

Historical chemical annotations of *Cinchona* bark collections are comparable to results from current day High-Pressure Liquid Chromatography technologies

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Running title:

Historical chemical annotations of *Cinchona* barks

ABSTRACT

Ethnopharmacological relevance: Species of the genus *Cinchona* (Rubiaceae) have been used in traditional medicine, and as a source for quinine since its discovery as an effective medicine against malaria in the 17th century. Despite being the sole cure of malaria for almost 350 years, little is known about the chemical diversity between and within species of the antimalarial alkaloids found in the bark. Extensive historical *Cinchona* bark collections housed at the Royal Botanic Gardens, Kew, UK, and in other museums may shed new light on the alkaloid chemistry of the *Cinchona* genus and the history of the quest for the most effective *Cinchona* barks. **Aim of the study:** We used High-Pressure Liquid Chromatography (HPLC) coupled with fluorescence detection (FLD) to reanalyze a set of *Cinchona* barks originally annotated for the four major quinine alkaloids by John Eliot Howard and others more than 150 years ago. **Materials and Methods:** We performed an archival search on the *Cinchona* bark collections in the Economic Botany Collection housed in Kew, focusing on those with historical alkaloid content information. Then, we performed HPLC analysis of the bark samples to separate and quantify the four major quinine alkaloids and the total alkaloid content using

fluorescence detection. Correlations between historic and current annotations were calculated using Spearman's rank correlation coefficient, before paired comparisons were performed using Wilcoxon rank sum tests. The effects of source were explored using generalized linear modelling (GLM), before the significance of each parameter in predicting alkaloid concentrations were assessed using chi-square tests as likelihood ratio testing (LRT) models. **Results:** The total alkaloid content estimation obtained by our HPLC analysis was comparatively similar to the historical chemical annotations made by Howard. Additionally, the quantity of two of the major alkaloids, quinine and cinchonine, and the total content of the four alkaloids obtained were significantly similar between the historical and current day analysis using linear regression. **Conclusions:** This study demonstrates that the historical chemical analysis by Howard and current day HPLC alkaloid content estimations are comparable. Current day HPLC analysis thus provide a realistic estimate of the alkaloid contents in the historical bark samples at the time of sampling more than 150 years ago. Museum collections provide a powerful but underused source of material for understanding early use and collecting history as well as for comparative analyses with current day samples.

Keywords: collections; alkaloid; malaria; Cinchona; HPLC; quinine

1. Introduction

Barks have been used as medicines for thousands of years and are deeply embedded in traditional knowledge (Rajamurugan et al., 2016; Senkoro et al., 2014; Turner and Hebda, 1990; Williams, 2004). First reported from Peru in 1630 as a traditional remedy for the treatment of malaria, the bark of trees from the *Cinchona* genus (Rubiaceae) is considered the most influential bark medicine in human history (Prendergast and Dolley, 2001).

The barks of *Cinchona* contain an array of about 35 different alkaloids (Kacprzak, 2013), which are thought to be produced as defence compounds against diseases and herbivores (Maldonado et al., 2017). The four most prevalent alkaloids are quinine, quinidine, cinchonine and cinchonidine, diastereoisomers with four chiral centres (Figure 1A). The bark's total alkaloid content ranges between 7-12%, with quinine the most abundant alkaloid, accounting for up to 90% of the total alkaloid content (McCalley, 2002). However, considerable variation in content and composition of alkaloids is found between and within species, and both quinine, cinchonidine and the total content of the four major alkaloids appear to be correlated with phylogeny (Maldonado et al., 2017). In the period between the introduction to Europe in the mid-17th century and the creation of synthetic antimalarials during World War II, *Cinchona* bark, quinine or a mixture of quinoline alkaloids were the only known effective remedies for curing malaria (Honigsbaum, 2001; Kaufman and Rúveda, 2005; Deb Roy, 2017).

The molecular interaction between quinine and other active bark alkaloids with the parasite that causes malaria, *Plasmodium*, is through modification of haem-compounds that are by-products of the *Plasmodium* feeding on the iron-rich human red blood cells (White and Ho, 1992). A recent study showed that quinoline antimalarials bind to freely exposed sites of the actively growing hemozoins, hindering crystallization through a process referred to as "kink blocking" (Olafson et al., 2017). This in turn accumulates haem-buildup in the digestive vacuoles of *Plasmodium*. As such, the parasites end up as victims of their own metabolism

upon quinine administration. This is supported by a study combining confocal microscopy and quinine linked fluorophores. The experiments showed a concerted translocation of the quinine-molecules to the parasite's digestive vacuole (Woodland et al., 2017).

When it was first discovered that *Cinchona* barks were successful in the treatment of malaria, its aetiology was not even close to being understood. The word 'malaria' was not assigned to the disease until the middle of the 18th century and originates from 'bad air' in Italian: "mal'aria". The disease was attributed to the air surrounding bogs and swamps, but it was still unknown that malaria was a parasitic disease carried by mosquitoes which bred in stagnant water. Even today, the time and place of the discovery of the effect of *Cinchona* bark on malaria remains uncertain (Deb Roy, 2017; Crawford, 2016; Walker and Nesbitt, 2019). No certain records of Inca or Quechua peoples using the *Cinchona* tree against malaria have been found. Its long history of use against feverish episodes and shivering led to the popular name, fever tree (Lee, 2002). Explorers, merchants, physicians, botanists, and monks have written varied accounts of the first usage of this bark against malaria, none of which can be verified. These stories range from South American mountain lions chewing the bark and the indigenous tribes learning from it, told by La Condamine, to an ill native American drinking from a natural pool of water surrounded by *Cinchona* trees and recovering from the fever episodes, as told by Clements Markham (Markham, 1862).

The Peruvian bark probably first arrived in Europe, via Seville (Spain), introduced in the early 1630s by Jesuit monks and then popularised by an ecclesiastical figure, Cardinal de Lugo. He promoted the use of *Cinchona* against tertian and quartan agues, and bark extracts were given to hundreds of patients proving its efficacy (Lee, 2002). The bark's fame then spread across Europe, reaching England and the Netherlands, where it was first received with suspicion as it was regarded as a Popish remedy not to be trusted (Honigsbaum, 2001). *Cinchona* bark made its first official appearance in European archives in 1677. Its large-scale use in Europe started around 1650 and continued for around 200 years. As reported by Humboldt (Humboldt, 1795), more than 25,000 trees were harvested and destroyed in one year. By the middle of the 19th century there were claims that overharvesting would pose a threat to the native *Cinchona* forests ultimately impacting the drug's availability (Eyal, 2018).

After centuries of export of barks from South America to Europe, the threat of overharvesting along with the desire to control quality and quantity of supply led to attempts by the British, Dutch and French empires to start *Cinchona* plantations in other tropical regions, taking this Andean tree as far as India and Indonesia (Lee, 2002; Walker and Nesbitt, 2019). *Cinchona calisaya*, with a total alkaloid content up to 6.5% of which around 80% is quinine (Rusby, 1931), provided the most readily available bioactive alkaloid with barks in reliable supply, and the form extracted and administered with most ease (Achan et al., 2011).

In 1820, two French chemists, Joseph Pelletier and Pierre Caventou, first extracted two active constituents of *Cinchona*, quinine and cinchonine (Delepine, 1951). This gave physicians, botanists and chemists a tool not only for measuring dosage and efficacy but also a way to measure the alkaloid content of various species of *Cinchona* to enable targeting of species for transfer to plantations. As the *Cinchona* tree was relatively inaccessible to western scientists, bark samples were collected along the drug trade routes entering Europe and analysed in European laboratories.

Many of these bark samples representing several hundred years of collecting, and 150 years of experimentation in the plantations, are stored in museums in Europe and elsewhere, with the most extensive collections housed in the Economic Botany Collection of the Royal Botanic Garden, Kew, UK. Although most of the collected barks are annotated with origin, collectors were forced to rely on trade names and provenances, often more representative of ports of export from Latin America than the original harvesting location. Exceptionally, some bark specimens were analysed and annotated for quinine and other major alkaloids in the mid-late 19th century by John Eliot Howard, a partner in the pharmaceutical wholesalers Howards and Sons (Deb Roy, 2017; Walker and Nesbitt, 2019; Figure 1B). Howard was interested in analysing *Cinchona* to discover reliable sources of quinoline alkaloids for commercial purposes. He published prolifically on *Cinchona* botany and chemistry of those plants, and many of his bark specimens can be cross-referenced to his manuscript and printed texts. These historical bark collections provide an invaluable source of material and information, which can potentially be used for research within a range of sciences, from biodiversity and conservation science to collecting history and drug discovery. Although historical bark samples provide valuable sources of information to the study and mapping of early uses of *Cinchona*, it remains unknown whether they can also inform on the chemical quality of those samples, and how the concentration of alkaloids in those barks might have changed through time.

The aim of the present study was to assess if current day contents of the four major quinoline alkaloids analysed using High-Pressure Liquid Chromatography (HPLC) of historical *Cinchona* barks are correlated with the annotated historical analysis made 150 years ago. If current day and historic analyses prove to be similar, this implies that quinoline alkaloids are relatively stable in historic specimens, and that current day analyses of historic specimens are therefore representative of original alkaloid content. This would greatly increase the value of historic specimens for research.

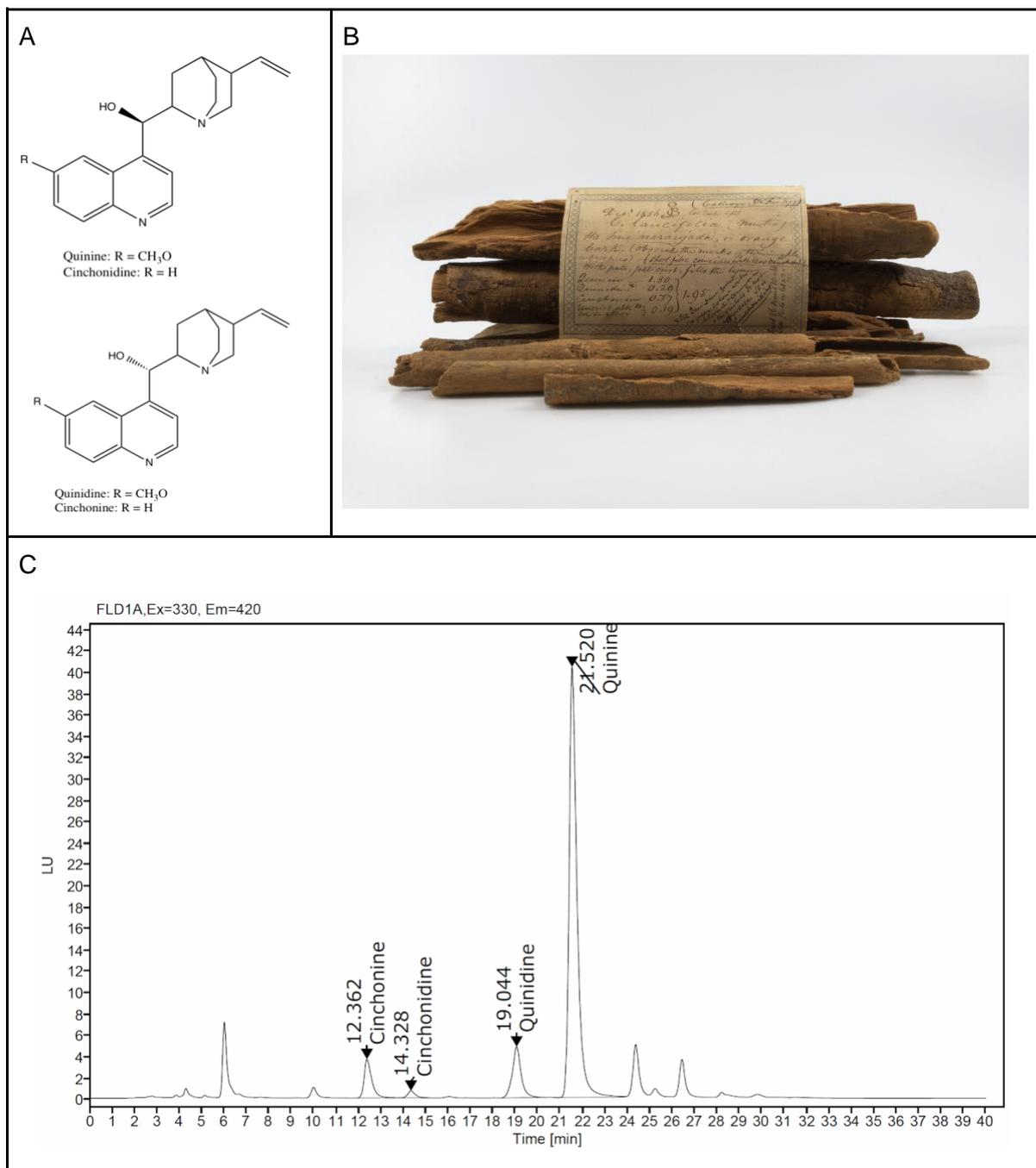


Figure 1. (A). Structures of the four major quinine alkaloids. (B). Piece of *Cinchona lancifolia* Mutis bark with chemical annotations of the four major quinine alkaloids provided by Howards and Sons, collected 1856, Kew Economic Botany Collection specimen #52935. (C). Example of current day HPLC chromatogram from analysis of *Cinchona lancifolia* Mutis, Kew collection #52935.

2. Material and methods

2.1. Sampling strategy of historical bark collections.

Bark samples were obtained in June 2018 from the Economic Botany Collection housed at the Royal Botanic Gardens, Kew, UK. We selected the specimens based on availability of associated meta-data on origin, species identity, and historical chemical annotation. The majority of specimens were from the mid to late 19th century chemically-annotated collections of John Eliot Howard, and other collections with chemical annotation donated directly to Kew or obtained later via the Royal Pharmaceutical Society or other collections. In total, 67 specimens that were historically annotated with one or more major alkaloids (or total alkaloids) were sampled for this study. For these samples, the average age was 159 years corresponding to a collection year being in the range of between 1850 and 1904. Details about the specimens used are presented in Table 1.

Table 1 - Details of material used in the present study.

[submitted as separate excel file]

2.2 Alkaloid extraction and analysis

Around 100 mg of each bark sample was powdered and homogenized using a purpose-built modified coffee grinder to reduce dead volume allowing minimum destructive sampling of the historical collections (Hansen et al., 2015). 50.0 mg homogenized and pulverized bark were used for further processing. Extractions were done according to a previously established protocol using DMSO and double-extractions with 70% methanol w/ 0.1% formic acid in an ultra-sonication bath (Holmfred et al., 2017). Supernatants were compiled, diluted to 50 mL using 0.1% formic acid in deionized water, and stored for up to three days at 5 °C prior to processing on HPLC. Before analysis, the diluted supernatants were vortexed to ensure proper homogenization of the samples, 1.5 mL homogenized extract was spun down, and 600 µL were added to HPLC-compatible vials and crimp-sealed.

For HPLC analysis, we followed a published method which we previously established for studies of current day *Cinchona* barks (Holmfred et al., 2017; Maldonado et al., 2017). The HPLC system consisted of an Agilent 1200 system (Agilent, USA), which included a degasser G1379B, a binary pump G1312B, an autosampler G1367C, a column oven G1316B, and a fluorescence detector (FLD) G1321A. The column used was a Kinetex XB-C18 (150 mm × 2.1 mm) with 2.6 µm particles. Two mobile phases were used for this analysis. Mobile phase A was 0.2 M ammonium formate buffer with 0.1% formic acid (pH 3.5) and water (10:90 v/v) and mobile phase B was 60:40 (v/v) acetonitrile:methanol. The flow was set at 0.2 mL/min. The gradient was 18% B from 0 to 10 min, then changed from 18% B to 35% B from 10 to 25 min and returning to 18% B after 26 min with a total run time at 40 min. The column oven temperature was 20 °C and the injection volume 3.0 µL. Fluorescence detection was performed with excitation of 330 nm and emission of 420 nm.

Quinine sulfate and cinchonine standards were obtained from Merck (Darmstadt, Germany). Quinidine and cinchonidine were both obtained from Fluka (Sigma-Aldrich, Denmark). Purity of standards were checked with NMR and ranged from 78% (quinine as sulphate) to 92% (quinidine) (Holmfred et al., 2017).

The limit of quantification (LOQ) and the limit of detection (LOD) was estimated from the standard deviation (RSD) of the lowest standard using 7(+1) calibration levels and 9 replicates (Supplementary material online). LOD: 2.38 µg/g (alkaloid/dried bark) and LOQ: 7.87 µg/g (alkaloid/dried bark). Blank samples were used to check for carry over and no carry over was detectable. A linear calibration equation was used, and tested against a second order calibration equation, and an F-test was performed on the residual variances of the two fits showing no significance on a 95 % confidence level. Furthermore, residual plots were made to check for nonlinearity and time drift. The identity of the target analytes was confirmed using LC HRMS (Thermo qExactive).

We performed several tests to ensure column integrity and reproducibility of results and to set a washing regime for the column during automated analysis. The washing step was set to 30-40 min with methanol followed by a 60:40 (v/v) acetonitrile:methanol plug injection. We processed the samples in three separate HPLC runs, with up to thirty samples in the autoloader. Three individual measurements were made per sample and 3 µl were injected by the autoloader each time.

2.3. Data processing and statistical analysis

We extracted all data from the Agilent Openlab (Agilent Software, USA) platform after inspection of integrated peaks and manual correction of faulty determinants, such as double-tops or false peak determination by the software.

We performed all statistical analyses using *R* Studio (v1.1.453) within the *R* statistical computing environment, (v3.5.0) and all figures produced using the package *ggplot2* (Wickham, 2016). Due to non-normally distributed data, correlation tests were performed between historic and current day annotations using Spearman's rank-order correlation (using *cor.test* function), before paired comparisons were performed using Wilcox rank sum tests (using the *wilcox.test* function). Meanwhile, the effects of source (which sub-collection each sample was part of), age, country/region of origin and species on bark alkaloids were explored using generalized linear modelling (GLM) using the negative binomial family as data was alkaloid over-dispersed. Initially, models were fitted using the *glm.nb* function, before the significance of each parameter in predicting alkaloid concentrations were assessed using chi-square tests as likelihood ratio testing (LRT) models using the *drop1* function. For each analysis, we analysed quinine, quinidine, cinchonine and cinchonidine individually, alongside total content of the four alkaloids. However, as historic annotations for cinchonine and cinchonidine were performed less frequently, and the number of samples included in each comparison varied substantially (Table 1).

3. Results

We successfully quantified the four major alkaloids from 67 *Cinchona* bark specimens collected between 1850 to 1904 (Table 1). Quinine was the most abundant of the major alkaloids, followed by cinchonine and cinchonidine, while quinidine was the least abundant alkaloid in both datasets (Figure 2). However, alkaloids were determined in greater abundance in the historical dataset, with the mean alkaloid content higher in historical annotations than

the current day for quinine (2.2% and 1.3%), cinchonidine (0.8% and 0.6%), and total alkaloid content (3.2% and 2.8%). Additionally, we found considerable discrepancy in quinidine annotations, which were found to have five-fold higher concentrations in the historical annotations than the current day annotations (0.5% and 0.1% respectively). Cinchonine was however present at a mean concentration of 0.8% in both datasets.

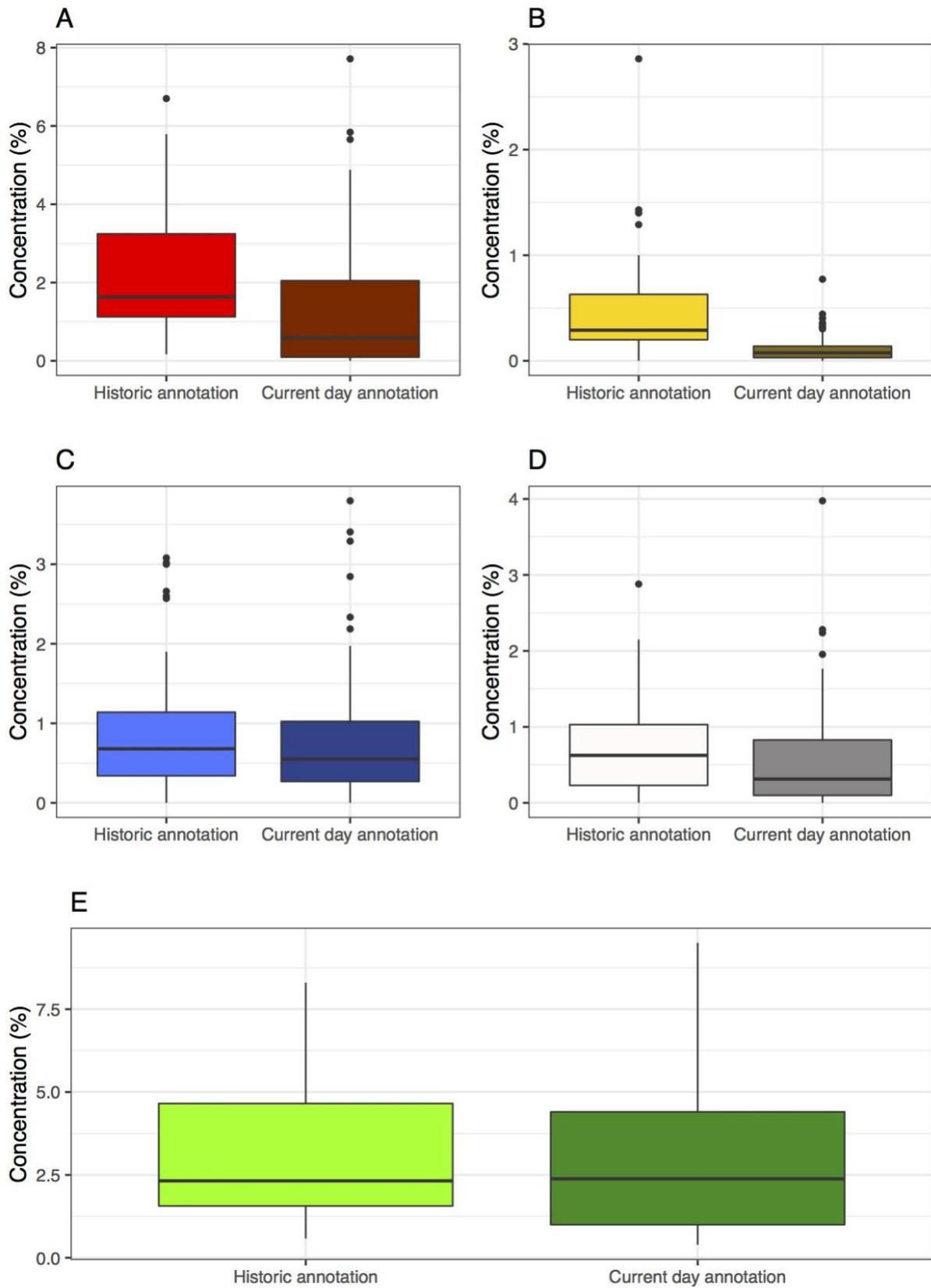


Figure 2. Boxplots comparing (A) quinine, (B) quinidine, (C) cinchonine, (D) cinchonidine and (E) total alkaloid content between historic and current day annotations (using HPLC). Correlations between historic and current annotations were calculated using Spearman's rank

correlation coefficient, while linear relationships were drawn on significantly correlating alkaloids for illustrative purposes only.

Initially, we calculated the general correlation between historical alkaloids using Spearman's rank correlation coefficient, with significant correlations for quinine ($S = 5157$, P -value < 0.001), cinchonine ($S = 11293$, P -value < 0.001) and total alkaloid content ($S = 17290$, P -value < 0.001), but not for quinidine and cinchonidine (Figure 3; Table 2). However paired analyses performed with Wilcoxon signed rank tests revealed less clear correlations between the historical and current day annotations (Figure 4), with only quinine ($V = 759$, P -value = 0.039) and quinidine ($V = 920$, P -value < 0.001) significantly correlating and not cinchonine, cinchonidine and total alkaloid content (Table 2).

We plotted the historic quantifications against the current day results and these graphs are shown in Figure 3. The plots containing fitted lines for (A) quinine, (C) cinchonine and (E) total alkaloids, showed statistically significant relationships. The equations for the graphs modelling the relationships between historical and current day quantifications for each of these were $y = 0.76x + 0.44$, $y = 0.48x + 0.44$ and $y = 0.71x + 0.68$ for quinine, cinchonine and total alkaloids, respectively. We found the same correlations to be significant using the Spearman Rank Correlation Test for quinine ($S = 5157.9$, P -value < 0.001), cinchonine ($S = 11293$, P -value < 0.001) and total alkaloids ($S = 17290$, P -value < 0.001).

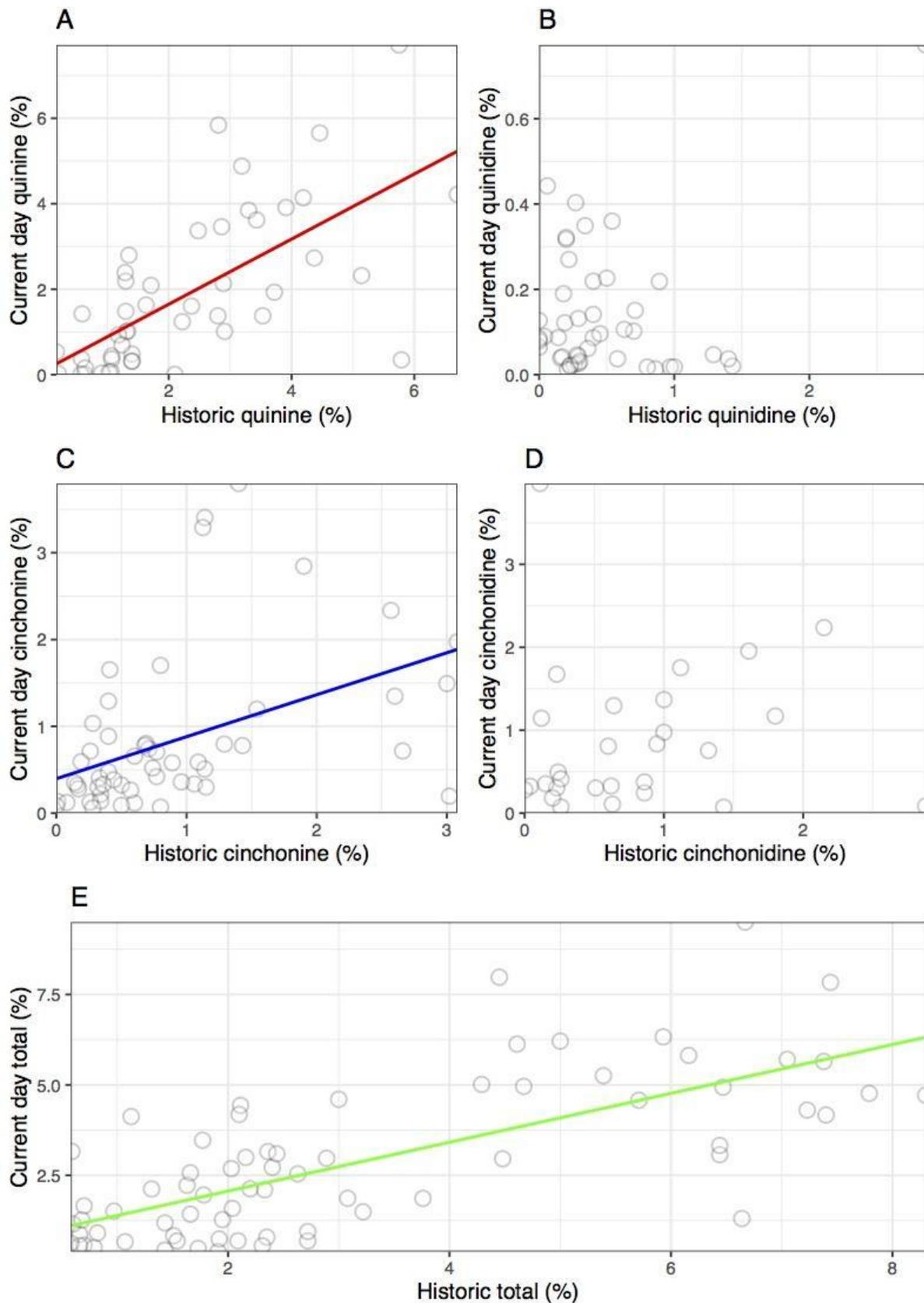


Figure 3. Linear relationship between historic and current day measurements of the four major alkaloids present in *Cinchona* barks. (A) quinine, (B) quinidine, (C) cinchonine, (D) cinchonidine, and (E) total alkaloids.

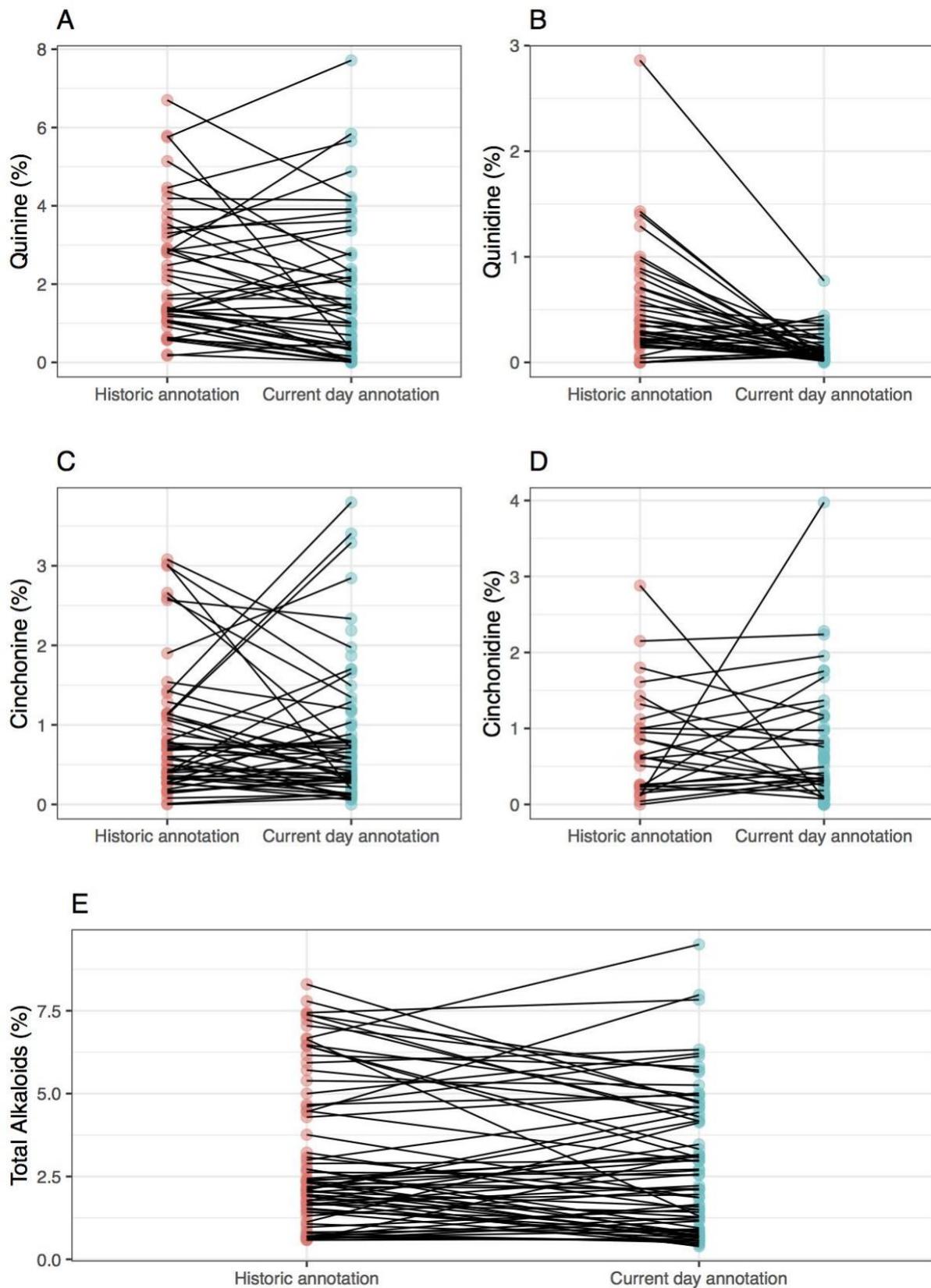


Figure 4. Individual current day HPLC quantifications (%) connected with their paired current day quantification (%) for (A) quinine, (B) quinidine, (C) cinchonine, (D) cinchonidine and (E) total alkaloids. Slopes of lines between individual points represent quantification differences in either positive or negative direction.

Table 2. Correlations between historical and current day annotations for each of the four major alkaloids

Alkaloid	Spearman's rank		Wilcoxon signed rank test (paired)	
	S-value	P-value	V-value	P-value
Quinine	5157.9	<0.001	759	0.039
Quinidine	17428	0.332	920	<0.001
Cinchonine	11293	<0.001	846	0.250
Cinchonidine	3025.7	0.382	185	0.695
Total	17290	<0.001	1491	0.147

Finally, we performed generalized linear modelling (GLM) to determine the significance of the samples source, country of origin, age and species on alkaloid concentration for the historical and current day datasets separately. The significance of each of these parameters on alkaloid concentration was assessed using likelihood ratio testing (LRT, chi-square) (Table 3). While we found no difference in the regulation of these alkaloids between datasets, we also found no significant factors affecting alkaloid concentrations in either the historical or current day annotations.

Table 3. Significance of sample, age, country of origin and species effects on each of the four major alkaloids and total alkaloid content on the current day and historical samples using likelihood ratio testing (LRT)

Alkaloid	Explanatory parameter	Historical		Current day	
		LRT	P-value	LRT	P-value
Quinine	Source	0.000	-	2.141	0.144
	Age	1.339	0.247	0.338	0.561
	Country	0.776	0.378	3.563	0.468
	Species	4.962	0.549	13.313	0.273
Quinidine	Source	0.000	-	0.019	0.889

	Age	0.185	0.667	0.196	0.658
	Country	0.410	0.938	0.233	0.994
	Species	3.654	0.979	1.602	1.000
	Source	0.005	0.943	2.633	0.105
Cinchonine	Age	0.003	0.960	0.010	0.921
	Country	0.015	1.000	0.285	0.991
	Species	7.985	0.630	7.941	0.719
	Source	0.000	-	0.037	0.848
Cinchonidine	Age	0.046	0.830	0.050	0.823
	Country	0.000	-	1.624	0.805
	Species	1.627	0.804	7.549	0.753
	Source	3.151	0.076	0.201	0.654
Total	Age	3.824	0.051	0.167	0.683
	Country	3.111	0.539	2.400	0.663
	Species	8.313	0.685	10.979	0.445

4. Discussion

4.1. Comparison of historical and current day chemical data

Statistical comparison of the results of the historical and current day chemical analysis showed a high level of consistency for both individual major alkaloids and the quantity of the four alkaloids combined. However, the level of quinidine was found to be five times higher in the historical data than with the HPLC analysis. All standards were checked for degradation using NMR and found to be of high purity as described in the methods, and there is no obvious expectation that quinidine should have been determined with less accuracy than the other alkaloids in the historical analysis. The cause of the relatively higher discrepancy in quinidine determination is therefore uncertain and needs further exploration. However, we can hypothesize that the historic methodologies possibly relying on differential solubility (e.g. van

der Hoogte & Pieters, 2014; Herapath et al., 1859), did not isolate quinidine efficiently from dihydro-quinidine, leading to overestimation of quinidine. Even the comparably pure commercial standards obtained today include dihydro compounds as impurities (Holmfred et al., 2017). Interference with other minor alkaloids is also possible, but less likely. Overall, our results suggest that current day analysis of historical *Cinchona* barks provide a reasonably reliable estimate of the content of the alkaloids in historical analysis. This allows for a better understanding of the history of selection of the most valuable *Cinchona* barks. Additionally, reliable chemical data from historical barks potentially enables inclusion of the historical barks that lack historical analyses in comparative analysis with present day collections, thereby expanding available data considerably both in numbers and geographic coverage (Maldonado et al., 2017).

4.2. Potential for finding new antimalarial leads from *Cinchona* barks

Malaria has been and is still a major issue for human health, with an estimated 219 million cases and 435 000 deaths globally in 2017 alone (World Health Organization, 2018). Quinine was largely replaced by other antimalarial drugs in the second half of the 20th century as first line therapy (Kaufman and Rúveda, 2005). However, consistent problems with development of resistance to all new drugs continues to be a major challenge in the treatment of malaria (White, 1992). Whereas the historical quest for *Cinchona* barks was focused on the high yield of the quinine and to some extent the other major alkaloids, more than 30 minor *Cinchona* type alkaloids have been identified (Kacprzak, 2013), and many more yet unknown and untested compounds are observed in the HPLC chromatograms which may present potential leads for future treatment. *Cinchona* bark extracts continue to be used as traditional medicines in South America (e.g. personal communications in Bolivia and Peru) and it is possible that development of parasite drug resistance may be less pronounced when using bark extracts, which includes multiple compounds in combination (Rasoanaivo et al., 2011).

4.3. The value of historical collections and data

Historical collections are invaluable records of data in time and space, which can both be used to understand historical trends and enable future predictions, as well as providing additional samples of rare or difficult to access species and locations (Foutami et al., 2018; Funk, 2018; Nesbitt, 2014). However, collection methods, storage conditions and data recording may vary considerably challenging comparative analysis (Maldonado et al., 2015). The value of historical collections for research is therefore dependent on the degree of meta-data available. In particular lack of information on species identity and origin may impair the research value of specimens. In some cases, additional information may be retrieved from archives and literature, which can be linked to the specimens improving their value. However, such archival work requires considerable time investment and a thorough understanding of collection history. In the present study, 67 specimens out of more than 185 chemically annotated specimens surveyed in the collections in Kew, were considered sufficiently annotated to be included in the study. The historical chemical annotations of the *Cinchona* barks are the result of analysis done by different laboratories, possibly using different methods over time. In addition, although alkaloids are considered to be relatively stable compounds, their degree of potential degradation of the alkaloids is unknown (Yilmaz et al., 2012). Previously, it has been found that the stability of almost 80 years old quinine injection solutions showed a content

decrease up to 13% over that period, which becomes quinotoxine and dihydroquinine. (Kudláček et al., 2017). However, quinoline alkaloids would be expected to be more stable in the dried barks than in solution.

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Author contributions

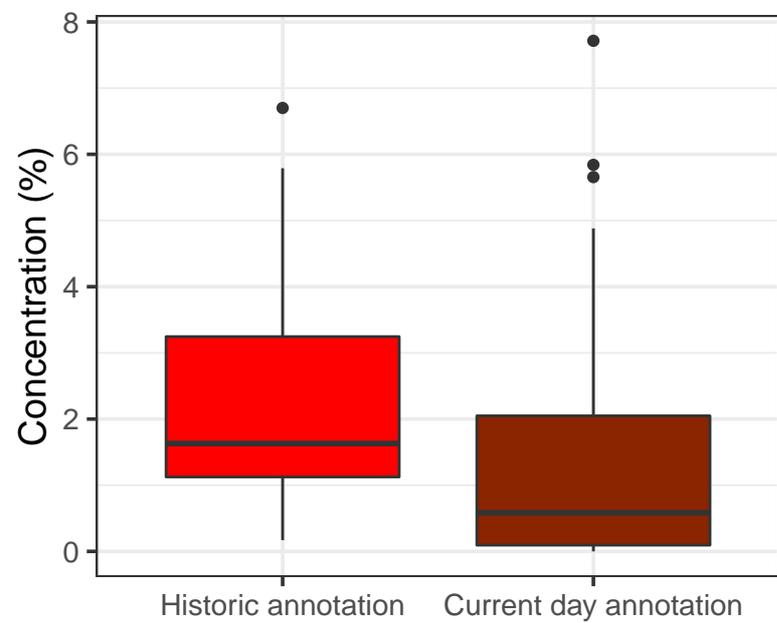
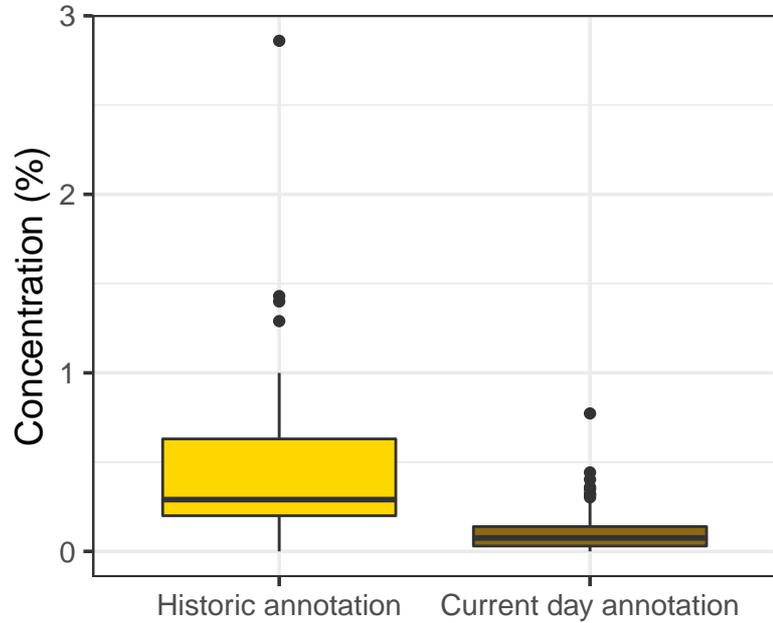
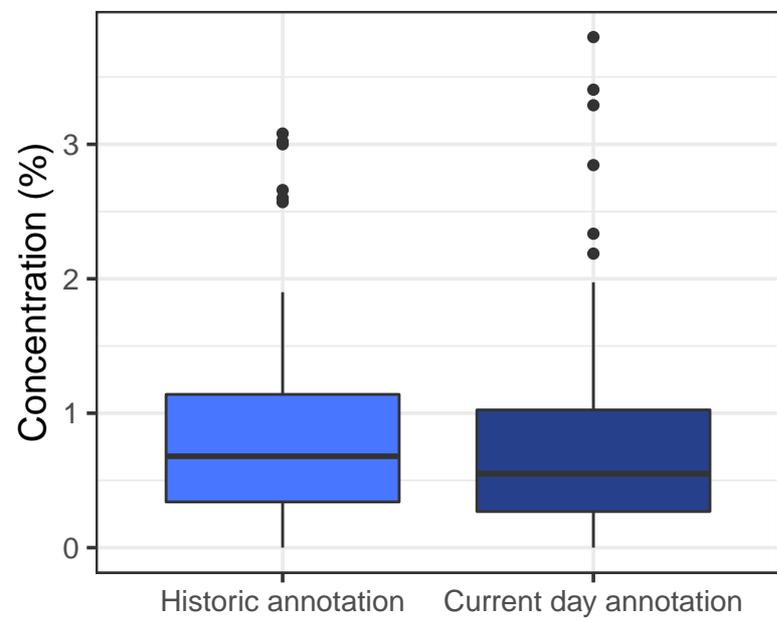
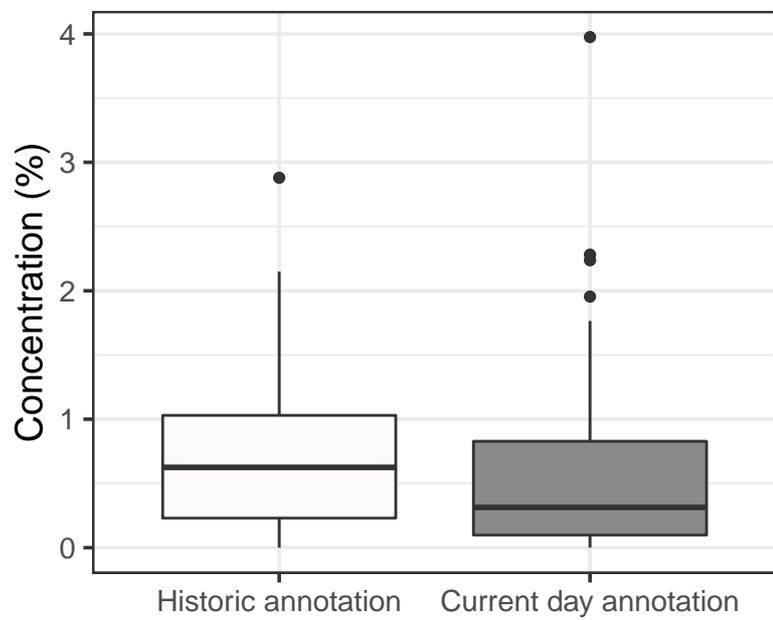
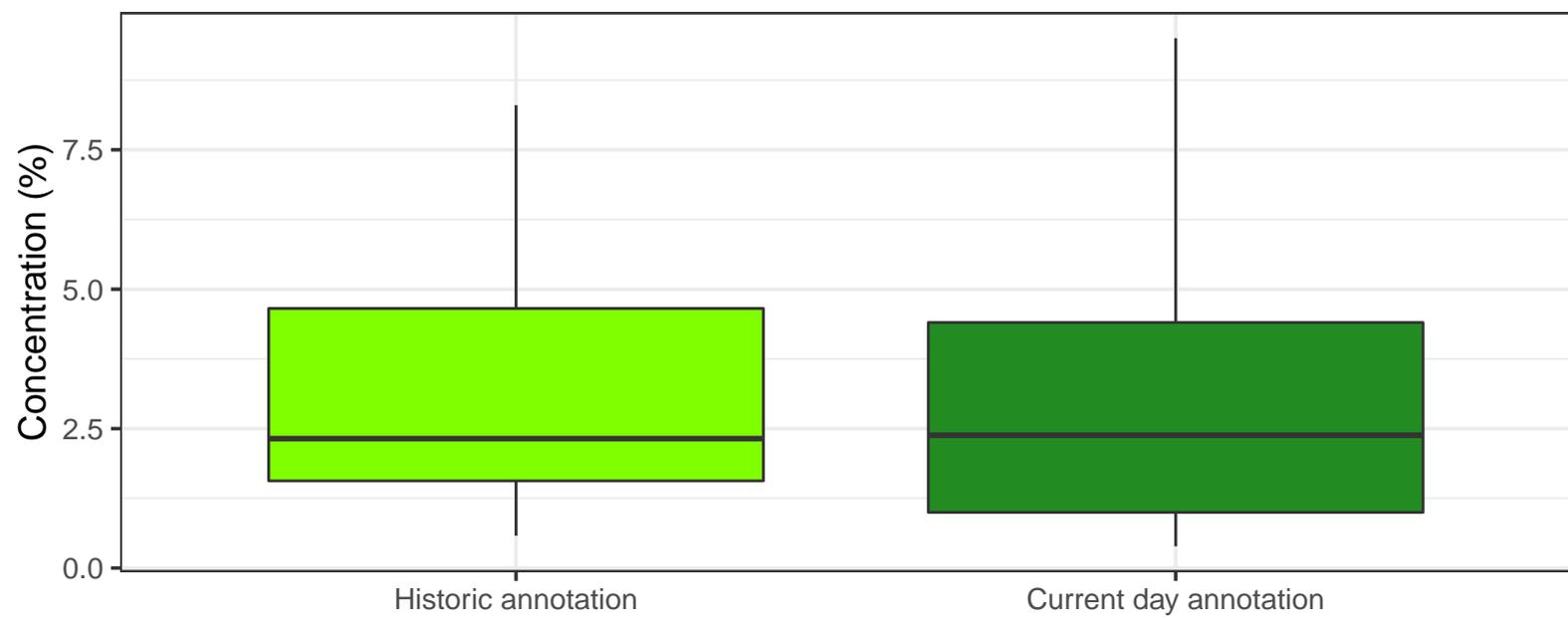
N.A.C., T.G.H., K.W., M.N., and N.R. jointly conceived and designed the project. N.A.C., K.W. and M.N. selected the samples. K.W. researched the archives and provided the metadata of the samples, supervised by M.N. and F.D.. N.C. and T.G. collected the HPLC data under supervision by C.C.. T.G.H and C.J.B. performed the statistical analysis. N.A.C. drafted the paper with T.G.H., C.J.B. and N.R. All authors commented on the manuscript and approved the final version.

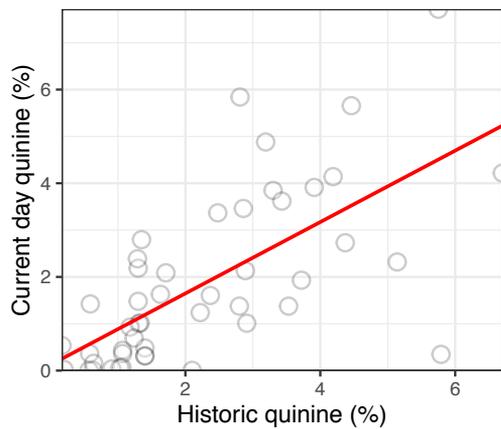
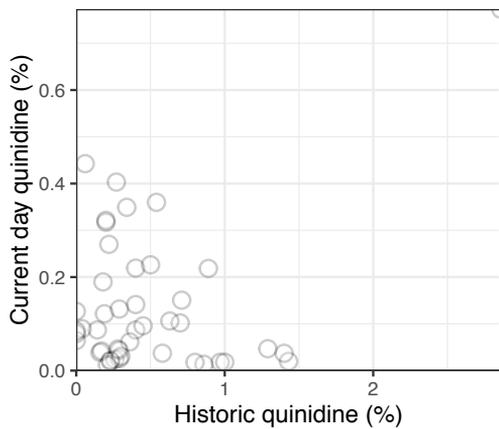
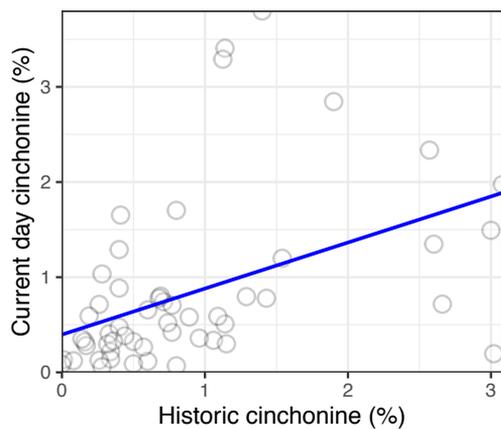
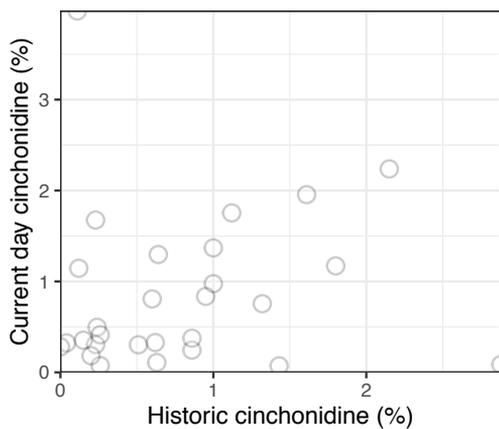
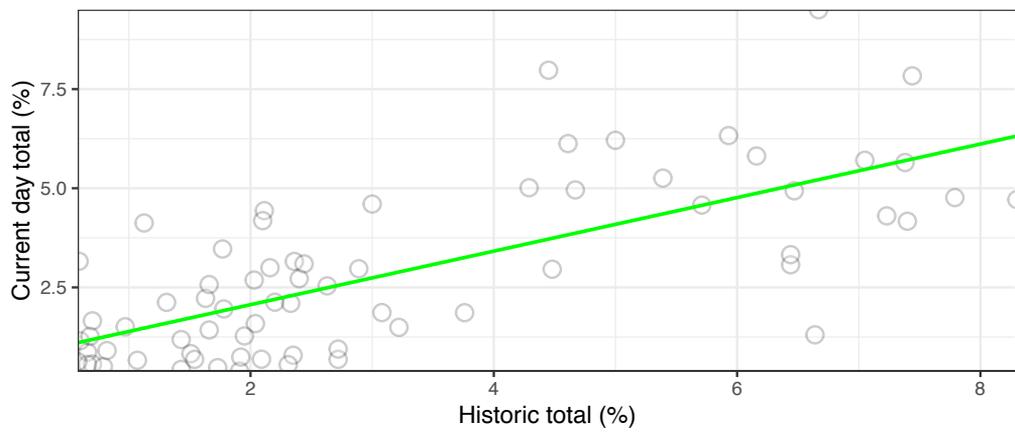
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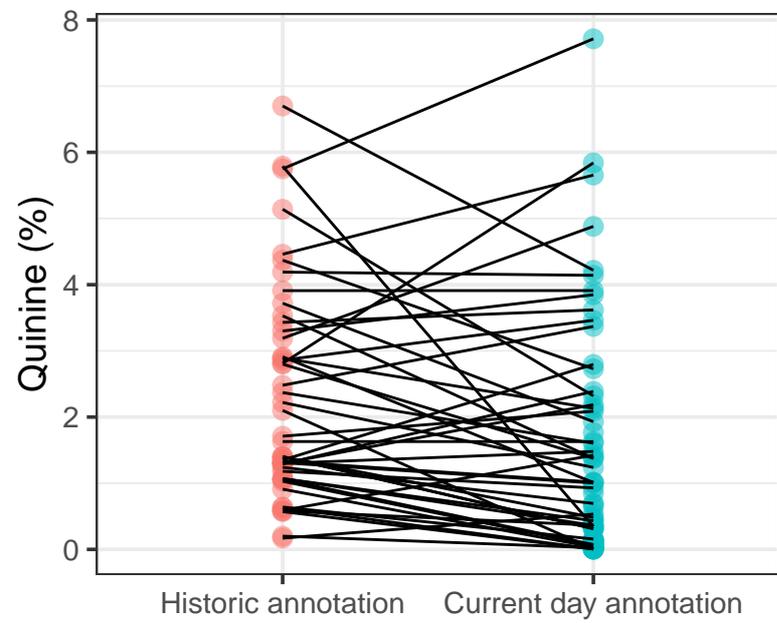
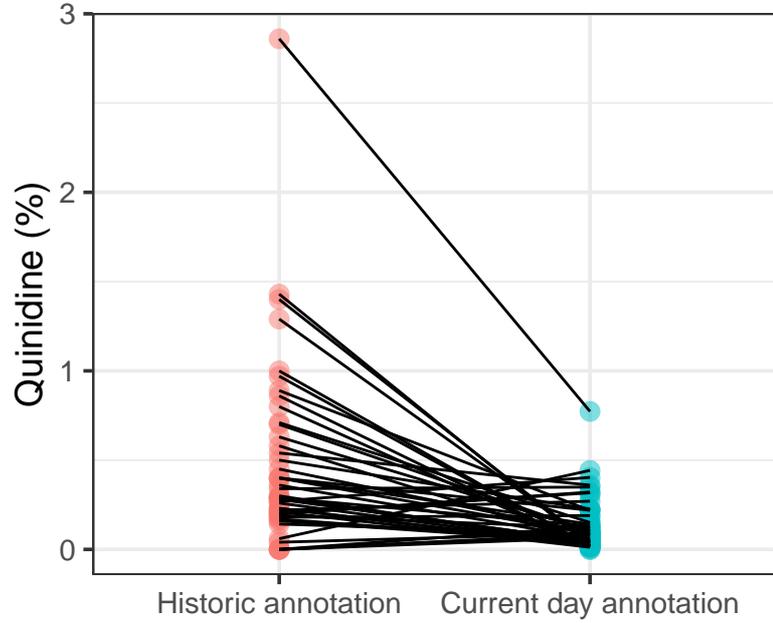
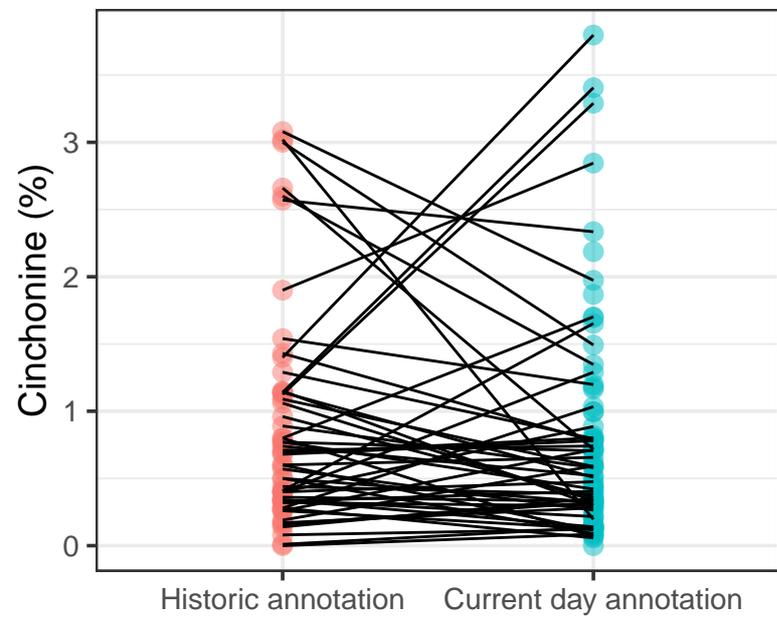
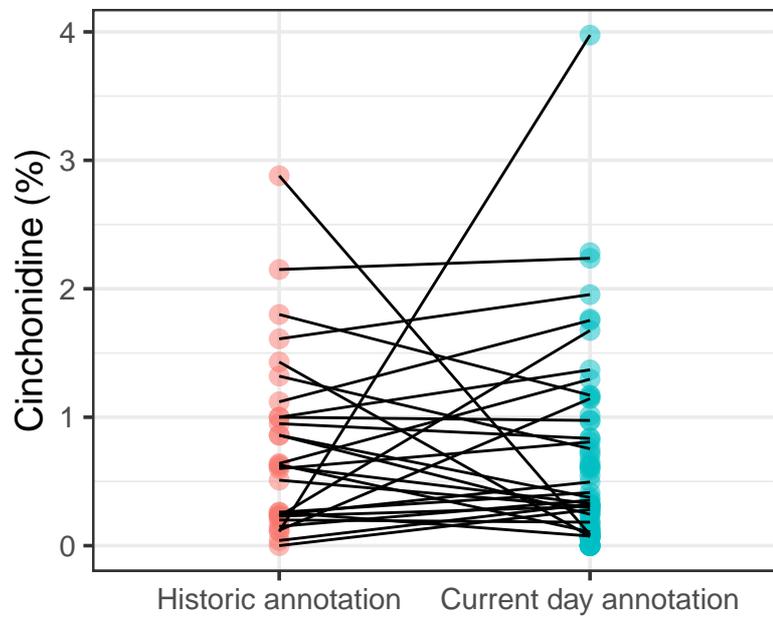
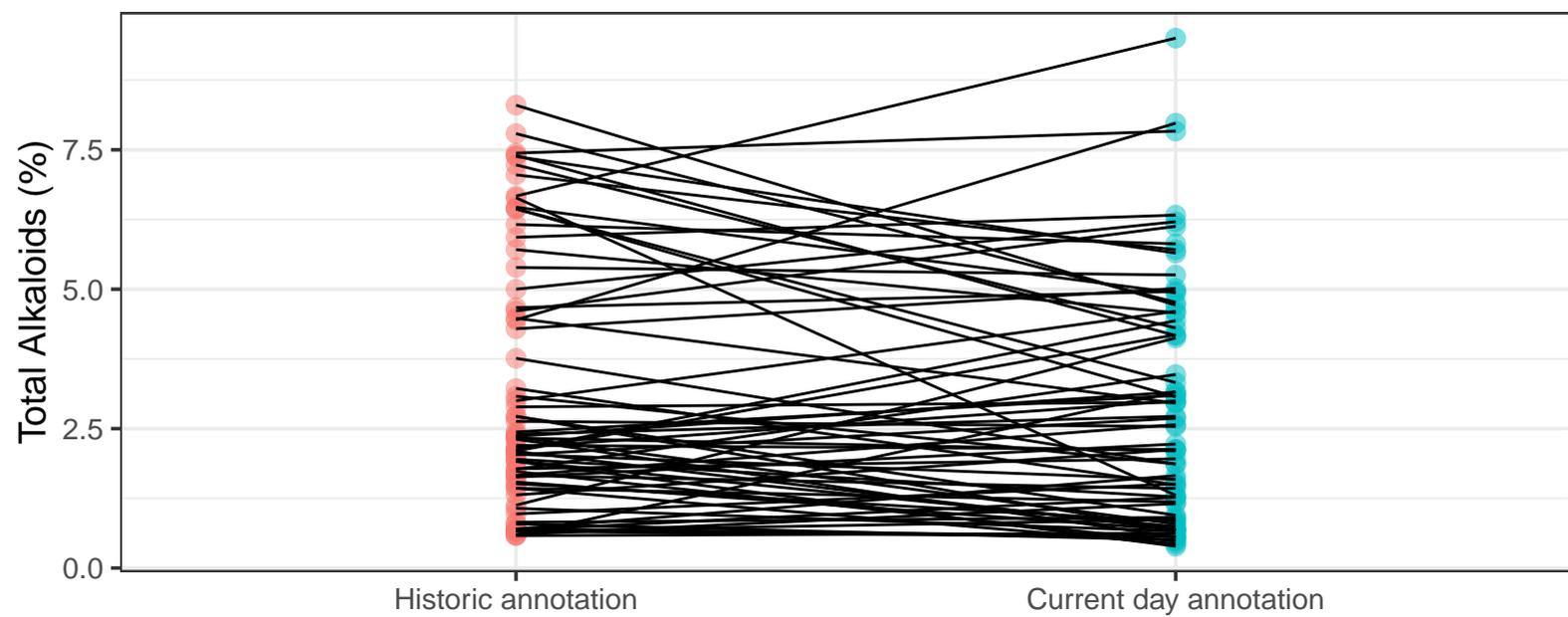
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Supplementary material online to:

Historical chemical annotations of *Cinchona* bark collections are comparable to results from current day High-Pressure Liquid Chromatography technologies

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Details of standards used for quantification

Standard number	Quinine (µg/mL)	Quinidine (µg/mL)	Cinchonine (µg/mL)	Cinchonidine (µg/mL)
1	(0.039) ^a	(0.046) ^a	(0.042) ^a	(0.046) ^a
2	1.56	1.84	1.69	1.83
3	3.91	4.61	4.21	4.58
4	7.82	9.22	8.43	9.16
5	15.63	18.43	16.85	18.32
6	39.08	46.08	42.14	45.8
7	78.16	92.16	84.27	91.60
8	156.32	184.32	168.54	183.2
Estimated LOD	7.87 * 10 ⁻³ (0.01)	1.36 * 10 ⁻² (0.01)	1.24 * 10 ⁻² (0.01)	1.63 * 10 ⁻² (0.02)
Estimated LOQ	2.38 * 10 ⁻² (0.02)	4.11 * 10 ⁻² (0.04)	3.74 * 10 ⁻² (0.04)	4.95 * 10 ⁻² (0.05)
R ₂	0.9998	0.9998	0.9995	0.9995

^a This level was not included in regression, but used for estimation of LOD and LOQ.

Additional information and details about the method used can be found in:

Hansen, S.H., 2015. An efficient, robust, and inexpensive grinding device for herbal samples like *Cinchona* bark. *Sci. Pharm.* 83, 369–376. <https://doi.org/10.3797/scipharm.1410-14>.

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