WHY FURTHER INVESTIGATION OF BRAZILIAN STRYCHNOS? (*)

L. Angenot (**)

SUMMARY

During the past fifteen years, African ***Strychnos*** *species have been pharmacologically and chemically screened by some European Universities, sometimes with the collaboration of some African Institutes.* ***Strychnos usambarensis*** *will be discussed here because of our interest in it, after the discovery of its use in the preparation of an arrow poison with curarizing activity.*

The European studies have shown that the African species of ***Strychnos*** *chemically are very similar to certain South-American species. Indeed, the occurrence in the African species of curarizing quaternary bases, and in the American species of tetanizing tertiary bases, such as akagerine, emphasizes the unity of genus* ***Strychnos*** *in terms of its alkaloids. Many biological activities (antimicrobial, hypotensive, spasmolytic, amoebicidal...) have been detected.*

Finally, the antimitotic properties of some alkaloids will be presented. Besides known antitumor drugs, e.g. alstonine and ellipticine, African species possess new antimitotic products, e.g. strychnopentamine present in ***Strychnos usambarensis*** *.*

Because of these findings, the main reasons why further investigate Brazilian ***Strychnos*** *species are the lack of knowledge about:*

- *the distribution of the species in the southern part of the great Amazon Basin. According to Krukoff, it is possible that new species may be found there.*
- *the distribution of alkaloids in many of known species. Indeed, there is little information on the variation in composition of the alkaloidal mixtures in the different parts of the plants.*
- *the biological activities of alkaloids. In the past, interest has been focused too narrowly on possible curarizing properties.*

Why should Brazilian species not contain alkaloids with properties similar to those found in the African species?

(*) Lecture given at the VIIIth Symposium of Medicinal Plants of Brazil - Manaus - September 1984.

(**) Laboratoire de Pharmacognosie et de Chimie structurale organique, Institut de Pharmacie - Faculté de Médecine de l'Université de Liège - Rue Fusch, 5 - B-4000 LIEGE (Belgica).

INTRODUCTION

The genus **Strychnos** is pantropical and comprises about 200 species, which may be subdivided into three geographically separated groups: one in Asia with 44 species, one in America with about 70 species and one in African with 75 species.

Although investigations into the genus **Strychnos** have been going on for a long time, the African members suffered a long period of neglect. The first systematic studies were undertaken in Liege by the late Professor Denoël on materials from the Belgian Congo (Zaire) (Denoël **et al.**, 1953). Other works include the taxonomic revision of the genus by Leeuwenberg (1969), the ethnobotany (Bisset, 1970) and a preliminary screening for alkaloids by Bisset and Phillipson (1971). During the past fifteen years, 48 African species have been screened by the Swedish University of Uppsala for muscle-relaxant and/or convulsant activity. In recent years, other chemical and biological studies have been undertaken in many Universities of Europe (Great-Britain, France, Italy, Sweden, Netherlands and Belgium). A review of the phytochemistry of African **Strychnos** species and their biological activities has been recently presented in the **Journal of Ethnopharmacology** (Ohiri **et al.**, 1983).

STUDY OF AFRICAN LOGANIACEAE AT THE UNIVERSITY OF LIEGE

The research programme of the University of Liege on **Strychnos** alkaloids is also an offshoot of an inventory of medicinal and toxic plants in the Eastern part of Rwanda carried out during the years 1969-70 (Angenot, 1970).

Among the toxic plants, those species which are ingredients of arrow poisons, are really worth studying. An intriguing account of a hunter named Kahijama, gamekeeper in the National Park of Akagera became a special inspiration to the initiation of our programme, Kahijama belongs to the tribe of Banyambo: it is a small tribe living along the Akagera river on the border between Rwanda and Tanzania. This tribe prepared in front of us an arrow poison (Angenot, 1971).

The main ingredients of this previously unknown poison were **S.usambarensis** leaves and roots, we had first examined.

So the objectives of our programme on **Strychnos** were: the increase of the limited knowledge of the African species, mainly a chemo-and pharmaco-taxonomic evaluation and the discovery of some pharmacologically active compounds.

The most active ingredients of **Strychnos** are indole alkaloids. They belong to the so-called Corynanthe/**Strychnos** type of terpenoids indole alkaloids. Condensation of tryptamine and secologanin units leads to gluco-alkaloids from which the various types of indole alkaloids are obtained by further reactions.

Let us examine in details **Strychnos usambarensis**. It is a climbing shrub in West Africa, while East and South African specimens are small trees.

The root barks, leaves and fruits of **S. usambarensis** collected in Rwanda and Tanzania, have been examined about tertiary and quaternary alkaloids.

Up to now, using various chromatographic methods including PTLC, LC and DCCC, we have

isolated twenty-one alkaloids from root barks. These alkaloids were divided into three groups according to their polarity and the characteristics of their amine functions. The identification and the structural determination of these compounds were achieved by spectroscopy and sometimes X Ray analysis.

The first group contains tertiary alkaloids. Apart from harman which was found for the first time in **Loganiaceae**, we have isolated three new alkaloids.

Two of them (usambarensine and dihydro-derivative) are bis-indole type biogenetically derived from two tryptamine and one mono-terpene units as some alkaloids of **Apocynaceae (Ochrosia)** and **Rubiaceae (Uncaria)**, two families very close to **Loganiaceae** from a chemotaxonomy point of view. They are unsymmetrical dimers. Their structure and configuration were proved either by X-Ray analysis for usambarensine (Fig. 1) or by synthesis for dihydro-usambarensine (Fig. 2).

Usambarensine offers atropine-like and spasmolytic activities (on the smooth muscles). Dihydro-usambarensine has amoebicide properties.

The third alkaloid of the first group is also new; akagerine(Fig.3) is a tetracyclic indole alkaloid possessing a perhydro-azepine ring coupled to tetrahydro-β carboline,by an original N_1 -C_{17} bond (Angenot **et al.**, 1975). Akagerine should be a precursor of some sophisticated alkaloids (bis-indole and heptacyclic) that would be obtained by reaction with tryptamine. Akagerine and derivatives have been recently recovered in some African and South American species studied by the Universities of Uppsala (Sweden) and Roma (Italy) (Marini-Bettolo **et al.**, 1980). Akagerine has exhibited tetanizing properties, but is a hundred times less active than strychnine.

The second group is represented by an anhydronium base (6,7-dihydro-flavopereirine) and mainly by two substances containing simultaneously a quaternary ammonium and a tertiary amine function. They are thus hybrid alkaloids with particular properties of solubility and extraction. Indeed, these products remain between the aqueous phase and the organic phase during the extraction procedure.

The last group contains quaternary ammonium compounds Monomeric alkaloids include: melinonine F (Caprasse **et al.**, 1983a), fluorocurarine (Fig. 4) (Caprasse **et al.**,1981b)and macusine B, three products formerly isolated from American Strychnos and curare. Macusine B (Fig. 5) has been shown to possess adrenolytic properties. These last years, we have also obtained N_b-methyl antirrhine previously found in **S. camptoneura** and malindine(Fig. 6) first isolated from **S. decussata** (Caprasse **et al.**, 1948a). Three new derivatives O-methyl-macusine B, O-methyl-19, 20 dihydro-macusine B and isomalindine were still detected in this group.

Dimeric quaternary compounds belong mainly to **Strychnos** type. The symmetrical dimers are very active products. Three of them (curarine, calebassine, dihydrotoxiferine) (Fig. 7) were previously isolated from Calabash-Curare and some South American Strychnos. They are found and isolated for the first time in a **Strychnos** species growing outside of America. These diquaternary ammonium compounds offer interesting and potent neuromuscular blocking properties, and we may conclude that the lethal action of the arrow poison prepared by the Banyambo hunters is certainly due to the curarizing properties of

these products (Angenot **et al.**, 1975a).

The last dimeric compound is a new product that we have called afrocurarine (Fig. 8). It is an unsymmetrical bisindole alkaloid less curarizing than the *symmetrical* dimers (Caprasse **et al.**, 1984b).

The leaves of the same Strychnos were therefore studied. *They contain* sixteen alkaloids, that are all bisindole alkaloids of Corynanthe type and different from these present in the roots. They are new and possess *the usambarane* skeleton but with a shift of the double bond from C_{19} -C_{20} to C_{18} -C_{19} or by lacking of this bond. (Caprasse **et al.**, 1983b). Moreover, many are *phenolic and amongst* them, strychnopentamine and isomers are curious alkaloids with five nitrogen atoms; a methylpyrrolidine group is joined to the benzene *ring of the Corynane* part of the molecules. Oxindole alkaloids (strychnofoline, strychnophylline and isomers) are also found for the first time in **Strychnos** genus.

Fruits of **S. usambarensis** such as cherries have caused the poisoning of children in Africa. We have then isolated their main alkaloid and identified it as descarbomethoxydihydrogambirtannine; that alkaloid had been never found in the family of **Loganiaceae** but was previously discovered in Ochrosia genus (**Apocynaceae**).

Among the other **Strychnos** recently studied at Liege, we will hold the attention on:

- **S. variabilis** (small tree endemic around Kinshasa in Zaire). The golden fruit pulp is deliciously sweet but the root bark is a *violent poison from which* twenty alkaloids have been isolated and especially two pairs of diastereomeric equilibrating alkaloids that could be the biogenetic *starting point of two* series of mono-and dimeric alkaloids which have a different stereochemistry for C_{16}: the retuline series and the epimer isoretuline series (Tits **et al.**, 1980). *These* alkaloids still contain a new group of **Strychnos** unsymmetrical dimers: the strychnobilines, characterized by a carbinolamine ether group *in a hexacyclic ring* similar to that of geissospermine (Tits **et al.**, 1983a, b). Moreover, the quaternary alkaloids - mavacurine and fluorocurine - (Fig. 9) have been *extracted from* this African species (Tits **et al.**, 1981).

- **S. icaja** used as ordeal and arrow poison in the North East of Zaire and in Gaboon. The root barks *sometimes contain* up to fifteen percent of alkaloids, out of which strychnine is the main constituent but again we have found a new dimeric product that we *have called sungucine* (Lamotte **et al.**, 1979).

- **S. gossweileri** very rich in a new glucoalkaloid:dolichantoside and in quaternary alkaloids (*diploceline (Fig.* 10) *with* antimicrobial activity) and anhydronium bases like alstonine and a new one: strychnoxanthine (Coune **et al.**, 1984).

- **S. scheffleri** from Zaire *with alkaloids related* to the tabascanine and spermostrychnine type bases, already known from the American **S. brasiliensis** and **S. tabascana** (Caprasse and Angenot, 1981*a*). $N_{(1)}$-acetyl-O-methylstrychnosplendine, main alkaloid in the leaves, was now found to have strong muscle-relaxant activity (Weeratunga **et al.**, 1984).

During our phytochemical screening on medicinal plants of Central Africa, we have

obtained samples of an other species of **Loganiaceae**; **Anthocleista grandiflora** is a tree (5-35 m high) unarmed and called "cabbage-tree" or "fever-tree" because used against fever. This species never contains alkaloids but always secoiridoid glycosides. The main constituent of the bark is sweroside, whose febrifuge activity was also performed during this study (Chapelle, 1976). More recently, an anti-amoebic activity has been demonstrated for this compound (Van Beek **et al.**, 1984). According to these results, we think that the biological activity of the iridoids related to loganin and quite common in the **Strychnos** as they take part in the biogenesis of alkaloids, has to be investigated in the future.

REASONS FOR THE STUDY OF **STRYCHNOS** IN THE FIELD OF TUMOR INHIBITORS

It seems that two fundamental approaches can be followed to identify plants having these properties:

Random selection screening

It is the method mainly practised by National Cancer Institute (U.S.A.). According to Suffness and Douros (1982), NCI screens about 10,000 new synthetic compounds and 400 pure natural products per year plus about 14,000 crude natural extracts (8,000 from fermentation; 5,000 from plant and 1,000 from marine animal). About 6-8 compounds enter clinical trials each year. Slighty less than half of them are natural products. The cost of bioassay procedures becomes greater with increasing number of plant parts tested, but this program offers an advantage in that it is providing some new structures — previously unassociated with tumor inhibiting activity —, that serve as model for synthesis of derivatives with could be more active, and hopefully have fewer side-effects, than the parent natural compound (Farnsworth and Kaas, 1981).

Screening of medicinal and toxic plants

The approach of testing plants with an account of use for any condition in traditional medicine has been most successful up to now and at a lower cost. One recent study describes an attempt to correlate results obtained from the NCI plant antitumor screening program with selected types of folkloric uses for the same plants.

From these data, it would appear that one would increase by a factor of two the number of plants species that would show experimental **in vitro** or **in vivo** cytotoxicity or antitumor activity if the plants were selected on the basis of alleged use as anticancer remedies, and by a factor of about five, for the plants alleged to be useful as arrow poisons (Farnsworth and Kaas, 1981). Now, the genus **Strychnos** is well known in the world as well as an arrow and dart poison (curare in South America, Ipoh in Asia, Banyambo's gift in Africa, as an ordeal poison (M'Boundou in Africa...).

Moreover, we have considered the published experimental data indicating antitumor activity for crude extracts from **Strychnos**:

- the alkaloidal extracts of **S. henningsii** were shown to be active against cancer cells (Hokanson, 1975).

- the aqueous extracts of **S. elaeocarpa** and **S. lucens** stem bark, and the chloroform extract of **S. usambarensis** leaves have all been shown to be active against lymphatic

leukemia P-388 in vivo in mice (personnal communication of the late M. Kupchan to W. Rolfsen (1980).

Since 1981 we have carried out experiments about cytotoxic and antitumor properties of alkaloids isolated from **Strychnos usambarensis**, since this species is used as arrow poison and has shown an antitumor activity from a crude extract.

METHODS

Cytotoxicity

Antimitotic activity tests have been carried out on cultured:

- hepatoma cells derived from HW 165 hepatoma of Wistar rats;
- B 16 melanoma cells derived from C 57 Bl mouse melanoma;
- Ehrlich ascites cells (line ELT) derived from a mouse mammary gland carcinoma and transplanted into C 57 Bl mouse peritoneal cavity.

The cells are cultured in a liquid nutrient (MEM Gibco medium 90% complemented with 10% foetal calf serum) and 100 U/ml penicillin. The agent to be tested is added to the culture medium at various concentrations and for a maximun of 48 h.

After the treatment, the cells are fixed and stained by Feulgen reaction for cytological analysis (light microscopy). Mitotic activity or degree of cell death is expressed as mitotic or pycnotic index (number of cells in mitosis of pycnosis for 1,000 cells). In each case, 5 to 10,000 cells are analysed. The percentages of mitotic phases are calculated in view of detecting possible mitotic disturbances. Mean index are compared with Student t test.

Antitumor activity tests "in vivo"

C 57 Bl 6J male mice were injected i.p. with 10^6 Ehrlich ascites cells. The tumor was allowed to grow for 4 days and at that time, the mice received the drug i.p. either in a single shot or in repeated injections at a day interval for 3 days. Various dosages of the drug were used. Mice were sacrificed by exsanguination. Peritoneal washings were performed to remove all tumor cells and cell count was done with hemocytometer or Coulter counter model B. Tests were controlled each time on 10 mice.

FIRST EXPERIMENTS ON ALKALOIDS ISOLATED FROM **S. USAMBARENSIS**

- Selection based on structure-antimitotic activity relationships:

a) Two alkaloids of the roots (melinonine F and dihydroflavopereirine) possess a tricyclic β carbolinium ring and are very close to pentacyclic anhydronium bases such as alstonine, serpentine (Fig. 11) and sempervirine whose patent as antitumor drug is pending (Beljanski and Bugiel, 1979). Another representative compounds of this family of planar alkaloids are ellipticine (Fig. 12) -found not only in **Ochrosia sp. (Apocynaceae)** but also in **Strychnos dinklagei** (Michel **et al.**, 1980) -and mainly 9-hydroxy-2 methylellipticinium (Fig. 13). This quaternary compound is less toxic than ellipticine

and used as anticancer drug in Europe.

Quaternary ammonium salts of anhydronium bases thus seem attractive candidates as potential agents. Various cytotoxic effects have been detected by cytological methods and compared to sempervirine (Table 1) (Bassleer **et al.**, 1982, 1983 and 1984).

Concomitantly with tests of biological activity, it appeared useful to investigate the interaction of these products with DNA, which has most frequently been found as the main target of many similar drugs in the cell. The differences between the behaviours of the three alkaloids so far investigated can be interpreted on the basis of different extent of penetration of the chromophore ring into the DNA helix (Caprasse and Houssier, 1983 and 1984).

b) Three alkaloids of the leaves (strychnofoline, dihydro-usambarine and strychnopentamine) possessing the usambarane skeleton were first tested. They are structurally similar to emetine (Fig. 14) and may be regarded as indole analogues. Emetine acts by means of protein synthesis inhibition and has been evaluated clinically as an antimitotic drug. The structural similarities prompted us to evaluate the cytotoxic properties of these alkaloids. Strychnofoline and dihydro-usambarine showed a certain degree of antimitotic activity at relatively high doses [10 µg/ml and mainly 50 µ/ml (Bassleer **et al.**, 1982)]. Strychnopentamine is about ten times more powerful than strychnofoline and 18,19 dihydro-usambarine as an antimitotic agent in animal tumor cell cultures, since it inhibits 50% of the cells in mitosis at a concentration of 1 µg/ml (1.8 µm). The comparison of their molecular structures induces us to think that the presence of a N-methyl-pyrrolidine group increases the antimitotic activity of these alkaloids (Table 2) (Tits **et al.**, 1984).

Considering this antimitotic activity, we have undertaken preliminary experiments to search for a possible antitumor activity of this molecule in the animal. Our experiments have been carried out on Ehrlich ascites tumor cells in male mice (Tits **et al.**, 1984). When they received strychnopentamine in a single injection of 1 mg, there was a medium decrease of cells number (about 50%). At a single dose of 2mg, a very strong decrease of tumor cell number was observed (more than 80%). The very rapid regression is obvious, but mice rapidly show signs of intoxication, since four mice out of ten died very quickly after the injection. In the coming future, similar tests will also be carried out with isomers of strychnopentamine and other derived alkaloids at various dosages in view of establishing a closer structure-activity relationships, and to look for less toxic compounds.

WHY FURTHER INVESTIGATION OF BRAZILIAN **STRYCHNOS**?

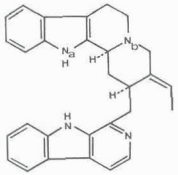
The main reasons why we should further investigate Brazilian **Strychnos** species are:

- the lack of knowledge concerning the distribution of alkaloids among many of them (Marini-Bettolo and Bisset, 1972; Bisset, 1977). Moreover, according to Krukoff, the distribution of species in Southern part of the great Amazon Basin — particularly from the upper Rio Xingu and Rios Tapajós and Madeira — is poorly known and it is possible

that new species may be found there (Krukoff, 1972). Indeed, there is little information available not only on the variation in composition of the alkaloidal mixture in the different parts of the plants (e.g. the leaves), but also on their biological activities too focused in the past on curarizing activity.

- the new results in the antimitotic field from African species of **Strychnos**, which chemically are very similar to certain South American species. Indeed, the occurrence in the African species of quaternary bases and in the American species of tertiary bases such as akagerine emphasizes the unity of the genus **Strychnos** in terms of its alkaloids. Besides known antitumor drugs, e.g. alstonine and ellipticine, African **Strychnos** species possess mainly new antimitotic products, e.g. strychnopentamine. Why should Brazilian species not contain alkaloids with similar properties? As far as I am concerned, I would be grateful to anyone who would accept to cooperate in this field.

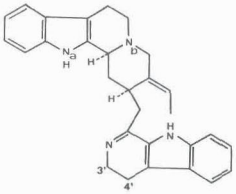
According to many results, it is a clear that research into arrow poisons and their ingredients remains closely connected with current activities in the never-ending search for new and better drugs, both naturally-occurring and synthetic. Natural products have previously been of great benefit in providing molecular structures for study by organic chemists and as models for study by pharmacologists. It is a part they still have to play at our present time.



USAMBARENSINE

Spasmolytic activity

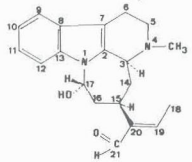
FIG. 1. Usambarensine.



DIHYDRO-USAMBARENSINE

Cytotoxic and amoebicide activities

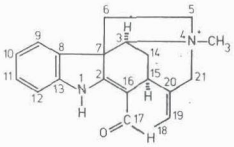
FIG. 2. Dihydro-usambarensine.



AKAGERINE

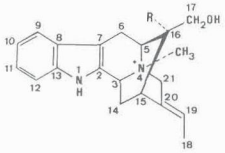
Convulsant activity (50 mg/Kg)

FIG. 3. Akagerine.



FLUOROCURARINE

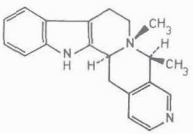
FIG. 4. Fluorocurarine.



MACUSINE B

Hypotensive activity

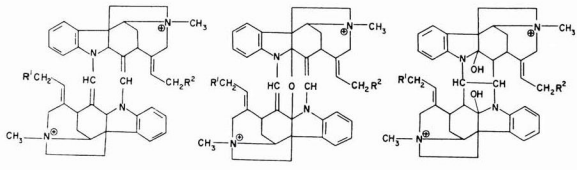
FIG. 5. Macusine B.



MALINDINE

Muscle-relaxant effect (75 mg/Kg)
not antagonized by neostigmine

FIG. 6. Malindine.



Very strong curare activity (1-150 µg/Kg)

FIG. 7. C-dihydrotoxiferine C-curarine and C-calebassine.

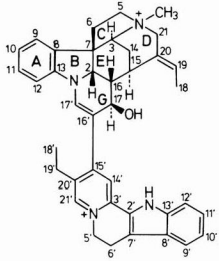


FIG. 8. Afrocurarine.

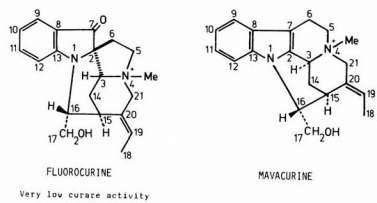


FIG. 9. Fluorocurine and Mavacurine.

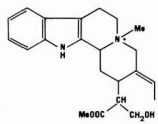


FIG. 10. Diploceline.

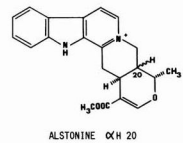
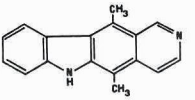


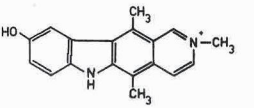
FIG. 11. Alstonine and Serpentine.



ELLIPTICINE

Antitumor and mutagenic activities

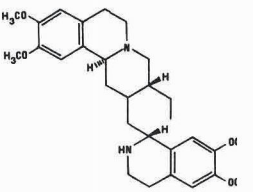
FIG. 12. Ellipticine



9-HYDROXY 2-METHYL ELLIPTICINIUM

Anticancer agent
(not mutagenic)

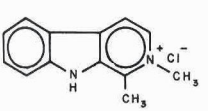
FIG. 13. 9-hydroxy-2-methylelliptic̆inium

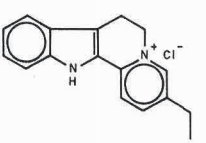


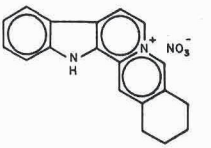
EMETINE

Antiamoebic and antitumor activities

FIG. 14. Emetine

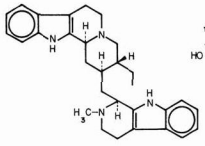


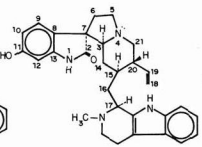


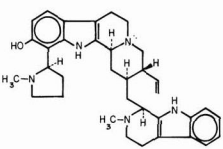


	Melinonine F	Dihydroflavopereirine	Sempervirine
a On B 16 Melanoma	After 72 h	After 24 h	After 72 h
	ED_{50} > 50 µg/ml <100 µg/ml	$ED_{50} \simeq$ 50 µg/ml	$ED_{50} \geq$ 10 µg/ml
b On Ascites Ehrlich cells	ED_{50} < 50 µg/ml	$ED_{50} \simeq$ 50 µg/ml (many pycnotic cells)	.....

Table 1







	18,19 dihydro-usambarine	Strychnofoline	Strychnopentamine
a **On B 16 Melanoma**	After 72 hours	After 72 hours	After 24 hours
	ED_{50} > 10 µg/ml	ED_{50} > 10 µg/ml	ED_{50} ≃ 1 µg/ml
	< 50 µg/ml	< 50 µg/ml	
b **On Ascites**	After 24 hours	After 24 hours	After 24 hours
Ehrlich cells	ED_{50} ≃ 10 µg/ml	ED_{50} ≃ 10 µg/ml	ED_{50} ≃ 3 µg/ml
c On HW 165 Hepatoma	.....	After 72 hours	After 24 hours
		ED_{50} > 10 µg/ml	ED_{50} ≃ 3 µg/ml
		< 50 µg/ml	< 1 µg/ml

Table 2

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