

Applying New Science for Old Medicines: Targeting Leukocyte-Endothelial Adhesions by Antiinflammatory Herbal Drugs

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During the last two decades, considerable progress has been made in understanding the molecular mechanisms of the various leukocytes and endothelial cell adhesion molecules (cell adhesion molecules - CAMs) involved in cell-cell and cell matrix interactions. This understanding has opened up a new avenue of novel chemotherapeutic targets and bioassay models for inflammatory diseases. Recently developed *in vitro* bioassays on leukocyte/endothelial cell adhesions can now offer rapid and inexpensive assessment methods for herbal medicines with claimed antiinflammatory uses. Through the use of these robust *in vitro* methods, active principles of herbal drugs can also be isolated thereby providing the opportunity of standardizations based on a known chemical standard(s) and pharmacology. This review highlights relevant leukocyte/endothelial CAMs targets, available *in vitro* methods and our strategic approach for herbal standardizations.

Keywords: inflammatory targets-adhesion molecules expression-cell adhesion-bioassays-herbal medicines standardization.

HERBAL MEDICINES WITH HOLISTIC ORIENTATION: THE CHALLENGE

The role of medicinal plants as a source of numerous clinically useful medicines has been well demonstrated. Approximately 70% of new chemical entities reported during the last two decades resulted from studies on natural products [1] of which about 48% are from plants [2]. Likewise, herbal medicines as a holistic therapy and with over 5000 years of history, have recently gained more worldwide recognition within the scientific and health care communities.

Most of the recent scientific researches on herbal drugs focus on the modernization of therapeutic practices by addressing the *quality*, *safety* and *efficacy* issues [3-5]. The "European Directive on Traditional Herbal Medicinal Products" regulation which requires traditional and over-the-counter herbal remedies to be based on an established safety and quality standards is a perfect example of recent developments in this sector. There still exists, however, a large gap between herbal practices and contemporary medical sciences in philosophy, theories and applications. Novel bioassay methods are desperately required to facilitate the process of scientific evidence provision and also

eliminate the notion of herbal medicines working through realm of myth. In view of this development, researches in our laboratories are devoted to pharmacological evaluations of herbal medicines, identification of active constituents, and standardisation of herbal products through established in-house pharmacological and chemical assays. Our strategic approach is presented here by using our interest in the identification of leukocyte-endothelial cell adhesion inhibitors from herbal sources.

GENERAL METHODOLOGY AND STUDY APPROACH

Our approach is based on an understanding that there is no contradiction between the holistic and single chemical entity principles. As with the discovery of many useful drugs of single chemical entity from plants have been proven beyond doubt, we believe that in some cases no single active constituent is responsible for the overall efficacy or quality. Thus, we try to validate either option through scientific studies.

Challenges in bioassay screening remain an important issue in providing the scientific basis of herbal medicines. Bioassays based on *in vitro* responses of

stable cell lines are common assay of choice as they provide pharmacologically relevant information with less variability, expense and labor-intensity than *in vivo* assays [6]. The ever growing stringent restriction in the use of experimental animals for medical research does also mean that sufficient *in vitro* data need to be generated to demonstrate the justification for moving experiments to animals. In our laboratories, we use a variety of rapid cell-based bioassay methods customized for fast preliminary screening of herbal drugs. This is followed by detailed mechanism of action studies using protein-based assays. While many scientists and pharmaceutical industries tend to isolate active ingredients and test them one at a time, our approach has been a systematic bioassay-guided isolation and characterization of active constituents. We use a variety of analytical tools including NMR, mass spectrometry (MS), liquid chromatography and hyphenated (GC-MS, LC-MS, etc) methods for rapid detection and identification of herbal components.

LEUKOCYTE TRAFFICKING AS TARGETS FOR INFLAMMATORY DISEASES

General overview: Inflammation is a necessary host defence mechanism that involves leucocytes emigration into extravascular tissues to deal with tissue injury, infection or other cellular/biochemical insults. In a regulated acute inflammatory response, the inflammatory reactions and associated cardinal symptoms (see Figure 1) are short lived and ultimately lead to the restoration of normal tissue architecture and function. During chronic inflammatory conditions, however, abnormally high level of leucocyte trafficking results in extensive tissue damage and pathologies such as those seen in asthma [7], arthritis [8,9], atherosclerosis [10-12], inflammatory bowel disease [13-15], ischaemia-reperfusion injury [16-18], multiple sclerosis [19,20] and sepsis [21]. Hence, down-regulating leucocyte mobilization is a key therapeutic target for chronic inflammatory diseases [22].

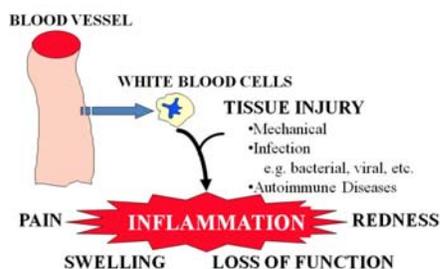


Figure 1: Schematic diagram showing an acute inflammatory process and some of its cardinal signals.

Adhesion molecules expression, cell-cell adhesion and cell-matrix interactions: Under normal physiological

conditions, leucocytes circulate freely owing to the maintenance of a delicate homeostatic balance between proinflammatory and antiinflammatory hormonal/local mediators. In chronic inflammatory conditions, where the homeostatic balance is tipped in favor proinflammatory mechanisms, leucocytes and endothelial cells are subjected to continuous activation by a plethora of mediators. Among the most relevant and best characterized mediators of chronic inflammatory diseases are tumor necrosis factor- α (TNF), interleukin-1 β (IL-1) and interleukin-6 (IL-6). These cytokines induce the activation and expression of various cell adhesion molecules (CAMs) on leucocyte/endothelial cell surfaces leading to coordinated sequential leucocytes-endothelial and leucocyte-matrix interactions. Cell-cell and cell-matrix interactions are thus the prerequisite to leucocyte emigration to inflamed tissues. The various CAMs expressed on endothelial/leucocyte cell surfaces and their role in inflammatory diseases have been extensively reviewed in recent years [12,14,22-25]. The scope of this section is thus limited to a brief review of the major cell adhesion-based therapeutic targets for herbal medicines.

The first phase of leucocyte endothelial interaction is characterized by 'rolling' which is mainly mediated by the lectin- and epidermal growth factor-containing CAMs called selectins (see Figure 2). The predominant endothelial and leucocyte selectins responsible for this weak adhesive interaction are P-selectin (CD62P, stored in Weibel-Palade bodies inside the cell) and L-selectins (CD62L, exist in their inactive form) respectively. Endothelial cells do also express E-selectins (CD62E) when they are stimulated by proinflammatory cytokines like TNF but the time course of its expression (from 2 h) suggests that its role is also extended to the stage of firm leucocyte-endothelial cell adhesion [11,26]. The lectin- and EGF-like domains of selectin CAMs are known to mediate interaction with sialylated and fucosylated oligosaccharides (e.g. sialyl-Lewis^X tetrasaccharide, CD15s).

The immunoglobulin family of CAMs are by far the most important mediators of proinflammatory cytokines-stimulated endothelial-leucocyte adhesions. The most common examples of this CAMs group with relevance to chronic inflammatory diseases are the intercellular adhesion molecule-1 (ICAM-1, CD54) and the vascular cell adhesion molecule-1 (VCAM-1, CD106) which are known to play pivotal role in firm leucocyte-endothelial adhesions followed by transigrations [22, see also Figure 2].

The ligands for ICAM-1 are the heterodimeric glycoproteins known as integrins which are expressed

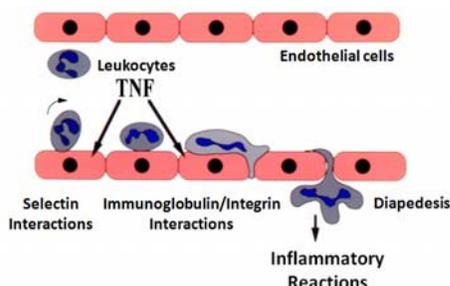


Figure 2: The three stages of leukocyte-endothelial adhesions are shown: rolling of leukocytes through weak interactions, strong leukocyte-endothelial adhesions and morphological changes leading to leukocyte emigration to extravascular tissues. Note that principally TNF but also many other proinflammatory cytokines such as IL-1 and IL-6 mediate firm endothelial-leukocyte adhesions.

on leukocytes cell surface. These integrins are composed of the common β_2 -subunit (CD18) linked with α -subunits designated as CD11a, CD11b, CD11c, or CD11d. Similarly, the counterpart receptor for VCAM-1 is an integrin adhesion molecule VLA-4 (very late antigen-4) which is an $\alpha_4\beta_1$ (CD49d/CD29) heterodimer expressed by leukocytes. Agents which either directly interact with the endothelial immunoglobulin class or leukocyte integrin CAMs have great therapeutic potential for uses in chronic inflammatory diseases [14,22]. There are also several integrin CAMs known to mediate leukocyte interaction with matrix proteins such as laminins, collagens and fibronectin [12].

A variety of inflammatory mediators including TNF, IL-1, bacterial products such as lipopolysaccharide (LPS) and reactive oxygen species are known to induce the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cell surface [27,28]. Since these CAMs are primary mediators of firm endothelial-leukocyte adhesions and their expression in chronic inflammatory diseases is highly upregulated, there has been a growing interest to target their expression [22-25]. The induced expression of ICAM-1, VCAM-1 and E-selectin is regulated by transcription factors such as NF- κ B and AP-1. In the past two decades, an explosive level of interest has been expressed in NF- κ B as it is also linked to upregulation of a variety of inflammatory mediators. NF- κ B proteins occur in the cytoplasm of cells as a heterodimers with bound inhibitory I κ B family proteins. Stimulation of endothelial cells by the above mentioned inflammatory mediators result in the phosphorylation of I κ B by I κ B kinase with subsequent ubiquitination and degradation of I κ B [29,30]. The free NF- κ B is then translocated to the nucleus where it binds to κ B binding sites in the promoter regions of target genes. Thus, NF- κ B mobilization has been regarded as a key therapeutic target for inflammatory and many other disease conditions including cancer [31-34].

In vitro Experimental Models of Adhesion Molecules Expression and Cell Adhesions:

The measurement of endothelial cell adhesiveness and expression of CAMs require *in vitro* cell culture and maintenance. Endothelial cells seed culture can be initiated from various blood vessels of experimental animals' origin but the most widely used are those originated from human umbilical vein. The maintenance of primary human umbilical endothelial cells (HUVEC) culture however is challenging as it requires the use of various growth factors and cultured cells being relatively slow to grow. Inherently, primary cell cultures have also a limited usable passage time (normally less than 5 for HUVEC) as continuous culturing leads to a loss of their endothelial characteristics. Hence, an alternative cell lines that allows fast screening of herbal medicines is a key for developing a good bioassay capability. Out of the various primary culture sources that we standardized for routine screening assays, bovine aortic endothelial cells (BAEC) were the cheapest and relatively easiest to handle. These endothelial cells can easily be scraped of from the inner lumen of the aorta and cultured until confluence. Treatment of BAEC with TNF and other mediators results in upregulation of expression of ICAM-1, VCAM-1 and E-selectin [35-37]. Interestingly, TNF-treated BAEC support the adhesion of leukocytes from other species, e.g. murine origin [38]. In our experiments, the adhesion of TNF-treated BAEC to human monocytes (see Figure 3) has successfully been used for initial screening of herbal drugs. In search of a more robust, cheap and relevant functional assay, however, our recent efforts were focused on commercially available endothelial cells derived from HUVEC. One of the most productive cells lines that we extensively characterized for its profile of CAMs expression and adhesiveness to leukocytes has been the EAhy 926 cells. This cell line is a product of hybridization between HUVEC and A549 carcinoma cells and displays the various features of endothelial features [39].

We have shown that exposure of EAhy 926 endothelial cells to TNF, LPS or PMA can induce a concentration- and time-dependent expression of ICAM-1 [40-45]. The cell surface expression of such CAMs on endothelial cells can be easily measured by an ELISA [40,44,46; Figure 4] or flow cytometry [30] methods. Classical example of the concentration-dependent expression of ICAM-1 by TNF is shown in Figure 4a. In parallel with the measurement of the induced expression of CAMs, the adhesiveness of endothelial cell surface to leukocytes needs to be measured. As a source of leukocytes, we routinely use human monocytic cells lines (e.g. U937 cells) thereby avoiding the burden of leukocytes isolation from healthy human volunteers. Since integrin adhesion molecules on leukocytes cell

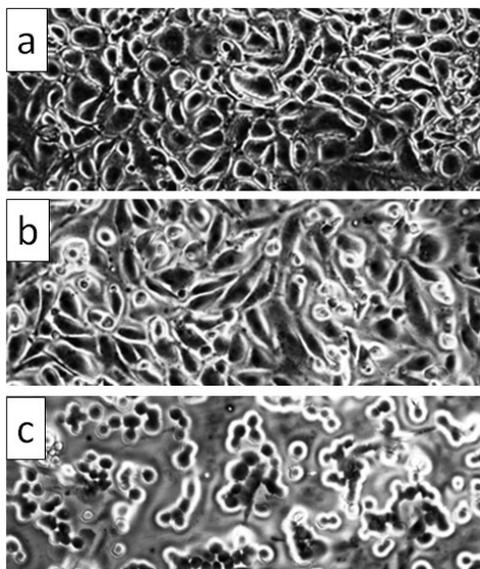


Figure 3: Adhesion of monocytic PMA-activated U937 cells to BAEC. Cultured BAEC were grown to confluence (a) and subjected to monocyte adhesion after no treatment (b) or treatment with 1 ng/ml TNF for 24 h. Note the small number of monocyte adhesion to untreated BAEC as compared with the TNF-induced carpeting of BAEC with U937.

surface normally exist in their inactive form, we use phorbol-12-myristate-13-acetate (PMA) for rapid cell activation. As shown in Figure 4, activated endothelial cells greatly increase their adhesiveness to U937 cells. We have shown that over 60% of the TNF-induced EAhy 926 endothelial cell's adhesiveness to monocytic U937 cells was due to the expression of the major endothelial cell adhesion molecule, ICAM-1 [40]. The further advantage of using stably transformed cells, like U937 cells, is the ease of their labelling with less hazardous radioisotopes like [^3H]-thymidine [40,42,45; Figure 4a] or fluorescent probes [47, see also Figure 4b]. By using this model of cell-cell adhesion, we have screened several medicinal plants known to be used in traditional medicine for inflammatory diseases. Some of our key findings are discussed below as case studies.

We [41,42] and others [48] have also shown that prolonged activation of monocytes by specific agonistic-antibodies or other stimuli (e.g. PMA) results in β_2 integrins-mediated homotypic cell aggregations. Integrin CAMs-mediated monocyte adhesion with matrix proteins, especially fibronectin, is another common feature of leukocyte functions [41,42,45,49]. The recognition of fibronectin by monocytes is predominantly mediated through interaction *via* β_1 integrins [41], while interaction with fibrinogen is a predominant function of β_2 integrins [45]. The activation/expression processes of these CAMs and adhesion-based cellular functions are further examples of targets for inflammatory diseases and *in vitro* bioassay screens.

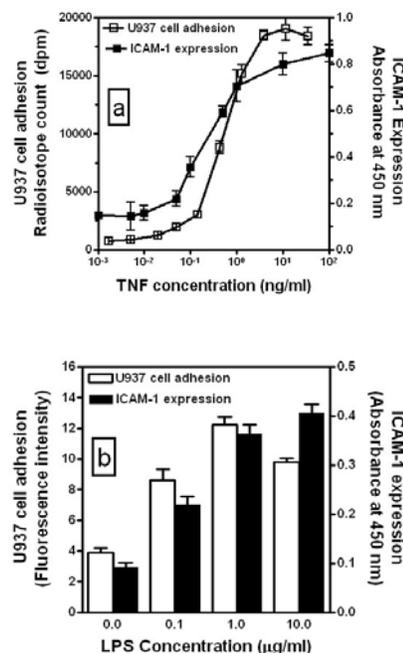


Figure 4: Representative results from our in-house cell-cell adhesion and endothelial CAMs expression assays. EAhy 926 endothelial ICAM-1 expression and adhesiveness to monocytes was measured following treatment with TNF for 4 h (a) or LPS for 24 h (b). ICAM-1 expression was measured by ELISA while endothelial-leukocyte adhesion was assessed by using [^3H]-thymidine (a) or fluorescent (2',7'-Bis-(2-Carboxyethyl)-5-(And-6)- carboxyfluorescein) (b) labelled monocytes. Results are mean values and SEM from three or four replicates.

STRATEGIES FOR HERBAL STANDARDIZATION THROUGH BIOASSAY-PHYTOCHEMISTRY CROSSTALK

In order to make sure that potentially heat-labile compounds are not destroyed during the extraction process, our herbal medicines are always extracted with cold alcohol. Under this circumstance, one should consider maximizing the yield of extraction by repeating the extraction process and/or prolonging the extraction period (2 weeks). Before extracts are tested for their antiinflammatory effect, their cytotoxicity profile in endothelial cells and leucocytes need to be established. Through this pre-screening assay procedure, we often identify herbal drugs with acute toxicity to mammalian cells [50,51]. Non-toxic concentrations of crude extracts are then tested in a functional leukocyte-endothelial *primary adhesion assay*. The suppressive effect of extracts on the expression of CAMs on endothelial/leukocyte surfaces [40-45], leukocyte/endothelial adhesions with single-purified CAMs of interest or other functional studies including, homotypic cell adhesion and leukocyte-matrix interactions [41,42] can serve as *secondary assays* for confirmation of biological activities. The overall strategy of our approach in this field is summarized in Figure 5.

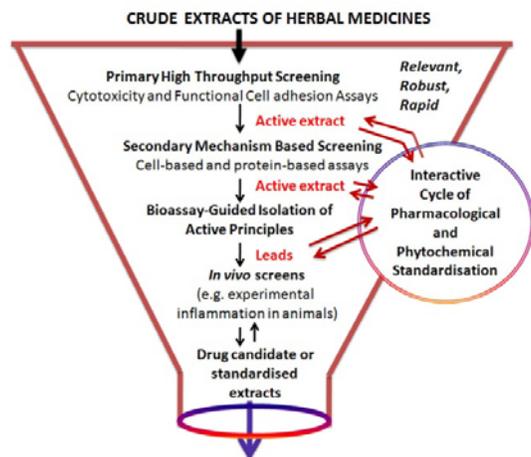


Figure 5: General strategy for phytochemical and cell adhesion-based pharmacological characterizations of antiinflammatory herbal drugs.

By using a functional endothelial-leukocyte adhesion assay as a guide, attempt should be made to isolate the active principles of crude herbal drug extracts. The science and art of bioassay-guided fractionation requires the use of chromatographic techniques hand in hand with bioassays. In the first instance, one should consider a simplified fractionation procedure by using solvents of ascending polarity (e.g. petroleum ether, chloroform, ethyl acetate and *n*-butanol partitioning from extract suspensions in water) followed by detailed activity-guided chromatographic analysis. We have successfully used this art to isolate active compounds from a number of herbal drugs [51-57 are just few to mention]. Once active compounds are isolated and identified, their activity profile can be established by the above mentioned assays. Further *in vivo* or clinical studies can also be considered depending on the promise of the demonstrated activity for the active principle(s) and/or crude extracts.

SELECTED CASE STUDIES

Of the many European medicinal plants screened for *in vitro* suppression of leukocyte trafficking, *Eupatorium purpureum* L. of the family Asteraceae was by far the most interesting. The root of this plant, commonly known as ‘gravel root’, has been specifically used in traditional medicine for treating rheumatic conditions [58]. Since scientific evidence in support of its indicated medicinal uses was not available, we studied the *in vitro* inhibitory effect of gravel root extract on leukocyte trafficking. The extract did not modify the induced-expression of CAMs on endothelial cell surface nor did it alter the adhesiveness of stimulated endothelial cells [41]. When the extract was added to monocytic U937 cells either during activation by PMA or at post-activation level, however, it potently inhibited monocyte adhesion [41]. As evidenced by a number of

other functional studies, including homotypic cell aggregation, it appeared that the extract directly interacts with monocyte integrin CAMs including the ligand for ICAM-1, lymphocyte function associated antigen-1 (LFA-1, CD18/CD11a; 41).

By using a systematic bioassay-guided fractionation study, the active principle responsible for the inhibition of monocyte adhesion by gravel root extract was isolated and characterized [42, Figure 6]. We have demonstrated that the active principle, inhibits integrin-mediated monocyte adhesion to activated endothelial cells [42]. In order to further ascertain the promise of the purified compound prior to moving our experiment to animal studies, a number of secondary assays were used. These include the demonstration of inhibitory activity on PMA-activated monocyte adhesion to purified ICAM-1, matrix proteins including fibronectin and fibrinogen, and homotypic cell aggregation [41,42,45]. Finally, an effect both by the crude extract and the active principle were demonstrated in the classical carrageenan rat paw edema (*in vivo*) model of inflammation [42]. As the carrageenan edema model reflects more of an acute inflammatory process, gravel root is expected to have a more pronounced effect in chronic *in vivo* models of inflammation where integrin CAMs play a principal role [9]. Thus, our studies not only established the scientific basis of gravel root’s use in traditional medicine but also provided evidence of a single chemical entity compound to be exploited from the herbal medicine.

While gravel root extract was directly interacting with integrin adhesion molecules on activated leucocytes, the activation process of integrin CAMs has been shown to be suppressed by extracts of some other medicinal plants. The β_1 integrin-mediated U937 homotypic aggregation has been shown to be inhibited by *Cinnamomum camphora* extracts [59] while the active principle (magnolol) of the traditional medicinal plant, *Magnolia officinalis*, with proven anti-inflammatory activities was shown to suppress β_2 integrin (CD11b/CD18, Mac-1) activation [59].

‘Corn Silk’, stigma/style of *Zea mays* L (Gramineae) is another European medicinal plant listed in various monographs [58]. The traditional uses of the plant include a range of disease conditions with specific reference to acute and chronic inflammation of the urinary system. Unlike the case of gravel root, the crude ethanolic extract of corn silk did not modify the adhesion of monocytes to activated endothelial cells [40]. When the extract was added together with potent inflammatory mediators (e.g. TNF or LPS), however, the expression of adhesion molecules (e.g. ICAM-1) and endothelial adhesiveness to monocytes were

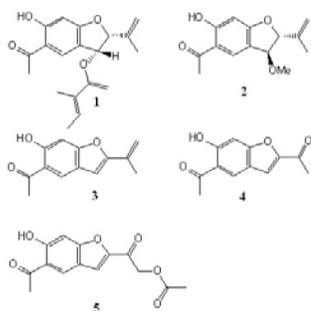


Figure 6: Compounds isolated from the most active fraction of gravel root extract. Bioassay-guided fractionation led to the identification of the active antiinflammatory principle as compound **1** [42].

potently suppressed. A bioassay-guided fractionation studies on the crude active extract of corn silk however did not result in the identification of a single more potent antiinflammatory component (unpublished results). These results suggest that the use of the crude extract in this particular case is better than a single chemical entity of corn silk-origin.

Several related studies from our laboratories [43,44] and others have shown that suppression of the induced expression of CAMs on endothelial cell surface is a valid therapeutic target for herbal medicines. In this regard, it is worth noting that many traditional Chinese medicines with indicated herbal uses related to inflammation have recently shown to suppress cytokines-stimulated CAMs expression on endothelial cells [61-64]. The antithrombic effect of several tea [65] and citrus [66] flavonoids have also reported to be mediated through their suppressive effects on cytokines-stimulated expression of VCAM-1 on endothelial cell surface. Several other flavonoids containing herbal medicines such as *Scutellaria species* are known to have similar effects [67,68]. These reports were further in good agreement with our previous findings where certain classes of polyphenols and/or flavonoids inhibit the biological effects of TNF [47,69,70]. Other plant

extracts with established suppressive effect on the expression of endothelial CAMs include garlic [71], *Curcuma longa* [72] and *Canscora decussata* [73]. As demonstrated for *Piper longum* and various other herbal products, the suppressive effect of induced expression of CAMs could be due inhibition of NF- κ B activation [74,75]. All these scientific evidences obtained through *in vitro* endothelial-leukocyte adhesions and CAMs expression can be used to validate the traditional uses of herbal drugs as antiinflammatory agents.

CONCLUSIONS: Overexpression of CAMs is a feature of many inflammatory diseases that can be targeted by antiinflammatory herbal drugs at the level of their expression or adhesive functions. *In vitro* assay models of leukocyte/endothelial adhesions can be used as *cheap, rapid* and *robust* methods of assessment for herbal drug extracts. The use of established cell lines further reduces the time and costs associated with routine isolation and culturing of primary endothelial cells and leucocytes. For herbal extracts that display promising activity in the leukocyte-endothelial assay, further evidence of proof of efficacy can be obtained through protein-based adhesion studies and *in vivo* models of inflammation. Such bioassay capabilities hand in hand with phytochemical analysis offer a unique opportunity for herbal medicines standardization and modernization. Since inflammation can also be modulated through other targets (e.g. mediators' release), one should bear in mind that 'lack of activity' in cell adhesion bioassays can not be taken as an absolute proof of 'lack of efficacy'.

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