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Antimalarial Use of Malagasy Plants is Poorly Correlated with Performance in Antimalarial Bioassays¹

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Abstract

Bioassay screening of plant extracts can identify unique lead compounds for drug development, but the “hit rate” from random screening is very low. Targeted screening of medicinal plants has been repeatedly reported to increase the percentage of samples displaying bioactivity. Contrarily, Maranz (2012) suggested that African antimalarial plants were unsuitable sources of antimalarial drugs because high prevalence of malaria would result in rapid evolution of resistance to active compounds that directly targeted the parasite. As malaria is highly prevalent in much of Madagascar, it was of interest to determine whether Malagasy antimalarial plants would outperform randomly selected plants in conventional antimalarial assays being conducted as part of a discovery program. Of 1294 plant samples screened for antimalarial activity, 39.6% had an $IC_{50} < 50 \mu\text{g/ml}$ and 21.1% had an $IC_{50} < 20 \mu\text{g/ml}$ (the minimum to qualify as a first-pass “hit”). Ethnobotanical uses were coded at both the generic and the species level, as neither samples nor use reports in literature were always identifiable to species level. The 526 samples belonging to genera having reported uses for malaria were slightly more likely than average to display activity (44.3% with $IC_{50} < 50 \mu\text{g/ml}$, $p < .01$; 23.2% with $IC_{50} < 20 \mu\text{g/ml}$). Of these, 67 samples from individual species with documented use were still more likely to be modestly active (49.3% with $IC_{50} < 50 \mu\text{g/ml}$), yet less likely to be highly active (17.9% with $IC_{50} < 20 \mu\text{g/ml}$). Thus, in this specific context, ethnobotanically directed screening would not have substantially improved screening efficiency, and would have missed most of the potential hits.

Keywords

Madagascar; malaria; natural products discovery

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Introduction

Plants provide human health benefits both through use in traditional medicinal products and as sources of pharmaceutical drugs or lead compounds for drug development. Early plant-derived drugs were extracted from plants that are useful for the same purpose as the drug (e.g., analgesic drugs from analgesic plants, antimalarial drugs from antimalarial plants). However, cases such as that of vincristine, useful for childhood leukemia but derived from a plant without traditional use for cancer, inspired modern researchers to view any species of plant as a potential source for a novel bioactive compound. In recent decades, broad-scale screening programs have therefore run many thousands of plant extracts through in vitro assays (e.g., Cragg et al. 1993; Miller 2011).

Natural products discovery programs provide the only means of discovering highly bioactive molecules that are too complex to be synthesized and tested in screening programs (e.g., Newman and Cragg 2007), but they suffer from the incompatibility of crude extracts with high-throughput screens and from the need to isolate the bioactive compounds from a complex matrix. In addition, even the first-pass “hit rate” of random screening, i.e., the percentage of samples possessing promising levels of activity, is often low enough that many companies consider screening programs to be economically unfeasible. Various approaches to increasing the hit rate have therefore been suggested. In particular, it is often proposed that the “ethnobotanical method” – screening plants with known medicinal uses, and especially uses related to the bioactivity of interest – will increase the percentage of samples that display high levels of activity (e.g., Balick 1990; Cox 1994; Lewis and Elvin-Lewis 1995).

Indeed, a number of studies support that belief. In a very early example, Spjut and Perdue (1976) reported that 52.2% of arrow poisons, 38.6% of fish poisons, and 29.3% of vermifuges tested were active in cytotoxicity assays, versus only 10.4% of all species tested. Ethnobotanically directed plant selection is also shown to improve hit rates in assays for antimicrobial activity (Taniguchi and Kubo 1993; it should be noted that this study involved East African traditional knowledge), anti-HIV activity (with plants selected for their use against less novel infectious diseases; Lewis and Elvin-Lewis 1995) and muscle relaxant activity (Sligh et al. 1999). However, results are inconsistent. In a large study involving screening of Laotian and Vietnamese medicinal plants and randomly collected Vietnamese flora using multiple bioassays, Gyllenhall et al. (2012) reported that the per-collection (i.e., per-species) hit rate was an absolute 2.5% higher in random collections, although the per-sample hit rate (i.e., per individual plant part) was an absolute 5.8% higher in ethnomedical samples. This result was certainly affected by the greater number of samples per collection in random flora (2.3, versus 1.0 for ethnomedical collections); however, Gyllenhall et al. found that though the increased hit rates for ethnomedicinal samples were highly significant in certain assays (e.g., anti-TB), random samples actually performed better in others (e.g., anti-HIV).

Further, as Miller (2011) noted, because of the very common phenomena of pharmacokinetic and pharmacodynamic synergy among compounds produced by a plant, the demonstration of activity in a complex extract usually does not lead to the discovery of a

single novel molecule that retains a useful level of activity. Albuquerque et al. (2014) suggested that the ethnobotanical method was inefficient for reasons including biased or misunderstood ethnobotanical data and use of inactive plants as placebos, but also briefly touched on the point that previously known, commonplace active compounds would be of little interest to drug development programs, whose requirements generally include patent protection and exclusivity. Hence the cultural values and interests embedded in screening programs make the challenge of “finding useful bioactivity” far more difficult. Still, if ethnobotanically directed screening did substantially increase the hit rate in first-pass screening, this could plausibly be assumed to increase the efficiency and economic feasibility of screening programs.

Malaria imposes a particularly large global health burden and rapidly evolves resistance to single-compound drugs. As a result, the development of new treatments, including herbal treatments (see Willcox et al. 2004), is of great interest, and studies that screen medicinal plants for antimalarial activity are fairly common. Despite those research efforts, no new pharmaceuticals have been derived from antimalarial plants since the artemisinin derivatives obtained from the Asian antimalarial plant *Artemisia annua* L. Recently, Maranz (2012) proposed the provocative hypothesis that the reason no antimalarial drugs have been developed via screening of African antimalarial plants is that the particularly high prevalence of malaria in affected African regions, compared to other tropical or subtropical areas, allows parasites to rapidly evolve resistance to any substance that directly kills them, even if it contains multiple active compounds. Hence, African medicines (and, he further plausibly suggests, bitter wild foods) are proposed to affect malaria only indirectly, by increasing host resistance or by inhibiting non-essential parasite activities such as cytoadhesion. Unquestionably, such indirect benefits can exist, and are virtually always overlooked in antipathogenic screening programs that seek only to maximize toxicity.

Maranz (2012) offered two major points of empirical evidence for the lack of direct antiplasmodial activity of African antimalarial plants: the low rate of discovery of highly active crude extracts, or highly active single molecules, in a few African screening studies or reviews of antimalarial plants (Soh and Benoit-Vical 2007; Pillay et al. 2008; Rukunga et al. 2009), and the fact that local malaria death rates drop substantially when pharmaceutical antimalarials become available (which shows, minimally, that the latter are more potent, until resistance develops). Another point he might have added is that, while Western physicians expect the result of antimalarial treatment to be rapid total parasite clearance, in a high-transmission area this may be clinically unimportant (Willcox et al. 2011) or actually detrimental, as persistent low-level infection may reduce the risk of significant illness in the likely event of future exposure (Sondén et al. 2015). Hence, if a choice between more and less potent traditional medicines were available, the latter might usually be preferable when reduced future illnesses and lesser side effects were considered. One implication of Maranz’ hypothesis is that, if antimalarial drugs with direct antiplasmodial toxicity are sought, there is effectively no point in conducting research on African traditional medicines.

Nevertheless, Maranz’ hypothesis regarding rapid evolution of resistance remains unproven and might be questioned by more than merely those believers in African traditional medicine whom he accuses of covering for a “sense of failure.” He only discusses the results of three

publications that reported small numbers of promising results, and does not seem willing to give those results any favorable attention. Rukunga et al.'s (2009) results were dismissed as "the majority of extracts had low or no activity, with a best IC₅₀ value of 3.65 µg/ml" (that is, the concentration required to result in 50% inhibition of parasite growth; Maranz 2012: 2). Yet they reported that seven of 23 crude extracts made from 12 antimalarial plants had an IC₅₀ of less than 20 µg/ml, which in a crude extract is considered worthy of further study, with 3.65 µg/ml being quite good; this seems like a decent return on research effort. Soh and Benoit-Vical (2007) identified one compound, gedunin, that was apparently as potent as artemisinin and had a desirable selectivity index, but Maranz (2012: 2) rejects the potential utility of this compound because "extracts from plants in this family have been found *in vivo* to act as abortifacients." Since the related plant referred to was *Azadirachta indica* A. Juss. (neem; cited references, Talwar et al. 1997 and Boeke et al. 2004), which is very widely and usually safely used as a medicinal plant, this does not seem an adequate objective reason to abandon further study of gedunin.

Other African studies that have reported good hit rates in screening of crude extracts of antimalarial plants, or larger numbers of promising isolated molecules, were not discussed by Maranz but do exist. In particular, Clarkson et al. (2004) screened 134 medicinal plants native to or naturalized in South Africa and used for malaria or fever, which were selected from an initial list of 623 such taxa based on a semiquantitative analysis that incorporated chemotaxonomic criteria and the strength of ethnobotanical use data (e.g., plants native to the malarial endemic area and plants sold in more than one regional market received more points; Crouch, pers. comm.). They reported that 49% of these highly selected antimalarial plants had "promising" activity, defined as an IC₅₀ < 10 µg/ml, while 17% were "highly" active, defined as an IC₅₀ < 5 µg/ml. (This is indeed a very good IC₅₀ for a crude extract, one that is easily achieved *in vivo* provided that the active compounds in the traditional medicine are even moderately, directly or indirectly, bioavailable.)

One might also note a pair of recent reviews (Onguène et al. 2013; Ntie-Kang et al. 2014) of some 290 isolated compounds extracted from African medicinal plants that have *in vitro* antimalarial activity, though not always to a degree that would make them appealing drug discovery candidates. Very few of those compounds have been subjected to the level of basic research that artemisinin enjoyed, much less the production and investigation of multiple derivatives that might lead to the development of superior compounds such as the artemisinin derivatives now in frequent use. It is therefore reasonable to suspect that the lack of an African-developed antimalarial pharmaceutical owes as much to a local lack of research money as to a lack of usable compounds.

One of the activities of the long-running International Cooperative Biodiversity Group program in Madagascar was to screen plant extracts for antimalarial activity. Since malaria caused by *Plasmodium falciparum* Welch is also highly prevalent in much of Madagascar, Maranz' hypothesis would predict that local antimalarial medicines would not have strong antiplasmodial activity. It was therefore of interest to determine whether or not plants with known antimalarial uses performed better than average in these assays. Ethnobotanical coding of an existing data set including over a thousand fully screened samples was therefore performed.

Material and Methods

Plant tissues were collected from several localities in Madagascar, including mostly eastern humid forests, as well as one littoral forest area and one low-altitude extreme-northern dry forest; *Plasmodium falciparum* malaria is prevalent over most of this entire region, except at high altitudes, as well as in much of the rest of the country. Voucher specimens were deposited at the herbaria of the Parc Botanique et Zoologique de Tsimbazaza (TAN), the Missouri Botanical Garden (MO), the Centre National d'Application des Recherches Pharmaceutiques (CNARP), and the Muséum National d'Histoire Naturelle in Paris (P). Field determinations were frequently supplemented or amended following examination of vouchers by botanists at MO, P or TAN.

Plant tissues were ground, using a hammer mill for tissues such as roots, and extracted in EtOH via 24-hour percolation at room temperature. Plant extracts were assayed for antimalarial activity using the SYBR Green I method (Bennett et al. 2004); the malaria strain used was chloroquine-resistant *Plasmodium falciparum* FCM29. Briefly, parasites are incubated for at least one life cycle, with and without tested extracts, in human erythrocytes, and their growth quantified by a dye that fluoresces when intercalated into DNA. Since mature human erythrocytes lack DNA, differences in fluorescence between treated and control cultures are attributable to variation in the quantity of plasmodial DNA and hence survival and replication of the plasmodia. For extracts initially showing a meaningful degree of growth inhibition in a single test, testing multiple concentrations of the extract permits calculation of the IC₅₀, reported in µg/ml.

Complete antimalarial testing data were available from a set of 1294 samples. After screening had been completed, ethnobotanical use data for those samples were coded. Two reviews of Malagasy plants used for malaria (Rasoanaivo et al. 1992, Randrianarivelojosia et al. 2003), as well as several other relevant journal articles and reference works related to Malagasy ethnobotany (Pernet and Meyer 1957; Andriamihaja 1986; Boiteau and Allorge-Boiteau 1993; Rakotobe et al. 1993; Novy 1997; Samyn 2001; Gurib-Fakim and Brendler 2004; Norscia and Bogognini-Tarli 2006; Kaou et al. 2008; Razafindraibe et al. 2013; Rabearivony et al. 2015), were reviewed for reports of use of the screened species or genera to treat malaria. Ethnobotanical data were coded as presence or absence of recorded use at both the species level and the generic level. It was not always possible to determine whether a specific species had a recorded history of use, both because literature reports of plant use sometimes identify a plant only to the generic level and because some plant samples, especially those collected when sterile, could be identified only to the generic level. Hence, many of the species with recorded use at the generic level were coded as having “unknown” use at the species level.

The number of samples in each use category that had at least moderate activity (IC₅₀ < 50 µg/ml) and the number that had activity considered worthy of further investigation (i.e., “hits”; IC₅₀ < 20 µg/ml) were tabulated. The statistical significance of observed differences in likelihood of activity between samples from genera with and genera without known use were calculated by chi-square for each use category separately.

Results and Discussion

Of 1294 tested samples, 39.6% displayed some level of direct antimalarial activity ($IC_{50} < 50 \mu\text{g/ml}$) and 21.1% were considered to be highly active (“first-pass hits”) at $IC_{50} < 20 \mu\text{g/ml}$. Over 40.6% of the samples came from genera with prior use for malaria in Madagascar; 5.2% of samples came from species known to have such use. Samples from genera with use data had modestly increased activity rates as compared to samples from genera without such data (Table 1); though that result was statistically significant ($p < .01$), the observed increase in the likelihood of high activity was not ($p > .1$). Samples from individual species known to be used had an even higher likelihood of moderate activity, but were actually slightly less likely to be highly active than samples from genera without known use for malaria. Thus, these data offer little support for the hypothesis that ethnobotanically directed screening would increase the efficiency of antimalarial screening in Madagascar, and fail to rule out Maranz’ (2012) hypothesis.

While efficiency (percentage of screened samples that generate first-pass hits) is valued in discovery projects, the total number of hits is also important, given the low percentage of hits that may ultimately supply a promising single lead compound for drug development. There were 273 “hits” among all samples, of which 122 (44.7%) were from genera for which we located a recorded use for malaria. Therefore, if screening in this study had been limited to those genera, about 55% of the first-pass hits would not have been discovered. If screening had been limited to the exact species that had known antimalarial use, assuming that all were identified and deliberately collected in the field, 95% of the first-pass hits in this data set would not have been discovered (only 12 of 273, or 4.4%, came from those species). From the drug discovery perspective, a very large reduction in the total number of hits surely outweighs a modest increase in hit rate. Therefore, in this context, ethnobotanically directed screening would clearly have been inadvisable.

These results are potentially affected by several limitations. First and probably most importantly, when species or genera with known use were screened, the plant organs screened were not limited to those traditionally used, and preparation of plant extracts did not follow traditional methods; both of those facts could dilute or obscure any increased likelihood of efficacy in medicinal taxa. Gyllenhall et al. (2012) found an increased per-sample hit rate in medicinal plants for which, almost always, only one commonly used part was collected. In this study, as many parts as possible were collected from all plants without regard for their medicinal uses (which were not known to collectors), and in part because use data are sometimes quite limited, taxa rather than samples were categorized as medicinal or not. It would be impossible to categorize individual plant parts as definitely used or not used without discarding a great deal of data. Hence, while these results do not contradict Maranz’ (2012) hypothesis, they also do not provide substantial support for it.

Second, the original samples do not truly represent a random selection from the flora. As is common in such studies, due to the volume of material of individual plant organs needed for the methodology in use at the time, large woody plants and common plants were far more likely to be included than small herbs or rare plants, and the former may be more likely than the latter to have interesting chemistry. Third, both species mentioned in ethnobotanical

literature and vouchers for collected material are sometimes incompletely or perhaps erroneously identified (though some incorrect field determinations of vouchers were corrected, others might persist). Fourth, in floristically diverse areas where malaria and other febrile illnesses are very common, it is likely that many plants will be at least occasionally tried as treatments, but when a use category includes numerous species, it is inevitable that some will be of less value than others. The approach of Clarkson et al. (2004), in which only the most promising quarter of species with reported use were selected for testing, did generate much better hit rates, although in some biodiverse rural areas, the necessary data to support such ranking may not be available.

Finally, it should be emphasized that these results do not support a claim that most Malagasy traditional medicines for malaria do not offer any benefits to users, nor do they even demonstrate that they do not function via direct antiparasitic activity. First, as noted, the extracts tested from those species were not comparable to traditional medicines. Second, some natural products may be active only in vivo; the extract of the African *Nauclea pobeguinii* (Pobég. ex Pellegr.) Merr. ex E.M.A. Petit, which is effective for treatment of malaria in humans (Mesia et al. 2012), was found to be poorly active in vitro, as were its main suspected active constituents, but more active in mice, suggesting that one or more of its components is rendered active by gut flora or host metabolism after consumption (Mesia et al. 2010). Thus, it would remain desirable to conduct in vivo studies of those medicines considered most effective by Malagasy traditional healers, if funding were available.

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Table 1

Percentage of specimens demonstrating moderate or high antimalarial activity according to ethnobotanical categorization

Categorization	# of samples	IC ₅₀ < 50 µg/ul	IC ₅₀ < 20 µg/ul
All samples	1294	513 (39.6%)	273 (21.1%)
Samples from genera <i>without</i> known use in Madagascar for malaria	768	280 (36.5%) ^a	151 (19.7%) ^b
Samples from genera with known use in Madagascar for malaria	526	233 (44.3%) ^a	122 (23.2%) ^b
Samples from species with known use in Madagascar for malaria	67	33 (49.3%)	12 (17.9%)

^a Statistically significant difference (p < .01)

^b Not statistically significant (p > .1)