FOOD & FUNCTION

Bioavailability study of a polyphenol-enriched extract from *Hibiscus sabdariffa* in rats and associated antioxidant status

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The aqueous extracts of *Hibiscus sabdariffa* have been commonly used in folk medicine. Nevertheless, the compounds or metabolites responsible for its healthy effects have not yet been identified. The major metabolites present in rat plasma after acute ingestion of a polyphenol-enriched *Hibiscus sabdariffa* extract were characterized and quantified in order to study their bioavailability. The antioxidant status of the plasma samples was also measured through several complementary antioxidant techniques. High-performance liquid chromatography coupled to time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) was used for the bioavailability study. The antioxidant status was measured by ferric reducing ability of plasma method, thiobarbituric acid reactive substances assay, and superoxide dismutase activity assay. Seventeen polyphenols and metabolites have been detected and quantified. Eleven of these compounds were metabolites. Although phenolic acids were found in plasma without any modification in their structures, most flavonols were found as quercetin or kaempferol glucuronide conjugates. Flavonol glucuronide conjugates, which show longer half-life elimination values, are proposed to contribute to the observed lipid peroxidation inhibitory activity in the cellular membranes. By contrast, phenolic acids appear to exert their antioxidant activity through ferric ion reduction and superoxide scavenging at shorter times. We propose that flavonol-conjugated forms (quercetin and kaempferol) may be the compounds responsible for the observed antioxidant effects and contribute to the healthy effects of *H. sabdariffa* polyphenolic extract.

Keywords:
Antioxidant capacity / Bioavailability / Flavonol glucuronides / *Hibiscus sabdariffa* / HPLC-ESI-TOF-MS

The demonstration of the beneficial effects of dietary polyphenols on health is becoming an important issue. Although most cellular studies have proven their biological effects at the in vitro level, the correlation between the in vivo observed effects and the presence of the responsible metabolites are still a great challenge. Nevertheless, bioavailability of phenolic compounds in humans is very low due to poor intestinal absorption and because gut microbiota, which reflect a complex interplay of diet and genetics, heavily metabolize the phytonutrients. After acute consumption of up to 100 mg of a single compound, the maximum concentration in plasma rarely exceeds 1 μM [1].

Hibiscus sabdariffa L. (family: *Malvaceae*), called bissap, karkade, or roselle, is a tropical plant commonly used as local soft drink. The calyces are rich in polysaccharides [2] and phenolic compounds [3, 4].

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Abbreviations: FRAP, ferric reducing ability of plasma; **PEHS**, polyphenol-enriched *Hibiscus sabdariffa* extract; **SOD**, superoxide dismutase

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The aqueous extracts of *H. sabdariffa* have been commonly used in folk medicine to treat hypertension, fever, inflammation, liver disorders, and obesity. Previous studies have shown that *H. sabdariffa* possesses antitumor and antioxidant properties [5, 6]. In humans, *H. sabdariffa* significantly decreases Monocite Chemoatractant Protein 1 (MCP-1) plasma concentration, suggesting that *H. sabdariffa* may be a valuable traditional herbal medicine for treating chronic inflammatory diseases and ameliorate metabolic disturbances [7]. The antioxidant and antihyperlipidemic properties of *H. sabdariffa* aqueous extract have been previously reported by our group using a mouse model [4]. Moreover, the strong antioxidative and hypolipidemic properties of a *H. sabdariffa* polyphenolic extract have been recently reported in a cell model for adipogenesis and insulin resistance [8]. In any case, the compounds or metabolites responsible for such activities have not yet been identified.

In this study, the major metabolites present in the rats plasma after acute ingestion of a polyphenol-enriched *Hibiscus sabdariffa* extract (PEHS), prepared as previously reported [8], were identified and quantified by high-performance liquid chromatography with diode-array detection coupled to electrospray time-of-flight mass spectrometry (HPLC-DAD-ESI-TOF-MS).The chromatographic, UV, and ESI-TOF-MS conditions were performed as previously reported [4]. C_{max} and t_{max} were determined by the experimental observation. The half-life ($t_{1/2}$) was calculated as ln 2/ k_{el}, where k_{el} (elimination rate constant) was established from the slope of the elimination phase in the concentration vs. time plot. The antioxidant status of the plasma samples was also measured through several complementary antioxidant techniques: the thiobarbituric acid reactive substances assay which measured malondialdehyde (MDA) by HPLC coupled to fluorescence detection (Supporting Information) [9], the ferric reducing ability of plasma (FRAP) method as reported [10], and the superoxide dismutase (SOD) activity assay as previously described [11]. Throughout the experiments, the animals were processed according to the suggested ethical guidelines for the care of laboratory animals [12] and under the authorization of the animal experimentation ethics committee (permission number CEEA 2011–359). The blood samples were withdrawn via cardiac puncture into heparinized tubes at 0 (control group), 20, 60, and 120 min postdosing (four animals per time). All blood samples were centrifuged at 1000 *g* for 15 min at 4°C, and then plasma was stored at –80°C.

The detailed composition of the PEHS used for the study has been reported previously (Supporting Information, Table S1) [8].

A detailed material and methods section is available as a Supporting Information Table S2.

A total of 17 compounds were detected in plasma samples using HPLC-ESI-TOF-MS (Fig. 1). Supporting Information Table S3 lists the retention times of these compounds, the accuracy mass, error, molecular formula provided by the TOF-MS, the quantification technique and the standard used for this quantification. Eleven of these compounds were metabolites, including hydroxycitric acid. Nevertheless, hydroxycitric acid is naturally present in *H. sabdariffa* raw material, but PEHS was devoid of this compound since this extract was obtained after reverse chromatographic separation in nonpolar solvent. Therefore, we assume that the hydroxycitric acid found in the rat plasma derived most probably from metabolized hibiscus acid, or hibiscus acid ester derivatives.

After oral administration, deglycosylation of flavonoids is likely to occur either pre- or postabsorption in rats [13]. Therefore, all quercetin glucuronide conjugates determined in this study (phase II metabolites) must derive from the pool of quercetin aglycone to which different quercetin glycosylated and nonglycosylated forms present in PEHS contribute (Supporting Information, Table S1). Similarly, kaempferol glucuronides found in rat plasma must derive from kaempferol derivatives present in the extract.

The pharmacokinetic parameters and the quantification of each compound appear in Table 1. The organic acids hibiscus acid, hibiscus acid hydroxyethyl ester, and the metabolite hydroxycitric acid reached high concentrations in plasma at $t_{max} \geq 120$ min (>100 μM for hibiscus acid), contributing to micromole amounts of organic acids in plasma. Phenolic acid derivatives found in rat plasma were chlorogenic acid, methyl digallate, and *N*-feruloyltyramine. Chlorogenic acid reached 3.8 μM at 60 min and methyl digallate and *N*-feruloyltyramine registered concentration values below 1 μM, at 60 and 20 min, respectively. These compounds also exhibited low elimination half-life values most probably indicating a lack of tissue accumulation.

Considering a total extract dose of 1200 mg/kg, all quercetin derivatives in PEHS accounted for 16.8 μmol, whereas kaempferol derivatives in PEHS totaled 2.0 μmol equivalents. Most quercetin-conjugated forms were within the low micromolar range in rat plasma. The highest concentrations were found for quercetin glucuronide (isomer 1) and quercetin (Table 1). The methylated forms of quercetin have been found as phase II metabolites after quercetin administration in previous studies [14]. All kaempferol derivatives were also found in concentrations lower than 1 μM. The total concentration of flavonol-conjugated forms (quercetin + kaempferol) in plasma obtained from the concentrations determined at t_{max} yielded about 4.3 μM at 20–60 min and 5.5 μM at 120 min. These values agree with those reported for the oral administration of other quercetin-enriched extracts in rats [13].

Among all the quercetin derivatives, quercetin-3-glucoside reportedly shows better absorption than quercetin aglycone or other glycosides bearing a rhamnose moiety do [15]. Hence, this compound in PEHS must be the one that contributes most to the different glucuronidated forms of quercetin in rat plasma. However, we cannot rule out that some of the quercetin conjugates found in plasma could also derive from glycosylated kaempferol derivatives present in PEHS through oxidative conversion and conjugation, as reported elsewhere [16]. It is also remarkable that quercetin conjugates have longer elimination half-life values than those of

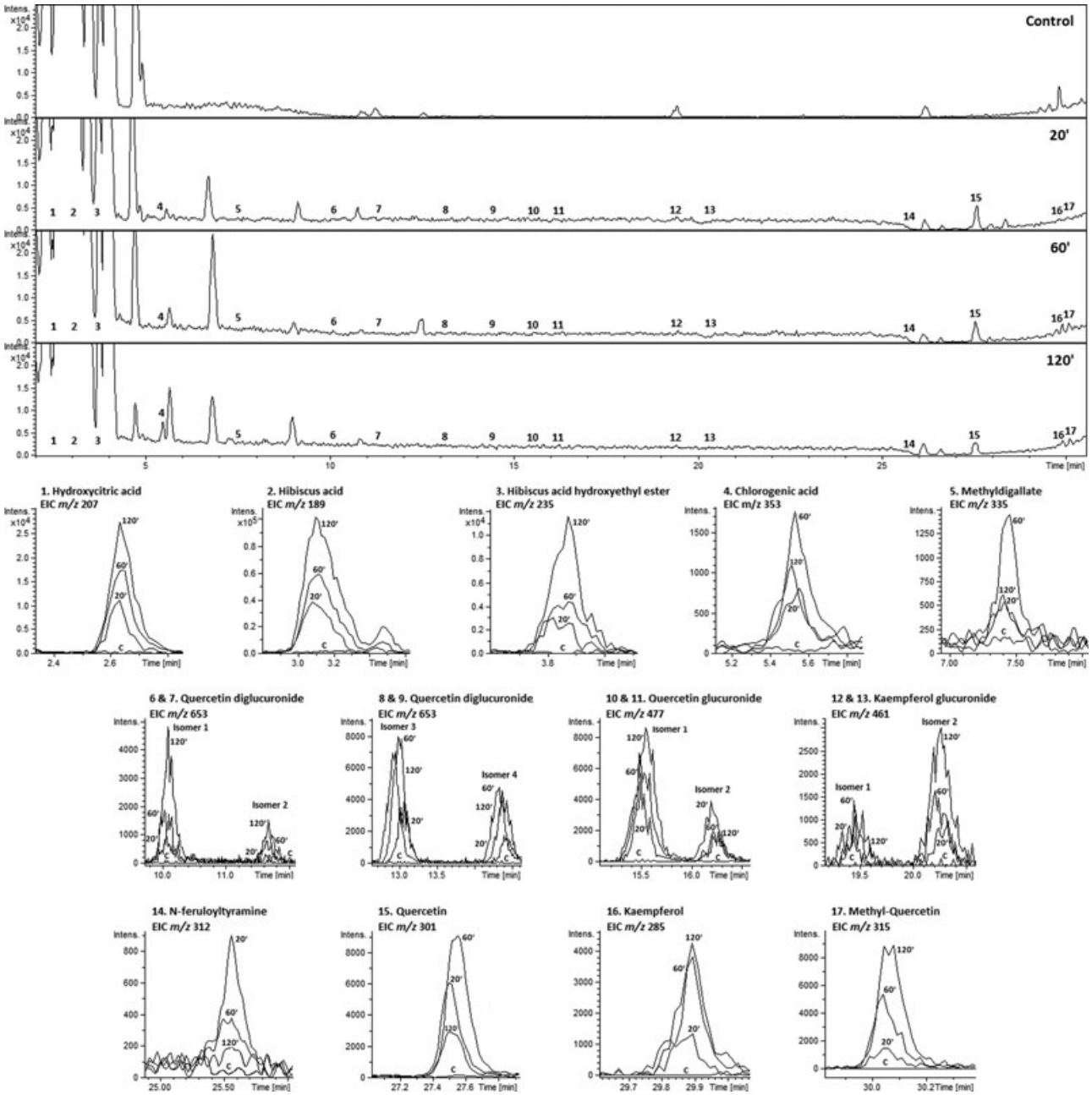

Figure 1. Representative base peak chromatograms (BPC) obtained by HPLC-ESI-TOF-MS of rat plasma samples at 0 (control, C), and 20, 60, and 120 min following the oral ingestion of *Hibiscus sabdariffa* polyphenols. Figures in lower panel represent the extracted ion chromatograms of each compound identified by numbers 1–17 in the upper BPC. Peaks corresponding to the same compound but found at different times after ingestion (C, 20, 60, and 120 min) are overlapped in each graph: hydroxycitric acid (1), hibiscus acid (2), hibiscus acid hydroxyethyl ester (3), chlorogenic acid (4), methyl digallate (5), quercetin diglucuronide isomers (6–9), quercetin glucuronide isomers (10, 11), kaempferol glucuronide isomers (12, 13), *N*-feruloyltyramine (14), quercetin (15), kaempferol (16), and methyl-quercetin (17).

phenolic acids, especially quercetin diglucuronide (isomer 4), indicating that these compounds may accumulate in the tissues and could be responsible for long-term effects.

Consequently, the total flavonol concentration found in rat plasma after a dose of 1200 mg/kg of hibiscus polyphenols was about 5 μM within the 60–120 min period following ingestion. Since the elimination rate of quercetin metabolites has been reported to be very low [14], higher plasma quercetin concentration could be easily maintained in rats with a subchronic administration of hibiscus polyphenolic extract.

Despite of the above-mentioned low bioavailability of most polyphenols, some studies have reported the

Table 1. Total administered and circulating plasma moles, and pharmacokinetic parameters of rat plasma phenolic compounds and their metabolites after oral administration of PEHS

Compound	Administered dose (μmol)[b]	Plasma max. amount (nmol)[c]	C_{max} (μM)	t_{max} (min)	$t_{1/2}$ (min)
Hibiscus acid	244.1	1749.6 ± 71.2	112.50 ± 4.57[d]	≥120[d]	—[e]
Hibiscus acid hydroxyethylester	125.3	94.8 ± 12.1	6.07 ± 0.77[d]	≥120[d]	—[e]
Hydroxycitric acid[a]	—	196.5 ± 24.1	12.59 ± 1.55[d]	≥120[d]	—[e]
Hibiscus acid derivatives	499.8	2041.0			
Chlorogenic acid	108.2	58.8 ± 5.4	3.77 ± 0.35	60	77.0
Methyl digallate	3.0	5.0 ± 0.8	0.32 ± 0.05	60	38.3
N-feruloyltyramine	1.0	8.3 ± 2.5	0.54 ± 0.16	20	46.8
Quercetin	6.9	24.4 ± 2.7	1.57 ± 0.18	60	81.5
Quercetin diglucuronide (isomer 1)[a]	—	11.1 ± 2.1	0.71 ± 0.13[d]	≥120[d]	—[e]
Quercetin diglucuronide (isomer 2)[a]	—	3.6 ± 0.4	0.23 ± 0.02[d]	≥120[d]	—[e]
Quercetin diglucuronide (isomer 3)[a]	—	14.4 ± 0.3	0.92 ± 0.02	60	70.7
Quercetin diglucuronide (isomer 4)[a]	—	11.4 ± 1.9	0.73 ± 0.12	60	433.2
Quercetin glucuronide (isomer 1)[a]	—	33.9 ± 1.9	2.17 ± 0.12[d]	≥120[d]	—[e]
Quercetin glucuronide (isomer 2)[a]	—	12.4 ± 0.5	0.79 ± 0.03	20	80.6
Methyl-Quercetin[a]	—	13.7 ± 1.2	0.88 ± 0.08[d]	≥120[d]	—[e]
Quercetin derivatives	16.8				
Kaempferol[a]	—	12.7 ± 1.3	0.81 ± 0.08[d]	≥120[d]	—[e]
Kaempferol glucuronide (isomer 1)[a]	—	4.2 ± 0.2	0.27 ± 0.01	60	43.3
Kaempferol glucuronide (isomer 2)[a]	—	11.4 ± 1.5	0.73 ± 0.09[d]	≥120[d]	—[e]
Kaempferol derivatives	2.0				

a) Metabolites derived from the compounds of *H. sabdariffa* polyphenolic-enriched extract.
b) Quantification of total administered polyphenol amount was made on the basis of the concentration (w/w) of each compound in PEHS extract (Supporting Information) and total administered extract.
c) Total moles at t_{max} considering an average plasma volume of 60 mL/kg (6% of bodyweight).
d) t_{max} was not calculated because C_{max} was not achieved. Data indicate the time and concentration at maximum time assayed.
e) $t_{1/2}$ was not calculated since descent curve was not achieved.

enhancement of plasma antioxidant status after oral administration of polyphenol-enriched extracts in animal models. MDA, a thiobarbituric reactive substance, is considered as a reactive species that derives from oxidation of polyunsaturated lipids causing toxic stress and advanced lipoxidation end products. This compound is generally accepted as a biomarker of oxidative stress [17]. Upon PEHS ingestion, the amount of plasma MDA, determined by HPLC coupled to fluorescence detection, was decreasing with the time of sample extraction showing a minimum value at the latest time tested, i.e. 120 min (Fig. 2A). This result indicates that antioxidant compounds promoting a maximum antioxidant activity against lipid peroxidation would be those having the highest concentration at the longest times of the study (60–120 min), such as some quercetin glucuronide or kaempferol glucuronide conjugates (Table 1).

The FRAP method measures the capacity of antioxidant compounds to reduce the ferric ions by an electron transfer mechanism. The results show that the maximum FRAP was achieved fairly fast, i.e. in plasma samples taken at 20 min after oral ingestion (Fig. 2B). At 60 and 120 min, FRAP values were decreasing, but significant differences still appeared compared with control samples ($p < 0.05$). The fast response of this free-radical scavenging activity in plasma may be related to phenolic compounds that promote a fast absorption process in the small intestine. This correlates with the presence of phenolic acids such as chlorogenic acid, methyl digallate, or even *N*-feruloyltyramine, which reach their maximum concentration at 20–60 min.

The capability of rat plasma samples to scavenge superoxide radicals was determined by using the xanthine/xanthine oxidase system coupled to SOD (Fig. 2C). The results showed that maximum capacity to inhibit the formation of the superoxide anion radicals in plasma, i.e. SOD-like activity, was also reached at 20 min after oral ingestion of PEHS. Significant differences in SOD activity were also observed at 60 min compared to control ($p < 0.05$). This result agrees with the result found in the FRAP determination, which may indicate that both activities are due to similar metabolites. It has been reported that chlorogenic acid was the main contributor among other phenolic compounds to SOD-like activity in apple vinegar [18].

Many different bioavailability studies on phenolic acids, anthocyanins, and flavonols contained in different food matrixes are available in the literature. Nevertheless, studies focusing on the particular combination of polyphenols in *H. sabdariffa* may be necessary because their interaction and absorption in the gastrointestinal tract may have particular determinants. The identification of the flavonol metabolites after the ingestion of *Hibiscus* polyphenols in humans requires further research in order to identify the compounds responsible for its healthful effects. Such studies may

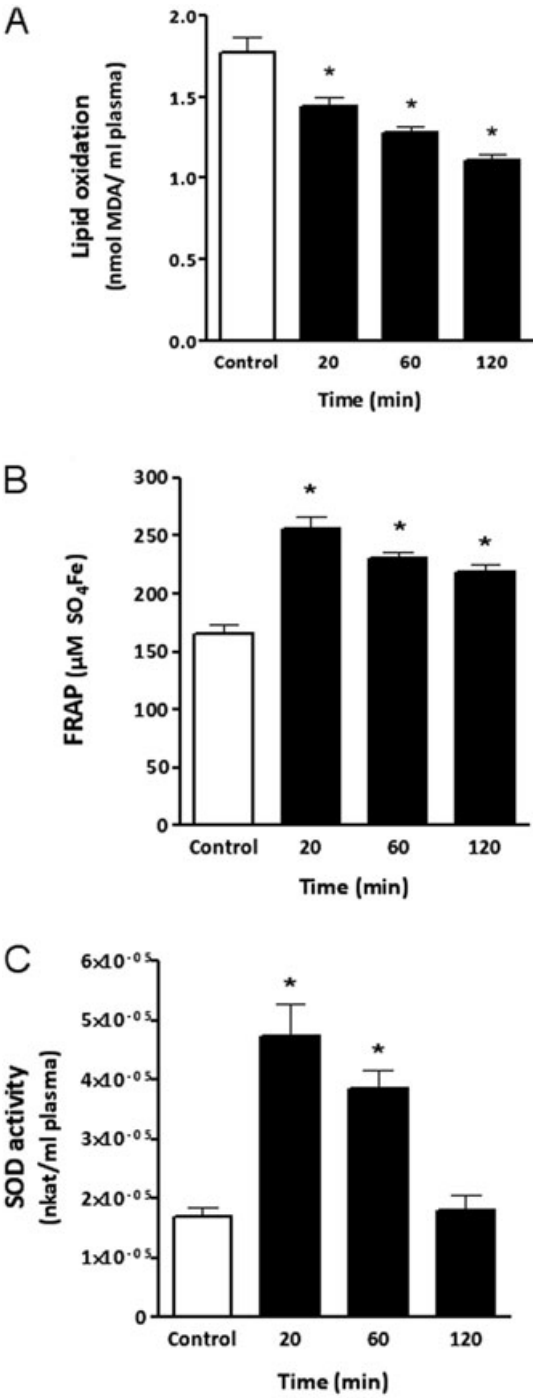

Figure 2. (A) Variation of MDA concentration determined by HPLC-fluorescence in rat plasma samples at 20, 60, and 120 min after the oral ingestion of *Hibiscus* polyphenols. Each bar represents the mean ± SD ($n = 6$). (B) Ferric-reducing capacity of plasma (FRAP) in rats at 20, 60, and 120 min after being orally treated with *Hibiscus* polyphenols. Each bar represents the mean ± SD ($n = 8$). (C) Superoxide dismutase activity in plasma of rats at the same time intervals than those above-mentioned after the oral ingestion of *Hibiscus* polyphenols. Each bar represents the mean ± SD ($n = 8$); $^{*}p < 0.05$ indicates statistically significant differences compared to control.

help to rationally develop nutraceuticals containing higher content in bioactive compounds, which can be used with greater guaranties in human clinical trials focused to cardiovascular related pathologies.

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The authors have declared no conflict of interest.

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