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Assay Development for the Discovery of Semaphorin 3B Inducing Agents from Natural Product Sources

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Abstract

Semaphorins are a class of membrane-bound and secreted proteins. They have been found to regulate basic cell functions such as axonal growth cone guidance and recent studies have focused on their effect on tumor progression. Semaphorin 3B (Sema 3B) particularly is a secreted protein that has been known to modulate proliferation and apoptosis, processes that are critical for tumor progression and development. In spite of its importance, there is yet no high-throughput screening assay available to detect or quantify the expression of Sema 3B for natural product anticancer drug discovery purposes. Therefore, the development of a new high-throughput bioassay for the discovery of Sema 3B inducing agents from natural product sources is described herein. A wide variety of pure compounds and extracts from plants and microorganisms has been found suitable for screening using this Sema 3B assay to detect and quantify the effect of Sema 3B inducing agents and thereby identify new selective bioactive Sema 3B lead compounds for anticancer drug discovery and development. Also, this new bioassay procedure is based on a high-throughput platform using an enzyme-linked immunosorbent assay that involves the optimization of sensitivity and selectivity levels as well as accuracy, reproducibility, robustness, and cost effectiveness.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Keywords

Semaphorin; ELISA; anticancer; natural products

1. Introduction

Semaphorins (Semas) are membrane-bound and secreted proteins that influence axonal growth cone formation during neuronal development.¹ Also, Semas are known to regulate cell migration, axon guidance factors, immune responses, angiogenesis, and tumor progression.^{2,3} Semas are classified into three classes based on their origin, namely, invertebrate (Sema 1, 2, and 5C), vertebrate (Sema 3, 4, 5A and B, 6 and 7), and virus (Sema 8A and B).^{4,5} Among them, there are the two forms of secreted (Sema 2s, 3s, and 8s) and membrane-anchored or transmembrane Semas. Sema receptors are divided into two groups based on receptor binding properties: neuropilins and plexins.^{6–8} Neuropilins are receptors for Sema 3 and require other factors such as plexin A1–4 and plexin D1 to transduce Sema signals.^{9–12} In contrast, plexins A, B and C1 are able to bind Semas to transfer signals in cells without binding neuropilins.^{9–12} Plexin D1 binds to Semas 3E and 4A alone or with Sema 3C and neuropilin 1 or 2.¹³ The functions of Semas are pleiotropic and the signaling of Semas can impact the etiology of various human diseases.^{14,15} Structural and functional information on Semas has been well documented.^{2–4} Especially, the modulation for the enhancement of tumor progression and the inhibition of tumor progression have been researched based on various perspectives. Semas can also have a positive or a negative effect on cancer proliferation. Figure 1 shows the relationship between Semas and their binding receptors related to positive and negative effects on tumor progression. Also, the secondary associated receptors such as tyrosine kinases in Semas 4D, 6D, 5A and 3E and plexins in Semas 3A, 3B, and 3F are explained as inducers and inhibitors of tumor progression.^{8,16–23} Sema 3B is expressed in both neuronal and non-neuronal cells. In neuronal cells, Sema 3B primarily acts as an axon repellent and at the non-neuronal tissues Sema 3B modulates cell growth, survival, migration and proliferation.²⁴ In addition to the literature documenting the role of Sema 3B in neuronal cells, there is a growing body of research on Sema 3B as an inhibitor of breast, hepatocellular, lung, and ovarian cancers, and cholangiocarcinoma.^{25–26} Sema 3B is able to suppress major leading cancer types and its importance is gradually increasing because its induction on cancer cells is a key way to control malignant cell proliferation and to induce the process of apoptosis during tumor progression and development.^{26–31} In order to discover new inducers of Sema 3B, however, optimized methods to economize time-consuming and expensive experimental procedures involved are required.

High-throughput screening (HTS) is used extensively as a core technology in drug discovery and is relevant to the life science fields of research. HTS robots are able to test up to 100,000 compounds per day.³² HTS is available to select quickly from millions of biologically effective chemicals. The ability of reliable screening to identify active compounds provides the basic foundation for the discovery of new lead compounds to advance into drug design and development. In spite of Sema 3B's importance there is not yet a high-throughput screening assay available to detect or quantify the expression of Sema

3B for natural product cancer drug discovery. For this reason, an authentic screening method for the selection of active compounds or extracts is required. Therefore, this research focuses on the method development of a new high-throughput bioassay for the discovery of Sema 3B inducing agents from natural product sources. The new bioassay is based on a high-throughput platform employing an enzyme-linked immunosorbent assay that involves the optimization of sensitivity and selectivity levels.

2. Materials and methods

2.1 Chemicals

RIPA buffer, *p*-nitrophenyl phosphate, protease inhibitor cocktail, sodium azide, levamisole hydrochloride, actinomycin D, 1,25-dihydroxyvitamin D₃, and diethanolamine were purchased for this study (Sigma-aldrich Co., St. Louis, MO). Other reagents used were of analytical grade. Trypsin EDTA solution (0.5%), the primary antibody of sema 3B [SEMA 3B(L-20)-R], and the secondary antibody (Goat anti-rabbit IgG-AP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Extraction and Isolation of test compounds

All extraction procedures were performed as previously reported.^{33–34} Briefly, the procedure employed is described. In case of the fruits of *Elaeocarpus chinensis*,³³ the ground material was extracted with methanol three times at room temperature. Extracts were then suspended in a mixture of 80% methanol in H₂O, partitioned with hexane and CHCl₃, and the non removal of solvents, separate hexane- and CHCl₃-soluble extracts were obtained. The CHCl₃-soluble extract exhibited an IC₅₀ value of 0.4 µg/mL against HT-29 cells. Then, it was subjected to a LC-MS dereplication procedure. Bioassay-guided fractionation was used to facilitate the isolation process of the active plant isolates. 5Z-7-Oxozeanol was separated initially with 1:1 MeOH/CHCl₃. The mixture was shaken, filtered, and the solvent was evaporated. The extract was then partitioned between CH₃CN/MeOH/hexanes and the CH₃CN/MeOH soluble extract was chosen. The CH₃CN/MeOH extract was subjected to HPLC using normal and reversed phase C₁₈ column for final purification.³⁴ NMR spectroscopy and mass spectrometry were employed to elucidate the structures of compounds used in the present investigation. NMR spectroscopic data were obtained at room temperature on Bruker Avance DRX-400 or 600 MHz spectrometers (Bruker BioSpin Corporation, The Woodlands, TX) and the data were processed using MestReNova 6.0 software (Mestrelab Research SL, Santiago de Compostela, Spain). LC-MS experiments were performed on a liquid chromatography/autosampler system that consisted of a Waters Alliance 2690 Separations Module (Waters, Milford, MA, USA) and a Micromass LC-TOF II mass spectrometer (Micromass, Wythenshawe, UK) equipped with an orthogonal electrospray source (Z-spray). Accurate mass values were performed on a Micromass LCT ESI spectrometer (Micromass, Wythenshawe, UK). The chloroform extracts of the plant species presented in table 1 were also prepared following the same protocol as in the case of the fruits from *Elaeocarpus chinensis*.

2.3 Cell culture

Human colon (HT-29, ATCC # HTB-38), breast (MCF-7, ATCC # HTB-22), and cervical (HeLa, ATCC # CCL-2) cancer cell lines were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) or Dulbecco's modified Eagle's medium (DMEM)(Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotics (100 x) including penicillin and streptomycin, at 37 °C in humidified air and 5% CO₂. The number of cell passages was no more than ten to maintain actively growing conditions. The number of cells and the cell conditions were optimized for the cell exhibiting the lowest Sema 3B mRNA expression. Selected cells (10⁵) were seeded in a 96-well and were treated at 100 ug/mL for 0, 6, 12, 18 and 24 hours. Experimental samples were tested in triplicate and in two separate experiments.

2.4 Quantification of protein solution

The total extracted protein solution of treated and non-treated cells was quantified using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL) and the quantification procedure was performed following the manufacturer's protocol. Briefly, a mixed solution (95µL) of reagents A and B (50:1) supplied by the manufacturer was added to the protein solution (5µL per well) in a 96-well plate, incubated for 30 min with sealing tape at 36.5°C and then absorbance measured at a wavelength of 560nm. The concentration of the protein solution per well was normalized to 100ng/well.

2.5 Qualitative and quantitative method

The method used to screen for inducing agents of Sema 3B was based on an indirect-ELISA method. Briefly, the procedure is described below for the extraction and quantification of Sema 3B. The assay for Sema 3B detection was designed for a 96-well plate format. The cell density standardized for this assay was 10⁵ cells/well, when using HT-29 human colon cancer cells. In the case of cell treatment and protein quantitation, first cells were seeded in 100µl of culture medium in a CO₂ incubator overnight at 37°C and then treated with or without experimental compounds by incubation for 18h, next the medium was carefully removed, 50µl PBS were added to each well, and cells centrifuged at 1500 rpm for 10min, then cells were washed. After this, cells were treated for lysis with 50µl of the RIPA buffer including a protease inhibitor cocktail, the 96-well plate was shaken for 30 sec incubated at 4 °C during 30 min and then the 96-well plate was centrifuged at 4000 rpm (4°C) for 45min. Last, the supernatant was transferred into a new 96-well plate and an aliquot of the protein solution (100ng protein) was quantified from each well using the BCA assay.

In order to quantify the amount of Sema 3B before and after treatment, 100 ng of protein from each well were added into a new ELISA 96-well plate to normalize the amount of protein in each well. Next, a carbonate buffer (pH 9.0) was added into each well to adjust to the total volume per well to 250µl, and the 96-well ELISA plate was also incubated in a refrigerator with agitation at 4 °C overnight and then the antigen solution was removed by tapping the 96-well plate upside down on paper towels. Then, cells were blocked with 200 µl blocking buffer (PBS containing 1% bovine serum albumin and 0.02% azide), incubated for 1 h at room temperature, with the blocking buffer (200 µl/well) removed, and washed with PBS containing 0.02% azide. Detection and quantification of Sema 3B was pursued as

follows. Sema 3B primary antibody (50 μ l/well) was added, and the 96-well Elisa plate incubated at room temperature for 1hr, and next, washed with PBS including 0.05% Tween-20 (100 μ l/well, three times). After, 50 μ l of alkaline phosphatase conjugated secondary antibody were added, with the plate incubated for 1h at room temperature, secondary antibody solution removed, the wells washed with washing buffer (100 μ l, three times) and the plate slapped to dry. The wells were washed once with 10mM diethanolamine including 0.05mM $MgCl_2$ at pH 9.5 (100 μ l/well), and *p*-nitrophenol phosphate (50 μ l/well) including diethanolamine (100mM) and levamisole (0.1mM), to reduce background alkaline phosphatase activity in this assay involving detection signal amplification by intestinal alkaline phosphatase. Next, the 96-well Elisa plate was incubated in the dark for 30 min at room temperature. In order to stop the reaction between the secondary antibody and the substrate, 50 μ l of stop solution (0.75N NaOH solution) was added per well and the absorbance was read at 405 nm. The fold induction (FI) was calculated by using the following formula: (treatment absorbance-blank absorbance)/(non-treatment absorbance-blank absorbance). The larger the value the more significant was the Sema 3B induction (Figures 2). Depending on the disease state of interest, the potential index (PI) can be calculated by including values from cytotoxicity data (ED_{50}) such as calculating ED_{50}/FI .

3. Results and Discussion

The purpose of this method is to detect secondary metabolite inducers of Sema 3B from diverse natural sources and to quantify their relative induction levels. To evaluate the potential of this assay in fulfilling its purpose, two libraries were screened using this assay, including a library of pure natural products compounds and a library of chloroform-soluble plant extracts. The library of compounds was selected on previous cytotoxic activity on colon cancer cells, structural diversity, and on the availability for further biological studies. It is hoped this assay will be of great importance for Sema 3B natural product drug discovery, because such compounds are highly diverse chemicals with the potential for having new mechanisms of action. In order to reduce time and cost for screening of biologically active compounds, various attempts using different methods and platform approaches have been carried out. Among them, ELISA is a very useful method because it is very flexible and logistically easy to be employed in various research fields as a diagnostic tool and in drug discovery screening. It is very sensitive due to the use of an antigen and antibody and is able to be adjusted accordingly to the number of samples available. In order to screen for inducers of Sema 3B, the libraries of extracts (table 1) and compounds were tested using an ELISA assay with a 96-well platform. The method developed as part of this investigation is well suited as user-friendly and cost effective. The method and principle of the assay are easy to understand and adapt by the users. The use of antigen binding and antibody is not specific at general laboratories, and so the optimization for sensitivity and selectivity of this particular assay has been possible through adjustments and standardization. To determine the induction levels of Sema 3B and to standardize the Elisa method, the pure compounds (including those in figure 3) and crude extracts (table 1) from different natural sources were used.

For protein quantification, different amounts of protein solution were used to standardize the technique. The findings suggest that lower amounts of protein solution would tend to

generate a larger error; hence, 5µl of protein solution (cell extract solution) was selected as the most optimal for this assay. The protein solution was also quickly transferred into a new 96-well plate including the assay solution of BCA assay to reduce the error rate between samples, which was significantly reduced with the use of an electronic twelve-channel pipette (with a volume range from 1 to 10µl).

The cell density chosen for HT-29 human colon cancer cells in the 96-well plate was that of almost 90% confluence, in order to obtain enough protein solution (100ng) for Sema 3B induction detection and quantitation. For the selected cell treatment and protein quantitation, the cell lysis time was optimized at 30 min at 4°C and a centrifugation procedure was applied to allow floating the cells in the pellets. The incubation time for induction of Sema 3B was standardized and confirmed. Two important factors that needed to be studied before deciding on the incubation time required for Sema 3B induction included the loss of floating cells on top of the medium due to the effect from highly cytotoxic active compounds and the loss of Sema 3B expressed in the medium. These two factors might affect the final concentration of Sema 3B in the total protein solution used to run the assay. The incubation time in the presence of test compounds was better if less than 24 h on considering the number of cells and the effects of the test compounds. In the case of 25-*O*-acetylcucurbitacin F (7), the concentration of Sema 3B decreased almost as much as 25% when compared with the induction values between 18 and 24 h of treatment. 25-*O*-Acetylcucurbitacin F has been shown to exhibit significant cytotoxicity against HT-29 cells.³³ Hence, these results suggest that the dose used for highly cytotoxic compounds should be reduced in order to keep the time standardized for general screening or the treatment time with these drug leads should be reduced and adjusted in a flexible way. As can be seen on Figure 4, the incubation time for this assay was standardized to an 18 h incubation.

Different components used as part of the reaction in each well were also adjusted to reduce error and standardize the technique. Carbonate buffer was added to attach cell proteins to the bottom of the plate. The total volume used was up to the maximum level allowed for each well, because all protein in solution needed to be dispersed and evenly attached to the plate bottom. The covering of the plate with adherent film was carried out to avoid change in pH of the carbonate buffer due to loss of CO₂ into air. Since an alkaline phosphatase secondary antibody was selected for the development of this assay, levamisole was added to reversibly and non competitively inhibit most isoforms of alkaline phosphatase. The storage condition of the reagents, the cell condition and types, and the incubation time of the cells should be strictly controlled to minimize artifact influence on the assay procedure and on the final data obtained. Optimal conditions should be adjusted dependent on the cell type, well plate, the type of secondary antibody, and the experimental time for the application of this method to other cancer cell lines.

To determine the detection range limits for the total extracted protein in this assay protocol, concentrations between 0 and 6.0 µg/mL were tested. The lowest detection limit calculated for total protein using this procedure was in the range of 0.01µg/mL and the highest detection limit is over 6.0µg/mL. Figure 2 shows a dose-dependent curve for sema 3B detection limits. Detection above 2µg/mL was not concentration-dependent, suggesting a lack of sensitivity at concentrations of 2µg/mL or higher. For screening purposes, it is

desirable to design a highly sensitive method. Additionally, this method is designed to be used to detect and quantify Sema 3B induction levels, suggesting a prioritization for cells with a lower mRNA expression of Sema 3B. Since our antitumor drug discovery program has had colon cancer as a main focus, different colon cancer cells were evaluated including HT-29, LoVo, and HCT116 cells as well as a human lung fibroblast cell line (SV 80) and a non-small cell lung carcinoma cell line.³⁵ Expression of Sema 3B in mRNA is not significantly expressed in HT-29 cells when compared to other cell lines such as LoVo, SV 80 and HCT116. The level of Sema 3B in mRNA is slightly higher in HT-29 than in non-small cell lung carcinoma.³⁵ Hence, this method was developed using HT-29 colon cancer cells. Choosing the cells with the lowest mRNA expression among the colon cells will allow for a wider range of detection and quantitation since the minimum detectable dose on total extracted protein solution for human Sema 3B was below than 0.01 µg/mL. Other cell types having similar Sema 3B mRNA expression levels include hepatocellular carcinoma (Hep-G2) and osteosarcoma (SAOS-2) cells; hence, it might be possible to apply this method directly to Hep-G2 and SAOS-2 cells.³⁵

In order to normalize the data obtained, a coefficient of variation (2.01%) was calculated. With this method, the analysis was performed using the compounds shown in Figures 3 and 4 and extracts from plants shown in Figure 5. Extracted protein solutions of treated and untreated cells were used to identify Sema 3B induction agents based on this indirect enzyme-linked immunosorbent assay after the stability and reproducibility were optimized. This new method is cost and time effective to detect and quantify the induction of Sema 3B because it is optimized in a 96-well plate format with high sensitivity, reproducibility, and selectivity. It is also a suitable method to apply to different monolayer cancer cell lines and can be adjusted to the use of floating cells.

Examples of natural product compounds (**5–10**) exhibiting significant induction after 18 and 24 h of treatment using this assay are presented in figures 3 and 4. Compounds exhibiting no induction at all are not presented as part of this manuscript. Induction of Sema 3B was observed in the cucurbitacin type of compounds such as cucurbitacin D (**5**), 3-*epi*-isocucurbitacin D (**6**) and 25-*O*-acetylcucurbitacin F (**7**). According to previous report, the level of cytotoxicity of compounds **5–7** was as follows: **6** > **5** > **7**.³³ The level of induction was 10 to 20% higher in relative activity than the other cucurbitacins investigated (**1–4**) (Figure 3). Also, 5Z-7-oxozeanol (**8**), related to the catalytic activity of TAK1 MAPK kinase kinase³⁶ and wortmanin (**9**), a furanosteroid metabolite from fungi and used as a specific covalent inhibitor of phosphoinositide 3-kinases (PI3Ks)³⁷, exhibited similar activity. Compound **10** (1,1',3,3',5,5'-hexahydroxy-7,7'-dimethyl-[2,2'-bianthracene]-9,9', 10,10'(4aH,9aH)-tetraone) was found to be a stronger inducer than compounds **5**, **8**, and **9**, being the third most active compound among the five most active from the fifty compounds tested as part of the standardization of this assay. Some plant extracts were also shown to exhibit significant activity on the Sema 3B induction assay.

For the plant extracts tested (see table 1 and figure 5), the induction effect of Sema 3B was significantly higher in four species, including the branches of *Syzygium polyanthum*, a species with also a reported effect on DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity³⁸, the stems and leaves with twigs of *Litsea mollis* and the roots of *Gardenia*

angusta. In particular, because phytochemical studies on *L. mollis* and *G. angusta* were not previously reported, additional research on these species may be suggested, to test for various biological activities. Also, the whole parts of *Aralia vietnamensis*, the fruits and branches of *Symplocos lucidia*, the leaves with twigs of *Vernonia arborea*, and the roots of *Symplocos glomerata* exhibited some inductive effect when compared with other species.

4. Conclusion

The aim of this method was to detect and quantify the induction of Sema 3B levels in cancer cells upon treatment with different concentrations of plant extracts and pure compounds from natural sources. Each step in the protocol was optimized based on its co-relationship with other available Elisa methods. In other to validate the utility of this assay in natural product drug discovery, plant extracts and pure compounds were tested. About forty chloroform extracts from plants and fifty natural product pure compounds were tested to determine their ability to induce Sema 3B. Ten compounds (cucurbitacins, furanosteroid, and resorcylic lactone types) exhibited the most significant induction and are presented in this paper. At 18 hours of incubation, compounds **6**, **7**, and **10** exhibited the most significant activity in this assay. Extracts from *Litsea mollis* cf. (Lauraceae) and *Syzygium polyanthum* (Myrtaceae) exhibited the most significant activity among those tested as part of this study. Based on the results of pure compounds and plant extracts, this method is to be used for the screening of inducing agents of Sema 3B. This assay has great value in terms of time reduction, cost, reproducibility, and effectiveness. It should be a useful tool for the screening of Sema 3B agents by using the ELISA technique in a 96-well plate format. This method could also be further optimized to the use of different cell lines and for high throughput screening applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic views of semaphorin effects based on receptor binding. Primary and secondary bindings with semaphorins play key role in selectivity and tumor progression [13].

Figure 2.

Detection limit depending on Sema 3B concentration. Data are plotted using absorbance units versus concentration units.

Figure 3.
Structures of compounds tested using the Sema 3B ELISA assay.

Figure 4.
Representative data of a library of compounds from natural sources with diverse structures, exhibiting relative induction of Sema 3B.

Figure 5. Relative induction of Sema 3B in the presence of selected plant extracts (100 µg/mL). NT refers to untreated sample (control).

Table 1

Plant chloroform extracts tested using an indirect ELISA assay.***

Code	Family	Genus	Species	Plant part	Activity
1	Myrtaceae	<i>Syzygium</i>	<i>polyanthum</i>	BR	S
2	Lauraceae	<i>Litsea</i>	<i>mollis</i> cf.	LF+TW	S
3	Lauraceae	<i>Litsea</i>	<i>mollis</i> cf.	ST	S
4	Rubiaceae	<i>Gardenia</i>	<i>angusta</i> cf.	RT	S
5	Araliaceae	<i>Aralia</i>	<i>vietnamensis</i>	FL+FR+LF+TW	M
6	Araliaceae	<i>Aralia</i>	<i>vietnamensis</i>	ST	W
7	Symplocaceae	<i>Symplocos</i>	<i>lucida</i>	LF+TW	W
8	Symplocaceae	<i>Symplocos</i>	<i>lucida</i>	SB	W
9	Symplocaceae	<i>Symplocos</i>	<i>lucida</i>	FR	M
10	Symplocaceae	<i>Symplocos</i>	<i>lucida</i>	BR	M
11	Symplocaceae	<i>Symplocos</i>	<i>lucida</i>	RT	M
12	Myrtaceae	<i>Syzygium</i>	<i>lineatum</i> cf.	LF+TW	W
13	Myrtaceae	<i>Syzygium</i>	<i>lineatum</i> cf.	SB	B
14	Myrtaceae	<i>Syzygium</i>	<i>lineatum</i> cf.	FR	W
15	Myrtaceae	<i>Syzygium</i>	<i>lineatum</i> cf.	BR	B
16	Myrtaceae	<i>Syzygium</i>	<i>lineatum</i> cf.	RT	B
17	Asteraceae	<i>Vernonia</i>	<i>arborea</i>	LF	M
18	Asteraceae	<i>Vernonia</i>	<i>arborea</i>	BR	W
19	Asteraceae	<i>Vernonia</i>	<i>arborea</i>	LF+TW	M
20	Asteraceae	<i>Vernonia</i>	<i>arborea</i>	ST	B
21	Asteraceae	<i>Vernonia</i>	<i>arborea</i>	RT	B
22	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	FL+LF+TW	B
23	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	SB	B
24	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	BR	B
25	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	RT	M
26	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	FR+LF+TW	W
27	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	BR	W
28	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	SB	W

Code	Family	Genus	Species	Plant part	Activity
29	Symplocaceae	<i>Symplocos</i>	<i>glomerata</i> cf.	RT	B
30	Lauraceae	<i>Litsea</i>	<i>cubeba</i>	FB+LF+TW	B
31	Lauraceae	<i>Litsea</i>	<i>cubeba</i>	FR	B
32	Lauraceae	<i>Litsea</i>	<i>cubeba</i>	LF	B
33	Sapotaceae	<i>Sarcosperma</i>	<i>kontumensis</i>	TW	W
34	Sapotaceae	<i>Sarcosperma</i>	<i>kontumensis</i>	RT	W
35	Euphorbiaceae	<i>Balakata</i>	<i>baccata</i>	FR+LF+TW	W
36	Euphorbiaceae	<i>Balakata</i>	<i>baccata</i>	SB	B
37	Euphorbiaceae	<i>Balakata</i>	<i>baccata</i>	BR	B
38	Euphorbiaceae	<i>Balakata</i>	<i>baccata</i>	RT	B
39	Sterculiaceae	<i>Commersonia</i>	<i>commersonia</i>	BR	B

* LF=leaves, TW=twigs, ST=stems, RT=roots, FR=fruits, FL=flowers, BR=bark, SB=stem bark

** S=Strong activity over 40%, M=Medium activity over 30%, W=Weak activity over 20%, B=Below 20% induction when compared with control.

*** Voucher samples of each one of the plant species used in the collection of the data presented in this manuscript have been deposited at the Field Museum of Natural History, Chicago, IL.