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Running title: Catechins from from ready-to-drink tea.

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Abstract

Objective: As consumption of teas may be associated with potential health benefits due to its content in polyphenols and as in Western countries the consumption of tea is equally divided between the hot and the ready-to-drink (RTD) cold version of this typical beverage, aim of this work is studying absorption and metabolism of flavan-3-ols in human volunteers after the ingestion of a commercial RTD tea.

Research Methods & Procedures: A feeding study was carried out on 20 healthy human volunteers and urine samples were collected for 24 hours after tea ingestion. Flavan-3-ols derived molecules were identified and quantified in urine samples by HPLC with tandem mass spectrometric detection.

Results: Eight relevant metabolites were identified in urine, all modified flavan-3-ols with the exception of unmetabolised gallic acid. The urinary excretion of flavan-3-ols was equal to the 7.2% of the intake with tea. Gallic acid, which was abundant in the RTD tea used in this study, reached a 4.5% of the drunk amount.

Conclusions: The bioavailability values observed are in agreement with previous reports even though the dosage of polyphenols ingested in this study is remarkably lower. Moreover, the use of a group of twenty volunteers, more than the average number of subjects used for usual human acute feeding studies involving polyphenols, gives additional credibility to the results. After drinking the ready-to-drink ice tea used in this study, the internal compartments are exposed to non-marginal doses of flavanols and flavanol metabolites up to 24 hours.

Keywords: Tea, flavan-3-ols, catechins, polyphenols, bioavailability

Introduction

Tea is one of the world's most popular beverages second only to water, prepared by water infusion of dried leaves from *Camellia sinensis*. Consumption of teas may be associated with potential health benefits. Teas are a rich source of polyphenols, with the phenolic content dependant upon the degree of fermentation. Green tea is unfermented and the dried leaves are a rich source of flavan-3-ols (catechins) [1]. During preparation of black tea, leaves are crushed allowing fermentation by polyphenol oxidase. Oxidation followed by polymerisation diminishes catechins levels while theaflavins, procyanidins and other compounds of higher mass are formed.

The major phenolics found in teas are catechins (flavan-3-ols) and flavonols. There are three predominant families of catechins in teas, the free catechins, including gallocatechins and gallate esters, the theaflavins and finally the thearubigens. The main flavonols are conjugates of quercetin and kaempferol [1]. The conjugation varies from mono- to di- and tri- glycosides. In addition to these two families, flavones and phenolic acids are also reported in teas, in particular the quinic esters of gallic, coumaric and caffeic acid.

Polyphenols in general and catechins in particular are reported to exert various biological activities. A significant body of evidence relating to the protective effects of tea polyphenols against cardiovascular disease, stroke and cancer incidence has been amassed [2]. The proposed mechanisms for the protective effects of tea against coronary heart disease include inhibition of oxidation of LDL, known to be involved in the development of atherosclerosis, anti-hypercholesterolemic activity and inhibition of platelet aggregation [3]. Consumption of tea polyphenols has been linked with prevention of a variety of cancers using animal models, including prevention of cancers of the skin, digestive tract, liver, bladder and prostate [4]. Inhibition of tumorigenesis observed in these models may be applicable to human systems, however epidemiological evidence is again inconclusive. Moreover, protective effects of teas against cancers are more commonly observed in studies carried out in Asian countries where predominantly green tea is consumed [3].

In Western countries, with probably the only exception of the United Kingdom, the consumption of tea is equally divided between the hot and the Ready-to-drink (RTD) cold version of this typical beverage [5]. During spring and summer, moreover, this balance falls drastically towards an increased consumption of RTD teas, easily available and characterised by several special features beside the cold beverage nature, such as different tastes, vitaminic fortification, and sweeteners as substitutes of sugar. Among RTD products, however, the quality in terms of polyphenol content is strongly varied [6], usually depending on the mode of preparation (soluble extract or proper infusion), on the original tea leaves chosen by each brand and on the technological treatments used for safety issues. Moreover, little information exists on the bioavailability of tea catechins in RTD teas.

This paper reports on absorption and metabolism of flavan-3-ols in 20 healthy human volunteers after the ingestion of 500 mL of a commercial RTD tea. The work was performed by analysing flavan-3-ols and their metabolites in urine samples collected over 4 periods in 24 h after intake. Catechin metabolites were identified by HPLC-MS/MS and subsequently quantified using HPLC with MS in the selective ion recording (SIR) mode.

Materials and methods

Tea and chemicals

Five hundred mL bottles of ready-to-drink tea beverage were supplied by Soremartec Italia S.r.l. (Alba, CN, Italy). This brand represents the 25% of the Italian market of RTD tea [5]. The product is made from Sri Lanka tea leaves. The tea is produced by a unique technological system that reproduces the traditional tea infusion since leaves are dipped into boiling water. The final product is composed by tea infusion (water, tea), sugar, dextrose, lemon juice, ascorbic acid, flavour. The tea is produced in Italy (Alba, CN).

Pure (–)-epicatechin, (–)-epigallocatechin, (-)-epigallocatechin-3-gallate, (–)-epicatechin-3-gallate and gallic acid were obtained from Sigma (St. Louis, MO, USA). All the solvents and reagents were purchased from Carlo Erba reagents (Milano, Italy)

Human feedings study

The feeding study was carried out on 20 healthy human volunteers (17 men and 3 women) selected according to exclusion criteria including diabetes mellitus, cardiovascular events, chronic liver diseases or nephropathies, cancer, organ failure and taking antioxidant or vitamin integrators. The volunteers were 31 ± 15yo (mean ± SD) and with an average BMI of 23 ± 3 kg/m^2. Each volunteer signed an informed consent and the study protocol was approved by the Ethics Committee for Human Research of the University of Parma.

For two days prior to, and 24 h after the ingestion of tea, the subjects followed a diet almost deprived of flavonoids and phenolic compounds by avoiding fruit and fruit juices, chocolate, nuts, vegetables, tea and any kind of herbal tea, coffee, wine and dietary antioxidant supplements. To check for compliance, the volunteers were asked to fill a 3-day weighed food record during the two days before the study and the study day. On the day of the study, after an overnight fast, each subject drank 500 mL of the tea beverage. Urine was collected before the volunteers drank the tea and 0-4, 4-7, 7-10 and 10-24h after ingestion. The volume of urine collected during each period was

measured and aliquots stored at –80ºC prior to analysis of 0.45μm filtered samples by HPLC-MS/MS without further processing.

HPLC- ESI-MS/MS analysis

Flavan-ols and their metabolites in tea and urine were analysed using a Waters 2695 Alliance separation module equipped with a Micromass Quattro Micro Api mass spectrometer fitted with an electrospray interface (ESI) (Waters, Milford, MA, USA). Separations were performed using a Waters Atlantis dC18 3 μm (2,1 x 150 mm) reverse phase column (Waters). The mobile phase, pumped at a flow rate of 0.17 mL/min, was a 15-min gradient of 5-30 % acetonitrile in 1 % aqueous formic acid. The tuning of the mass spectrometer was optimised by infusing a standard of (–)-epicatechin into the source along with the 5% acetonitrile in 1 % aqueous formic acid, the initial HPLC mobile phase, at a flow rate of 30μL/min. The ESI source worked in negative ionisation mode. Source temperature was 120ºC, desolvation temperature was 350°C, capillary voltage was 2.8 kV, cone voltage was 35V. The collision energy for MS/MS identifications was set at 25 eV. Following HPLC separation and MS/MS identification, flavan-3-ols and their metabolites were quantified using HPLC with the MS operating in the selected ion recording (SIR) mode. Unmetabolised flavan-3-ols were quantified using calibration curves of the appropriate standard compound while metabolites of epicatechin and epigallocatechin in urine were quantified using epicatechin and epigallocatechin, respectively.

Results

Analysis of tea

The relevant flavan-3-ol content of 500 mL of RTD tea was 22.9 ± 1.7 μmol (mean ± SD) (–)-epigallocatechin; 25.7 ± 3.1 μmol (–)-epigallocatechin-3-gallate; 14.1 ± 1.0 μmol (–)-epicatechin; 24.8 ± 2.7 μmol (–)-epicatechin-3-gallate; making a total of 87.4 ± 8.4 μmol of total flavan-3-ols. The content of gallic acid was equal to 59.4 ± 4.7 μmols. A sample chromatogram is reported in figure 1.

Identification of flavan-3-ols and their metabolites in urine.

Urine samples collected at different time points after the ingestion of 500 mL of tea were analysed by HPLC-MS/MS. Eight relevant compounds were identified in urine, all modified flavan-3-ols with the exception of unmetabolised gallic acid. No metabolites of either epicatechin-3-gallate or epigallocatechin-3-gallate were detected in urine. The MS/MS criterion of identification is mainly based on previously reported mass analyses [7] and is reported in table 1 together with the number of detected peaks for each metabolite. Methyl-epigallocatechin was identified based only on molecular ion (m/z = 319) and retention time.

Quantitative analysis of urine

After the initial qualitative analysis, urine samples were analysed by MS in the selected ion recording (SIR) mode and typical HPLC-SIR traces obtained and used to quantify metabolites are reported in figure 2. The excretion of tea-related components in urine are reported in figure 3. The main excreted flavan-3-ol metabolites were epigallocatechin-O-glucuronide and methyl-epicatechin-sulphate, whereas the sulphate metabolite of epigallocatechin was almost negligible along all the 24 hours (not shown in figure). It is interesting to note that, while most of the catabolites reach their peak excretion within the first 4 hours, the methylated compounds show their maximum excretion after 10h (figure 3).

Bioavailability was calculated as a ratio between the total excretion and the total intake of flava-3-ols and gallic acid. However, to try to distinguish between absorption rate of epicatechin and epigallocatechin (being the metabolites derived from this two molecules), the assumption that EGC-catabolites derived from both EGC and EGCG (after degalloylation) and EC-catabolites derived from both EC and ECG (after degalloylation), was made. It is in fact impossible to discern between EC as a product of ECG and EGC as a product of EGCG degalloylation, reaction that has been reported to occur also in the mouth [8]. Following this assumption, the bioavailability (table 3) of EC-components (namely EC and ECG) was equal to approximately 7.1%, similarly to the bioavailability of EGC-related compounds (namely EGC and EGCG), which excretion accounted

for almost the 7.3% of the total intake. This leads to a total flavanols excretion equal to the 7.2% of the intake with tea. Excretion of gallic acid, which was abundant in the RTD tea used in this study, reached a 4.5% of the amount ingested.

Discussion

The consumption of ready-to-drink tea, generally called iced tea, is increasing in the western countries and is often overcoming the intake of traditional hot tea [5]. Considering the evidence that tea is one of the most significant sources of polyphenols in the human diet and that tea consumption [2] and polyphenol intake in general [9] are strongly related to reduced risk for several chronic diseases, the aim of our investigation was to assess the actual exposure of human volunteers to the most representive and bioavailable tea polyphenols (namely, flavan-3-ols) after acute consumption of a ready-to-drink tea prepared by infusion of tea leaves. The tea used in this study was marketed as black, but contained a relevant amount of flavan-3-ols which are known to be highly bioavailable [10] and linked to most of the health benefits demonstrated for this beverage and its non-fermented counterpart, green tea.

Before conclusions can be drawn on the potential *in-vivo* effects of tea polyphenols on human health, a more complete understanding of the mechanisms of absorption, bioavailability and biotransformations is necessary. Recent studies indicate that following tea consumption catechins are metabolised and circulate as sulphated, methylated or glucuronidated derivatives [11-13]. Absorption is believed to occur through the small intestine as bacterial degradation within the colon is hypothesised to break down the flavonoids into smaller phenolic acids [14].

The ready-to-drink tea used in this study is a source of flavan-3-ol monomers, contained at a level of 87 ± 8 μmol/500 mL. Based on this data, the present work represents the feeding and bioavailability study with the lowest dosage of this class of components present in the literature. However, urine excreted over a 24 h period after ingestion of the tea contained several flavan-3-ol metabolites of EC and EGC. In fact, neither EGCG or ECG were detectable in urine (as metabolites

or aglycones), in agreement with previous studies [15]. This is probably due to biliary excretion which these galloylated catechins undergo, investigated and clarified in rats after intravenous administration of (-)-[4-^{3}H] EGCG [16].

In total, 6.3 ± 3.1 μmols of metabolites were excreted, corresponding to 7.2 % of the ingested dose of tea flavan-3-ols. Moreover, considering that epicatechin metabolites derive not just from epicatechin but also from its galloylated form, and making the same argument for epigallocatechin, this demonstrates that the bioavailability of these two subclasses does not differ (table 2). Previous works have reported higher bioavailability for epicatechin [17], but excluding the galloylated forms as potential source of EC and EGC is at least questionable, in particular given the evidence that human saliva contains an esterase that can convert epigallocatechin-3-gallate to epigallocatechin [8].

By observing the excretion profiles peculiar behaviours for different catabolites emerge. Most of the metabolites show their peak excretion during the first 4 hours of urine collection, while two of them (namely methyl-epigallocatechin and its sulphated version) reach their peak excretion later in time, during the 7 to 10 hour period.

A limitation of the study is the absence of colonic metabolites of polyphenols from the analysis, as there is a growing appreciation that a significant portion of ingested dietary flavonoids and related compounds are not absorbed in the small intestine but pass to the large intestine where they are degraded by the colonic microflora to simple phenolic acids which can be absorbed into the circulatory system. Flavan-3-ols can interact with the human colon microbiota, giving rise to 5-(3’,4’,5’-trihydroxyphenyl)-γ-valerolactone, 5-(3’,4’-dihydroxyphenyl)-γ-valerolactone and 5-(3’,5’-dihydroxyphenyl)-γ-valerolactone [18] all of which also appear as sulphated and glucuronidated derivatives. These ring fission metabolites are found at high concentrations in urine and plasma of human volunteers after drinking green tea. However, due to the low amount of catechin ingested and to the absence of commercially available pure standards, they were not investigated in this study. A second study with RTD green tea is presently ongoing.

Conclusion

After drinking the ready-to-drink ice tea used in this study, the internal compartments are exposed to non-marginal doses of flavanols and flavanol metabolites up to 24 hours. Whether the excretion in urine continues beyond such period can only be hypothesised considering literature data on ring fission metabolites generated in the colon. The bioavailability values observed in this study are in agreement with previous reports [17] even though the dosage of polyphenols ingested in this study is remarkably lower. Moreover, the use of a group of twenty volunteers, more than the average number of subjects used for usual human acute feeding studies involving polyphenols, gives additional credibility to our results.

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281 Table 1: MS/MS identification of phenolic compounds and metabolites in human urine collected 0-

282 24 h after the ingestion of 500 mL of ready-to-drink tea.

Compound	***Molecular ion (ESI-)***	***MS/MS identification fragments***	***Number of isomers detected in urine***
Gallic acid	169	125	1
Methyl-epigallocatechin	319		2
Epicatechin-O-sulphate	369	289 (EC)	3
Methyl-epicatechin-O-sulphate	383	303 (Methyl-EC)	4
Epigallocatechin-O-sulphate	385	305 (EGC)	2
Methyl-epigallocatechin-O-sulphate	399	319 (Methyl-EGC)	2
Epicatechin-O-glucuronide	465	289 (EC)	1
Epigallocatechin-O-glucuronide	481	305 (EGC)	1
Methyl-epigallocatechin-O-glucuronide	495	319 (Methyl-EGC)	2

283 ESI – Electron Spray Ionisation; EGC – Epigallocatechin; EC - Epicatechin

284

285 Table 2: Quantification of flavan-3-ols and gallic acid in tea, total excretion of metabolites and
286 gallic acid in urine and bioavailability of these compounds after the ingestion of 500 mL of ready-
287 to-drink tea by twenty human volunteers. Data expressed as mean values in μmol ± standard error
288 (n = 20) with the exception of bioavailability values, expressed as percentage of ingested
289 component.
290

	Tea (500 ml)	***Urine***	***Bioavailability (%)***
Gallic acid	59.43 ± 4.67	2.62 ± 1.01	4.49 ± 1.69
EC + ECG	38.90 ± 3.66	2.77 ± 1.23	7.13 ± 3.16
EGC + EGCG	48.53 ± 4.69	3.54 ± 2.04	7.30 ± 4.21
Total flavan-3-ols	87.43 ± 8.34	6.32 ± 2.68	7.23 ± 3.06

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293 Figure captions.

294

295 Fig 1: HPLC-SIR chromatograms of flavan-3-ols in tea. Chromatograms represent gradient reversed
296 phase HPLC analysis with detection of flavan-3-ols using the selected ion recording mode.

297

298 Fig 2: HPLC-SIR chromatograms of principal flavan-3-ol metabolites in urine. Chromatograms
299 represent gradient reversed phase HPLC analysis with detection of metabolites using the selected
300 ion recording mode.

301

302 Fig 3: Excretion profiles of flavan-3-ol metabolites in urine during the 0-4, 4-7, 7-10 and 10-24h
303 periods in 20 volunteers. Values are expressed as mean ± standard error.

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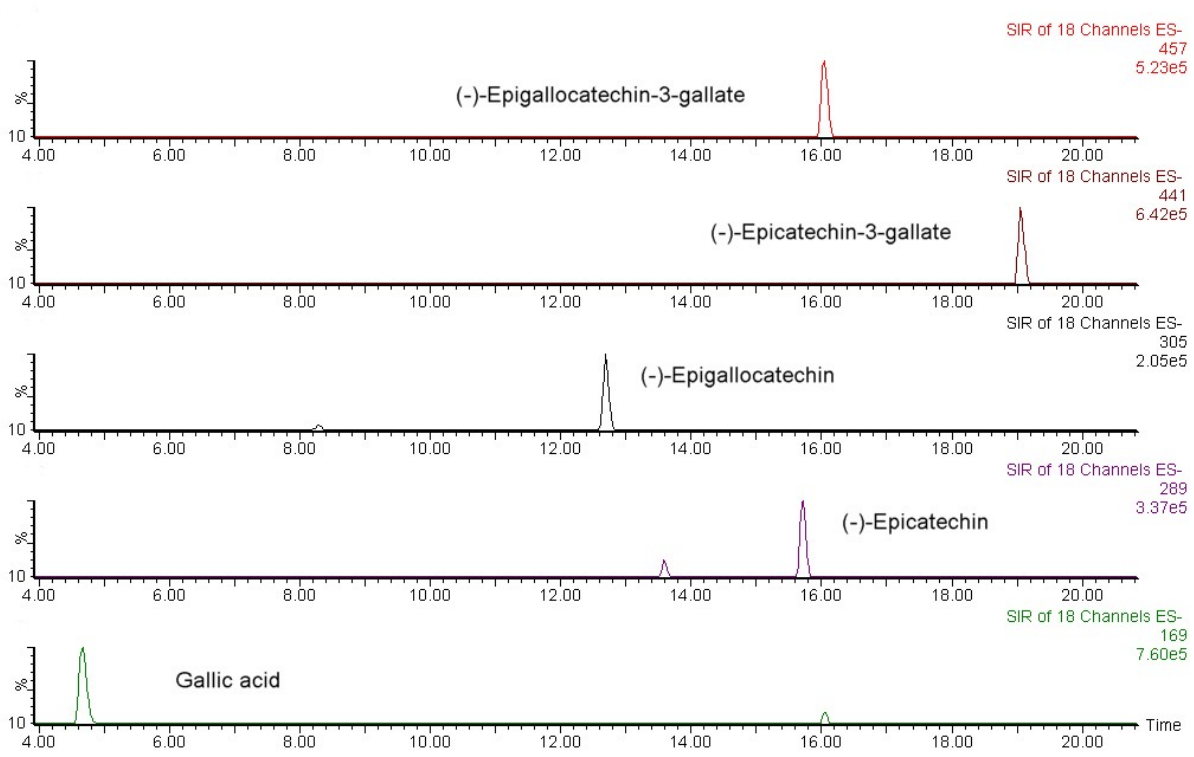
306 Figure 1



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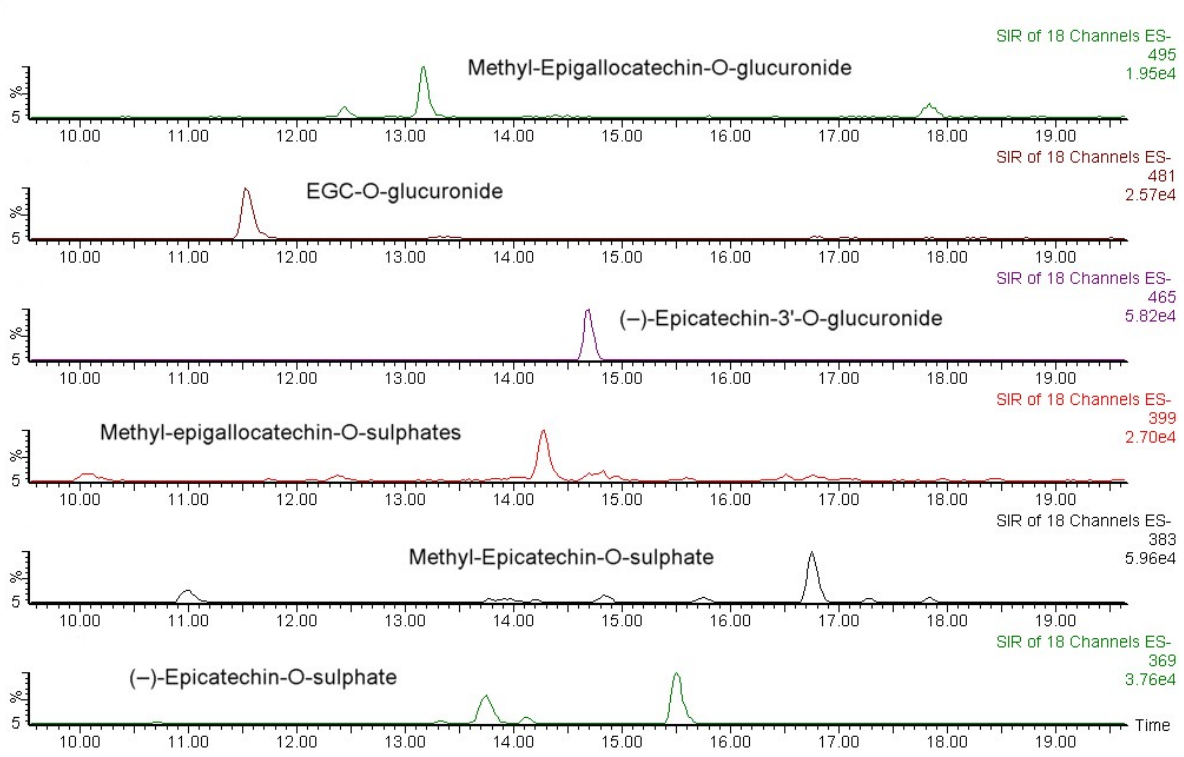
311 Figure 2



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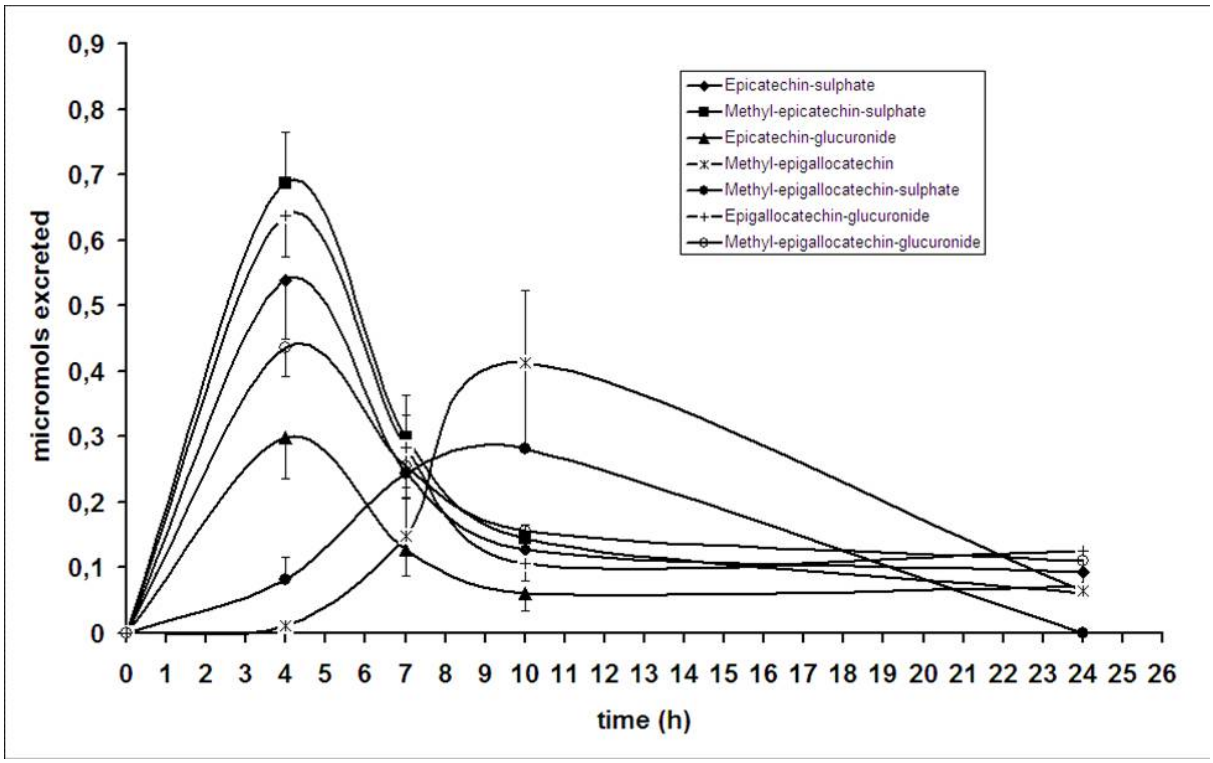
316 Figure 3



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