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Research Paper

Assessment of *in vitro* pharmacological effect of Neotropical Piperaceae in GABAergic bioassays in relation to plants traditionally used for folk illness by the Yanesha (Peru)

G. Picard [a], C. Valadeau [b], J. Albán–Castillo [c], R. Rojas [d], J.R. Starr [a,e], R. Callejas-Posada [f], S.A.L. Bennett [g], J.T. Arnason [a,*]

[a] Department of Biology, University of Ottawa, Ottawa, ON, Canada K1N 6N5
[b] Centre EREA, UMR 7186, CNRS-Bat. D, Haudricourt, 7 rue Guy Moquet, 94801 Villejuif cedex, France
[c] Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Av. Arenales 1256, Jesús María, Lima, Perú
[d] Laboratorio de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado, San Martin de Porres, Lima, Perú
[e] Canadian Museum of Nature, Ottawa, ON, Canada K1N 6P4
[f] Facultad de Ciencias Exactas y Naturales, Instituto de Biología, Universidad de Antioquia, Apartado postal 1226, Medellín, Colombia
[g] Neural Regeneration Laboratory and Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON, Canada K1H 8M5

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ABSTRACT

Ethnopharmacological relevance: A previous pilot ethnobotanical and ethnopharmacological study with the Q'echi' Maya identified the family Piperaceae, as an important taxonomic group traditionally used for the treatment of epileptic and culture-bound anxiety disorders and possessing activity in the GABA system. Following that lead, a botanical survey was conducted in Peru, where 47 species of Piperaceae were collected including 21 plants traditionally used for folk illnesses by the Yanesha of Peru, an indigenous Amazonian group.

Materials and methods: Two high throughput bioassays were used to quantify the in vitro activity of botanical extracts on the GABA system.

Results: Plant extracts demonstrated moderate to high affinity to the γ-aminobutyric acid benzodiazepine (GABA-BZD) receptor. In addition, extracts demonstrated low to moderate activity in the inhibition of the GABA-transaminase, with select plants exhibiting significant activity. Plants indicated by the Yanesha showed comparable activity to the other Piperaceae plants collected. *Piper cremii* was the most active plant in the GABA-BZD receptor assay, and *Drymaria cordata* (Caryophyllaceae) in the GABA-T assay.

Conclusion: The study provides evidence that there is a pharmacological basis behind the use of plants in the treatment of *susto* and *mal aire* in both Central and South America, and we propose that the possible mechanism of action includes an interaction with the GABA-T enzyme and/or the $GABA_A$-BZD receptor.

1. Introduction

Each year, mental and neurological disorders account for over 1.2 million deaths and contribute to 28% of the total burden of non-communicable diseases. On a worldwide basis they surpass cancer and cardiovascular diseases (WHO, 2005). Epilepsy, one of the most serious neurological disorders, is more prevalent in the neotropics than in other more developed countries (deBittencourt et al., 1996). Unfortunately, the inadequate distribution of primary health care, combined with the scarcity of psychiatric care obliges the majority of indigenous peoples suffering from mental, behavioral, and neurological disorders in the neotropics to depend almost entirely on the medicinal flora and ritual treatments.

In addition to the Quechua people of the Andes, Peru has over 300,000 people belonging to 59 different indigenous ethnic groups within its Amazonian region (IBC, 2006). The traditional medicinal use of plants by several of these Peruvian indigenous groups has been documented (Schultes and Raffauf, 1990); however, with the exception of a few studies, information explicitly focusing on the use of plants for mental and neurological disorders is rather rare (Shepard, 1998; De Feo, 2003; Bussmann et al., 2010). The lack of plants recorded in Peruvian pharmacopoeias to treat

* Corresponding author.

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disorders such as anxiety or epilepsy could potentially be explained by the fact that classification of these illnesses, often categorized as "culture-bound syndromes" or folk illnesses, does not conform to a conventional medicine definition and they have therefore been overlooked in ethnobotanical studies. Nonetheless, a recent ethnobotanical survey documenting plants used by an indigenous people of the Chazuta Valley, considered to be one of the largest of Peruvian Amazonian groups, has identified numerous plants used to treat folk illnesses associated with mental health disorders, such as *susto* ("fright sickness") and *mal aire* ("malevolent wind" or "bad air"), highlighting the importance of including culturally significant terminology in studies (Sanz-Biset et al., 2009). Indeed, several outstanding descriptions of what are currently termed culture-specific syndromes of mental disorder (Kennedy, 1973) or "folk" illnesses have been done (Fabrega, 1971). According to Rubel (1964), *susto* is a complex illness and its interpretation depends directly on several social factors. The Spanish term of *mal aire* is perhaps the most frequent Spanish–American explanation for illness although its exact nature has an elusive quality.

Among the Yanesha, an indigenous group of Amazonian Peru, *yoreñets* is a generic term that the informants define as *susto* or in other cases as *mal aire* (Valadeau et al., 2010). There does not seem to be a clear difference between those two nominative illness terms. The symptoms appear because of a fright or an attack caused by a "walking shadow soul", *choyeshe'mats*, or "wind living being", *morranesha*'. These two types of spiritual beings can either be seen, as in *gacha'teñets* illness, or just simply heard, as in *macatsteñets* illness. Both these illnesses can lead to several symptomatic problems described as depression, weakness, and apathy (Valadeau et al., 2010). In the present study, we attempt to determine a pharmacological mechanism of action for plants traditionally used for folk illnesses among the Yanesha, as recently documented by Bourdy et al. (2008).

We have previously reported that neurological and mental conditions such as epilepsy and *susto* are well recognized by the Q'eqchi' Maya of Belize (Bourbonnais-Spear et al., 2005). The Maya are a similar culture to the Yanesha, and both groups believe that culture-bound syndromes are serious illnesses leading to severe conditions if ignored, and both therefore use a variety of medicinal plants to treat them. Our group has also reported that some of the species used by the Q'eqchi' Maya exhibit potent anxiolytic properties in animal behavioral models (Bourbonnais-Spear et al., 2007) and have been shown to act on the γ-aminobutyric acid (GABA) system (Awad et al., 2009), a key pathway involved in anxiety related disorders (Lydiard, 2003). Out of the plants available to the Q'eqchi' Maya, Piperaceae species not only seem to be preferentially selected for the treatment of such conditions but are among the most active species tested (Awad et al., 2009). The Yanesha pharmacopeia also includes many Piperaceae species (16.9% of collected plants, Valadeau et al. (2010)), some of which have been recently proven to have great ethnopharmacological importance (Cabanillas et al., 2012). Similar to the Q'eqchi' Maya, the Yanesha consider Piperaceae as important source of medicinal plants for folk illnesses such as *mal aire* and *susto*. Consequently, the focus of this study was the bioactivity of Piperaceae.

Piperaceae, the pepper family, is a highly diverse, pantropical plant family composed of two primary genera, *Piper* L. (ca. 2000 species) and *Peperomia* Ruiz & Pav. (ca 1700 species). The Amazonian Lowlands, as well as the eastern slopes of the Andean Cordillera in the upper Amazon, where the Yanesha live, are considered to be one of the areas with the greatest richness of *Piper* species (Quijano-Abril et al., 2006) and can be found in numerous other documented pharmacopoeias of the region, (e.g for the Ashaninka, Asháninka and shipibo'ones) (Lenaerts, 2005; Tournon, 2006; Luziatelli et al., 2010). However, only a small fraction of the Neotropical species have been studied in an ethnopharmacological context and very few species have been studied for their effect on the Central Nervous System (Felipe et al., 2007). Thus, we have undertaken the evaluation of an extensive collection of Piperaceae plants found throughout Peru as well as those selected by the Yanesha.

The evaluation of potential anxiolytic and antiepileptic properties of the crude extracts from the plants collected in the field was accomplished using two well-established high-throughput bioassays:the $GABA_A$-BZD receptor binding assay and the GABA-T inhibition assay (Awad et al., 2009). This approach allowed us to assess the anxiolytic and antiepileptic activity of plants in relation to the two main targets in the GABAergic system (Treiman, 2001; Lydiard, 2003). Therefore the objective of this research was to determine whether plant extracts exert their activity through the mode of action of binding to the BZD site of the $GABA_A$ receptor or by inhibiting GABA-T.

2. Materials and methods

2.1. Plant collection

A field trip for collection of a wide variety of Peruvian Piperaceae was undertaken from April to July of 2009. While participating in a variety of field expeditions throughout Peru, a total of 55 plants, 47 of which are from Piperaceae family, were collected. The first collection was carried out in Tsachopen (Fig. 1, Pasco Region, Oxapampa province). Yanesha plants were collected based on their recorded traditional uses as previously reported by Valadeau et al. (2010) and compiled in the book; *Yato' Ramuesh*: *Plantas Medicinales Yaneshas* (Bourdy et al., 2008). Plants used to treat epilepsy, anxiety related disorders, and culture bound illnesses such as *susto* or *mal aire* (Table 1) were targeted due to their potential interaction with the GABAergic system (Bourbonnais-Spear et al., 2007; Awad et al., 2009). The remaining plants were collected during various field expeditions throughout Peru with and organized by collaborator Joaquina Alban-Castillo (Museo de Historia Natural. Universidad Nacional Mayor de San Marcos (UNMSM), Lima Peru) (Table 2). Plants were collected according to the issued permit by INRENA (Instituto Nacional de Recursos Naturales, Ministerio de Agricultura, Lima, Peru, 124-2011-AG-DGFFS-DGEFFS). Plant vouchers have been deposited at the UNMSM herbarium in Lima, Peru, and at the University of Antioquia, Colombia. Most specimens have been identified to species level by Dr. Ricardo Callejas with the exception of some specimens from the *Peperomia* genus, some damaged or incomplete vouchers, and one particular specimen that could be a new species.

2.2. Plant extraction and sample preparation

During plant collection, leaves were immediately stored in ethanol for the duration of the fieldwork. The plants were eventually stored in Dr. Rosario Rojas laboratory at the Universidad Peruana Cayetano Heredia, where they were ground, extracted with approximately 10 × (*m*/*v*) 95% EtOH for a period of 24 h at room temperature, then filtered. For each sample, the filtrate was combined with the solvent originally used to store leaves during fieldwork and then roto-evaporated to prepare the solid extract. The resulting material was freeze dried to remove remaining water and stored at 4 °C until needed. Prior to experiments, the extract was reconstituted in the desired ratio of EtOH/dH_2O and filtered through a 0.2 μm polytetrafluorethylene (PTFE) Chromspec filter.

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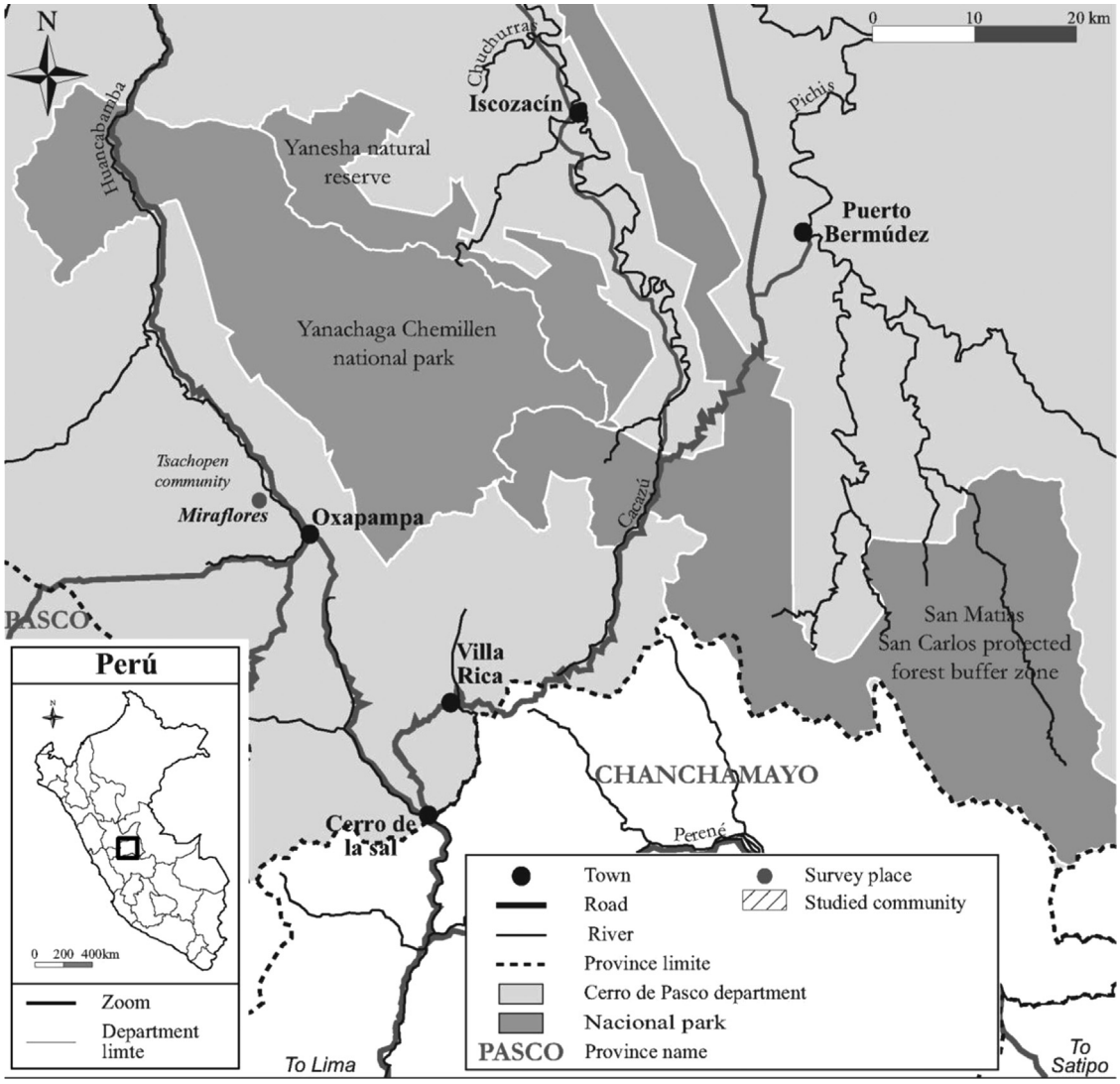

Fig. 1. Area of study where Yanesha plants were collected.

2.3. $GABA_A$-BZD receptor binding assay

The following radioligand binding assay is based on previously described protocols (Snodgrass, 1978; Benke and Mohler, 1999), which were later adapted by our research group (Awad et al., 2009) to be suited for 96 well Multiscreen FB filter plates (1.0/0.65 μm) (Millipore, Billerica, MA, USA).

First, brains from male adult Sprague-Dawley rats were obtained from Dr. Zul Merali (Department of Psychology, University of Ottawa) and were homogenized in 50 mM Tris buffer (50 mM Tris HCl, 50 mM Tris base, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) using a glass homogenizer. The membranes were then isolated by centrifuging the homogenate for 15 min (32,000*g*, 4 °C), rehomogenizing the pellet in the buffer, and finally collecting the pellet obtained after a second centrifuge round. A standard protein assay was then conducted to determine protein concentration in the pellet (Bradford, 1976). Note that the results for GP049 in the assay were not reliable and not reported.

Second, the plates were prepared by adding triplicates of the plant extracts prepared with 95% EtOH to attain a concentration of 10 μg/ml in the well with the protein (homogenized pellet) and the radioligand ^{3}H-flunitrazepam (Perkin-Elmer, MA, USA). The total binding was measured by adding the protein in the wells with 20 nM ^{3}H-flunitrazepam. The non-specific binding was measured by adding the protein with 20 nM ^{3}H-flunitrazepam and flumazenil (Sigma-Aldrich, MO, USA), a ligand with greater affinity. The plates were then incubated on ice for 75 min before washing the wells three times with the buffer and removing the content under vacuum.

Finally, plates were incubated for 24 h with 25 μL of scintillation fluid (Supermix cocktail, Perkin-Elmer, Waltham, MA, USA) in each well. The affinity of the plant extract to bind to the receptor was measured with a microplate scintillation counter (Wallac MicroBeta Trilux, Perkin-Elmer, Waltham, MA, USA), and the rate at which ^{3}H-flunitrazepam was displaced was calculated using the following formula:

$$\%\text{displacement} = 100 - \left(\frac{\text{total binding} - \text{non specific binding}}{\text{total binding}_{\text{control}} - \text{non specific binding}_{\text{control}}} \times 100\right)$$

2.4. GABA-T inhibition assay

The ability of plant extracts to inhibit GABA-T was evaluated using our validated and previously described spectrophotometric method (Awad et al., 2007). The plates were prepared by adding triplicates of the plant extracts (prepared with 80% EtOH to attain a concentration of 0.5 mg/mL in the well) with the protein

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Table 1
Voucher data for plants used traditionally by the Yanesha of Tsachopen and identified to have the potential of interacting with the GABAergic system. The local name of plants assayed in this study is given in Yanesha (Yan.), Spanish (Esp.) or in both languages when known. All vouchers were collected by the first author and are housed at USM.

Col.#	Local name	Family	Species	Location	Latitude	Longitude	Alt. (m)
GP001	Palo Santo (Esp.)	Undetermined	Undetermined	Tsachopen, Miraflores	S10.53507	W075.4472	1830.9
GP002	Senollechap (Yan.)	Caryophyllaceae	*Drymaria cordata* (L.) Willd. ex Schult.	Tsachopen, Miraflores	S10.53507	W075.4472	1830.9
GP003	Corarnopan (Yan.)	Piperaceae	*Piper subflavispicum* C.DC.	Tsachopen, Miraflores	S10.53507	W075.4472	1830.9
GP004	Corarnopan (Yan.)	Piperaceae	*Piper acutifolium* Ruiz & Pav.	Tsachopen, Miraflores	S10.53531	W075.4464	1793.6
GP005	Corarnopan (Yan.)	Piperaceae	*Piper* aff. *euriphyllum* Trel.	Tsachopen, Miraflores	S10.53978	W075.4447	1791.9
GP006	Corarnopan (Yan.)	Piperaceae	*Piper cremii* Trel.	Tsachopen, Miraflores	S10.53978	W075.4447	1791.9
GP007	Sentsopan po'senempan (Yan.)	Piperaceae	*Piper denisii* Trel.	Tsachopen, Miraflores	S10.54645	W075.4472	2049.8
GP008	Orranapan (Yan.), Garponia (Esp.)	Piperaceae	*Piper quimirianum* Trel.	Tsachopen, Miraflores	S10.54645	W075.4472	2049.8
GP009	Popnor, or Antacopa (Yan.), Té de monte (Esp.)	Chloranthaceae	*Hedyosmum* sp.	Tsachopen, Miraflores	S10.54645	W075.4472	2049.8
GP012	Mecha'tentsopar, (Yan.), matico (Esp.)	Piperaceae	*Piper* sp.	Tsachopen, Miraflores	S10.54633	W075.4475	2071.4
GP013	Pashenorren (Yan.)	Piperaceae	*Piper longifolium* Ruiz & Pav.	Tsachopen, Miraflores	S10.54633	W075.4475	2071.4
GP014	Muecho'tpar (Yan.)	Dryopteridaceae	*Elaphoglossum* sp.	Tsachopen, Miraflores	S10.54633	W075.4475	2071.4
GP016	Puesen (Yan.)	Piperaceae	*Piper* cf. *adreptum* Trel.	Tsachopen, Miraflores	S10.54505	W075.4478	2157.0
GP018	Acenacapar (Yan.), Flor de picaflor torsido (Esp.)	Undetermined	Undetermined	Tsachopen, Sipizu	S10.53301	W075.4466	1803.0
GP019	Tepeshpan (Yan.)	Verbenaceae	*Lantana camara* L.	Tsachopen, Sipizu	S10.52714	W075.4462	1786.9
GP020	Puetse'llompar (Yan.)	Undetermined	Undetermined	Tsachopen, Sipizu	S10.52826	W075.4529	1832.1
GP021	Ollocharetspar (Yan.)	Piperaceae	*Peperomia pertomentella* Trel.	Tsachopen, Sipizu	S10.53969	W075.4520	2047.1
GP023	Orranapan pashenorrer (Yan.)	Piperaceae	*Piper carpunya* Ruiz & Pav.	Tsachopen, Sipizu	S10.32.31	W075.26.88	1895
GP024	Corarnopan (Yan.)	Piperaceae	*Piper aduncum* L.	San Ramon	S11.1118	W075.4029	1518.0
GP045	Corarnopan (Yan.)	Piperaceae	*Piper peltatum* L.	Puerto Moldenado	S12.49611	W069.2121	169.1
GP062	Muentsopar (Yan.)	Fabaceae	*Mimosa pudica* L.	Reserva IIAP, Iquitos	S3.97194	W073.4211	124.6

(homogenized pellet) and the buffer (100 mM/L potassium pyrophosphate, 5 mM/L α-ketoglutarate, 4 mM/L nicotinamide adenine dinucleotide, 3.5 mM/L 2-mercaptoethanol, 10 μM/L pyridoxal-5′-phosphate, pH 8.6). Plates were subsequently incubated (15 min, 37 °C) before adding 115 mM/L GABA. The rate of the enzymatic reaction (V_{max}) was determined by measuring NADH production at an absorbance of 340 nm at 37 °C for 10 min within the linear range (Spectramax M5 with SoftMax Pro Software version 4.8, Molecular Devices Corporation, CA, USA). Relative enzyme activity was calculated and compared to the solvent control. Gamma-vinyl-GABA (GVG; vigabatrin, Sigma-Aldrich, MO, USA), a well-known GABA-T inhibitor was used as the positive control and completely inhibited the enzyme at 1 mM and had an IC_{50} of 84 μM. Some plants could not be tested and were removed from the experiment due to the formation of precipitate in the well interfering with the spectrophotometric part of the assay (plants GP006, GP026, GP034, GP039, GP040, GP042, and GP043)

2.5. Determination of EC_{50} and IC_{50}

The crude extracts of the most active plants were tested at concentrations (in well) of 1, 3, 10, 30, 100 and 300 μg/ml for the $GABA_A$-BZD receptor binding assay in order to determine the half-maximal effective concentration (EC_{50}) and of 0, 0.125, 0.25, 0.5, 0.75 and 1 mg/ml in order to determine the half-maximal inhibitory concentration (IC_{50}). Linear regressions were then completed with SigmaPlot (Systat Software Inc., CA, USA) in order to obtain these values.

3. Results and discussion

The biological activity was assessed using bioassays targeting two important aspects of the GABAergic systems. The bioassay of the crude ethanolic extracts of Peruvian Piperaceae plants and selected plants traditionally used by the Yanesha demonstrated that they possess moderate to high activity in the $GABA_A$-BZD receptor-binding assay (Fig. 2A). This bioassay was based on displacement of competing radioligand ^{3}H-flunitrazepam, and is the recognized in vivo target for many anxiolytic substances. In addition, the bioassay for the inhibition of the GABA-T enzyme, an important antiepileptic drug target, showed low to moderate activity for most plant extracts, with a few plants exhibiting promising activity (Fig. 2B). These assays appear to work reliably with botanical extracts in this study and in other published studies (Awad et al., 2007, 2009).

All but four of the 47 Piperaceae plants tested, and 18 out of the 21 plants selected by the Yanesha, were able to inhibit the binding of the radioligand to the $GABA_A$-BZD receptor by more than 50% at the tested concentration. The top four plants and their respective EC_{50} evaluated in a concentration dependent assay (Fig. 3) are as follows: *Piper longifilamentosum* Trel. [EC_{50} (± 95% CI)] = 18.3 μg/ml (13.2, 24.6)], *Piper loretoanum* Trel. [EC_{50} (± 95% CI)] = 16.6 μg/ml (10.8, 24.1)], *Piper cremii* Trel. [EC_{50} (± 95% CI)] = 17.5 μg/ml (10.6, 26.7)], and *Piper barbicuspe* Trel. [EC_{50} (± 95% CI)] = 31.1 μg/ml (23.1, 41.8)]. The most active plants within the Piperaceae family were of the genus *Piper*. However, the activity does not seem to be genus-specific since both active and inactive plants were also found within *Peperomia*. No significant

Table 2
Voucher data for Peruvian Piperaceae assessed for their activity within the GABAergic system, but not used by the Yanesha of Tsachopen. All vouchers were collected by the first author and are housed at USM.

Species	Col.#	Date collected	Location	Latitude	Longitude	Altitude (m)
Peperomia sp.	GP010	05/22/09	Tsachopen, Sector Miraflores	S10.54645	W075.44728	2049.8
Peperomia sp.	GP015	05/22/09	Tsachopen, Sector Miraflores	S10.54505	W075.44788	2157.0
Peperomia sp.	GP017	05/22/09	Tsachopen, Sector Miraflores	S10.54505	W075.44788	2157.0
Piper sp.	GP022	05/23/09	Tsachopen, Sector Sipizu	S10.32.314	W075.26.882	
Piper ferruginispicum Trel.	GP026	05/24/09	San Ramon	S11.12.130	W075.23333	1794
Peperomia galioides Kunth	GP028	05/30/09	Pamparomas, Ancash	S9.07312	W077.9752	2763.5
Piper aff. *cupreatum* Trel.	GP029	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46262	W069.13781	257.3
Piper dumosum Rudge	GP030	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46454	W069.1352	223.6
Piper pseudoarboreum Yunck.	GP031	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46454	W069.1352	223.6
Piper margaritanum Trel.	GP032	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46455	W069.13519	257.3
Piper allardii Trel.	GP033	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46455	W069.13519	257.3
Piper propinquum C.DC.	GP034	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46528	W069.13204	223.4
Piper celer Trel.	GP035	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46538	W069.13204	281.5
Piper sp.	GP036	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46362	W069.11715	237.8
Piper cf. *dilatatum* L.C. Rich.	GP037	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46367	W069.11725	273.6
Piper bermudezense Trel.	GP038	06/04/09	Reserva UNAMAD, Puerto Moldenado	S12.46367	W069.11725	273.6
Piper sp.	GP040	06/05/09	Reserva UNAMAD, Puerto Moldenado	S12.64651	W069.33395	257.7
Piper pseudoarboreum Yunck.	GP041	06/05/09	Reserva UNAMAD, Puerto Moldenado	S12.64651	W069.33395	257.7
Piper longifilamentosum Trel.	GP042	06/05/09	Reserva UNAMAD, Puerto Moldenado	S12.64181	W069.33611	223.6
Piper setulosum Trel.	GP043	06/05/09	Reserva UNAMAD, Puerto Moldenado	S12.39.449	W069.19810	225
Piper callosum Ruiz & Pav.	GP046	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP047	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP048	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP049	06/25/09	Tamshiyacu, Loreto	S4.01115	W073.14061	110.7
Piper sp.	GP050	06/26/09	IIAP, KM2.5 Quinones, Iquitos	S3.7665	W073.27498	91.0
Piper sp.	GP051	06/26/09	Reserva UNAP, Iquitos, Loreto	S3.83155	W073.37358	147.4
Piper loretoanum Trel.	GP052	07/03/09	Reserva IIAP, Iquitos	S3.97071	W073.41995	125.6
Piper soledadense Trel.	GP053	07/03/09	Reserva IIAP, Iquitos	S3.97136	W073.42054	119.1
Peperomia sp.	GP055	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Piper barbicuspe Trel.	GP056	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Peperomia sp.	GP057	07/03/09	Reserva IIAP, Iquitos	S3.97161	W073.42038	158.2
Piper leucofuscum Trel.	GP059	07/04/09	Reserva IIAP, Iquitos	S3.97166	W073.42271	147.2
Piper pavonii C.DC.	GP060	07/04/09	Reserva IIAP, Iquitos	S3.97213	W073.42313	96.3
Peperomia pellucida (L.) Kunth	GP061	07/04/09	Reserva IIAP, Iquitos	S3.97194	W073.42119	124.6

difference was observed when comparing the activity of all plants within genera ($F_{1,45}=1.5035$, $P=0.2267$). Plants selected by the Yanesha showed comparable activity to the other Piperaceae plants ($F_{1,53}=3.6396$, $P=0.0619$) with *Piper cremii* Trel. being the third most active plant. Thus the Yanesha appear to recognize the pharmacological potential of the family, but do not preferentially select the most active Piperaceae at the species level.

In the second bioassay, only four plants were able to inhibit GABA-T by more than 50% at the concentration tested, with two of them coming from the Yanesha pharmacopoeia. The top four plants and their respective IC_{50} were *Piper pavonii* C.DC. [IC_{50} ($\pm$ 95% CI)]=0.28 mg/ml (0.26, 0.3)], *Piper barbicuspe* Trel. [IC_{50} ($\pm$ 95% CI)]=0.28 mg/ml (0.21, 0.35)], *Drymaria cordata* (L.) Willd. ex Schult (Caryophyllaceae) [IC_{50} ($\pm$ 95% CI)]=0.46 mg/ml (0.41, 0.52)], and *Hedyosmum sp.* (Chloranthaceae) [IC_{50} ($\pm$ 95% CI)]=0.37 mg/ml (0.31, 0.43)] (Fig. 4). Similar to the previous assay, the activity does not seem to be genus-specific ($F_{1,40}=0.7712$, $P=0.385$). No significant difference was observed when comparing the activity of Yanesha plants to the other Piperaceae species ($F_{1,48}=0.3178$, $P=0.576$). Therefore there is no evidence to suggest that they select overall the most active Piperaceae at the species level.

In general, the results show comparable activity to plants used by the Q'eqchi Maya of Belize, where *Piper amalago* L., the most active Piperaceae plant had an EC_{50} of 18.6 μg/ml (14.2, 24.1) in the $GABA_A$-BZD receptor binding assay and *Piper tuerckheimii* C.DC. ex Donn. Sm. had an IC_{50} of 0.51 mg/ml (0.40, 0.70) in the GABA-T inhibition assay (Awad et al. 2009). These results are also comparable to a study of Danish folk medicines used for epilepsy where *Primula eliator* (Primulaceae) and *Tanacetum parthenium* (Asteraceae), two of their most active plants, had an EC_{50} of 18.45 μg/ml and 40.05 μg/ml respectively in the $GABA_A$-BZD receptor binding assay (Jäger et al. 2006).

A thorough review of the published literature suggests that the top ranking *Piper* species identified here have never been studied before. In fact, most Piperaceae collected in this study have never been investigated in an ethnopharmacological or phytochemical context. There are a few exceptions of course, such as the widespread species *Piper aduncum* L., *Piper acutifolium* Ruiz & Pav., *Piper peltatum* L., the Andean species *Peperomia galioides* HBK, and the introduced species *Peperomia pellucida* (L.) HBK. Nevertheless, none of the species have been reported to exhibit activity within the GABAergic system. Here we report for the first time a pharmacological basis for the ethnopsychiatric use of plants by the Yanesha of Tsachopen, and underline the promising biological activity of the Piperaceae on two specific sites of the GABAergic system.

The Piperaceae are well known for the presence of piperamides (Parmar et al., 1997), a unique group of nitrogenous secondary metabolites thought to play an important role in the traditional use of these plants for the treatment of various CNS disorders (DHooge et al., 1996; Wattanathorn et al., 2008; Pedersen et al., 2009). The recent finding that the amide piperine acts as a positive $GABA_A$-BZD modulator (Zaugg et al., 2010), and the observation of in vivo anxiolytic activity from such compounds (Felipe et al., 2007; Yao et al., 2009), highlights the importance of Piperaceae as CNS-depressant plants acting within the GABAergic system. The presence of piperamides in the selection of Neotropical Piperaceae collected could explain their activity. Nevertheless, other classes of secondary metabolites such as naturally occurring flavones and

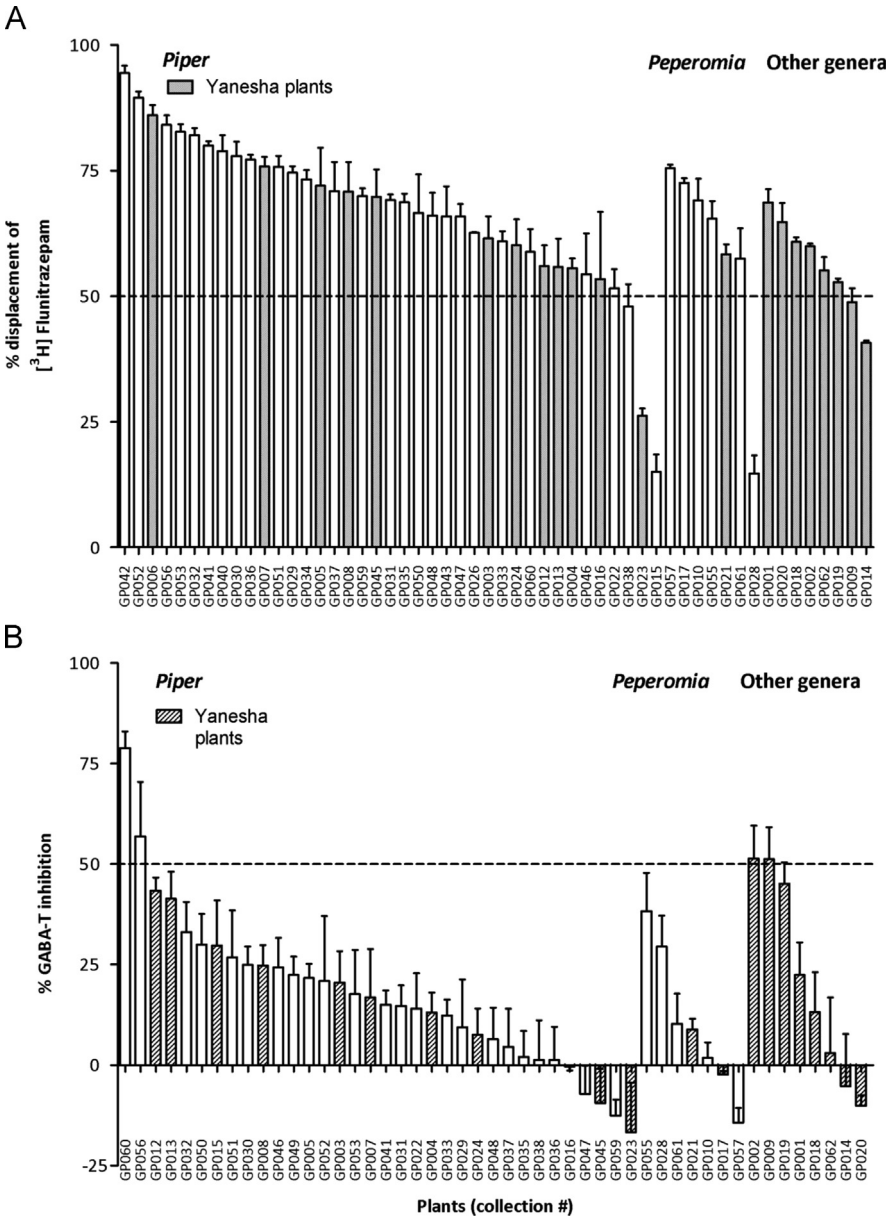

Fig. 2. The ability of selected Piperaceae species and plants used by the Yanesha to (A) displace the radioligand [^{3}H]-Flunitrezepam from the GABA$_A$-BZD receptor (± SEM) at 0.1 mg/ml and (B) to inhibit GABA-T (± SEM) at 0.5 mg/m. See Tables 1 and 2 for voucher details.

related compounds (i.e. flavonoids) isolated from traditionally used anxiolytic, sedative and anticonvulsant plants have been shown to bind with great affinity to the GABA$_A$-BZD receptor (Medina et al., 1997) and pharmacophore models since have been used for the design of potent synthetic molecules (Kahnberg et al., 2002), such as triazoloquinazolinediones, a novel class of BZD receptor ligands (Nilsson et al., 2011). Flavonoids from Pipereaceae have been isolated and tested for their antifungal (Lago et al., 2004), antiplasmodial (Portet et al., 2007), antioxidant (Velozo et al., 2009), and antinociceptive properties (Da Silva et al., 2010); however, to our knowledge none have been tested for their antiepileptic and anxiolytic properties.

The extracts of *Drymaria cordata* (L.) Willd. ex Schult and *Hedyosmum sp*., the most active non-Piperaceae GABA-T inhibitors selected by the Yanesha, offer promising leads for the discovery of anti-epileptic natural compounds. Interestingly, *Drymaria cordata* was the only plant to be specifically chosen for the treatment of epilepsy, whereas most of the other plants were used for anxiety related disorders and culture bound syndromes such as *susto*. A previous study has shown *Drymaria cordata* to exhibit anxiolytic activity in an in vivo experiment (Barua et al., 2009). The highly aromatic Neotropical genus *Hedyosmum* has also been investigated for its analgesic and sedative effects, which have been attributed to the presence of flavonoid glycosides (Cardenas et al., 1993) and sesquiterpene lactones (Tolardo et al., 2010) respectively. In addition to the ethnobotanical data available for these two plants and the observed in vitro activity, we present data here that show that these plants act on the CNS via the GABAergic system by interacting with GABA-T. It is important to note that Yanesha plants (i.e. *Elaphoglossum sp. Dryopteridaceae*) that showed weak activity in the GABAergic assays ehre could still have anxiolytic or antiepileptic effects by other mechanisms of actions such as the glutamate/NMDA receptors (Milton and Jung, 2003). Nevertheless, the data presented here provides us with interesting leads for the potential isolation of potent neuroactive plant-derived natural products. *Piper*, *Drymaria*, and *Hedyosmum* spp should be collected

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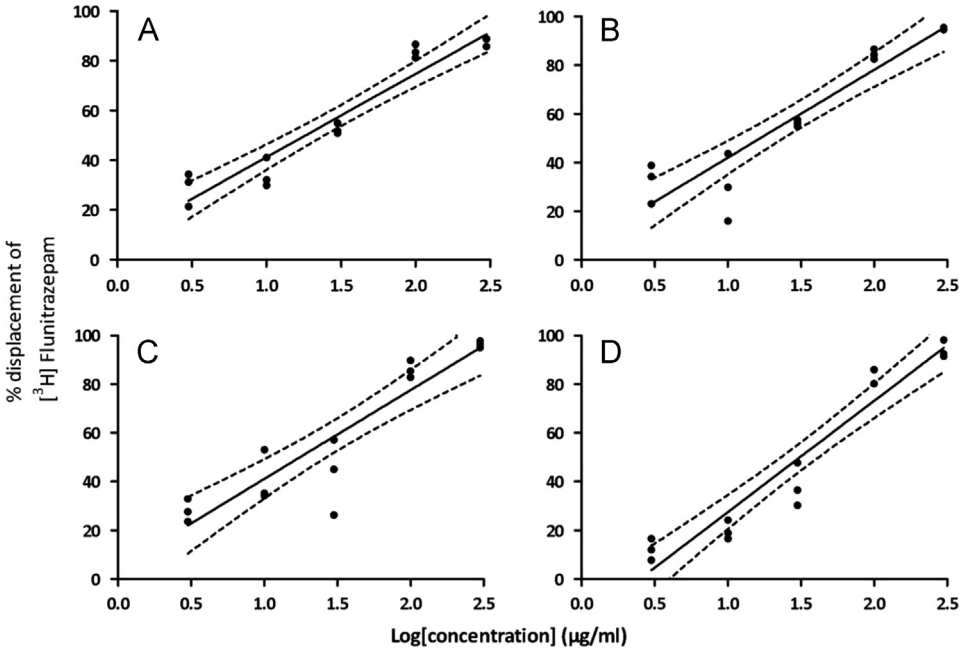

Fig. 3. Concentration (Log 10) dependant Linear regression analysis (95% CI) of the four most active plant extracts; (A) *Piper longifilamentosum* Trel. (GP042), (B) *Piper loretoanum* Trel. (GP052), (C) *Piper cremii* Trel. (GP006), and *Piper barbicuspe* Trel. (GP0056) in the displacement of [^{3}H] Flunitrazepam. Fifty percent effective concentration [EC_{50} (± 95% CI)] = 18.3 µg/ml (13.2, 24.6), 16.6 µg/ml (10.8, 24.1), 17.5 µg/ml (10.6, 26.7) and 31.1 µg/ml (23.1, 41.8) for A, B, C and D respectively (n = 3).

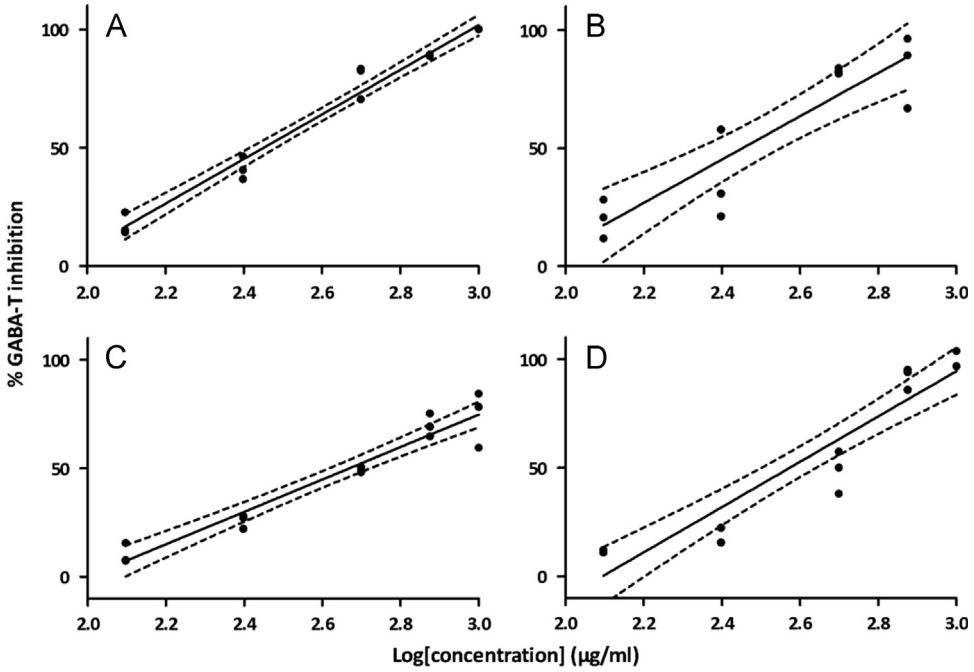

Fig. 4. Concentration (Log 10) dependant Linear regression analysis (95% CI) of the four most active plant extracts; (A) *Piper pavonii* C.DC. (GP060), (B) *Piper barbicuspe* Trel. (GP056), (C) *Drymaria cordata* (L.) Willd. ex Schult. (GP002), and *Hedyosmum sp.* (GP009) and their in vitro inhibition of GABA-T. Fifty percent inhibition concentration [IC_{50} (± 95% CI)] = 0.28 mg/ml (0.26, 0.3), 0.28 mg/ml (0.21, 0.35), 0.46 mg/ml (0.41, 0.52) and 0.37 mg/ml (0.31, 0.43) for A, B, C and D respectively (n = 3).

in bulk and subjected to bioassay-guided fractionation in order to identify the active constituents. Finally, the activity should be confirmed in *in vivo* models.

The purpose of our study was to screen a variety of Neotropical Piperaceae species, and plants used for ethnopsychiatric disorders by the Yanesha people, for their activity on the GABAergic system, thus identifying potential active antiepileptic and anxiolytic plants. Our results show that Piperaceae plants can exhibit great activity in $GABA_A$-BZD receptor binding assay but somewhat less activity in the GABA-T inhibition assay, and that most plants used by the Yanesha showed moderate to high activity, especially for GABA-T inhibition. The folk illness, or culture-bound syndromes of *susto* (fright) and *mal aire* (malevolent wind) have both been documented as an important and often severe source of ailment throughout the Neotropics (De Feo, 2003; Bourbonnais-Spear et al., 2005; Sanz-Biset et al., 2009). For the Yanesha, being struck by such illnesses can lead to sadness, depression, anorexia, apathy, insomnia, and convulsion (Valadeau et al., 2010), among other symptoms that can be attributed to anxiety related disorders or attacks of epilepsy. The presence of *susto* and *mal aire* in Latin

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America has been extensively documented in an anthropological context, yet plants used for these folk illnesses have rarely been studied for their biological activity in this context. In addition to the work with the Q'eqchi' Maya of Belize (Bourbonnais-Spear et al., 2007; Awad et al., 2009), our study provides evidence that there is pharmacological basis behind the use of plants in the treatment of *susto* and *mal aire*, and offer the interaction with the GABA-T enzyme and/or the $GABA_A$-BZD receptor as potential mechanisms of action.

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