The *in vitro* Anti-denaturation Effects Induced by Natural Products and Non-steroidal Compounds in Heat Treated (Immunogenic) Bovine Serum Albumin is Proposed as a Screening Assay for the Detection of Anti-inflammatory Compounds, without the use of Animals, in the Early Stages of the Drug Discovery Process

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ABSTRACT

*There are emerging ethical issues with regards to the use of animals in the early stages of drug discovery for anti-inflammatory and degenerative diseases from natural products using the activity-directed isolation pathways when many compounds (*eg *> 100) are present in the crude extract or fraction and are to be tested. The above-mentioned is the main reason for proposing the use of the in vitro anti-denaturation (stabilization) effects of heat treated (immunogenic) bovine serum albumin (BSA) as an assay. Current methods used for detecting and isolating a wide range of anti-inflammatory compounds in the early stages of the drug discovery process utilize a large number of animals. When BSA is heated and is undergoing denaturation, it expresses antigens associated to Type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Thus, the assay that is being proposed should be applicable to the discovery of drugs for treating the above mentioned diseases and others, once the compounds stabilize the denaturation process.*

Los Efectos de la Anti-desnaturalización *in vitro* Inducida por Productos Naturales y Compuestos no Esteroidales en la Albúmina Sérica Bovina (Inmunogénica) Tratada con Calor, se Proponen aquí como Ensayo de Pesquizaje para la Detección de Compuestos Inflamatorios sin el uso de Animales en las Etapas Tempranas del Proceso de Descubrimiento del Medicamento

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RESUMEN

Actualmente surgen problemas éticos en relación con el uso de animales en las etapas tempranas del descubrimiento de medicamentos anti-inflamatorios y contra enfermedades degenerativas, a partir de productos naturales, usando vías de aislamiento dirigido por actividad, cuando muchos compuestos están presentes (p.ej. > 100) en la fracción o extracto crudo, y deben ser probados. Lo anterior representa la razón principal para proponer el uso de los efectos de la anti-desnaturalización (estabilización) in vitro *de la albúmina sérica bovina (inmunogénica) tratada con calor (ASB) como ensayo. Los métodos corrientes usados para detectar y aislar una amplia gama de compuestos anti-*

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inflamatorios en las etapas tempranas del proceso de descubrimiento del medicamento, utilizan un gran número de animales. Cuando la ASB es calentada y sometida a un proceso de desnaturalización, expresa antígenos en relación con la reacción hipersensitiva de tipo III, relacionada a su vez con enfermedades tales como la enfermedad del suero, la glomerulonefritis, la artritis reumatoide, y el lupus sistémico y eritematoso. De este modo, el ensayo que aquí proponemos debe ser aplicable al descubrimiento de medicamentos para el tratamiento de las enfermedades anteriormente mencionadas y otras, una vez que los compuestos estabilicen el proceso de desnaturalización.

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INTRODUCTION

There are inherent problems in using animals in research such as the lack of rationale for their use when other methods are available or could be investigated (1). In addition, cross species differences make extrapolation very difficult due to lack of analogy-isomorphism with human species, especially at the cellular and molecular level where disease occur (1). The numbers and hence cost of animals required for the isolation of an anti-inflammatory prototype from complex natural product extracts using the activity-directed isolation pathway is alarming. It is for the above reason that Roach and Sufka (2) have proposed the chick carrageenan response assay for the discovery of molecules with anti-inflammatory nociception properties. However, the Bovine Serum Albumin (BSA) assay seeks to eliminate the use of live specimens as far as possible in the drug developmental process. Grant *et al* (3) have reported that one of the features of several non-steroidal anti-inflammatory drugs *eg* indomethacin, ibufenac, flufenamic acid and salicylic acid is their ability to stabilize (prevent denaturation) heat treated BSA at pathological pH [pH 6.2 – 6.5] (3, 4). The authors have been examining the ability of several natural products (complex extracts and pure compounds) to stabilize BSA at pathological pH for a number of years (1997 – 2007). Based on the findings, the effect is hereby proposed as an assay for the detection of extracts, not only for anti-inflammatory activity, but for a wide range of biological action for isolating the active compounds which can serve as drug prototypes for final testing in animals. It is also interesting to note that some of the extracts and pure compounds *eg* the extract of *Boehmeria jamaicensis* (Urb) (Urticaceae), phenyl propanoid; eugenol and the poly-sulphide, dibenzyl trisulphide (DTS), which prevent the denaturation of BSA, have *in vitro* anti-oxidant activity (5, 6) which is also a feature of some anti-inflammatory drugs. In these studies, DTS demonstrated no *in vitro* anti-oxidant activity using the 1,1-diphenyl-2-picrylhydrazine (DPPH) assay but has high anti-cancer activities (7, 8) with implications for treating various forms of human leukocyte antigen-DR (HLA-DR) immune system dysfunction diseases (9). Thus, the authors are of the view that when compounds stabilized BSA at low doses such as 0.012 to 1.0 µg/mL or lower, they should be channelled into a broad spectrum of *in vitro* assays to evaluate their potential as drug prototypes. Williams *et al* (10) also strongly support the broad spectrum screening of compounds for maximum therapeutic development but their philosophy is based on the diverse structures of secondary metabolites (natural products).

MATERIALS AND METHODS

The methods used for assessing the anti-denaturation effects of natural products with anti-inflammatory properties is simple and inexpensive. Grant *et al* (3) reported, for the first time, the above mentioned protocol, however Williams *et al* (11, 12) have reported on modified versions of Grant's protocol in an effort to reduce cost by eliminating solubilizers *eg* dimethylformamide (DMF) and Triton X-100, and instead used pure methanol. Methanol was selected for dissolving the test compounds in the assays of Williams *et al* (11,12) based on the fact that it will not affect the gross molecular configuration of albumin at concentrations up to 30 % [w/v] (13). Williams *et al* (11, 12) used 1.0 % to 10.0 % methanol in the albumin.

Assay No 1 reported by Grant *et al* (3)

The test system contained 2.5 ml of buffered 1% BSA and 2.5 ml of buffer or test solution (unless otherwise specified, the BSA used was fraction V). The buffer was 0.05M Tris-acetate; dimethylformamide (DMF) was added to a maximum of 2.5% (v/v) which was necessary for solubilization of the test compounds. Control and test systems were heated at 69°C for exactly 4 minutes, cooled and their turbidities read at 660 mµ. Aggregates which decreased turbidities by less than 20% at 1 mM were tested a second time at the same level; those which decreased turbidities by more than 20% were judged active and were tested at 0.8, 0.6, 0.4, 0.2 and 0.1 mM. Compounds retaining activity at 0.1 mM were run at still lower concentrations.

Assay No 2, the modified versions of Grants *et al*, 1970 published by Williams *et al* (11)

Stock solution of 0.5% (w/v) BSA, fraction V of 96% purity (Sigma Chemical Co) without further purification was prepared in a mixture of 0.05 M tris-phosphate buffer saline which was adjusted to pH 6.5 with glacial acetic acid. Triton X-100 (0.01% w/v, in distilled water) was added to the tris-acetate buffer in a ratio of 5:95 (v/v). The following stock solutions 50.0 ppm, 5.0 ppm, 0.5 ppm and 0.05 ppm of compounds were prepared in methanol. From each of the above mentioned stock solutions 500 µL was added to 5.0 mL of the 0.5% (w/v) stock BSA in tris-acetate buffer Triton-X 100

mixture to produce concentrations of 0.1 to 10.0 µg/mL of each compound. Each sample was heated for 5 minutes at 70°C in a test tube placed in a water bath, cooled and its turbidity measured at 660 nm on a Beckman Spectrophotometer in 4.0 mL cuvettes in two replicates. The degree of inhibition of denaturation or precipitation of the BSA from the solution by each compound was calculated as shown in equation 1:

Equation 1:

$$\frac{\text{Absorbance of control minus Absorbance of treated}}{\text{Absorbance of control}} \times 100$$

In the above assay, compounds inhibiting denaturation greater than 20% over the range of concentrations were considered as having anti-inflammatory properties and could be of value for drug development (11).

NOTE: Williams *et al* (11) have also eliminated Triton X-100 (0.01% w/v) as a solubilizer from the buffer solution. In the above situation, a stock solution of 0.2% (w/v) BSA is prepared in the tris-acetate phosphate buffer, pH 6.5. The test compounds should then be applied to 5.0 mL BSA solution in 50 µL of methanol and the mixture heated for 4 minutes at 73°C and then cooled for spectrophotometric analysis.

Assay No 3, published by Williams *et al* (12)

This method was designed to reduce the volume of BSA and stock solution of compound or extract to be used for assessing the anti-denaturation (anti-inflammatory) activity. This assay (12) is therefore appropriate when small quantities *eg* < 2.0 mg of extract or compound is to be analyzed.

In this method, stock solution of 0.2% (w/v) of BSA was prepared in tris-buffer saline and adjusted from pH 8.53 to 6.74 using glacial acetic acid. Two replicates of 500 µL of the BSA stock solution were pipette and 5.0 µL of the test compound or extract dissolved in methanol and added to the BSA solution from two stocks of 50 ppm (0.005%; w/v) and 0.5 ppm (0.00005%; w/v). The control consisted of 500 µL BSA with 5 µL methanol. The samples were then heated at 72°C for 5 min in 2.0 mL Eppendorf tubes in metal racks, then cooled for 20 minutes under laboratory conditions and absorbance read using a Spectrofluorimetric wavelength parameter of Ex 480/Em 520 in 1.0 mL glass cuvettes or at 660 nm in a Spectrophotometer. The percentage inhibition of precipitation (stabilization of the protein) was determined on a percentage basis relative to the controls as in equation 1 shown above.

RESULTS

The results presented below were derived using method number 2 stated above. The data presented in Figures 1 and 2 give graphical representation of two extracts: *Boehmeria jamaicensis* and *Gliricida sepium* (Jacq) Kunth ex Griseb (Papilionaceae) which protect BSA from denaturation.

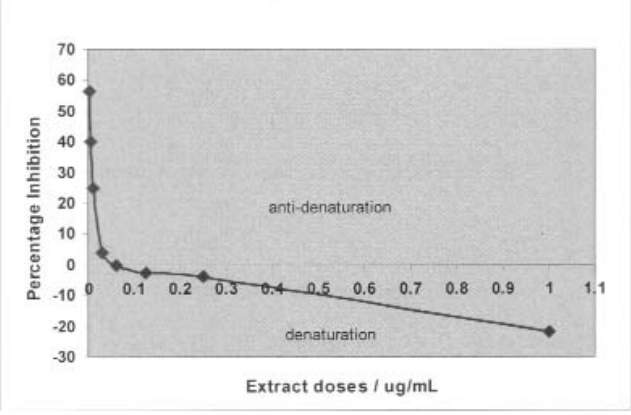

Fig. 1: Percentage denaturation of bovine serum albumin *in vitro* due to *Boehmeria jamaicensis* extract.

Figure 1 revealed that the ethanolic extract of *Boehmeria jamaicensis* leaves and stems which stimulated gamma interferon above 24 fold at 25 µg/mL (14) protected BSA from denaturation at a concentration less than 0.012 µg/mL (> 20% anti-denaturation effect). The crude ethanolic extracts of *B jamaicensis* demonstrated high level (> 90%) antioxidant activity in the *in vitro* DPPH free radical scavenging assay (5). In addition, tea made from the leaves of *B jamaicensis* is effective against the common cold (14). Thus, it is evident that extracts which stabilize BSA seem to have interesting multiple biological action.

Figure 2 shows the anti-denaturation/denaturation profile of the ethyl acetate fraction of a crude ethanolic extract from the leaves and stems of *Gliricida sepium*. The data

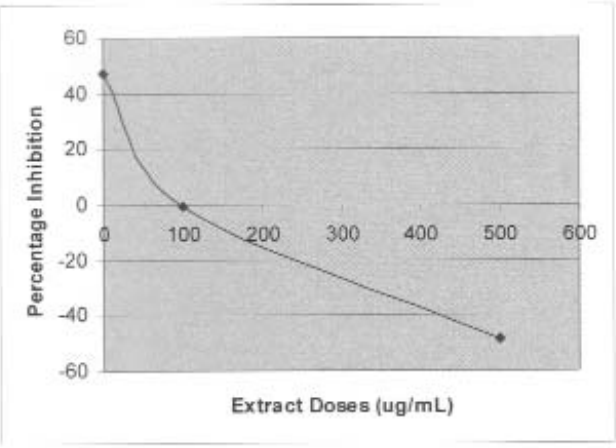

Fig. 2: Percentage of denaturation of bovine serum albumin *in vitro* due to *Gliricida sepium* extract.

presented in Figure 2 revealed that the extract of *G sepium* protects BSA from denaturation (> 20% anti-denaturation) at concentration lower than 1.0 µg/mL. The extract also demonstrated high level of anti-proliferation/ cytotoxic activity (50 µg/mL inflicted > 90.0% anti-proliferation/cytotoxic action) on the human SH-SY5Y neuroblastoma cells *in vitro* (unpublished data).

Various fractions of the crude ethanolic extracts of the dried green leaves of *Artocarpus altilis* (S Parkinson) Fos-

berg, (Moraceae) (breadfruit) stabilized BSA (prevent denaturation) at concentrations < 1.0 µg/mL which Grant *et al* (3) stated is a feature of non-steroidal anti-inflammatory drugs. Caribbean folklore states that the leaves of *A altilis* are used to prevent pain and inflammation (15). Singh *et al* (15) have demonstrated anti-inflammatory activities of *A altilis* leaves using the carrageenan rat paw oedema and isolated organ section assays, thus further supporting the validity of the use of the anti-denaturation effects of BSA as a potential therapeutic parameter for finding anti-inflammatory compounds without the use of animals. The crude ethanolic extracts of *A altilis* which failed to stabilized BSA demonstrated low anti-proliferation/cytotoxic and *in vitro* anti-oxidant activities. However, upon fractionation of the crude ethanolic extract, high level of cytotoxic activities (50 µg/mL inflicting > 90% anti-proliferation activity) were found in the medium polar fraction (ethyl acetate fraction) on the human SH-SY5Y neuroblastoma cells *in vitro*. This fraction also stabilized BSA. Today several geranyl flavonoids have been isolated from the leaves of *A altilis* with cytotoxic activity (16).

In a series of experiments, the phenyl propanoids: iso-eugenol and eugenol demonstrated 51.03% and 58.73% anti-oxidant activity using the DPPH assay at 20µg/mL, respectively, while methyl eugenol was inactive. Eugenol and iso-eugenol are present in the leaf oil of *Pimenta dioica* (L.) Merr. (Myrtaceae) which gave 83.84% anti-oxidant activity at 50 µg/mL in the DPPH free radical assay (5) and is used for treating inflammation in Jamaica. Williams *et al* (11) have demonstrated that eugenol and iso-eugenol were effective in preventing the denaturation of BSA at concentration < 10.0 ppm while methyl eugenol was weak to inactive. It is also interesting to note that eugenol is now formulated commercially with diclofenac to enhance its anti-inflammatory action.

DISCUSSION

In utilizing the anti-denaturation *in vitro* assay which is being proposed for isolating compounds from plants, it is advisable to prepare a crude ethanol extract of the selected plant part(s) under investigation. A hexane fraction (non-polar fraction), ethyl acetate fraction (medium polar fraction) and a methanol-water fraction can then be prepare from this crude extract by freeze drying for testing (17) in order to find the most active fraction(s) from which the compounds are to be isolated. Several modifications of these extraction pathways exist today. These fractions should normally be tested at concentrations ranging from 50.0 µg/mL to 0.0035 µg/mL over a range of six doses. It is worthy of note that the anti-denaturation action of the biologically (therapeutic) interesting compounds and extracts is greatest when the concentrations are lowest. This means only a small quantity (2 mg) of the crude extracts and their fractions and compounds are required for testing. Once there is anti-denaturation activity, the fractions and compounds isolated should be incorporated into a broad spectrum of *in vitro* biological screens (*eg* anti-oxidant, anti-cancer, immuno-modulation *ie* cytokine release, angiotensin converting enzyme inhibition using enzyme link immunosorbent assay *etc*) and the best activity found for the pure molecule(s) explored for the development of drugs. The above drug developmental process seeks to use animals after the pure compounds are isolated and their biological activities confirmed, thus excluding the use of animals in testing of crude extracts at early stages of drug exploration.

Williams *et al* (11), based on one and two dimensional proton nuclear magnetic resonances (1D and 2D ^{1}H NMR) assignments published by Sadler and Tucker (18) for BSA, made some predictions on the possible interaction/binding sites of molecules with anti-denaturation action on bovine serum albumin. Two of the interesting binding sites are in the aromatic tyrosine rich and aliphatic threonine and lysine residue regions of the BSA (11). From these interactions on BSA, Williams *et al* (7, 11) predicted that some of the interesting therapeutic molecules could be activating the tyrosine motif rich receptor dually with threonine that regulate signal transduction biological pathways for their overall biological action (7,12). Those compounds interacting with the aliphatic regions around the lysine residue on the BSA could be interesting as anti-oxidants with anticancer activity such as the polyphenols, phenyl propanoids and the disulphides (*eg* alpha-lipoic acid) and could also play important roles as anti-glycation compounds (11, 12, 19). Crystallization of molecules *eg* dibenzyl trisulphide and eugenol have also been achieved for X-ray crystallographical analyses which the authors hope will further validate the binding sites of the compounds in BSA (12).

CONCLUSION

In conclusion, it would appear that the anti-denaturation effects observed for BSA when interacted with biologically active compounds could be manipulated as first stage screens for selecting natural products for therapeutic drug development. The molecules or extracts demonstrating anti-denaturation activity at the lowest possible doses (nanogram per mL concentration) are to be selected for broad spectrum screens towards developing a wide range of therapeutic drugs.

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