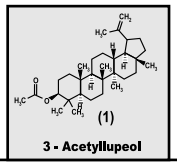
Artigo Científico/Cientific Article

Competitive enzyme immunoassay for antibodies to 3-Acetyllupeol in brazilian *Vernonia scorpioides* grown under domestication

Márcia de Fátima Inácio Freire[a] & Ronald Bastos Freire[b]

SUMMARY – A solid phase indirect competitive enzyme immunoassay using polyclonal IgY antibodies raised to 3-Acetyllupeol (3-AL) was applied to quantify the content of 3-AL in crude extracts obtained from stalks, leaves and roots of *Vernonia scorpioides* domesticated in individual vessels containing brazilian yellow-red podsolic soil added of different combinations Ca $(NO_3)_2$ and of $CaHPO_4$. Test sensitivity for 3-AL decreased more than five orders of magnitude as the number of 3-AL ligands on the processed plant material reduced antibody recognition of the 3-AL conjugated to a carrier protein. Soil $CaHPO_4$ concentration was directly related to crescent root production of lupeol-acetate, suggesting a phosphoenzyme metabolic activation, warranting further studies on this region of synthesis. The technique is designed for qualitative and quantitative studies and allows ready identification of 3-AL. It may also be further applied in the study of other plant bioactive substances lending support to certify raw products from natural sources.

GRAPHICAL ABSTRACT – A competitive ELISA was applied to quantify the triterpene named 3-Acetyllupeol (1), an important secondary metabolite with medicinal activity, in crude extracts of cultured stalks from *Vernonia scorpioides* added of different macroonutrient concentration. The technique allows ready identification of 3-Acetyllupeol and may be further applied in the study of other bioactive substances giving support to certify raw products from natural sources.



KEYWORDS – *Vernonia scorpioides*; 3-Acetyllupeol; Hapten, competitive ELISA.

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Ensaio imunoenzimático competitivo para anticorpos anti-3-Acetil-lupeol *Vernonia scorpioides* brasileira cultivada sob condições de domesticação

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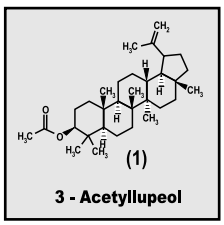
RESUMO *– Aplicou-se um ensaio imunoenzimático competitivo indireto em base sólida, utilizando-se anticorpos IgY policlonais anti-3-Acetil-lupeol (3-AL), para a quantificação de 3-AL endógeno em extratos vegetais diferenciados (raízes, caules e folhas) a partir de* Vernonia scorpioides *domesticada em vasos individuais contendo lato-solo brasileiro adicionado de diferentes concentrações de Ca $(NO_3)_2$ e $CaHPO_4$. A magnitude da sensibilidade do ensaio decresceu ao quíntuplo quando os anticorpos foram previamente neutralizados pelo hapteno livre contido nos extratos e deixaram de reconhecer o 3-AL conjugado à proteína carreadora presos à base sólida. A quantificação dos extratos das plantas crescidas sob os diferentes regimes de tratamento indicou que a maior fertilidade do solo foi proporcional à recuperação do 3-AL. A concentração de $CaHPO_4$ mostrou-se diretamente proporcional à maior produção de 3-AL nas raízes, sugerindo uma ativação metabólica de fosfoenzimas, indicando a possibilidade de futuros estudos nessa região de síntese. A técnica mostrou-se indicada para ensaios qualitativos e quantitativos e permite uma identificação bastante precisa de 3-AL endógeno. Esta metodologia pode, também, ser aplicada para estudos futuros com outras substâncias bioativas vegetais, oferecendo suporte para a certificação de matérias-primas oriundas de fontes naturais.*

SUMÁRIO GRÁFICO *– Um ensaio de ELISA competitivo foi aplicado para a quantificação do triterpeno 3-Acetil-lupeol (1), importante metabolito secundário com atividade medicinal, em extratos brutos de* Vernonia scorpioides *crescidas por estaquia e adicionada de diferentes concentrações de macro-nutrientes. A técnica permite a identificação precisa de 3-Acetil-lupeol e poderá ser aplicada no estudo de outras substâncias bioativas, como suporte para a certificação de matérias-primas obtidas a partir de fontes naturais.*



PALAVRAS-CHAVE *–* Vernonia scorpioides*; 3-Acetil-lupeol; hapteno, ELISA competitivo.*

INTRODUCTION

Trends have been developped in many countries as good alternative to preserve medicinal plants (Freire *et al*, 1996, Lans *et al,* 2001, Calixto, 2000). During the two past decades, biotechnological and genetic studies of medicinal plants were made to improve methods to the analysis of medicinal constituents. GC-MS when used with stable isotopically labeled carriers (Morris *et al.,* 1991) is specific, precise and

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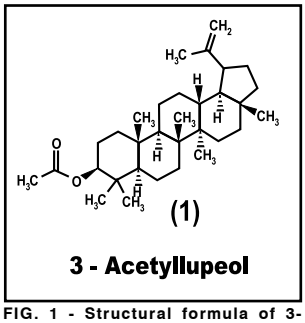

FIG. 1 - Structural formula of 3-acetyllupeol (1)

sensitive. On the other hand, capture ELISA is fast, very sensitive, and multiple samples can be processed. While each technique offers advantages over the other, the speed, sensitivity, and sample handling capability of the immunological methods are attractive, and they have been widely applied to the analysis of bioactive substances with variable degrees of success (Honda *et al.,* 1981, Morris *et al.*, 1991, Avrameas *et al.,* 1992, Abramson *et al.,* 1995, Ribeiro-Neto *et al.,* 1997, Strachan *et al*., 2002, Almquist *et al*., 2004, Lapc̆c´k *et al.,* 2004).

Competitive enzyme immunoassays (competitive ELISA) are widely used because they are rapid, simple, accurate and specific. They are also a cost-effective way to quantify many biologically important molecules such as steroids (Lapc̆c´k *et al.,* 2003), peptides and nucleic acids (Brown-Augsburger *et al*., 2004).

Recently we reported an accurate detection method for a rapid recognition to 3-Acetyllupeol (3-AL), a biologically active triterpene (1) (Fig. 1) naturally occurring in brazilian *V. scorpioides,* using IgY (egg antibodies) raised in hiperimunized hens (Freire *et al*., 2004). We now report a competitive ELISA to determine the relative quantitation of LAc produced by greenhouse propagated *Vernonia scorpioides* stalks in brazilian yellow-red podsolic soil added of phosphorus ($CaHPO_4$) and nitrogen ($Ca(NO_3)_2$). The specificity of IgY selection and resolution adds a biosynthetic origin information.

2. RESULTS AND DISCUSSION

It was the first time a coompetitive ELISA was carried out to detect the relative concentration of 3-AL content in domesticated *V. scorpioides*. The success of this kind of assay to quantify different haptens is well documented (Suzuki *et al,* 2000) and is accomplished by the sample-antibody preincubation before addition to microtiter plates.

The competitive ELISA can be used to measure bioactive chemicals in this study quickly and accurately, without sample pretreatment or dilution. Being sensitive, rapid and accurate with a simple plant fragment sample homogenate; this *in vitro* assay appears to be a rapid medicinal source monitoring and a good method for domesticated plants certification (Freire *et al.,* 2006).

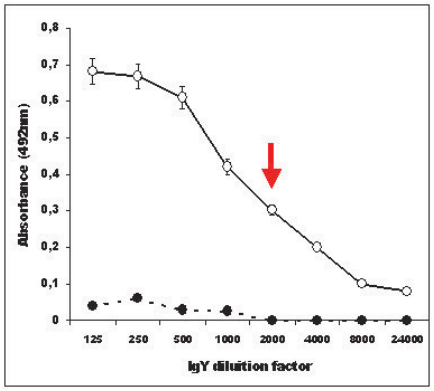

FIG. 2 - Influence of specificity in indirect ELISA. Phytohapten-conjugated to chicken egg albumin (OVA-3LAc, 250 ng/mL in PBS) or control chicken egg albumin (OVA, 250 ng/mL PBS) were adsorbed to plastic wells of immunoplates before blocking. Diluted anti-3-AL IgY antibodies were incubated on the plate and presence of immobilized antibodies was revealed by a three-step amplification procedure. The serum dilution factor (1,000) produced a non detectable background signal to OVA and a sensitive specific OVA-3LAc signal (arrow). n = 4, error bars indicating ± SD.

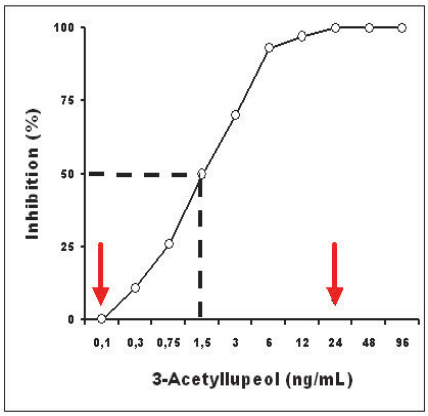

FIG 3 - Competitive inhibition of IgY antibody binding to OVA-3-AL-coated plates by free 3-AL. Phytohapten-protein conjugates (OVA-3-AL, 250ng/mL in PBS) were adsorbed to immunoplates before blocking. Antibodies (1/1,000 in PGT) were incubated on the plate and after washing steps, immobilized antibodies were revealed using the two-step procedure. Results are expressed as inhibition percent from control. Lines are indicative of the concentration of 3-AL that caused 50% of signal inhibition. Arrows indicate the lowest (0.1ng/mL) and the highest (24ng/mL) detection considered as detection limits for the test (n = 4, in all cases SD was <5% of the value).

The time required for plant has been a problem. Many variations for rapid assays have been proposed, but most require extensive preparation and solvent extraction. The sample size also has been a substantial problem overcome by the immunological methods, allowing many more samples to be handled at one time. This in effect provides for multiple parallel analysis of different (or replicate) samples. The anti-3-AL IgY at the 1: 2000 work dilution were 100% specific to the hapten conjugated to chicken egg albumin (OVA), a carrier protein different from bovine serum albumin (BSA), the carrier protein used to elicite the anti-hapten response in the immunized animals (Fig. 2).

The 3-AL competitive ELISA showed a strict compatibility with the therpene presence in each extract assayed. The signal inhibition, not detectable when 3-AL were absent, was ever inversely proportional to the amount of antigen in solution and correlated with the amount of 3-AL present in the sample. It was not a surprise, once the prediction values for the competitive ELISA were settled every time as high as 80%. Because of that, it could be determined high lupeol-acetate concentrations even when the extract yield was not that big, and *vice versa.*

Thus a substantial time element is reduced by enabling direct measurement of 3-AL in its biologic sources. For pure grade 3-AL, the LOD was found to be 100pg/mL (5pg/well). The 50% intercept of the calibration curve was 1.5ng/mL (75pg/well). The working range of the assay for pure grade 3-AL, as with all other matrices (roots, stalks and leaves), was 0.1–24ng/mL (5–1,200pg/well), corresponding to 0.01-4.8ng 3-AL/500mg *V. scorpioides* stalking in soil added of different macronutrient concentration (Figure 3).

The intra and interassay variation-coefficients established through *V. scorpioides* extracts and pure grade 3-AL spiked fractions did show 100% of concordance, with no significant difference to the estimation of 3-AL concentration obtained for both, the pure grade 3-AL and the 3-AL content in all the extract samples. In all cases, 3-AL alone or in mixture produced the same inhibition curves (Figure 4).

In all plants, there exists a complex conjunction of metabolites, which may change according to their environmental and genetic profiles in order to guarantee their best survival (Emerenciano *et al*., 1998, Gottlieb & Kaplan, 1993).

The plant response to the environmental stress con-

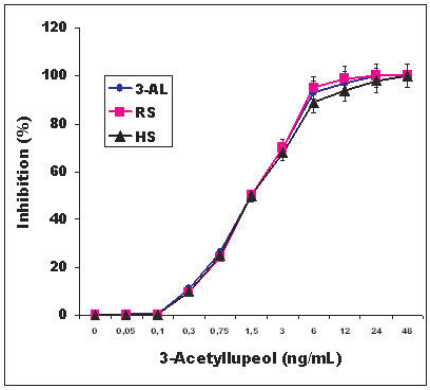

FIG. 4 - Competitive inhibition of IgY antibody binding to BSA-LAC-coated plates by pure grade 3-Acetyllupeol (3-AL), homogenate (HS) and spiked recovered samples (RS) from both, aerial parts and roots produced the same inhibition curves. Results are expressed as inhibition percent median value (n = 48, error bars within symbols size; in all cases SD was <5% of the value).

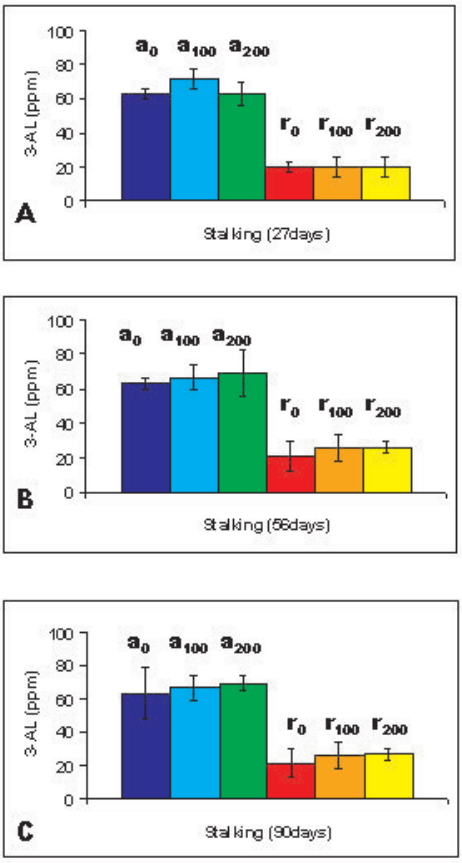

FIG. 5 - *Vernonia scorpioides* crude extracts yelding of 3-Acetyllupeol (3-AL) in extracts from the aerial parts (a) and the roots (r) of stalks propagated for (A) 27 days, (B) 56 days and (C) 90 days in podsolic soil amended of 0,100 and 200kg.ha^{-1} $Ca(NO_3)_2$ kg. ha^{-1} plus 80kg.ha^{-1} $CaHPO_4$. Experiments with 0 and 60kg.ha^{-1} $CaHPO_4$ originated identical results. Results are the median value taken of 4 repetitions± SE (bars within symbols size), p<0.01.

ditions should be unpredictable in many aspects (Watermam & Mole, 1989). As the metabolic condition to generate bioactive terpenes seems to be a result of the individual constitution (Dovjak & Andrascik, 1987; Corrêa-Júnior, 1998), the environmental nutrients disposition may be strategically modified to benefit the desired metabolism.

Unfortunately, the knowledge of how to cultivate and, at the same time, maintain the bioactive delivering without depriving their natural behaviour continues to be a problem to be solved, due to the few accessible information about the best irrigation condition, the soil pH correction needed and the fertilization of the ground necessary for a successful cultivation.

We found in these samples differences correlated the soil treatment with macronutrients but neither related to the intrinsic qualities of the soil or the matrices processing. As the yellow-red podsolic soil, the natural soil from which samples were taken from, has a poor nutritional value there was no over loading of nutrients to the plants, approaching the stalking condition very closely to the natural behavior *V. scorpioides* presented at the time it was collected.

Stalkings propagated in soil added 200kg.ha-1 Ca (NO3)2 presented a great aerial part development, while those added 80 kg.ha-1CaHPO4, great rooting. Nevertheless, the plant relative biomass production seems to exert no interference on the 3-L.Ac yielding in the processed aerial part of the plant. Based on the high accuracy of the test assay we observed inorganic nitrogen added to the soil, despite of influencing on the total weigh of fresh collected stalks and leaves did not interfere augmenting the relative 3-AL concentration of in any sample (Fig. 5).

Similarly, the yielding of the triterpene for each gram of dry root was constant without a significant correlation to the overall biomass production, independently of the time plant samples were haversted.

The inorganic phosphorus disposition is an essential feature of *Vernonia scorpioides* artificial growth on driving the secondary metabolism to generate a cumulative yield of 3-AL which decreased with the nitrogen augmentation, probably favoring 3-AL migration to the leaves under nitrogen feeding circumstances.

In fact, ATP is definitive for phosphatase to generate therpenoids from isoprene unities (Bramley, 1997) and iso-penthenyl-pyrophosphate formation to which the high-energy inorganic phosphorus is intrinsically necessary. The clear needs of ATP seemed to be obvious since, as it was already demonstrated, deficiency of this constituent leads to sugar accumulation in all parts of the plant (Maia, 1998; Shuman, 1994).

Terpenoids have a preferential radicular origin in several natural sources of terpenes as observed under natural conditions (McCaskill & Croteau, 1998; Flores & Curtis, 1992; Flores *et al.* 1999). Being an independent variable, this macronutrient might exert a down regulation on the genetic expressions of terpenes in cultured plants, during the developmental stages of the plant organism or tissue leading the youngest stalkings to concentrate more 3-AL in the roots and the eldest to acumulate higher 3-AL content in their leaves and stalks.

Unfortunately we could not establish a long term and a short period exposure, warranting a further time-course experiment to determine the 3-AL production in the different parts of the plant. Previous experiments, however, demonstrates there is a seasonal pattern that directs the terpene content and emission in plants naturally grown under field conditions (Llusià and Pañuelas, 2000). Our observations corroborate with these findings and use that plants domestication to give good results if stalks cultured in the presence of ideal nutrients concentration. Finally, it must be emphasized that anti-hapten antibodies, constitute themselves a very sensitive tool for tracking bioactive micro molecules. Being reproducible, it certainly will be helpful on generating, further comprehensive and less expensive methodology for accessing the expression of important medicinal drugs as well as from substances, warranting further studies of metabolic pathways that have not been yet characterized.

3. EXPERIMENTAL

3.1. General

An indirect competitive enzyme immunoassay was used to detect 3-AL in the plant samples. The polystyrene microplates were filled with pure grade 3-Acetyllupeol conjugated to a carrier protein diluted in a carbonate–bicarbonate buffer (pH 9.6; 100µl/well) and incubated overnight at 4±1°C for absorption. The sensitised plates were washed with a 5% (200µL.L^{-1} – v/v) PBS-Tween 20 (PBS-Tw) and kept at 4°C until further use. Fristly, 50µL of each of the 1:2 serial dilution of the competitive substance from 96 to 0.1ng/mL hapten was added to 25µL of 3x strength of antibody (3x the giving 50% inhibition on direct ELISA), in sealed tubes. The hapten-anti-IgY antibodies reaction was incubated at 37±1°C for 2 hours and added (50µL per well) to the sensitised micro plates previously washed four times with 200µL per well of 1% casein PBS –Tw. Plates were incubated again at 37±1°C for 2 hours. After been newly washed, the plates were added of 50µL goat anti-IgY conjugated to peroxidase (Sigma, St Louis, MO). After incubation, the plates were washed again, added of 100µL substrate (0.01g OPD, 10µL 30 volumes H_2O_2 in 25 mL 11.9g.L^{-1} Na_2HPO_4) and kept in the dark at room temperature for 10 minutes. The enzyme reaction was stopped through addition of 50µl of 2 M H_2SO_4, and absorbance was measured at 492nm by a Labsystem Multiscan MCC/340. The limit of detection (LOD) for the competitive ELISA was calculated as the analyte concentration that reduced absorbance to 85% of the maximum.

3.2. Plant material

V. scorpioides as identiffied by Prof. Maria Mercedes Rosa Teixeira was collected during October, November and December, in Seropedica City, Rio de Janeiro State (22º45′ South latitude, 43º41′ West longitude) and deposited in the with the vauch number RBR 4140. Stalks containing the apical gemule and, at least, one lateral gemule were cutted at 20 cm, added of indol-butylic acid (IBA) at different concentrations (0; 2000; 8000 and 10000ppm) and submitted to rooting in a washed-sand-containing box coupled to an automatic sprinkler system (Freire *et al.,* 1995). Each treatment corresponded to one different box leading to four rooting boxes, with 60 stalks in each one. After 40 days, the medium number of successful rooting was taken, serving to determine the best IBA formulation to be further added. The propagules that developed the best rooting were selected at random and further used for nursing at the greenhouse experiments.

3.3. Greenhouse growths

Samples of 10cm long stalks of *V. scorpioides* were distributed at random, with a 3^2 factorial arrangement, on the 10-20 superior crust yellow-red podzolic soil fractions (Anjos *et al.,* 1998). Each group of five samples was added of 0, 100 and 200 kg.ha^{-1} Ca $(NO_3)_2$ and 0, 60 e 80 kg .ha^{-1} CaH (PO_4), respectively. Propagules were daily poured on double-distilled deionised water. At intervals of 27, 56 and 90 days propagation, the aerial parts (leaves and stalks) and roots were separated and dryed at environmental conditions, in a shadowed place. Additionally, the collected samples were wrapped in kraft paper and incubated under controlled temperature of 41 ± 1°C under forced air. All the dry samples were granulated to 40 and 80 mesh diameter, weighted and kept deep-frozen until lyophilized to further use.

3.4. Extraction

We prepared homogenates of *V. scorpioides* and authentic green-house samples for analysis using two methodologies, either including solvent extraction or not. The dry material was disintegrated in a grinder and extracted for 4h with 80% ethanol. The extracts were filtered and adjusted to a final volume of 25ml of extract per 1.0g dry matter. Before the analysis, the extracts were diluted 100 times with the assay buffer. Powdered plant parts (0.5g) were fluxed with 66% acetonitrile in water (20ml) containing hydrochloric acid (3.5M, 5ml) at 95 8°C for 45 min. The mixture was filtered, and the volume of filtrate was adjusted to 25ml with 66% acetonitrile. Before the analysis, the extracts were diluted with water and re-extracted with diethyl ether. The ether was evaporated, and the residue was reconstituted in distilled water for ELISA. We also used the resulting slurry for all subsequent preparation of standard spiked homogenate and negative control homogenates. Homogenate was prepared from dry material by direct extraction with methanol from plant parts into glass vials (0,5g plant/vial). The samples were spiked with standard 3-AL ranging from 0 to 4.0ìg/vial to generate a final 3-AL concentration ranging from 0 to 80ìg/100g dry plant in log 2 dilutions. Spiked samples were incubated for 1h at room temperature and then were homogenized with 20mL of phosphate-buffered saline (PBS) for 3min at high speed on a commercial blender to generate a final dilution of the homogenate at 0.025g/mL. The homogenates were then frozen at –20°C until use.

3.5. Conjugation

3-AL was conjugated to egg-ovalbumin (OVA, Sigma Co, St. Louis, USA) as described elsewhere (Abramson *et al.*, 1995 and Freire *et al.,* 2004). In a brief, 40mg of OVA was carefully added under constant agitation of 19mg 3-LAc in the presence of N-N-dimethyl-aminopropil-N-etil-carbodiimide chloridrite (EDPC) at room temperature, with a pH adjustment from pH 5 to pH 8 with 2N NaOH. The mixture was incubated for 12 hours in a light protected shaker. The reaction was adjusted to pH 4.2 by adding sodium acetate. The mixture was kept to settle during 4 hours at room temperature to stabilize and submitted to dialysis at 4°C against 0,01 M PBS (8,18g NaCl; 1,051g Na_2HPO_4; 0,310g NaH_2PO_4; H_2O *q.s.p.* 1000mL pH 7.2) with two daily changes for 72 hours. The conjugated material was characterized by UV spectrophotometer and considered done when the proportional number of moles of LAc bound to each mole of OVA was between 230 and 360nm, according to the molar extinction coefficient, in a quartz 1cm of optical pass container, using PBS 0,01 M, pH 7.2 as a blanc. The relative concentration of each molecule was calculated in function of its exact individual slop of absorption (Hermanson, 1996, Freire *et al.,* 2004).

3.6 Antibodies anti-3-AL-BSA

The anti-3-AL chicken polyclonal antibodies were produced and purified in the Immuno-chemistry Laboratory in the Environmental and Parasitary Immunoto-

xicology Section, Department of Animal Biology at the Federal Rural University of Rio de Janeiro. Anti-3-AL constructs were obtained against 3-AL conjugated to bovine serum albumin (BSA) as previously described (Freire *et al.,* 2004). The anti-3-AL-BSA IgY specificity of the anti-3-AL-BSA IgY preparations were calculated by competitive ELISA in the presence of 3-L.Ac (0.1; 0.3; 0.75; 1.5; 3; 6; 12; 24; 48 and 96ng 3-AL per mL). The anti-3-AL-IgY sensitivity was calculated by an indirect capture ELISA in function of the highest antibody dilution which could exclusively recognize the hapten linked to a different carrier protein (OVA). Only preparations that matched predictive values equal or superior to 85% were taken in consideration for the further assays (Crowther, 1995, 1998).

3.7 Sensitisation of micro plates

It was used an adaptation of the previously described methodology (Crowther, 1995, 1998). Egg ovalbumin conjugated to 3-AL (OVA-3-AL) was diluted in 0.06M carbonate buffer (5.0406g Na2HCO3; 6,359g NaH_2CO_3; H_2O q.s.p. 1000mL, pH 9,6) in order to generate a 10mg.mL^{-1} solution per 100mL buffer and were added to each one of a 96 wholes polystyrene plane bottomed micro plates (Immulon-2, Dynatech, USA). Plates were covered with parafilm and incubated for 2 hours at 37°C, followed by an overnight incubation at 4°C. The plates remained for a month.

3.8 Competitive ELISA with pure grade 3-AL

The coated plates were washed four times with 5% (200mL.L^{-1} – v/v) PBS-Tween 20 (PBS-Tw). Sample solution with aliquots (50ml) in PBS-Tw (extracts homogenate solution in the range of 0.1 – 24 ng/ml ([5–1,200 pg/well]) and 50ml of antibody (working dilution 1/ 2,500) in the same buffer containing 0.1% casein were transferred into wells of the precoated plates (100ìL/ well). After the incubation and washing, the antibodies associated with the plates visualized using the amplification procedure described above. The signal obtained in presence of inhibitor and maximal signal are referred to as L and Lo, respectively. Plotting the percent inhibition (100%–% L/Lo) generated inhibition curves against the log of the free 3-AL concentration, representing 85% of the maxime absorbance as the lowest limit detection (LOD). The 3-AL reactivity was expressed as the ratio of 50% intercept of 3-AL. As the recovery of bioactive substances from plants and natural sources depends on the material used, we minimized matrix effects performing competitive experiments in which phyto-hapten concentration in the sample varied by dilution and were compared with the results from matrix controls (lives, stalks and roots extracts) without 3-AL. All the competitive assays were carried out with four repetitions of each dilution.

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