metallic ion.

The FT-IR spectra of the ligand showed major bands around 1618 cm^{-1} assigned to $\nu_{C=N}$, which could also be found in the spectrum of the $Mn(BSB)_2$, which suggested the involvement of the nitrogen atom from the C=N group in the coordination process. A more significant modification appeared in the 1040 cm^{-1} band, attributed to the -OH of the phenolic group, which was absent in the complex. That indicated the involvement of the oxygen anion into a δ bond with the metal cation. More than that, a peak appeared in the spectrum of the complex at 520 cm^{-1} that could be attributed to the metal-N bond, and another at 456 cm^{-1} attributed to the metal-O bond.

The recorded FT-IR spectrum confirmed the hypothesis of the formation of the complexes by the coordination of manganese to the azomethinic nitrogen and to the phenolic oxygen.

In order to evaluate the toxicity of the BSB and their complexes $Mn(II)_2$ the following doses were tested: 100, 200, 400 and 800 mg/kg. At doses of 100 and 200 mg/kg, all compounds were nontoxic. At dose of 400 mg/kg, BSB and its complex induced central phenomena such as shaking and fast breathing. It was also noticed that, at 800 mg/kg dose, all compounds induced sudden death due to convulsive phenomena. In conclusion, BSB and the corresponding complexes are basically nontoxic.

The antimicrobial activity was estimated by measuring the diameter of the area inhibited by the tested compounds: BSB and its Mn(II) complex. The results from table 1 could be attributed to the structure of the tested compounds that seemed to be the main factor influencing the antibacterial activity. That was certainly correlated to the ability of a compound to diffuse through biological membranes to reach its site of action.

A good antimicrobial activity of the BSB was noticed when compared to *Chloramphenicol*, *Tetracycline*, *Ampicillin*, *Ofloxacin* and *Nystatin* on *Candida albicans* and *Pseudomonas aeruginosa*. The BSB did not have a great antimicrobial activity on *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Escherichia coli*. The best antimicrobial activity was noticed against *Candida albicans*. The Mn(II) complex was efficient against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli Pseudomonas aeruginosa* and *Candida albicans*. The cation involved in the complexes might intensify the antibacterial activity. The Mn(II) complex was most effective against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans.*

Anti-inflammatory activity

The results from table 2 revealed that the tested compounds had a significant anti-inflammatory effect in reference to the control group and the effect was comparable with that of indomethacin, which was used as a reference drug. The effect started at 4 h, increased at 6 h and 8 h and then it decreased. For the group treated with 10 mg/kg BSB, the maximum effect was observed after 8 hours, when the volume of paw edema was 28.83±1.57 in reference to the control 38.66±1.32.

The Mn(II) complex showed a higher effect at a 10 mg/k dose than at a 5 mg/kg dose. 8 h after the administration of the compound, in 10 mg/kg doses, the volume of paw edema was 20.65=66.52, which meant that was 1.4 time

Table 1

ANTIMICROBIAL ACTIVITY OF THE TESTED COMPOUNDS

Antimicrobial agents	BBS 10 µg/mL	$Mn(BBS)_2$ 10 µg/mL	Ampicillin 10 µg/mL	Tetracycline 30 µg/mL	Chloram-phenicol 30 µg/mL	Ofloxacin 30 µg/mL	Nystatin 100 µg/mL
	diameter of inhibition zone (mm) as mean of three replicate ± standard deviation						
Staphylococcus aureus	33.66±0.57	38.66±0.52	22.66±0.52	32.33±0.57	25.33±0.57	29.66±0.32	-
Bacillus subtilis	16.33±0.52	40.32±0.70	34.33±0.52	29.66±0.52	34.66±0.52	29.66±0.52	-
Bacillus cereus	24.33±0.52	37.33±0.57	17.33±0.57	31.33±0.52	34.33±0.57	34.33±0.57	-
Escherichia coli	17.33±0.57	34.66±0.52	15.66±0.57	27.33±0.57	22.00±0.00	27.66±0.52	-
Pseudomonas aeruginosa	34.33±0.52	43.33±0.70	0	31.66±0.52	38.66±0.52	34.33±0.70	-
Candida albicans	28.66±0.52	35.66±0.52	-	-	-	-	25.33±0.52

Table 2

IN VIVO ANTI-INFLAMMATORY ACTIVITY OF THE SYNTHESIZED COMPOUNDS IN CARRAGEENAN-INDUCED PAW EDEMA

Time	0	1h	2h	4h	6h	8h	24h
Control	19.05±1.02	28.26±0.62	22.66±0.52	31.13±0.37	39.13±2.17	38.66±1.32	23.87±1.15
Indometa-cin 10mg/kg	21.33±4.02	25.32±2.51	25.83±2.02	25.26±2.52	25.04±2.15	25.66±3.52	24.85±2.57
BBS 10mg/kg	17.33±0.52	26.35±1.57	28.53±0.55	29.33±0.62 *P<0.05	29.53±2.67 *P<0.001	28.83±1.57 *P<0.001	21.52±0.67 *P<0.01
Mn(BBS)2 10mg/kg	18.63±1.57	27.66±0.82	29.86±2.38	27.33±2.75 *P<0.001 **P<0.001	21.63±5.23 *P<0.001 **P<0.001	20.66±6.52 *P<0.001 **P<0.001	21.26±1.77 *P<0.001 **P<0.001
Mn(BBS)2 5mg/kg	19.43±0.72	26.63±0.65	27.05±1.32	31.66±0.52	30.56±6.52 *P<0.05	28.35±8.70 *P<0.001	21.83±0.27

* P values compared with control group

** P values compared with the group receiving BSB

more active than BSB (28.83±1.57), and slightly higher than indomethacin (25.66±3.52). In the same conditions, the Mn(II) complex at a dose of 5mg/kg also presented strong anti-inflammatory effect at 4h, 6h and 8h ($P \leq 0.001$), compared with the BSB (10mg/kg).

Conclusions

The research study reports the successful synthesis and antimicrobial activity of a new Schiff bases complex with Mn(II) ions. The complex was physic-chemically characterized through elemental analysis, UV-Vis and FT-IR analysis, and the ratio of metal/ligand combination, the melting point and the solubility were evaluated.

The antimicrobial activity of the complex was tested in comparison to the Bis-Schiff base against the Gram-positive and Gram-negative bacteria The comparative study of the antimicrobial activity of a Bis-Schiff base and its new complex $Mn(BSB)_2$ proved the fact that the BSB as well as its complex manifested an antimicrobial activity, similar to *Chloramphenicol*, *Tetracycline, Ampicillin*, *Ofloxacin* and *Nystatin.*

The study of the anti-inflammatory activity of the manganese(II) Bis-Schiff base complex proved that it induced effects comparable to that of Indomethacin. The anti-inflammatory effect of Mn(II) complex was stronger than the anti-inflammatory effect induced by the free Bis-Schiff base.

References

1.MCNAUGHT, A., WILKINSON, A., IUPAC Compendium of Chemical Terminology 2nd Ed. (the Gold Book). Royal Society of Chemistry, Cambridge, 1997.

2.PUI, A., PERREE-FAUVET, M., KORRI-YOUSSOUFF, H., BREBAN, I.G., Rev. Chim. (Bucharest), **60**, no. 8, 2009, p. 763.

3.QIAO, X., MA, Z.Y., XIE, C.Z., XUE, F., ZHANG, Y.W., XU, J.Y., J. Inorg. Biochem., **105**, no. 5, 2011, p. 728.

4.VENKATESH, P., Asian J. Pharm. Hea. Sci., **1**, no. 1, 2011, p. 8.

5.HUSSEIN, M.A., OMAR, R.H., FARGHALY, H.S., Int. J. Acad. Res., **3**, no. 1, 2011, p. 454.

6.SATHE, B.S., JAYCHANDRAN, E., JAGTAP, V.A., SREENIVASA, G.M., Int. J. Pharm. Res. Dev., **3**, no. 3, 2011, p. 164.

7.PANDEY, A., DEWANGAN, D., VERMA, S., MISHRA, A., DUBEY, R.D., Int. J. Chem. Tech. Res., **3**, no.1, 2011, p. 178.

8.KUMAR, S., DHAR, D.N., SAXENA, P.N., J. Scient. Ind. Research, **68**, 2009, p. 181.

9.CHINNASAMY, R.P., SUNDARARAGAN, R., GOVINDARAJ, S., Soc. Pharm. Edu. Res., **1**, no. 3, 2010, p. 342.

10.HARINATH, Y., KUMAR, R., KUMAR, B.N., APPARAO, C.H., SESHAIAH, K., Spectrochim. Acta A. Mol. Biomol. Spectrosc., **101**, 2013, p. 264.

11.VINOKUROV, V., STAVITSKAYA, A., CHUDAKOV, Y., IVANOV, E., SHRESTHA, L.K., ARIGA, K., DARRAT, Y., LVOV, Y., Sci. Technol. Adv. Mater., **18**, no. 1, 2017, p. 147.

12.SIGEL, A., FREISINGER, E., SIGEL, R.K.O., CARVER, P.L. Met. Ions Life Sci., **19**, 2019, p. 267.

13.SINGH, D.P., KUMOR, K., SHARMA, C., Eur. J. Med. Chem., **44**, no. 8, 2009, p. 3299.

14.ALAM, M.S., CHOI, J.H., LEE, D., Bioorg. Med. Chem., **20**, 2013, p. 4103.

15.ALI, M.M., JESMIN, M., AZAD, A.K., ISLAM, K., ZAHAN, R., Asian Pac. J. Trop. Biomed., 2012, S1036-S1039.

16.MUMTAZ, A., MAHMUD, T., ELSEGOOD, M.R.J., WEAVER, G.W., Rev. Chim. (Bucharest), **69**, no. 7, 2018, p. 1678.

17.TRANDAFIR, L.M., BACIU, G., CONSTANTIN, M.M.L., MASTALERU, A., TEMNEANU, O.R., MIHAI, B., NOVAC, O., FRASINARIU, O.E., IVAN, A., TUDORACHI, N.B., AL HIARY, R., MOSCALU, M., Rev.Chim. (Bucharest), **69**, no.11, 2018, p. 3048-3051.

18.TANTARU, G., NECHIFOR, M., APOSTU, M., VIERIU, M., PANAINTE, A.D., BIBIRE, N., Rev. Chim. (Bucharest), **66**, no. 12, 2015, p. 1965.

19.DOLOCA, A., TANCULESCU, O., TRANDAFIR, L., CIONGRADI, I., STOLERIU, S., MOCANU, R., IFTENI, G., Mat. Plast., **53**, no. 3, 2016, p. 370-374.

20.BENGHIAC, A.G., BUDACU, C., MOSCALU, M., IOAN, B.G., MOLDOVANU, A., HABA, D., ROMANIAN JOURNAL OF LEGAL MEDICINE, **25**, no. 2, 2017, p. 174.

21.DOROFTEI, B., MAMBET, C., ZLEI, M., PLOS ONE, **10**, no. 4, 2015, Article Number: e0125216

22.ANTON, E., BOTNARIUC, N., ANCUTA, E., DOROFTEI, B., CIOBICA, A., ANTON, C., MEDICAL-SURGICAL JOURNAL-REVISTA MEDICO-CHIRURGICALA, **119**, no. 2, 2015, p. 410.

23.TIANG-RONG, L., ZHENG-YIN, Y., BAO-DUI, W., Chem. Pharm. Bull., **55**, no. 1, 2007, p. 26.

24.NIRMAL, R., PRAKASH, C.R., MEENAKSHI, K., SHANMUGAPANDIYAN, P., J. Young Pharm., **2**, no. 2, 2010, p. 162.

25.ABDEL-RAHMAN, L.H., ISMAIL, N.M., ISMAEL, M., ABU-DIEF, A.M., AHMED, E.A., J. Mol. Struct., **1134**, 2017, p. 851.

26.RAMAN, N., PITCHAIKANI RAJA, Y., KULANDAISAMY, A., Proc. Indian. Acad. Sci., **113**, 3, 2001, p. 183.

27.CASCAVAL, AL., RO Patent no. 105122, 31.01.1995.

28.COLLMAN, J.P., ZENG, L., BRAUMAN, J.I., Inorg. Chem. 2004, 43: 2672.

29.TANTARU, G., DORNEANU, V., STAN, M., J. Pharm. Biomed. Anal., 27, 2002, p. 827.

30.SALGA, M., ALI, H.M., ABDULLA, M.A., ABDELWAHAB, S., Int. J. Mol. Sci. **13**, 2012, p. 1393.

31.MURRAY, P.R., BARON, E.J., PFALLER, M.A., TENOVER, F.C., YOLKE, R.H., Manual of Clinical Microbiology 6th Ed. American Society of Microbiology, Washington D.C., 1995.

32.***Directive 86/609 European coalition for biomedical Research, 1986.

33.BROWN, D.F.J., BLOWERS, R., Disc method of sensitivity testing and semiquantitative methods in Laboratory methods in Antimicrobial chemotherapy. Churchill Livingstone, London, 1979.

34.***NCCLS National Committee for Clinical Laboratory Standard Document M 45-A, vol. 26, no. 19, Villanova, PA, USA, 1999.

35.***AVMA Guidelines on Euthanasia, 2007.

36.WINTER, C.A., RISLEY, E.A., WHESS, G.W., Proc. Soc. Biol. Med., **111**, no. 5, 1962, p. 44.

RAVISHANKAR, A.C., PRIYA GANDIGAWAD, N., HIREMATH, S.V., Pharmacologyonline, **3**, 2011, p. 729

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