Original Research Article

Ex vivo antiplasmodial activity of plant extracts used in traditional medicine in Côte d'Ivoire

Abstract

In view of increased resistance of *Plasmodium falciparum* to almost all conventional antimalarial drugs, it has become necessary to search for new classes of molecules from traditional medicinal plants. Indeed, the best antimalarial drugs for which there are fewer cases of resistance today are derived from medicinal plants (quinine, artemisinin and its derivatives). In this study we evaluated *ex vivo* effectiveness of various extracts of *Terminalia glaucescens* and *Erigeron floribundus* on the growth of *P. falciparum* isolates sensitive or resistant to amodiaquine according to the optical version of the WHO microtest. Aqueous extracts activity of various organs of plants was first measured and then three organic extracts of each part of plant.

Leaves and roots aqueous extracts of *T. glaucescens* were the most active and showed respectively IC_{50s} values of 2.69 µg/ml and 0.99 µg/ml on amodiaquine-sensitive isolates (AQ-S) and 2.74 µg/ml and 1.56 µg/ml on amodiaquine-resistant isolate (AQ-R).

Moreover with regards to the organic extracts, methanol extracts of leaves and roots of *T. glaucescens* showed the best activity with respective IC50s average values of 2.69 µg/ml and 1.39 µg/ml on isolates AQ-S.

The high antiplasmodial activity showed by extracts of *T. glaucescens* would favor their use as candidates for the development of improved traditional antimalarial drugs.

Keywords: *Plasmodium falciparum, ex vivo, Terminalia glaucescens, Erigeron floribundus.*

Introduction

Malaria remains a serious public health problem. 198 million cases were recorded in 2013 causing 584,000 deaths, according to the WHO report in 2014 **[1]**. Control strategies put in place to fight against the disease, although having shown some effectiveness, still have shortcomings in the face of *Plasmodium falciparum* resistance to almost all conventional antimalarial drugs including artemisinin derivatives (**[2]**, **[3]**, **[4]**, **[5]**, **[6]**). This situation that constitutes a threat to malaria eradication implies among other solutions, the search for new classes of molecules derived from natural substances through the study of traditional therapies. This option remains promising and very hopeful in two ways. First, the best antimalarial drugs for which there are fewer cases of resistance today are mostly from the traditional pharmacopoeia. This is the case of quinine (**[7]**, **[8]**, **and [9]**) as well as artemisinin and its derivatives (**[3]**, **[4]**, **[10]**). Secondly according to the WHO **[11]**, 42-90% of the population in Europe and 70-90% in Africa rely on traditional medicine for treatment. It would therefore be of great interest to promote this type of medicine by the implementation of Improved Traditional Medicines (ITM) and modernized herbal medicines to make it cheaper and therefore accessible to all regardless of social status, especially in Africa where the majority of the population is very poor. This will contribute to the development of stable forms and standardized dosage to be taken.

However, before developing these ITM, it is important to highlight the activity of plants used in the preparation of traditional medicinal plants.
Indeed, in Côte d'Ivoire, several plants are used to treat fevers associated or identified with malaria. Our study aimed at evaluating the *ex vivo* efficacy of various extracts of *Terminalia glaucescens* and *Erigeron floribundus* on the maturation of *P. falciparum* isolates.

Material and Methods

We conducted an experimental study which took place from 2009 to 2012 at the Malaria Research and Control Centre (CRLP) of the National Institute of Public Health (NIPH) and at the Diagnostic and Research Centre on AIDS and its opportunistic diseases (CeDReS) in Abidjan.

Collection of plants

Plants sampling sessions were held in September 2008 in the mornings for a week. The following sites were selected for plants harvesting: along the road linking the northern highway to Lamto geophysics station, along road linking Taabo-village and Kotiessou; three localities in central Côte d'Ivoire in open forest and savannah area. We harvested leaves, stem and roots bark for *Terminalia glaucescens* (*Combretaceae*) then the entire plant for *Erigeron floribundus (Asteraceae).*
Plant organs harvests were conducted in accordance with Ivorian law governing the management of the national flora. A voucher specimen of each plant has been made on-site for archiving at the Herbarium of the National Floristic Centre in Abidjan.

Antimalaria and plant extracts solutions

Amodiaquine (amodiaquine dihydrochloride dihydrate A2799-5G, Lot 038F0993V), used as a reference molecule, was prepared as stock solution in 70% methanol in a way as to obtain a final concentration of 1600 nM in the inoculum. We then conducted dichotomous dilutions of this solution then giving a concentration range of 1600 nM to 12.5 nM.
The collected plant organs were allowed to dry in air conditioned at (18°C) for 3 days in the laboratory and then sprayed in the crusher (Impact Crusher RETSH® M-26951).
Four types of extracts (aqueous, ethanol, methanol and chloroform) were prepared for each plant.
Thus, for the aqueous extract, 100g of powder was boiled in 1 liter of distilled water for 45 minutes and filtered through gauze. The filtrates obtained from decoction were then frozen and dried in lyophilizer (Telstar ™ LyoQuest-55).
As for organic extracts, they have been obtained from the stripping method for successive solvents followed by evaporation on Rotavapor (BÜCHI® Rotavapor R-114).
Like the reference molecule, stock solutions of extracts were prepared from freeze-dried or evaporates and diluted dichotomously in order to obtain final concentration range from 100 µg/ml to 0.78 µg/ml in the inoculum.

Isolates of Plasmodium falciparum

25 field isolates of *P. falciparum* were collected in an aseptic condition in EDTA tubes obtain from bend of the elbow in patients with uncomplicated malaria. Parasitemia was greater or equal to 4000 trophozoites per microliter (uL) of blood. Those isolates came from the communitarian hospital of Anonkoua-Kouté in the north of Abidjan.

After their delivery in the laboratory, samples of parasitized red blood cells were washed 3 times in sterile Roswel Park Memorial Institute medium (RPMI 1640 medium) and blood smears were made for verification of the state of trophozoites, determination of parasite density and confirmation of species diagnosis. When the parasite density is greater than 0.2% a dilution was done with healthy red cells of group O previously washed in RPMI 1640 medium. *Ex vivo* culture tests were performed within 12 hours of sampling of parasitized blood.

Ethical Issue

The study protocol was approved by the Scientific Committee of the National Institute of Public Health of Côte d'Ivoire. Patients gave their consent before making blood samples

Ex vivo culture tests

The tests were conducted in two stages. The first stage consisted of screening of aqueous extracts on 20 *Plasmodium falciparum* isolates. The second stage was to test on 5 *P.falciparum* isolates organic extracts from plant parts with aqueous extracts gave a 50% inhibitory concentration (IC_{50}) relatively low. Amodiaquine was used as the standard antimalarial drug.

The activity tests were performed according to the optical variant of RIECKMANN microtest recommended by the WHO **[12]**.

An inoculum was prepared, consisting of RPMI 1640 buffered to pH 7.4 with 25 mM $NaHCO_3$ and 25 mM HEPES and supplemented with 10% bovine serum albumin and the pellet of parasitized red blood cells (1.5% hematocrit).

The various dilutions of amodiaquine or plant extracts were distributed in duplicate or triplicate in sterile culture plate of 96 wells of 50 µl per well. Three drug-free wells were used as negative controls.

The inoculum was distributed in each well in quantity of 200 µl per well. The plates were then shaken gently for one minute to ensure thorough mixing of drugs in the medium. All plates were finally incubated in an oven (SELECTA™) at 37°C for 42 hours in an environment of 5% CO_2 in air.

At the end of incubation, thick films were produced from the culture pellets after assembling pellets with same concentration. The test is validated only if we have at least 20% of maturation in the control wells. IC_{50} threshold for amodiaquine was 60 nM. **[13]**

Statistical Analyses

Reading the slides after staining has enabled us to calculate the various trophozoites maturation rate for each drug concentration compared to controls. IC_{50}s values were measured through nonlinear regression analysis of the logarithmic curve of the concentration - based on the growth inhibition percentage using the software "ICESTIMATOR" version 1.2 (**[14]**, **[15]**). *Ex vivo* response of *P. falciparum* was expressed as geometric mean of IC_{50} value with confidence intervals at 95%

Results

Of the 25 culture tests made, 20 were validated; a proportion of 80% success rate. There were 95% of the isolates that were sensitive to amodiaquine with an IC_{50} mean value of 10.70 nM against only 5% resistant to amodiaquine with an IC_{50} mean value of 68.93 nM.

All tested extracts showed significant antiplasmodial activity on sensitive (AQ-S) and resistant isolates (AQ-R) to amodiaquine.

On the AQ-S isolates the aqueous extract of the roots barks of *T. glaucescens* showed the highest activity followed by the aqueous extract of leaves with IC_{50}s mean values of 0.99 µg/ml and 2.69 µg/ml respectively (**Table 1**). The same trend was observed on the AQ-R isolate with IC_{50}s means values of 1.56 µg/ml and 2.74 µg/ml (**Table 1**).

All organic extracts (made with the parts of plants whose aqueous extracts gave the best results) had good activity on the AQ-S isolates of the second stage. Three of these extracts showed higher activities. They are methanol extracts of leaves (TG-f_Met) and root bark (TG-er_Met) of *T. glaucescens* and the ethanol extract (TG-er_Eth) of these root bark. The best activities were observed with the methanol extracts of leaves (TG-f_Met) and root bark (TG-er_Met) with IC_{50}s means values of 2.69 µg/ml and 1.39 µg/ml respectively (**Table 2**).

Discussion

Due to high level of resistance to chloroquine in more relatively high malaria endemic areas, this molecule has been removed from the antimalarial drugs for the treatment of uncomplicated *P. falciparum* malaria **[16]**. Also, with regard to the 4-amino quinolines, amodiaquine is used as an antimalarial in combination with artemisinin derivatives. Thus, it seems wise to compare the anti-malaria activity of new antimalarial drug candidates based on the sensitivity of P. *falciparum* isolates to amodiaquine instead of chloroquine. In this context, the use of monodesethylamodiaquine (the active metabolite of this molecule) for measuring the *ex vivo* sensitivity of *P. falciparum* would be appropriate (**[6]**, **[17]**, **[18]**). Meanwhile, like ours, several studies have reported significant activity of amodiaquine with IC_{50}s ranging from 3.93 nM to 39.55 nM **[19]**. Our results are similar to those of Okombo **[20]** who reported a mean IC_{50} value of 8 nM with a study population of 62 amodiaquine-sensitive isolates. Olasehinde **[21]**, in a study of 100 isolates of *P. falciparum* from the south-western Nigeria, reported an IC_{50} mean value of 6.3 nM, but with a higher percentage of resistant than ours; 13%.

Although in lower to moderate proportions, the presence of isolates resistant to amodiaquine or its active metabolite could jeopardize with time the effectiveness of combined therapies based on derivatives of artemisinin containing this molecule because of the selection pressure due to the large-scale use of these drugs.

The medicinal plants used in our study were collected from the sites mentioned above according to their frequent use by local population for the treatment of diseases related to malaria. Parts of plants collected were chosen following the indications of the ethnobotanical survey. In this study, regardless of the types of extracts or isolates, the two plants tested had antiplasmodial activities with lower IC_{50}s less than 20 µg / ml **[22]**.

Table 1: IC_{50} values of amodiaquine and aqueous extracts on *Plasmodium falciparum* isolates

Drugs	Amodiaquine-sensitive isolates					Amodiaquine-resistant isolates	
	N_1	Geometrical Mean IC_{50} (µg/ml) (standard deviation)	Interval of Confidence at 95% (µg/ml)	Extreme		N_2	Geometrical mean IC_{50} (µg/ml)
				Minimum	Maximum		
Amodiaquine (nM)	14	10,70 (4,61)	8,29 – 13,11	2,26	39,74	1	69,93
EF extract	14	6,78 (1,97)	5,75 – 7,81	0,81	47,22	1	26,17
TG-F extract	14	**2,69 (0,03)**	2,67 – 2,71	0,29	23,89	1	**2,74**
TG-ET extract	14	5,55 (0,43)	5,32 – 5,78	0,76	59,57	1	5,71
TG-ER extract	14	**0,99 (0,13**)	0,92 – 1,06	0,36	8,93	1	**1,56**

N_1: number of amodiaquine-sensitive isolates tested
N_2: number of amodiaquine-resistant isolates tested
EF: *Erigeron floribundus*
TG-ER: *T. glaucescens* leaves
TG-ER: *T. glaucescens* stem bark
TG-ER: *T. glaucescens* roots bark

Table 2: IC_{50} values of amodiaquine and organic extracts of leaves and roots barks of *T. glaucescens* on *Plasmodium falciparum* isolates

Drugs	N	Geometrical Mean IC_{50} (µg/ml) (standard deviation)	Interval of Confidence at 95% (µg/ml)	Extreme	
				Minimum	Maximum
Amodiaquine (nM)	05	10,70 (1,45)	9,43 – 11,97	7,07	25,13
TG-**f**_Eth	05	6.18 (1.09)	5.23 – 7.13	2.65	25.55
TG-**f**_Met	05	**2.69 (0.64)**	2.13 – 3.25	0.62	21.44
TG-**f**_Chl	05	5.99 (1.15)	4.98 – 7.00	2.4	33.94
TG-**er**_Eth	05	**3.06 (0.15)**	2.93 – 3.19	1.16	13.22
TG- **er**_Met	05	**1.39 (0.15)**	1.26 – 1.52	0.52	3.29
TG- **er**_Chl	05	6.15 (0.13)	6.04 – 6.26	2.62	20.73

N: number of tested isolates;
Met = Extract with Methanol;
TG-**f** = *T. glaucescens* leaves
Eth = Extract with Ethanol;
TG-**er** = *T. glaucescens* roots bark
Chl = Extract with Chloroform

Indeed, the first screening revealed that all the extracts had a significant *ex vivo* activity against *P. falciparum* sensitive to amodiaquine. As for the resistant isolate, only three extracts from *T. glaucescens* gave high activities. So we observed that whatever type of isolate (sensitive or resistant), the aqueous extracts of the leaves and root bark of *T. glaucescens* showed better anti-malaria activity.

A similar study conducted on 33 plants from Ivorian Pharmacopoeia showed that 24% of these plants had significant activity on the chloroquine-resistant plasmodium strain FcB1. Among these plants, *Nauclea latifolia*, *Fagara macrophylla*, *Funtumia elastica*, *Phyllanthus muellerianus* and *Rauvolfia vomitoria* were more active with IC_{50} values of 8.9; 2.5; 3.3; 9.4 and 2.5 µg/ml respectively **[23]**.

Also, among the organic extracts from leaves and roots barks of *T. glaucescens* the methanol extracts of the two parts have the best activities on isolates. Methanol seems to be here the best solvent that can extract enough active compounds against *Plasmodium falciparum*. Other authors have reported an IC_{50} of 2.43 µg/ml with the pentane extract of *T. glaucescens* **[24]** against values ranging from 4.3 to 10 µg/ml for that of *E. floribundus* **[22]**. Other studies have also shown relatively low IC_{50}s value with some plants used in traditional medicine; reflecting their good antiplasmodial activity. They are especially *Azadirachta indica* (6, 24 µg/ml) **[25]**, *Artemisia annua* (6, 25 µg/ml) **[26]**, *Alchornea cordifolia* (3, 36 µg/ml) **[22]**, *Tabernaemontana elegans* and *Vangueria infausta* (CI_{50}<2µg/ml) **[27]** and then *Chromolaena odorata* (4, 8 and 6, 74 µg/ml against *P. falciparum* HB3 and FCM29 respectively) **[28]**.

However, in our study, it should be noted that the antiplasmodial activities of organic extracts did not seem to be improved compared to that of aqueous extracts on AQ-S isolates. This profile suggests that either the compounds responsible for conveying these activities to the parts of the plants tested are more hydrophilic or the solvents selected could specifically extract, in addition to the active compounds, some non-hydrophilic compounds that may have an antagonistic effect. So, a bio-directed fractionation of these extracts shall allow us to confirm or deny these assumptions.

Nevertheless, studies performed on these extracts give us reason to continue our explorations for future conception of Improved Traditional Medicines (ITM). Indeed, the work of Tano **[29]** shows that both methanol extracts are not toxic *in vivo* within the quantitative therapeutic dose estimated at 13.90 mg/kg body weight. The LD_{50}s were respectively 2085 mg/kg body weight for the roots extract and higher than 2500 mg/kg body weight for the leaves extract. These values are interesting seeing that for artemisinin as reference substance, Phan **[30]** reported in its work a therapeutic dose of 10 to 20 mg/kg body weight with an LD_{50} of 4228 mg/kg of body weight. Thus, fractionation and purification of our organic extracts could enable us to isolate active ingredients with lower therapeutic dose values approaching that of artesunate witch goes from 2 to 4 mg/kg body weight with an LD_{50} of 1150 mg/kg body weight **[30]**.

Conclusion

The extracts that we tested here contain active ingredients capable of inhibiting the maturation of P. falciparum. The IC_{50}s obtained with aqueous and methanolic extracts of the leaves and roots bark of *T. glaucescens* indicates that these extracts possess significant antiplasmodial activity. These extracts could be good candidates for the development of their antimalarial ITM after suitable toxicology evaluation.

References

1. OMS
World Malaria report 2014

2. Djaman A. J., Basco L. K., Mazabraud A.
Etude in vivo de la sensibilité à la chloroquine et évaluation de la résistance à la pyriméthamine après analyse de la mutation ponctuelle du gène de la dihydrofolate réductase
Cahiers Santé, 2002, N°12 (4), 363-7

3. Noedl H, et al.
Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium: Evidence of artemisinin-resistant malaria in western Cambodia.
N Engl J Med, 2008, 359 (24) : 2619–2620.

4. Dondorp AM, et al.
Artemisinin resistance in Plasmodium falciparum malaria.
N Engl J Med, 2009, 361(5):455–467.

5. Plowe CV
The evolution of drug-resistant malaria.
Trans R Soc Trop Med Hyg. 2009, 103(Suppl 1):S11–S14.

6. Yavo W., Bla K. B., Djaman A. J., Assi S. B., Basco L. K., Mazabraud A., Koné M.
In vitro susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, quinine, mefloquine and halofantrine in Abidjan (Côte d'Ivoire)
African Health Sciences. 2010; 2: 111-116

7. Jane Achan, Ambrose O Talisuna, Annette Erhart, Adoke Yeka, James K Tibenderana, Frederick N Baliraine, Philip J Rosenthal and Umberto D'Alessandro
Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria
Malaria Journal. 2011 ; **10**(144)

8. Brasseur P., Kouamouo J., Moyou-Somo R., Druilhe P.
Multi-drug resistant falciparum malaria in Cameroon in 1987-1988. Stable figures of prevalence of chloroquine- and quinine-resistant isolates in the original foci.
The American journal of tropical medicine and hygiene. 1992; **46**(1): 1-7.

9. Jelinek T., Schelbert P., Löscher T., Eichenlaub D.
Quinine resistant falciparum malaria acquired in east Africa.
Tropical medicine and parasitology. 1995; **46**(1): 38-40.

10. Dondorp, A. M., Yeung, S., White, L., Nguon, C., Day, N. P., Socheat, D., von Seidlein, L.
Artemisinin resistance: current status and scenarios for containment.
Nat Rev Microbiol. 2010; **8**(4): 272-280.

11. OMS
Renforcement du rôle de la médecine traditionnelle dans les systèmes de santé : une stratégie pour la région africaine.
Comité Régionale de l'Afrique ; 63ème Session, Brazzaville, septembre 2013 P7

12. Basco L. K.
Field application of *in vitro* assays for the sensitivity of human malaria parasites to antimalarial drugs.
World Health Organisation; 2007
Available from: http://www.who.int/malaria/publications/atoz/9789241595155/en/index.html

13. Basco LK, Le Bras J.
In vitro activity of monodesethylamodiaquine and amopyroquine against African isolates and clones of Plasmodium falciparum.
Am J Trop Med Hyg. 1993; 48 (1): 120-125.
14. Kaddouri H, Nakache S, Houzé S, Mentré F, Le Bras J.
Drug Susceptibility of *Plasmodium falciparum* clinical isolates from Africa using plasmodium Lactate Dehydrogenase Immunodetection Assay and inhibitory Emax model for precise IC50 measurement.
Antimicrobial Agents and Chemotherapy, 2006, 50: 3343-3349.
15. Le Nagard H, Vincent C, Mentré F, Le Bras J.
Online analysis of *in vitro* resistance to antimalarial drugs through nonlinear regression.
Comput Methods Programs Biomed, Epub 2010.
16. OMS
World Malaria report 2011
17. Pradines B, Hovette P, Fusai T, Atanda HL, Baret E, Cheval P, Mosnier J, Callec A, Cren J, Amalvict R, Gardair JP, Rogier C.
Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among Plasmodium falciparum isolates from Pointe-Noire, Republic of the Congo.
J Clin Microbiol. 2006 Jul; 44(7):2404-8.
18. Menard D, Djalle D, Manirakiza A, Yapou F, Siadoua V, Sana F, Matsika-Claquin M.D, Nestor M, Talarmin A
Drud-resistant malaria in Bangui, Central African Republic: An *in vitro* assessment
Am. J. Trop. Med. Hyg, 73 (2). 2005. PP 239-243
19. Folarin O. A., Bustamante C., Gbotosho G. O., Sowunmi A., Zalis M. G., Oduola A. M., Happi C. T.
In vitro amodiaquine resistance and its association with mutations in pfcrt and pfmdr1 genes of Plasmodium falciparum isolates from Nigeria
Acta Trop. 2011 ; **120**(3): 224-230
20. Okombo J, Kiara S.M, Abdirahman A, Mwai L, Ohuma E, Borrmann S, Nzila A, Ward S
Antimalarial activity of isoquine against Kenyan *Plasmodium falciparum* clinical isolates and association with polymorphisms in pfcrt and pfmdr1 genes
J. Antimicrob Chemother. 2013. 68(4):786-8.20
21. Olasehinde G. I., Ojurongbe O., Adeyeba A. O., Fagade O. E., Valecha N., Ayanda I. O., Ajayi A. A., Egwari L. O.
In vitro studies on the sensitivity pattern of Plasmodium falciparum to anti-malarial drugs and local herbal extracts
Malar J. 2014 ; **13**: 63
22. Menan H., Banzouzi J. T., Hocquette A., Pelissier Y., Blache Y., Kone M., Mallie M., Assi L. A., Valentin A.
Antiplasmodial activity and cytotoxicity of plants used in West African traditional medicine for the treatment of malaria.
J Ethnopharmacol. 2006 ; **105**(1-2): 131-136.
23. Zirihi G. N., Mambu L., Guede-Guina F., Bodo B., Grellier P.
In vitro antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria
J Ethnopharmacol. 2005 ; **98**(3): 281-285.

24. Mustofa, Valentin A.; Benoit V. F.; Pelissier Y.; Kone Bamba D.; Mallie M.
Antiplasmodial activity of plant extracts used in West African traditional Medicine
Journal of Ethnopharmacology 2000, 73, N° 1-2, 145-151

25. Gathirwa J. W., Rukunga G. M., Mwitari P. G., Mwikwabe N. M., Kimani C. W., Muthaura C. N., Kiboi D. M., Nyangacha R. M., Omar S. A.
Traditional herbal antimalarial therapy in Kilifi district, Kenya.
J Ethnopharmacol. 2011; **134**(2): 434-442.

26. Ramazani A., Sardari S., Zakeri S., Vaziri B.
In vitro antiplasmodial and phytochemical study of five Artemisia species from Iran and in vivo activity of two species.
Parasitol Res. 2010 ; **107**(3): 593-599.

27. Bapela M. J., Meyer, J. J., Kaiser, M.
In vitro antiplasmodial screening of ethnopharmacologically selected South African plant species used for the treatment of malaria
J Ethnopharmacol . 2014. **156**: 370-373.

28. Ezenyi I. C., Salawu O. A., Kulkarni R., Emeje M.
Antiplasmodial activity-aided isolation and identification of quercetin-4'-methyl ether in Chromolaena odorata leaf fraction with high activity against chloroquine-resistant Plasmodium falciparum.
Parasitology Research. 2014. **113**(12): 4415-4422.

29. Tano Konan Dominique, Kouadio James Halbin, Yavo William, Djaman Alico Joseph, Menan Eby Ignace Hervé
Toxic effect study of methanol extract of Terminalia glaucesens leaves following single or short-term repetitive oral administration in Swiss mice.
American Journal of BioScience . 2013. **1**(4): 85-90.

30. Phan, V. T.
Artemisinine and artesunate in the treatment of malaria in Vietnam (1984-1999)
Bull Soc Pathol Exot . 2002. **95**(2): 86-88.