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PLANT PHENOLICS AND THEIR POTENTIAL ROLE IN MITIGATING IRON OVERLOAD DISORDER IN WILD ANIMALS

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Abstract: Phenolic compounds are bioactive chemicals found in all vascular plants but are difficult to characterize and quantify, and comparative analyses on these compounds are challenging due to chemical structure complexity and inconsistent laboratory methodologies employed historically. These chemicals can elicit beneficial or toxic effects in consumers, depending on the compound, dose and the species of the consumer. In particular, plant phenolic compounds such as tannins can reduce the utilization of iron in mammalian and avian consumers. Multiple zoo-managed wild animal species are sensitive to iron overload, and these species tend to be offered diets higher in iron than most of the plant browse consumed by these animals in the wild and in captivity. Furthermore, these animals likely consume diets higher in polyphenols in the wild as compared with in managed settings. Thus, in addition to reducing dietary iron concentrations in captivity, supplementing diets with phenolic compounds capable of safely chelating iron in the intestinal lumen may reduce the incidence of iron overload in these animal species. It is recommended to investigate various sources and types of phenolic compounds for use in diets intended for iron-sensitive species. Candidate compounds should be screened both *in vitro* and *in vivo* using model species to reduce the risk of toxicity in target species. In particular, it would be important to assess potential compounds in terms of 1) biological activity including iron-binding capacity, 2) accessibility, 3) palatability, and 4) physiological effects on the consumer, including changes in nutritional and antioxidant statuses.

Key words: Hemochromatosis, hemosiderosis, iron overload disorder (IOD), iron storage disease, phenolics, plant secondary compounds, tannins.

INTRODUCTION

Phenolic compounds are found in all vascular plants, and these compounds are very diverse structurally, ranging from simple phenolic compounds to heavy molecular weight polymerized tannins (Table 1). Phenolics function in plant metabolism, defend plants from competitors, pathogens, and herbivores as well as protect plants from ultraviolet radiation and desiccation.[55] As such, a plant's chemical profile can vary widely depending on factors such as plant part, species, age, light, season, nutrient status, and level of herbivory.[46,51,55] In particular, plant phenolic compounds can be feeding deterrents by eliciting an astringent taste and/or causing gastrointestinal distress and subsequent learned avoidance in consumers.[55] Furthermore, these compounds can induce physiologically taxing detoxification mechanisms, disrupt cellular and enzymatic function, exhibit pro-oxidative properties, and reduce the availability of nutrients, including protein, iron, and other minerals (e.g., calcium) upon consumption.[1,2,20,21,52,73] Conversely, depending on the chemical compound and dose, phenolic plant compounds are noted to be beneficial to consumers as phenolic compounds can be antioxidative, anticarcinogenic, antimicrobial, and antifungal.[10,46,51] Furthermore, food conversion efficiency is greater in ruminants fed moderate doses of phenolic compounds[5] as proteins can complex with phenolic compounds in the rumen,[88] thus escaping ruminal digestion and resulting in increased protein absorption by the animal.[5]

This review provides an overview of plant phenolic compounds in terms of methods of quantification, bioactivity, and recommendations for developing a phenolic dietary supplement for animal species susceptible to iron overload disorder (IOD).

QUANTIFYING PHENOLIC COMPOUNDS AND BIOACTIVITY, WITH AN EMPHASIS ON TANNINS

Analyzing forages for phenolic compounds, and in particular, tannins, is a challenging task as these compounds are very diverse structurally and functionally, and no ideal technique exists; thus, inconsistent quantification methods have been employed in the literature. Furthermore, the treatment of the plant material affects the phenolic compound composition and quantifica-

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Table 1. Plant phenolic compounds.[a]

Plant phenolic compounds
Phenolic acids
Hydroxybenzoic acids
Hydroxycinnamic acids (phenylpropanoids)
Stilbenes
Lignans
Tannins
Condensed (proanthocyanidins)
Hydrolyzable
Flavonoids
Flavones
Flavanones
Flavonols
Flavanolols
Isoflavones
Flavanolols (catechins)
Anthocyanidins

[a] Data adapted from Shahidi and Ho.[80]

tion;[7,40,47,68,86,99] samples that are flash frozen and subsequently lyophilized appear to have the greatest preservation of phenolics.[86,99] Assays used to quantify phenolics have been critiqued, and multiple shortcomings have been noted.[35,36,57,75] As the type of phenolic compound results in differential responses to various assays, results obtained by chemical quantification do not necessarily correlate with biological activity.[57] Thus, it has been suggested to use a combination of multiple chemical and biological assays in order to assess the composition and bioactivity of phenolics in extracts, bearing in mind the overall research goal (Table 2).[57,78]

Furthermore, it should be noted that the results from the majority of assays represent the ability of the extracts to form complexes relative to a standard (e.g., tannic acid equivalents) and should not be mistaken for concentrations on a dry matter basis. Results reported in reference to a standard can be problematic as reactivity can be variable depending on 1) the standard chosen and 2) the chemical profiles of the different species/cultivars tested. For example, quebracho tannin, which is often used as a standard for condensed tannins, has very low butanol-HCl activity and thus would overestimate condensed tannins in unknown samples if this assay were used with quebracho by means of comparison.[32,75] As a result, the use of internal standards derived from the study plant is more reliable[78] but labor intensive. As such, it may be preferable to utilize bioassays in lieu of attempting to quantify phenolics or phenolic fractions to avoid these various methodological challenges.[4]

Although potential phenolic supplements may be beneficial to consumers by reducing the incidence of IOD as well as eliciting other positive effects such as reduced parasitism[19] and improved antioxidant status,[77] it should be noted that potential negative impacts of plant secondary compounds exist depending on the type and dose of compound. For example, the non-protein amino acid mimosine (toxin isolated from *Leucaena* spp.) is a potent iron chelator;[50] however, this compound is an effective inhibitor of DNA replication,[90] and its relative toxicity is complicated by differential detoxification mechanisms by gut bacteria.[43] Studies investigating the effect of mimosine on wild animals *in vivo* are limited to an association with *Leucaena* consumption and alopecia in ring-tailed lemurs (*Lemur catta*);[42] hair loss and altered thyroid metabolism are most commonly noted in domestic species.[89] Its use as a supplement in wild animal diets is cautioned against at this time, as less toxic iron chelators likely are available, and *Leucaena* has also been noted to contain relatively high levels of iron.[56]

Because plant secondary compounds can interfere with the absorption of nutrients in addition to

Table 2. Methodology often used to characterize phenolic compounds in foliage.[a]

Chemical methods used to quantify phenolics in feeds (typically expressed in units of concentration relative to a standard)
Total phenolics: Folin-Ciocalteu method[45,46,57,58]
Total tannins: Folin-Ciocalteu/PVPP method[57,58]
Condensed/proanthocyanidins: butanol-HCl-iron method[57,69]
Gallic acid esters: rhodanine;[41] not specific to hydrolyzable tannins[36]
Precipitation/binding methods to assess the biological activity of phenolics in feeds
Protein complexing (concentration relative to a standard): BSA/ferric chloride assay,[34,59] radial diffusion assay[33,57]
PEG binding (PEG binding g/100 g dry matter; e.g., to assess percent nutrient digestibility)[22,83,84]
Iron complexing (micrograms of bound iron): method described by Wong and Kitts[95]

[a] PVPP, polyvinyl polypyrrolidone; BSA, bovine serum albumin; PEG, polyethylene glycol.

iron, such as calcium and protein, and can elicit other potential health effects as exemplified above with mimosine, additional bioassays would be important in the screening process for supplement selection. In particular, *in vitro* screens investigating the binding efficiency of the compound of interest to iron as well as to other nutrients would be crucial for finding a compound with high iron-binding specificity. Also, bioassays determining the health effects and toxicity of potential compounds in appropriate animal models dosed *in vivo* would be prudent to perform before testing such compounds on endangered or threatened species, although, as discussed below, polyphenolic bioactivity is species specific.[71,72]

POLYPHENOLIC COMPOSITION OF FREE-RANGING VERSUS CAPTIVE ANIMAL DIETS

Although comparisons of wild versus cultivated fruits indicated higher concentrations of polyphenolic compounds in the wild varieties,[87,91] and food items consumed by free-ranging herbivorous species such as gorillas (*Gorilla beringei*) can contain relatively high concentrations of phenolic compounds,[75] research comparing the phenolic composition of free-ranging versus captive wild animal diets is limited. Studies surveying the prevalence of polyphenolics such as condensed tannins in forages such as plant browse also are relatively rare;[3,44] furthermore, methodologies used are inconsistent and thus, difficult, if not impossible, to compare.[76]

It has been suggested that captive wild-animal diets contain lower levels of phenolic compounds than those consumed by free-ranging counterparts, and these low levels in captive diets may increase the bioavailability of iron in consumers.[11] Results based on a semi-quantitative colorimetric approach to approximate the condensed tannin content of wild and captive black rhinoceros' diets suggested comparable condensed tannin concentrations in African and North American browse plants, but both African and North American plant species appeared higher in condensed tannins than typical captive North American black rhinoceros' diets (pelleted feeds, hay, produce).[97] Likewise, Ward and Hunt[93] concluded that browse had higher concentrations of iron-binding phenolics than captive rhinoceros' diets based on a ferric chloride assay[34] using gallic acid as a standard. These authors also noted lower serum ferritin in animals fed diets containing a higher proportion of browse. Using the butanol-HCl assay and sorghum standard,[35] Helary et al.[38] found that condensed tannins in wild black rhinoceros' diets were 2.2–3.4% dry matter. Using the Prussian blue method,[70] total phenolic content in wild black rhinoceros' diets was 2–3% dry matter. In comparison, typical zoo diets were thought to be minimal (e.g., $<1.5\%$ dry matter) although remained to be quantified.[38,39] Other than these data, the phenolic composition of diets offered to wild species in captivity compared with free-ranging conditions remains unknown. Clearly, more information is needed using consistent methodology.

PHENOLIC COMPOUNDS AND IRON STATUS

Free-ranging wild animals typically ingest diets containing lower iron concentrations as compared to animals housed in managed facilities fed commercially available pelleted diets[23,30,67,74,98] with or without consumption of compounds that bind to iron, such as phenolic compounds. Plant phenolics may affect iron utilization by mobilizing iron from its intracellular storage protein ferritin, reducing iron to its ferrous state,[6] and the galloyl and catechol groups of phenolic compounds are thought to inhibit non-heme iron absorption presumably by forming complexes with iron in the small intestinal lumen of mammalian consumers.[9,29,49,65,92] Representative compounds from each class of phenolic compounds (Table 1; phenolic acids,[54] lignans,[26] stilbenes, tannins, and flavanoids[48]) have been noted to bind to iron in a dose-dependent manner.[92] Although certain browse species consumed by wild animals appeared to contain excessive iron,[24] high dietary iron in captive wild animal diets likely is involved in excessive hepatic iron deposition and associated tissue damage in multiple species held in captivity (IOD). Thus, in order to obtain iron balance in wild species sensitive to iron overload under human care, it is recommended to reduce iron concentrations in diets formulated for these species[81] based on this disorder's prevalence and pathogenetic factors,[85] as well as dietary treatment efficacy.[66,96] In addition to the primary strategy of reducing dietary iron, iron chelating phenolic plant compounds could be incorporated into the diet.

Research on feeding polyphenolic compounds and non-heme iron absorption largely is focused on humans and laboratory rats and abounds. For example, non-heme iron absorption was significantly reduced in humans dosed with tannic acid, and there was an inverse relationship between food total polyphenolic concentration and iron

absorption.[31] Phylogeny appears to be a factor, however, as tea markedly reduced iron absorption in humans but not in laboratory rats,[71] although certain other polyphenolic compounds reduced iron absorption in the rat.[8] An *in vitro* method simulating the gut of humans also was developed[63] to indicate iron availability from foods and matched the results of an *in vivo* study in humans, which found reduced iron absorption in the presence of polyphenolic-containing foods such as coffee and tea.[75]

Studies investigating the effects of polyphenols on *in vivo* parameters such as iron absorption in wild animals, however, are fairly limited. The addition of quebracho (a condensed tannin) but not tannic acid (a hydrolysable tannin) as 0.5–1.5% of diet dry matter (by weight; concentrations relative to a standard were not reported) significantly increased total antioxidant capacity in black rhinoceros[17] and reduced fecal *Enterobactericeae* colony-forming units[18] but did not affect iron apparent absorption,[13] water intake, or apparent digestibility.[12] Similarly, Ward and Slifka[94] found that the addition of quebracho (1% of diet dry matter) as a source of iron-binding polyphenolics (0.06 mg gallic acid equivalents [GAE]/g) did not appear to have an effect on iron absorption and iron stores in black rhinoceros. While it appeared that iron-sensitive straw-colored fruit bats (*Eidolon helvum*) absorbed labeled iron relatively extensively, the addition of tannic acid reduced iron absorption by 40% in these individuals.[53] Furthermore, lemurs (*Varecia variegata*) switched to a lower iron and vitamin C (which enhances nonheme iron absorption[37]) diet in conjunction with tamarind supplementation (*Tamarindus indica*; tannin concentration not quantified) showed improved iron status as measured by transferrin saturation.[96] When inositol and tannic acid or tea were added to diets enriched in iron, European starling (*Sturnis vulgaris*) liver iron concentrations did not increase compared to when birds were fed a diet high in iron without iron-chelating compounds.[66,79] Likewise, dietary modification including tea supplementation resulted in reduced hepatocellular hemosiderin in toco toucans[27] (*Ramphastos toco*). Indeed, tea, which is highly variable in composition, has been used historically and anecdotally as a supplement to prevent iron overload in wild animals under human care; however, peer-reviewed studies using consistent "doses" of tea with quantified and identified chemical composition followed by a rigorous documentation of the associated biological effects in these animals are lacking, making replication of historic supplementation nearly impossible.

Wild animals can avoid the potential negative consequences of consuming diets rich in tannins by selecting against particular plant parts or species with high tannin concentrations or by producing tannin-binding proteins (TBP) in saliva.[82] TBP, as the name implies, bind to tannins, reduce the antinutritive effects of tannins in the diet, and can be expressed in high levels constitutively[20] or induced by diet.[15,61,62] TBP have not yet been examined in birds other than chickens,[10] but they have been identified in various wild mammalian species,[64,82] evolutionarily adapted to diets containing tannins.[60] The presence, type, and binding affinity of TBP expressed in various species and individuals appear to be under both phylogenetic and environmental (diet-induced) control[64,82] and recently have been linked to the perceived astringency of the ingesta.[25]

A particular species may produce TBP that bind to a specific class of tannins; however, an animal's capability of producing TBP does not necessarily indicate that all dietary tannins, either by dose or class, are thus bound and not utilized by the animal. For example, both tannic acid–binding capacity and quebracho-binding capacity were induced in black rhinoceros (*Diceros bicornis*) when tannic acid was in the diet; however, dietary quebracho induced quebracho-binding capacity but not tannic acid–binding capacity.[15] The authors suggested that the greater induction of TBP capable of binding to hydrolysable tannins as compared to condensed tannins may be due to a lack of selection pressure in this species to adapt to condensed tannins in general. It is also plausible that the condensed tannin used was not sufficiently chemically similar to those condensed tannins found in this species' native habitat. Induction of TBP binding to a particular tannin does not necessarily indicate an induction of TBP binding to all tannins; thus, differential bioavailability/utilization of various concentrations and classes of tannins could potentially occur depending on multiple factors including animal species' evolutionary adaptations and captive diets.[72]

Additionally, it is possible theoretically to saturate all TBP binding sites, assuming the animal will consume relatively high concentrations of tannins. For example, presumably due to iron-binding properties, tannic acid supplementation appeared to lower hemoglobin concentrations in roe deer (*Capreolus capreolus*) despite the presence of TBP in this species.[16] Whether the

saliva of wild animals is capable of binding to the bioactive phenolic compound(s) of interest should be considered when determining the type and amount of the compound administered, as the presence of TBP capable of binding to the compound of interest may affect intake as well as bioactivity such as iron-binding potential.

RECOMMENDATIONS

Given the prevalence of IOD across multiple taxa, lowering dietary iron concentration is a primary strategy for reducing the incidence of this disorder. In conjunction with or secondary to this approach, is the aim to reduce dietary iron absorption in the gut of iron-sensitive species via supplementation of plant phenolic compounds. Likely, the extent of iron absorption inhibition is dependent on the structure and source of the phenolic compound owing to varying binding efficiencies. Although almost all polyphenolic compounds containing a hydroxyl group are expected to bind to iron,[80] it appears that iron binding is related directly to the number of hydroxyl groups.[49] Thus, depending on molecular structures, higher concentrations of larger phenolic compounds with multiple hydroxyl groups, such as condensed tannins, as compared with smaller compounds, such as phenolic acids, in supplements and plant browse fed to wild animals in captivity sensitive to IOD may be targeted for maximum iron chelation.

Given the noted challenges associated with quantifying phenolic compounds, in the context of addressing the issue of IOD, it may be preferable to direct efforts towards developing/using the method used by Wong and Kitts[95] or another iron-binding assay to compare feedstuffs, supplements, and browse plant species to minimize freely associated iron in the diet. Specifically, it is recommended to compare palatable,[14,16] low-cost, and commercially available products in terms of relative affinities for iron to determine if an appropriate dietary supplement exists for minimizing iron absorption in wild animals sensitive to IOD. Potential sources of phenolic supplements include grape pomace, wood extracts (e.g., chestnut, *Acacia* spp.), tea, pomegranate (*Punica granatum*), cranberry (*Vaccinium macrocarpon*), tamarind, sanfoin (*Onobrychis viciifolia* Scop.), lespedeza (*Kummerowia* spp.), and isolated soy protein.

As a particular phenolic compound and dose that safely binds to iron in the intestinal lumen has not yet been identified and rigorously tested, *in vitro* screenings of candidate compounds should be conducted to assess the effects on absorption of nutrients such as protein and other minerals. Although it would be ideal to assess the safety of potential polyphenolic compounds on model species (e.g., horse, starling) prior to supplementing threatened or endangered species, the effects of these compounds can vary based on species and thus may not be applicable to other taxa such as those that are iron sensitive.[71,72] As such, study animals (model species or otherwise) supplemented with phenolic compounds should be monitored for health parameters, including nutritional and oxidative status, because phenolic compounds, as noted above, can complex with nutrients other than iron and can exhibit both pro-oxidative and antioxidative properties. Example biomarkers to screen regularly as available depending on the animal species include those indicative of protein and mineral status (e.g., circulating albumin, mineral panel, ferritin, transferrin saturation, total iron-binding capacity), and oxidative status (e.g., circulating superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, malondialdehyde, 2-aminoadipic semialdehyde). Food consumption and animal body mass should also be monitored. In addition to diet nutrient analysis, total fecal collection could be conducted to measure nutrient and polyphenolic compound bioavailability, and it is advocated to investigate the presence and potential inducibility of TBP (as measured by Fickel et al.[28]) before and after initiating a diet change. Despite the need for additional information on the effects of polyphenolic compounds in wild animals, these compounds potentially have a large role in mitigating iron overload, and the practical use of polyphenolic compounds in the diets of these animals should be explored prudently.

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