Scientific Works. Series C. Veterinary Medicine. Vol. LIX (3)
ISSN 2065-1295, ISSN Online 2067-3663, ISSN-L 2065-1295

STUDIES ON CYTOTOXICITY AND ANTIBACTERIAL EFFECT OF ARTEMISININ

Dumitru MILITARU[1,2], Virgilia POPA[2], Daniela BOTUS[2], Beatrice STIRBU[2]

[1]Academy of Agricultural Sciences and Forestry, B-dul. Marasti Nr. 61, Sector 1, Bucharest, Romania, Postal code 011464 Phone: +40-21-3184450, +40-21-3184451; Fax: +40-21-3184478
E-mail: secretariat@asas.ro; [2]NS Pasteur Institute SA, Bucharest, Romania, Calea Giulesti 333, Sector 6, Bucharest, Romania, Postal code 060269, Phone: +40212206920, Fax: +40212206915,
E-mail: pasteur@pasteur.ro

Corresponding author E-mail: pasteurmili@yahoo.com

Abstract
Artemisinin, an extract of sesquiterpene lactone endoperoxide obtained from Artemisia annua, is routinely used in the treatment of malaria and various forms of human cancer. In order to extend its therapeutic range on animals and to set up models for testing similar extracts from other plants, studies were done on Artemisinin cytotoxicity on chicken embryo fibroblasts (CEF) in parallel with tests on Vero cells and the effect assessment on Salmonella spp strains of avian origin (4 Salmonella enteritidis, 2 S. typhimurium, 1 S. gallinarum). The cytotoxic effect was recorded for Artemisinin amounts higher than 177.10 nM on CEF and for 4.42 nM on Vero cells. In disk diffusion antibiogram the two concentrations had no antibacterial effect (inhibition diameters were of 6-9 mm).

***Keywords**: Artemisinin, CEF, Salmonella, MTT, Vero.*

INTRODUCTION

Artemisinin, discovered in 1972 and known as well as Qinghaosu, and its derivatives belong to a group of medicinal substances characterized by an efficient and rapid activity on malaria agent, *Plasmodium falciparum* (Protozoa Regnum), being included into standard therapy of the disease. Artemisinin is intensively studied / applied in various forms of human cancer, proving antitumoral and immunomodulator properties (di Felipe Avila Alcantara *et al*., 2013, Slade *et al*. 2009), and against other protozoozes as well (Dragan *et al*., 2010). Artemisinin is isolated from *Artemisia annua* L (annual absinthe wormwood), a herbaceous plant used in traditional Chinese medicine. The plant grows in temperate climates in both hemispheres of the globe, in dry or semi-arid habitats.

The genus Artemisia includes plants better known in terms of culinary and medicinal, tarragon, *Artemisia dracunculus*, wormwood, *Artemisia absinthium* (used as insecticide against mites and fleas), and green ginger or green tarragon, *Artemisia pontica*. The most species of the genus Artemisia are characterized by strong flavors and bitter taste due to terpenoids and sesquiterpene - lactones content, which removes herbivores, and probably bring a selective advantage.

From a chemical point of view, Artemisinin belongs to endoperoxide sesquiterpene-lactone group, being a secondary metabolite of the plant, and contains a special peroxide group (rather unstable), on which its mechanism of action is based. Although the mechanism of action is still not well described and accepted by the scientific community, it seems like Artemisinin, at least in the case of *Plasmodium falciparum*, disturbs redox homeostasis by inducing the appearance of free radicals, targeting cellular SERCA pump or by depolarization of mitochondrial membrane, but not inhibiting electron transport and respiration, and without action on mitochondria of mammalian host erythrocyt (Meshnick 2002, Slade *et al*. 2009).

In order to extend the therapeutic range of Artemisinin in animals and build a model for testing similar extracts obtained from other plants / related plants, within this study there were carried out experiments regarding the cytotoxicity of Artemisinin on chicken embryo fibroblasts (CEF) in parallel with tests on Vero cells, and assessed its effect on avian *Salmonella* spp strains.

MATERIALS AND METHODS

Artemisinin is poorly soluble in aqueous solutions, but soluble in organic solvents: 0.5 mg / mL in DMF (dimethyl formamide) and 100 mM in DMSO; the stock solutions were of 0.5 mg / ml, both in DMF and in DMSO (except for pre-experiments in which the stock solutions were of 100 mM in DMSO). Also, Artemisinin is unstable in aqueous solutions, and therefore the solutions tested were prepared on the day of each experiment.

The cytotoxicity tests were carried out on monolayers of chicken embryo fibroblasts (20,000 and 10,000 cells / ml), and Vero cells (10,000 cells / ml). The CEF monolayer was obtained from SPF chicken embryos of 9 – 10 days old. The Vero cell line (ATCC CCL-81), form I.Pasteur collection, p/10.2006/IP, was used to perform experiments on 6 passages, grown in MEM medium supplemented with 10% fetal bovine serum. The cell count was performed with Fuchs-Rosenthal counting chamber. The grown conditions were 37° C, 5% CO2, in 75cm2 tissue culture plates (Greiner 658 175) and 96-well plates (Linbro 76-008-04). The dissociation of the cells or cell detachment from the substrate was carried out with 0.25% trypsin solution in 0.02%EDTA, 0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.058% Na-bicarbonate and 0.002 ‰ phenol red. Artemisinin (dissolved in DMSO, DMF respectively, mixed with PBS or MEM) was placed in contact with the cells for 15 minutes and 1 hour, in concentrations of 177.10, 17.71, 8.85 and 4.43 nM. Evaluation of cytotoxic effect was performed by MTT assay, reading at 540 nm at intervals of 15 minutes, 1, 2 and 21 hours post-treatment. The average of absorbance of the control (cells untreated with Artemisinin) was regarded as 100% and the percentage of cells growth in wells treated with Artemisinin was calculated (Chiba *et al.* 1998). The antibacterial effect was tested by Kirby - Bauer disk diffusion assay, on 4 strains of *Salmonella enteritidis*, 2 of *S. typhimurium* and 1 strain of *S. gallinarum* of avian origin, in concentrations of 80 and 160 ug Artemisinin/ disk (Goswami *et al*., 2012), dissolved in the two organic solvents (DMSO and DMF).

The *Salmonella* spp strains from the I.Pasteur collection were isolated during 1994 - 1999 from industrial flocks in Romania, with one exception (one *S. enteritidis* received from the company Alltech, USA). The Kirby - Bauer test was conducted according to the 2010 recommendation of Antibiogram Committee of the French Microbiology Society. Along with Artemisinin disks, there were tested (to check the test and strains) amoxicillin (25ug), gentamicin (10ug) and enrofloxacin (5ug).

RESULTS AND DISCUSSION

The results obtained on the two types of cell culture regarding the cytotoxicity of Artemisinin are synthesized in Tables 1 and 2. The results from the tests on the anti-*Salmonella* effect of Artemisinin are shown in Table 3 and Figure 1.

Table 1. The evaluation of Artemisinin cytotoxicity on CEF monolayer by MTT assay.

	Artemisinin : DMSO				Artemisinin : DMF				Control CEF
	177.09 nM	17.70 nM	8.85 M	4.42 M	177.09 nM	17.70 nM	8.85nM	4.42 nM	
15'	101.00	118.40	111.99	128.21	105.49	116.96	140.86	134.24	100
1h	176.98	175.51	144.97	141.63	116.85	137.08	127.40	131.29	100
2h	135.65	174.51	104.10	93.58	106.51	112.30	116.76	110.52	100

Table 2. The evaluation of Artemisinin cytotoxicity on Vero monolayer, by MTT assay.

	Artemisinin : DMSO				Artemisinin : DMF				Control Vero
	177.09 nM	17.70 nM	8.85 nM	4.42 nM	177.09 nM	17.70 nM	8.85 nM	4.42 nM	
15'	73.88	86.46	95.23	103.90	77.29	118.30	133.20	104.02	100
1h	95.63	93.89	97.72	86.00	99.15	142.11	131.76	120.14	100
2h	80.21	111.60	85.54	129.41	118.75	109.37	105.38	120.27	100
20h	71.15	86.74	64.10	78.04	69.20	73.19	108.88	84.23	100

Table 3. The evaluation of antibacterial effect of Artemisinin on avian *Salmonella* spp. , by disk diffusion assay.

Crt. no.	Bacterial strain	AMOXICILLIN 25ug	GENTAMICIN 10ug	ENROFLOXACIN 5ug	ARTEMISININ:DMSO		ARTEMISININ:DMF	
					160ug	80ug	160ug	80ug
1.	S.typhimurium 179	30	19	27	6	6	6	6
2.	S.enteritidis 2488	32	25	27-31	6	6-7	6	6
3.	S. typhimurium 504	32	21	28-33	6-8	6	6	6
4.	S.enteritidis 13a	32	21	24-28	6-8	6-7	6	6
5.	S.gallinarum. 91	39	28	32	6	6-9	6	6
6.	S.enteritidis. 288	6.5	21-28	24-30	6-8	6	6	6
7.	S.enteritidis. 290	28-32	20	25-29	6	6	6	6

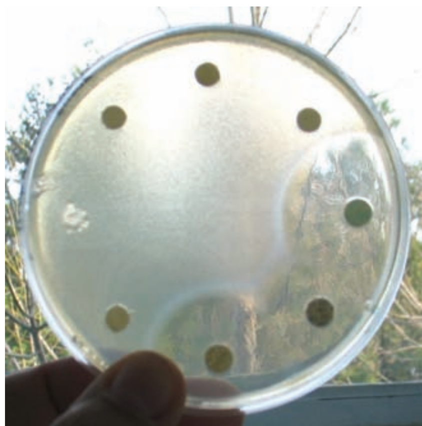

Figure 1. Evaluation of antibacterial effect of Artemisinin on avian *Salmonella* spp strains by disk diffusion assay (biodisk with Artemisinin 80ug and 160 ug, amoxicillin 25 ug, gentamicin 10 ug and enrofloxacin 5 ug).

On CEF monolayer, there were noticed differences due to the solvents used, for the same amount of Artemisinin. The cytotoxic effect on CEF was recorded at concentrations of Artemisinin higher than 177.10 nM.

The cytotoxic effect was recorded on Vero cells including concentration of 4.43 nM Artemisinin, regardless of the organic solvent used. Therefore, the mammalian cell monolayer seemed to be more sensitive to Artemisinin.

In disk diffusion antibiogram the two concentrations had no antibacterial effect against avian *Salmonella* spp strains (inhibition diameters were of 6-9 mm), results which are in agreement with those published by others authors (Slade *et al*. 2009), although there are studies with positive results against *Salmonella* spp strains of different origins (Appalasami *et al*., 2014).

CONCLUSIONS

Following this studies, there was made an experimental model for testing the cytotoxicity of extracts artemisinin type, as shown by MTT assay on chicken embryo fibroblasts and Vero cell monolayers (10,000 cells / well, readings at 15', 1h, 3h and 20h at 540 nm, Artemisinin stock solutions in DMSO and DMF, final concentrations of 177,097nM, 17,7097nM, 8,8548nM and 4,4274nM).

Differences were found between the two cell substrates tested in terms of Artemisinin cytotoxicity (Vero cell line proved to be about 40 times more sensitive than chicken embryos fibroblasts).

Within Kirby-Bauer assay, Artemisinin in concentration of 80 ug and 160 ug / disk has proven no anti-bacterial effect against seven avian strains of *Salmonella enteritidis*, *S. typhimurium* and *S. gallinarum*.

AKNOWLEDGEMENTS

Studies funded by the MNE by the Ctr. 110/2012 - PN-II-PT-PCCA-0274-2011-3.2: Development of a prevention strategy based on the use of Artemisia annua in coccidiosis of broilers (ARTCOC)

REFERENCES

Appalasami S., Lo KY, Ch'ng SJ., Nornadia K., Othman AS., Chan LK., 2014. Antimicrobial activity of artemisinin and precursor derived from in vitro plantlets of Artemisia annua L.. BioMed Research International Volume 2014, Article ID 215872, 6 pages, http://dx.doi.org/10.1155/2014/215872

Chiba K., Kawakami K., Tohyama K., 1998. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cell. Toxicology *in vitro*, 12, 251 – 258.

Commite de l'Antibiograme de la Societe Francaise de Microbiologie (CA-SFM) 2010, Recommandations 2010

di Felipe Avila Alcantara D., Ferreira Ribeiro H., Cerqueira dos Santos Cordoso P., Maira Thomaz Araujo T., Rodriguez Burbano R., Costa Guimaraes A., Salim Khayat A., de Oliveira Bahia M., 2013. *In vitro* evaluation of the cytotoxic and genotixic effects of artemether, an antimalarial drug, in a gastric cancer cell line (PG100). Journal of Applied Toxicology, 33, 151 – 156.

Dragan L., Titilincu A., Dan I., Dunca I., Dragan M., Mircean V., 2010. Effects of *Artemisia annua* and *Pimpinella anisum* on *Eimeria tenella* (Phylum Apicomplexa) low infection in chickens. Sci. Parazitol., 11 (2), 77-82.

Goswami S., Bhakuni R.S., Chinniah A., Pal A., Kar S.K., Das P.K., 2012. Anti-*Helicobacter pylori* potential of artemisinin and its derivatives, Antimicrobial Agents and Chemotherapy, 56(9), 4594-4607

Meshnick SR., 2002. Artemisinin: mechanisms of action, resistance and toxicity. International Journal for Parasitology, 32, 1655–1660

Slade D., Galal AM., Gul W., Radwan MM., Ahmed SA, Khan SI., Tekwani BL., Jacob MR, Ross SA., ElSohly MA. 2009. Antiprotozoal, anticancer and antimicrobial activities of dihydroartemisinin acetal dimers and monomers. Bioorganic & Medicinal Chemistry 17 (23), 7949-7957