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Evaluation of Biological Activity of *Uncaria tomentosa* (Willd.) DC. Using the Chicken Embryo Model*

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The biological activity of *Uncaria tomentosa* (Willd.) DC. (cat's claw) was evaluated by application of the chicken embryo model. Among three groups of eggs (n=360) with twelve-day old embryos, two were injected with different doses of cat's claw extracts (0.0492 and 0.492 mg/200 λ). To the third control group 200 λ of physiological salt was applied. All eggs were incubated in conventional forced-air apparatus until hatched. Hatchability, chicken weight and wholesomeness were analyzed. Selected parameters of blood including number of erythrocytes (RBC), number of leukocytes (WBC), mean red cell volume (MCV), hematocrit (HCT), hemoglobin concentration (HGB), mean amount of cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and embryo weight (MAS) were assayed and compared. Significant differences with ANOVA were observed for MCV (P=0.002), MCHC (P=0.00001) and MCH (P=0.02). Applying the chicken embryo model brought new information about the biological activity of *U. tomentosa* showing an unfavourable effect on some morphological blood parameters.

Key words: *Uncaria tomentosa*, cat's claw, una de gato, chicken embryo model.

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Uncaria tomentosa, commonly known as cat's claw or una de gato, is a wooded vine from the Amazon River basin in botanical nomenclature classified into the *Rubiaceae* family and *Cinchonoideae* subfamily (REINHARD 1999). For over two thousand years, many Amazonian tribes such as Asháninka, Aguaruna, Cashibo and Shipibo have believed that this species possesses magical and amazing healing powers (KEPLINGER *et al.* 1999). In Peru, Colombia, Guiana, Venezuela and Ecuador, decoctions from *U. tomentosa* have been traditionally used in treatment of several diseases including gastric ulcers, diarrhea, gonorrhea, arthritis, rheumatism, acne, diabetes and cancer (TAYLOR 2002; HEITZMAN *et al.* 2005). Generally, in these countries cat's claw is known as a contraceptive, anti-inflammatory and anticancer remedy. Despite its popularity in South America, cat's claw is still unappreciated in Western Medicine, however, in recent years several pharmacognosy investigations have been conducted. The biologically active constituents have been isolated (PHILLIPSON 1978; AQUINO *et al.* 1991; LAUS 1996; LAUS 1998; KEPLINGER *et al.* 1999) and their cytostatic, contraceptive, phagocytosis-stimulating, antiviral, antiedemal, antioxidant, antimutagenic and CNS-regulating properties extensively described (FALKIEWICZ & ŁUKASIAK 2001; BACHER *et al.* 2006; GARCIA PRADO *et al.* 2007; PILARSKI *et al.* 2007). Unfortunately, the majority of these studies have been carried out under simplified, artificial conditions and a significant part of the obtained results and the formulated theses have not been further confirmed on living organisms. Taking into account considerable limitations of such studies, we have applied the chicken embryo model for preliminary evaluation of the biological activity of *U. tomentosa*.

The employment of the chicken embryo model for bioactivity investigation on *U. tomentosa* is a very attractive methodological approach and may be recommended for other pharmacognosy studies

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under physiological conditions. Firstly, because of the sensitivity of this model, it is very useful for conducting any pharmacological assay. The presence of even the smallest quantity of any active substance apparently influences embryogenesis because the fast-dividing, non-differentiated cells possess little ability to maintain their homeostasis. Secondly, there is less risk of increment of variation in the obtained results by unknown external factors because all embryos develop under highly similar conditions (each egg is an autonomous organismal system with a significant limitation of energy and matter flow since the egg-shell is a sufficiently tight barrier). Finally, the chicken embryo model gives an investigator the possibility of administration of a precise dose of the preparation in a way similar to the most common administration of cat's claw.

Material and Methods

Plant preparation and its chemical characteristics

U. tomentosa bark originated from the Instituto Peruano de Investigacion Fitoterapica Andina (IPIFA) Lima, Peru and was supplied by Vilcacora Łomianki Centre, Warsaw, Poland. The characteristics of the bark are showed in Table 1. The preparation used in the studies was prepared in the following manner: 10 g of bark was extracted in 50 ml of water for 12 h in 37°C. After this time the sample was centrifuged at 4000 rpm and the supernatant was evaporated under vacuum to dry mass.

The oxindole alkaloid profile was determined as follows. To 100 mg of the preparation, 15 ml 2% sulphuric acid solution was added and sonified for 15 minutes in an ultrasonic bath (Bandelin Sonorex RK 103H). The mixture was then centrifuged at 3000 rpm for 10 minutes and extracted three times with 10 ml ethylacetate. The aqueous phase was separated and adjusted to pH 10 with 10% NH_4OH and then extracted three times with 10 ml of ethylacetate each. The organic extracts were combined, evaporated to dryness and the residue dissolved in 1 ml of methanol. The qualitative and quantitative content of alkaloids was determined by the HPLC fingerprint analysis [HPLC: L-7100 Intelligent Pump (Merck-Hitachi), L-7200 Autosampler (Merck-Hitachi), L-7450 Diode Array Detector (Merck-Hitachi); Software: D-7000 Chromatography Data Station Software ver. 4.0; Column: LiChrospher® 100 RP-18 (250 mm × 4 mm, Merck); Precolumn: LiChrospher® 100 RP-18 (4 mm × 4 mm, Merck); Solvents: A – phosphate buffer solution (10 mM, pH=6.6), B – methanol : acetonitrile (1:1); Gradient: (60% A and 40% B) to (30% A and 70% B); Time: 30 minutes; Washing: 20% solvent A and 80% B; Temp: 21°C; Flow Rate: 1.0 ml/min; Detection: 245 nm] (SHENG *et al*. 2000; STUPPNER *et al*. 1992).

The experiment was preceded by a determination of the microbiological purity of the studied preparation. 10% of cat's claw solution in physiological salt was made and a dilution series were prepared. The diluted samples were aseptically inoculated (1 ml) onto Petri plates with enriched agar medium (pH=7) and incubated 48 hours in 37°C. Since contamination with fungi and bacteria was observed, all samples were tyndalized by alternating and repeated heating up to 100°C and twenty-four hour incubation in 37°C. Control inoculations of the sterilized preparations did not show the presence of any microorganisms.

Table 1

Characteristics of *Uncaria tomentosa* bark according to the Laboratorios Induquimica S.A. (Peru)

Feature	Specification
Description	fine reddish or chest-nut brown powder with acrid-bitter taste
Foreign bodies	up to 0.3%
Particile size	40 mesh
Participation of alkaloids (as mitraphiline)	1.17%
Microorganisms	140 UFC/g
Aerobic	10 UFC/g
Yeast and moulds	non present
Salmonella	non present
Pseudomonas aeuruginosa	non present
Staphylococcus aureus	non present
Escherichia coli	non present

Solution Preparation

Three tyndalized solutions were prepared according to the scheme:

– Solution C – 40 ml of physiological salt;

– Solution I – 9.84 mg of cat's claw preparation in 40 ml of physiological salt (NaCl 0.09%);

– Solution II – 98.4 mg of cat's claw preparation in 40 ml of physiological salt.

Correspondingly, three groups of similar size eggs with embryos in the twelfth day of embryonic development were prepared: group I (n=120), group II (n=120) and control group C (n=120). Eggs were first candled to exclude incorrect developed embryos. Each was carefully washed with 80% ethanol and a small hole was made in the blunt end of the eggshell (above the air chamber). Next 200 λ of cat's claw solution was injected into the air space of each egg in accordance with the applied notations. Each hole was sealed with an adhesive tape. The eggs were incubated in a conventional forced-air apparatus maintained at 37.7°C and 50% relative humidity for 15 days. In the 19th day of embryonic development the eggs were transferred to a hatcher at 37.0°C and 85% relative humidity until hatched. All manipulations were performed under aseptic conditions.

Blood analysis

On the nineteenth day of embryogenesis 15 eggs from each group were taken. The eggs were broken, the chicks decapitated and samples of blood were collected into tubes, containing 0.07 mol/ml of EDTA anticoagulant, for morphology analysis.

Laboratory analysis

Complex analysis of blood morphology was performed by a Abbott Cell-Dyn 1700 hematology analyzer. The following routine hematological parameters in chicken blood were assayed: the number of erythrocytes (RBC), number of leukocytes (WBC), mean red cell volume (MCV), hematocrit (HCT), hemoglobin concentration (HGB), mean amount of cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) of one red cell.

Other analyses

Hatchability, chicken weight and wholesomeness were evaluated according to BEDNARCZYK *et al.* (1987).

Statistical analyses

The results were elaborated with the use of Q-Dixon, ANOVA, Duncan tests and χ^2 statistics.

Results and Discussion

Oxindole alkaloid profile

The fingerprint chromatogram is shown in Figure 1. Six pentacyclic oxindole alkaloids, including uncarine F (2), speciophylline (3), mitraphylline (4), isomitraphylline (5), pteropodine (5) and isopteropodine (6) were identified, however, isomitraphylline and pteropodine were eluted in one non-separated peak. Table 2 presents the calcu-

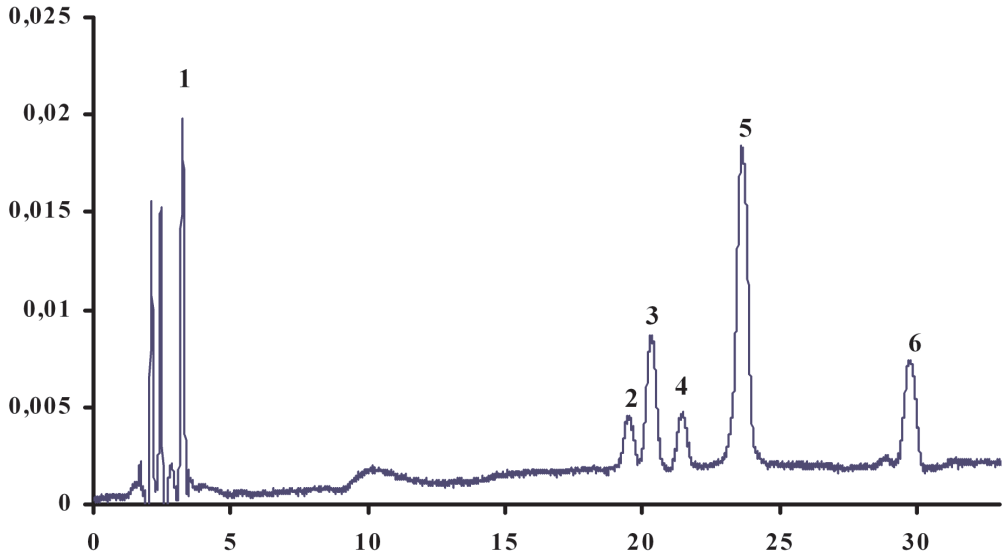

Fig. 1. HPLC-fingerprint analysis of the studied preparation of *U. tomentosa.* 1 – Caffeine, 2 – Uncarine F, 3 – Speciophylline, 4 – Mitraphylline 5 – Isomitraphylline & Pteropodine, 6 – Isopteropodine.

Table 2

Content of alkaloids in the *U. tomentosa* preparation [in mg/100 g]

Peak No.	Alkaloid	Content	
		mg/100g	%
1	Caffeine*	–	
2	Uncarine F	28.00	6.51
3	Speciophylline	68.03	15.81
4	Mitraphylline	33,01	7.75
5	Isomitraphylline & Pteropodine	230.05	53.55
6	Isopteropodine	70.44	16.38
	Total	429.53	100.00

*Internal standard

lated alkaloid contents. The determined total amount of oxindole alkaloids is in agreement with literature data **(**LEMAIRE *et al.* 1999**)**. It must be taken into account that these determinations did not show the presence of any tetracyclic oxindole alkaloids (e.g. rhynhophylline). Several studies have shown that *U. tomentosa* occurs in nature in two different chemotypes characterized by a pentacyclic or tetracyclic pattern of indole and oxindole alkaloids. It is suggested that both types of alkaloids exert antagonistic effects and, therefore, pentacyclic and tetracyclic mixtures of alkaloids are unsuitable for medicinal use (REINHARD 1999). Owing to this, the Ashaninka healers have used only plants representing the pentacyclic chemotype (KEPLINGER *et al*. 1999) and many pharmaceutical companies standardize their preparations for high dominance of pentacyclic alkaloids (above 95% of all alkaloids) (FALKIEWICZ & ŁUKASIAK 2001). Therefore, the obtained HPLC data confirm the authenticity and high quality of the used cat's claw preparation.

Blood morphology comparisons

Table 3 shows the results of chicken blood analysis. Significant differences were detected in MCV – Mean Corpuscular Volume, MCH – Mean Cellular Hemoglobin and MCHC – Mean Cellular Hemoglobin Concentration. The differences were observed between all tested groups. Slight differences were also noted for RDW – Red Cell Distribution Width. The extract of cat's claw did not have a significant influence on the rest of the analyzed blood parameters.

Table 3

Influence of the *U. tomentosa* preparation on the selected blood morphology parameters

Parameter	Group C n=14	Group I n=15	Group II n=15	P
RBC [M/ml]	2.76 ±0.30	2.66 ± 0.58	2.71 ± 0.40	0.770
WBC [K/ml]	15.17 ± 3.76	15.75 ± 6.05	15.84 ± 4.80	0.927
HGB [g/dl]	13.51 ± 1.31	12.97 ± 2.90	12.94 ± 2.02	0.733
HCT [%]	34.08 ± 3.27	34.70 ± 6.91	33.89 ± 5.26	0.913
MCV [fl]	123.82 ± 4.60	131.02 ± 6.89	124.83 ± 4.55	0.002*
MCH [pg]	49.10 ± 1.74	49.04 ± 1.12	47.65 ± 1.66	0.025**
MCHC [g/dl]	39.93 ± 0.74	37.18 ± 1.81	38.00 ± 1.097	0.000***
RDW [%]	12.37 ± 1.57	13.47 ± 1.77	12.27 ± 1.03	0.150
MASS [g]	41.09 ± 3.62	41.40 ± 3.85	41.14 ± 3.53	0.711

* Duncan $_{C/I}$ P=0.001; Duncan$_{C/II}$ P=0.62; Duncan $_{I/II}$ P=0.004
** Duncan$_{C/I}$ P=0.922; Duncan $_{C/II}$ P=0.021; Duncan $_{I/II}$ P=0.022
*** Duncan $_{C/I}$ P=0.000; Duncan$_{C/II}$ P=0.001; Duncan $_{I/II}$ P=0.048

The presented results of blood morphology give much information on the biological activity of the tested preparation because even the smallest change in biological or physicochemical parameters of this tissue have essential consequences for the whole organism. Additionally, due to the connection of blood with other parts of the organism, a change in its parameters may be secondary as a result of earlier changes in other functional systems. On the other hand, this tissue is a very comfortable study material due to the easiness of acquisition and the automated analytical procedure.

The statistical analysis was preceded by a Q-Dixon test in order to check for the smallest and the largest values of all chosen parameters and to reject those values that fell outside of the range of the general population. Due to full automatization of the analytical procedure and few occasions for making mistakes, only a small number of measurements were rejected: MCH_{min}= 42.6 (average = 49.04; SD=1.12) from group I, $MCHC_{min}$= 36.4 (average=39.93; SD=0.74) and RDW_{max}= 16.5 (average=12.27; SD=1.02) from group C.

The results of the statistical comparison including ANOVA and Duncan tests were in a few cases highly unexpected. An insignificant difference between the numbers of leukocytes (WBC) in the compared group was detected. It had been wrongly supposed that this parameter would be a pivotal indicator of the biological activity of *U. tomentosa* under *in vivo* conditions. The above assumption was in agreement with the literature data in which the immunostimulating activity of cat's claw is widely described (FALKIEWICZ & ŁUKASIAK 2001).

The results of our experiment do not necessarily testify to the lack of the postulated immunostimulating activity of cat's claw since the analyses were limited to the evaluation of leukocyte number. Further studies on all populations of white cells may bring more convincing conclusions. In this context the observations by BEDNAREK *et al.* (2002) are especially noteworthy. According to these authors, *U. tomentosa* does not significantly change the total number of leukocytes in calf blood, however it increases the percentage of lymphocytes T (CD2 and CD4) and decreases the quantity of lymphocytes B (WC4). On the other hand, the lack of significant increment of leukocyte number is a good argument for the effectiveness of the undertaken tyndalization. If the sterilization and the maintenance of aseptic conditions during the injections were not sufficient to prevent the infection of embryo tissues, possible changes in blood parameters would be easily questioned.

The above observations might be explained as evidence for the non-toxicity of *U. tomentosa*, since the intoxication of the biological preparation very often causes anemia appearing as a decrement of erythrocyte number (RBC), hemoglobin concentration (HGB) and hematocrit (HCT). The contrary conclusion that *U. tomentosa* has caused unfavourable changes in embryo bodies is supported by statistical evaluation of the other parameters. More or less significant differences with ANOVA were observed for MCV (P=0.002), MCHC (P=0.00001), MCH (P=0.02) and somewhat for RDW (P=0.15).

The influence of cat's claw on hatchability and wholesomeness measurements are presented in Table 4. The χ^2 statistic also confirms the non-toxicity of the applied preparations. In groups I and II the percentage of non-hatchable eggs was slightly higher than in the control group (α=0.1). Comparisons of debilitated chicks was unfeasible considering the small sample size.

Summarizing, cat's claw administration disturbed embryogenesis in both cases. In the first group it increased the mean corpuscular volume of erythrocytes and decreased the mean corpuscular hemoglobin concentration. In the second it decreased both mean corpuscular haemoglobin and mean corpuscular hemoglobin concentration. These results are good arguments for the null hypothesis that *U. tomentosa* may influence anemia during embryonal development. In available literature there is no explanation on the possible mechanism of this phenomenon beyond the brief information that it is often caused by B12 or folic acid deficiency (JANICKI 2001). Perhaps the discovered activity of cat's claw may be explained by its negative influence on absorption of these vitamins.

Table 4

Influence of the *U. tomentosa* preparation on the number of healthy and debilitated chickens, non-hatchable eggs and embryo weights

Trait	Group C	Group I	Group II
I series			
Healthy chickens	90	85	89
Crippled chickens	1	1	1
Non-hatchable eggs	4	8	5
II series			
Healthy chickens	88	79	83
Crippled chickens	0	1	1
Non-hatchable eggs	2	9	7

Test χ^2 for the both series has shown slightly difference in hatchability level among groups (α=0.1).

Methodological Considerations

Utilization of the proposed chicken embryo model seems to be a proper method for biological evaluation of *U. tomentosa* activity and may be useful for many pharmacological investigations. As was already noted, the traditional way of utilizing *U. tomentosa* is by drinking its decoctions, this form of consumption may be associated with some noteworthy implications. In the case of oral ingestion, certain active compounds contained in the extract are transported through the plasmalemma and into cellular metabolic pathways, whereas others are not. A living organism is able to absorb from the environment only those compounds that are necessary for keeping or restoring its homeostatic equilibrium. In this point of view the isolation of individual active compounds and their standardization is a needless and even harmful procedure. However, using extracts in experimental practice is much less comfortable than using intravenous injections due to the difficulty involved in standardization of doses and the unforeseability of its absorption kinetics. The described model combines the advantages of both methods of drug administration.

There are also some disadvantages that have considerably limited the usefulness of the proposed model in the context of this paper. If we assume that the divergence of birds and mammals had begun in the middle Mesozoic, both lines have evolved separately for at least 80 million years. From an evolutionary point of view this time is enough for rebuilding even the basic metabolic pathways. There are sensible apprehensions that some conclusions formulated on a bird organism may be erroneous for a human being. The other problem is the difficulty in interpreting the blood measurements with Cell Dynn. Since the calibration, calculation algorithms and measuring procedure of this apparatus are associated with human blood, there are many contraindications against strict extrapolation or comparisons of the obtained measurements. However, in zootechnical literature, indications of Cell-Dynns apparatus for chicken blood have already been cited (BEDNARCZYK *et al*. 1996).

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