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Demonstration of biological standardization of selected herbal extracts

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Abstract

Objective: To demonstrate biological standardization of selected herbal extracts using rapid and convenient bioassays. Methods *Boswellia serrata* alcoholic extract (resin), *Emblica officinalis* water extract (fruit) and *Salacia oblonga* 80%alcoholic extract (root) were tested with reference to lipoxygenase inhibition, anti-oxidant and α-glucosidase inhibitory activity respectively. Brine Shrimp lethality assay (BSLA) was used for standardization of *Bacopa monnieri* alcoholic extract (leaves). *Piper longum* extracts with varying concentration of piperine were also tested in BSLA. To evaluate the use of BSLA in detecting adulteration, samples of A*lpinia galanga* deliberately adulterated with *Acorus calamus* rhizomes were tested. Results : IC_{50} limits were established for extracts *of B.serrata, E.officinalis & S. oblanga.* Limits of acceptance were also achieved for *B. monnieri* in terms of LC_{50} values. BSLA was also capable of detecting the difference in *Piper longum* extracts, which were varying in Piperine content, the active constituent of *Piper longum*. BSLA was also sensitive to detect change in bioactivity of *Alpinia galanga (*rhizome*)* samples, which were deliberately adulterated with *Acorus calamus* rhizomes. Conclusion : The use of bioassays is suggested for effective quality control of herbal products.

Key words: Bioassays, Biological standardization, Herbal drugs, Quality Control.

1. Introduction

The use of natural medicines is a persistent aspect of present day health care. According to the American firm Frost and Sullivan, Europeans spent 560 million dollars on natural remedies and food supplements in 1986 [1], since then the natural medicine market has grown substantially. In a survey in 1998, it was estimated Americans spend 5.1 billion dollars on herbal products [2]. Phytomedicines being very complex substances offer special challenges in their quality control, thus, there is a need to evolve valid and reliable methods of standardization of quality control. Botanical products are "heterogeneous" due to the presence

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of mixtures of bioactive compounds either from the same or purposefully mixed botanical sources.

The commonly used analytical methods like chromatography have narrow scope in the analysis of heterogeneous botanical extracts. Most often a desired biological response is due to not one but a mixture of bioactive plant components and the relative proportions of single bioactive compounds can vary from batch to batch while the bioactivity still remains within tolerable limits [3].

Bioassays offer a special advantage in the standardization and quality control of heterogeneous botanical products. The reason being when an extract is assayed in a biological assay both the known and unknown bioactives express their effect to influence the assay results. However in a chemical assay only known bioactives can be assayed, the unknown bioactives are generally ignored.

In the present study an attempt is made to demonstrate biostandardization of certain commercial herbal extracts using specific and non specific bioassays. The specific assays were chosen based on the intended use of the extract and the pharmacological property related to the same. Thus, *Boswellia serrata* (used for arthritis) was standardized for lipoxygenase activity, since it is reported for the same [4].

Similarly, *Salacia oblonga* extract (used for hypoglycemic effect) was standardized with reference to α- glucosidase activity [5], *Emblica officinalis* (used as an anti-oxidant) was standardized for antioxidant activity [6]. Brine shrimp lethality assay (BSLA) was used as a non-specific assay for standardization of *Bacopa monnieri* extracts. BSLA is recommended for quality control of herbal products [3].

This assay is expected to detect variation in active constituent(s) in herbal extracts. To establish this, the bioactivity of *P. longum* extracts with varying levels of piperine (active constituent of *P. longum*) were tested. Adulteration is an important problem in herbal drugs. We were interested to know the use of BSLA to check such adulteration.

In order to establish this, we studied the sensitivity of BSLA to detect variation in bioactivity of *A. galanga* rhizome samples deliberately adulterated with *A. calamus* rhizome at varying concentrations. We choose to adulterate *A. galanga* with *A. calamus* because it is reported for the same in commercial samples (7).

2. Materials and Methods

2.1 Chemicals

Lipoxidase enzyme [type 1 from soybean, EC No. 1.13.11.12,], DPPH(1, 1 – diphenyl – 2-picryl hydrazyl) and Gallic acid (Sigma, USA), Linoleic acid, Tween 20, Sucrose, Disodium hydrogen phosphate anhydrous and Sodium dihydrogen orthophosphate, (Himedia Laboratories, India), Indomethacin (ICN, USA), Boric acid (Ranbaxy Fine Chemicals Ltd, India), Acarbose (Bayer, India) Glucose reagent Kit (Bhat Biotech, India.), Methanol (Ranbaxy Fine Chemicals, India).

2.2 Plant Material

Commercial Herbal Extracts of *Boswellia serrata* resin (alcoholic extract, batch no. BS/01001,BS/04,BS/01005,BS/06&BS/01007), *Salacia oblonga* root (80% ethanolic extract, batch no. GC/Sal/11, GC/Sal/12, GC/Sal/13, GC/Sal/16 & GC/Sal/17), *Emblica officinalis* fruit (water extract, batch no. EO/04, EO/01001, EO/06& EO/09) and *Bacopa monnieri* leaves (alcoholic extract, batch no. BM/02, BM/12, BM/16, BM/30, & BM/32), were procured from M/s. Natural Remedies, Bangalore, India (A phytopharmaceutical company).

Dried plant material of *Piper longum* (fruit), *Alpinia galanga* (rhizome) and *Acorus calamus* (rhizome) were procured from M/s. Amrut Kesari, Bangalore, India. These materials were authenticated by our Pharmacognosy department, the voucher samples are preserved in the same department. *A. galanga* and *P. longum* were powdered and extracted by percolation with alcohol, after filtration the solutions were evaporated to dryness under vacuum.

The yields were 21.9% w/w, (*P. longum*) and 5.8% w/w (*A. galanga*). Adulterated *A. galanga* samples were prepared by mixing powdered *A. calamus* with *A. galanga* @ 2.5%, 5.0%, 15.0% and 25.0%. The adulterated samples were extracted and dried as mentioned above.

2.3 Lipoxygenase Inhibition Assay

The samples of *Boswellia serrata* were assayed as per the method. [8] To test tubes were added: Varying volumes of test solution (750 µg/ml in 2% methanol) or reference standard (Indomethacin, 300 µg/ml in 3% methanol) to obtain different concentrations. This was followed by addition of 100 µl of 500-1000 units lipoxidase enzyme (type 1 from soybean, EC No. 1.13.11.12). The pre-incubation mixture was made up to 1 ml with 2M Borate buffer (pH 9.0) and incubated at 25°C for 5 min.

Following incubation, 2.0 ml of substrate solution (50 mg of linoleic acid dissolved in 50 µl Tween 20, then made up to 50 ml with 2M Borate buffer and diluted to a concentration of 166.6 µg/ml with 2M Borate buffer pH 9.0) was added, mixed well and incubated at 25°C - 30°C for 4 min. The absorbance was measured spectrophotometrically at 234 nm. Controls were run which were devoid of test samples.

The percentage inhibition exhibited by various concentrations of test samples was calculated and IC_{50} values were determined using Finney computer programme. Limits of acceptance were arrived after knowing the confidence intervals (CI) of IC_{50} values. The lowest acceptable IC_{50} limit referred to the least lower CI of the batches tested and vice versa.

2.4 α - Glucosidase Inhibition Assay

α- Glucosidase was prepared from rat small intestinal mucosa as per the method [9]. The samples of *Salacia oblonga* were assayed as per the method [10]. To test tubes were added: 50µl of enzyme solution in 80 mM phosphate buffer, pH 7.0 (protein concentration – 0.5 g/dl as per Lowry's method), varying volumes of test solution (100 µg/ml) or reference standard (Acarbose, 5 µg/ml in phosphate buffer) to obtain different concentrations.

The preincubation mixture was made up to 300µl with phosphate buffer (80 mM pH 7.0) and incubated at 37°C for 30 min. 500 µl of sucrose solution (37 mM in 80 mM phosphate buffer, pH 7.0) were added and incubated at 37°C for 20 min. The reaction mixture was heated for 2 min on a boiling water bath to stop the reaction. The amount of glucose liberated was measured by the glucose oxidase method.

Controls were run which were devoid of test samples. The percentage inhibition exhibited by various concentration of test sample was calculated and IC_{50} values were determined using Finney computer programme. Limits of acceptance were arrived as mentioned previously.

2.5 Free Radical Scavenging Assay using DPPH (1, 1 – diphenyl – 2- picryl hydrazyl)

The samples of *Emblica officinalis* were assayed as per the method of Vani *et al.* To the test tubes were added: Varying volumes of test solutions (1 mg/ml in distilled water) or reference standard (Gallic acid, 50 µg/ml in methanol) to obtain different concentrations.

This was followed by addition of 75 µl of DPPH (1.3 mg/ml in methanol). The total reaction mixture was made up to 3 ml with methanol. The tubes were mixed and incubated at 25°C for 15 min. The absorbance was measured at 510 nm. Control reaction was carried out without test sample.

The percentage inhibition exhibited by various concentrations of test sample was calculated and IC_{50} were determined using Finney computer programme. Limits of acceptance were arrived as mentioned previously.

2.6 Brine Shrimp Lethality Assay

The extract samples of *Bacopa monnieri*, *Piper longum*, *Alpinia galanga* and adulterated *Alpinia galanga* samples were assayed as per the method [11]. Briefly, Brine Shrimp (*Artemia salina*) eggs were hatched by placing them in artificial sea water. The assay was conducted with 20-32 h old larvae in multi welled culture plates.

The test solutions/reference standard (potassium dichromate, 1mg/ml in Brine) were added to culture wells to achieve various concentrations. To each well were added 15-20 shrimps. The plates were then incubated at 25°C for 24 h. The survivors were counted using a stereomicroscope. LC_{50} was calculated using Finney computer programme. Limits of acceptance for *Bacopa monnieri* were arrived as mentioned previously.

3. Results & discussion

The specific and non-specific bioassays used were effective in arriving limits of acceptance for a given extract (Table 1 - 4).

Thus, the range of acceptance limits were, *Boswellia serrata* - IC_{50}: 202.0-289.0 µg/ml (Lipoxygenase inhibition assay), *Salacia oblonga* - IC_{50}: 8.0-26.0 µg/ml (α - Glucosidase inhibition assay), *Emblica officinalis* - IC_{50}: 5.4-8.5 µg/ml (DPPH assay) and *Bacopa monnieri* - LC_{50}: 479.0-1152.0 µg/ml (Brine shrimp lethality assay) respectively. Brine shrimp lethality assay is suggested as an inexpensive, simple and rapid means for standardization and quality control of botanical products [3]. LC_{50} of a given plant extract in BSLA depends on the nature and potency of bioactive constituents in it.

Variation in these bioconstituents is expected to show up as a deviation from usual LC_{50} values for the given extract. In the present study,

Table 1
Lipoxygenase Inhibition Assay of commercial samples of *Boswellia serrata* extract

Batch No.	IC_{50} ± SD (µg/ml)*	95% Confidence interval(CI)	Acceptance limits** IC_{50} (µg/ml)
BS/01001	225.5 ± 1.6	202.8-243.8	
BS/04	236.8 ± 2.7	223.2-249.0	
BS/01005	278.9 ± 1.7	270.1-289.1	202.0 - 289.0
BS/06	222.9 ± 3.1	203.8-236.6	
BS/01007	230.1 ± 3.0	207.1-247.4	
Indomethacin†	55.7	50.0-65.0	

† Positive control
* Determinations were done in duplicate
**The lowest acceptable IC_{50} limit refers to the least lower CI of the batches tested and vice versa

Table 2
α-Glucosidase Inhibition Assay of commercial samples of *Salacia oblonga* extract

Batch No.	IC_{50} ± SD (µg/ml)*	95% Confidence interval(CI)	Acceptance limits** IC_{50} (µg/ml)
GC/Sal/11	18.3 ± 0.8	15.8 - 26.1	
GC/Sal/12	21.9 ± 0.5	18.9 - 25.6	
GC/Sal/13	14.5 ± 0.2	9.9 - 18.6	8.0 - 26.0
GC/Sal/16	12.0 ± 0.5	8.1 - 15.1	
GC/Sal/17	19.3 ± 0.5	15.6 - 23.6	
Acarbose†	0.5	0.3-0.9	

† Positive control
* Determinations were done in duplicate
**The lowest acceptable IC_{50} limit refers to the least lower CI of the batches tested and vice versa

Table 3.
DPPH Assay of commercial samples of *Emblica officinalis* extract

Batch No.	IC_{50} ±SD (µg/ml)*	95% Confidence interval(CI)	Acceptance limits** IC_{50} (µg/ml)
EO/04	7.1 ± 0.07	6.2-8.0	
EO/01001	6.5 ± 0.15	5.6-7.5	5.4 - 8.5
EO/06	7.51 ± 0.01	6.5-8.5	
EO/09	6.2 ± 0.22	5.4-6.9	
Gallic acid†	1.4	1.0-2.0	

† Positive control
* Determinations were done in duplicate
**The lowest acceptable IC_{50} limit refers to the least lower CI of the batches tested and vice versa

variation in the piperine concentration in Piper longum extracts showed up as variation in LC_{50} values dose dependently (Fig.1). This shows that BSLA is useful in identifying variation in active constituent of a plant extract. It is a well-known problem that herbal crude drugs could be adulterated with other herbs.

Some times this sort of adulteration can lead to toxic effects. For example: 70 Belgian individuals who had been treated with a slimming preparation, which supposedly included the Chinese herbs *Stephania terrandra* and *Magnolia officinalis*. Analysis of the preparation brought to light that the root of *Stephania tetranda* as in all probability substituted or contaminated with the root of *Aristolochia fangchi.*

This incident led to the development of the outbreak of fibrosing interstitial nephritis in these subjects. [12, 13] The role of quality control is of paramount importance in indicating such adulteration, in the present study BSLA was sensitive to detect the presence of an adulterant in *Alpinia galanga* samples (Table 5). Thus, BSLA may serve to be useful to check adulteration of plant material, if not exclusively it can complement chemical analysis.

It may be especially useful when the adulterant is a very toxic herb and present in small quantities. BSLA being a general bioassay could

be used for any extract since it detects general pharmacological activity [14]. BSLA being a simple assay could be adopted as a convenient tool of quality control of phytomedicines, especially in developing countries where sophisticated procedures may be difficult to adopt.

Wherever feasible, specific bioassays could be used for standardization. For instance, platelet aggregation assay is reported for biological standardization of commercial *Ginkgo biloba* extracts [15].

Similarly, serotonin re-uptake inhibition assay for Hypericum perforatum extracts, macrophage activation assay for *Echinacea purpurea*, Estrogen binding assay for black cohosh (*Cimicifuga racemosa*) and corticosterone release assay for *Panex ginseng* have been used for standardization. [16] The importance of bioassays over conventional methods of chemical analysis is clear by the studies [16].

In his studies several standardized commercial herbal extracts were acceptable with reference to the assay of an active principle but failed in a specific bioassay. For example, various commercial batches of *H. perforatum* extracts, were acceptable with reference to hypericin content, but were showing a great variation in 5-HT reuptake inhibition assay.

Table 4
Brine Shrimp Lethality Assay of commercial samples of *Bacopa monnieri* extract

Batch No.	**LC_{50} ± SD (µg/ml)***	**95% Confidence interval(CI)**	**Acceptance limits** LC_{50} **(µg/ml)**
BM/02	790.6 ± 7.7	558.9 - 1013.3	
BM/12	709.5 ± 0.86	478.8 - 901.0	
BM/16	794.11 ± 7.3	609.1 - 972.0	479.0 - 1152.0
BM/30	815.5 ± 2.2	552.2 - 1076.1	
BM/32	853.6 ± 9.8	579.7 - 1152.2	
Potassium dichromate†	26.8	20.00 - 40.00	

† Positive control
* Determinations were done in duplicate
**The lowest acceptable LC_{50} limit refers to the least lower CI of the batches tested and vice versa

Table 5
Effect of adulteration of *Alpinia galanga* samples with *Acorus calamus* rhizomes in Brine Shrimp Lethality Assay

Tested material	**LC_{50} ± SD (µg/ml)**	**95% C.I.**
A. galanga alone	75.5 ± 4.3	60.9 - 89.1
A. galanga with 2.5% *A. calamus*	84.4 ± 1.7*	65.4 - 98.0
A. galanga with 5% *A. calamus*	95.4 ± 0.3*	84.1 - 106.4
A. galanga with 15% *A. calamus*	141.2 ± 1.9*	112.1 -173.1
A. galanga with 25% *A. calamus*	302.9 ± 4.4*	259.3 - 398.5
Potassium dichromate†	29.6	19.2 - 36.2

† Positive control
*Significant difference in comparison to *Alpinia galanga* sample devoid of adultrate at $P < 0.05$ (Student's *t* - test). Determinations were done in triplicate

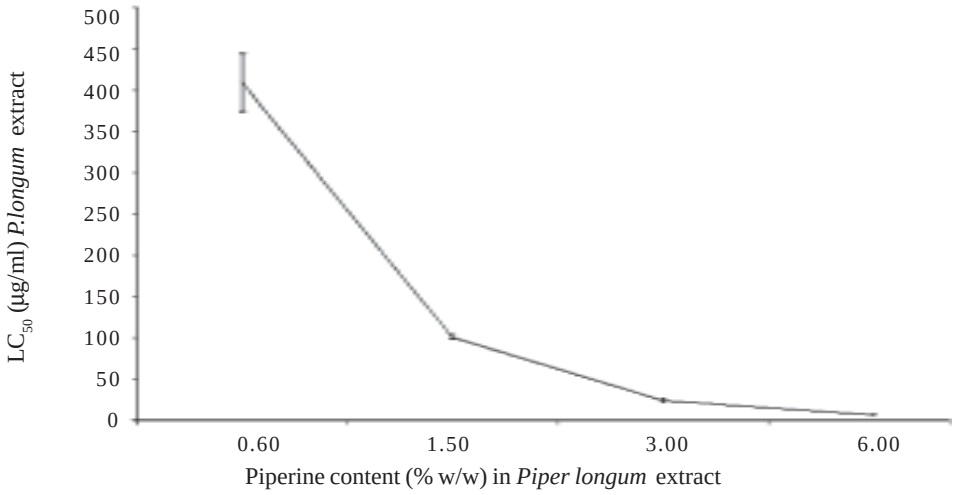

Fig.1. Effect of varying concentrations of Piperine in *Piper longum* extract on Brine Shrimp lethality

Similarly, samples of Echinacea extract were matching with reference to cichoric acid content but showed variation in lipoxygenase inhibitory effect. This indicates that standardization with reference to one or more markers may not represent comprehensive standardization, since some unknown bioactive compounds are not assayed.

Furthermore, it is known that the constituents within an extract can interact to influence the net biological effect. For instance, in Belladonna, the dried leaves of *Atropa belladonna* contain propane alkaloids, flavonoids, chlorogenic acids and others.

When plant constituents other than alkaloids are considered, flavonoids appear to be synergistic with the alkaloids in spasmolytic action, but antagonistic, to the alkaloids in action on urine retention. Chlorogenic acid may be synergistic with the alkaloids in anti- histaminic activity but antagonistic to alkaloids in CNS activity.

Therefore standardization of belladonna preparations assayed on selected marker constituents (Hyoscyamine, total alkaloid contents) remains questionable from a therapeutic standpoint [17].

Thus constituents with in an extract may be synergistic, additive or antagonistic with respect to a specific bioactivity. Bioassays may address such combinational effects since the whole extract gets assayed [18]. To sum it up, bioassays offer a special advantage in standardization and quality control of herbal products. These assays can provide meaningful information, which is not obtained by chemical analysis alone.

However, bioassays should not replace chemical assays but compliment them. The final interpretation on the quality of a phytomedicines should be based on the blend of data from biological and chemical assays. Bioassays chosen for quality control of herbal extracts should preferably be simple, rapid and economical to suit routine use.

When routine bioassays are done, positive controls should be run along with the test

samples, when the results of positive controls is not in acceptable range, the results of test samples should be rejected and the assay has to be repeated. This ensures proper application of bioassays and minimizes analytical errors.

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