# PHENOLIC COMPOUNDS, METHYLXANTHINES AND ANTIOXIDANT ACTIVITY IN COCOA MASS AND CHOCOLATES PRODUCED FROM "WITCH BROOM DISEASE" RESISTANT AND NON RESISTANT COCOA CULTIVARS

## Compostos fenólicos, metilxantinas e atividade antioxidante em massa de cacau e chocolates produzidos a partir de cultivares resistentes e não resistentes a "vassoura de bruxa"

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**ABSTRACT**

The "witch broom disease" caused by the fungus called *Moniliophthora perniciosa* is one of the most important cocoa diseases in Latin America, causing around 70% production reduction in the southern Bahia. In attempt to solve the problem, many cultivars resistant to the disease have been recommended to farmers. On the other hand, the chocolate flavour is composed by many compounds whose formation depends on the genetic background, environment where cocoa is grown and processing operations. Therefore, this work aimed at determining the monomeric phenolic compounds, methylxanthines and antioxidant activity of cocoa mass and dark chocolate from cocoa cultivars resistant to "witch broom disease" and non resistant to the disease. The total phenolic compounds in cocoa mass did not vary among cultivars with values ranging from 23.95mg $g^{-1}$ to 25.03mg $g^{-1}$. Chocolates made from non resistant cultivars showed higher total phenolic compounds (19.11mg $g^{-1}$) than SR162 and PH16 with 16.08mg $g^{-1}$ and 15.46mg $g^{-1}$, respectively. Epicatechin had higher content than catechin and the levels of these two compounds were higher in SR162. There were significant differences among samples of cocoa mass analyzed for caffeine. Chocolate made from SR162 had the highest amount of monomeric compounds due to its high concentration of catechin and epicatechin. The chocolate sample with the highest antioxidant activity was the SR162, followed by non resistant blend and PH16, showing relationship between the antioxidant activity and monomeric phenolics content.

**Index terms:** Functional food, theobromine, caffeine, epicatechin, catechin.

**RESUMO**

A "vassoura bruxa" causada pelo fungo *Moniliophthora perniciosa*, é uma das doenças mais importantes do cacau na América Latina, provocando uma redução de cerca de 70% na produção das amêndoas na Bahia. Para tentar resolver o problema, muitos cultivares resistentes à enfermidade têm sido recomendados para os agricultores. Por outro lado, as características do chocolate são oriundas de várias substâncias, cuja formação depende da origem genética do fruto, do meio ambiente onde o cacau é cultivado e das operações de processamento. Assim, neste trabalho, objetivou-se determinar os compostos fenólicos monoméricos, metilxantinas e a atividade antioxidante em massa de cacau e chocolates provenientes de cultivares resistentes à "vassoura de bruxa" e não resistentes à doença. Os compostos fenólicos totais na massa de cacau não variou entre os cultivares com valores que variam entre 25,03mg $g^{-1}$ a 23,95mg $g^{-1}$. Chocolates feitos a partir de cultivares não resistentes à doença apresentaram maior teor de fenólicos totais (19,11mg $g^{-1}$) que os cultivares resistentes, SR162 e PH16 com 16,08mg.$g^{-1}$ e 15,46 mg.$g^{-1}$, respectivamente. Os conteúdos de epicatequina foram superiores aos de catequina em todos os cultivares. Houve diferenças significativas entre as amostras de massa de cacau analisadas para a cafeína. Chocolates produzidos a partir do cultivar SR162 apresentaram maior quantidade de compostos fenólicos e metilxantinas. A amostra de chocolate com a atividade antioxidante mais elevada é a SR162, seguida dos cultivares não resistentes e PH16, mostrando uma relação entre a atividade antioxidante e o conteúdo de compostos fenólicos monoméricos.

**Termos para indexação:** Alimentos funcionais, teobromina, cafeína, epicatequina, catequina.

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## INTRODUCTION

The "witch broom disease" caused by the fungus called *Moniliophthora perniciosa* is one of the most important cocoa diseases in Latin America and Caribbean Islands causing huge economic losses. In southern Bahia, the mean productivity of cocoa has decreased around 70% since the disease emergence in 1989 (MANDARINO; GOMES, 2009). As a way to bypass the problem, many cultivars have been

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recommended by the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC) to farmers in order to have new resistant crops with higher productivity.

On the other hand, in the last few years, researches have been intensified the aiming at finding fruits, vegetables, plants, agricultural and agro-industrial residues as sources of bioactive compounds (MARTINS et al., 2011). However, the lack of knowledge about the content of active ingredients and their molecular composition restrict their use as natural antioxidant sources. Thus, the screening of various food products, with beneficial health properties is very important (SHAHIDI et al., 1994).

The first human clinical study with chocolate was performed in 1996, when it was found that 35 grams of defatted cocoa decreased the LDL oxidation between 2 and 4 hours after ingestion. Since 1996, other 38 human studies involving the use of cocoa in different forms have been performed and can be summarized under three main headings: antioxidant properties, cardiovascular protection and anticarcinogenic action (RUSCONI; CONTI, 2010).

The chocolate flavour is composed of many compounds whose formation depends on the genetic background, environment where cocoa is grown and processing operations that begin on the farm (harvesting, fermenting and drying) and continue in the industries that process cocoa and chocolate (BRUNETTO et al., 2007). In addition, there are also internal factors in the beans that can affect the flavour, such as polyphenol content (NOOR-SOFFALINA et al., 2009), which are responsible for the astringency and contribute to bitterness (MISNAWI et al., 2004).

Cocoa bean is one of best known sources of dietary polyphenols, which comprises on average 12-18% of total weight on a dry basis. Generally, cocoa contains significant content of procyanidin monomers, as catechin and epicatechin, and other dimer to tetradecamer molecules (MENG et al., 2009). Methylxanthines, such as caffeine and theobromine, are another group of bioactive compounds found in cocoa beans. These alkaloids have a stimulating effect on the brain and some works connect the presence of these compounds in chocolates with some effects, such as addiction and blood pressure reduction (BRUINSMA; TAREN, 1999).

This work aimed at determining the monomeric phenolic compounds and methylxantines content as well as the antioxidant activity of the cocoa mass and the dark chocolate (70% of cocoa) from cocoa cultivars resistant and on-resistant to "witch broom disease" (*Moniliophthora perniciosa*).

## MATERIALS AND METHODS

Dark chocolates, containing 70% cocoa, made from cultivars resistant to "witch broom disease", "SR162" and "PH16", and a blend of non-resistant cultivars composed by Pará, Parazinho and Maranhão all of them belonging to *Forastero* group were used. These *Forastero* cultivars are grown from decades in Bahia State; considered a material of high productivity and high fruit quality; however, non-resistant to the disease.

The "PH16" is a cultivar originated from a selection performed in a commercial area. It has unknowing parents and the plant was originally identified in 1996 in a hybrid cocoa population (crosses between *Amazônico* group and *Trinitario*) from "Porto Híbrido" farm, São José da Vitória municipality, Bahia, Brazil.

The "SR162" is a cultivar derived from genetic mutation of the common cocoa (*Alto Amazônico* group) with white seeds. The name came from the farm where there were identified, "São Roque" farm in Itagibá, Bahia, Brazil.

### Preprocessing of cocoa beans

The fermentation was carried out in 70cm x 70cm x 75cm wooden boxes. A total mass of 400Kg in each box were processed. Turnings were performed for oxygenation and mixing of the mass 48 hours from the beginning of each fermentation and after decreasing temperature until the end of the process. After fermentation, seeds were dried in the sun-roof surfaces with mobile timber for 5 to 7 days up to 8.0% of moisture.

### Chocolate processing

The fermented and dried beans were roasted in a circular roaster (Jaf Inox, Sao Paulo, Brazil) at 120° C, for 2 hours. Then, the toasted beans were crushed and removed the peel and germ (cocoa *nibs*). The *nibs* were ground in a knife mill and refined sugar was added. The cocoa mass was refined in a five roll mill to yield optimal particle size for the chocolate. The refined mass was taken to conching held in horizontal shell (Jaf Inox, Sao Paulo, Brazil) 60° C, for 48 hours. Commercial deodorized cocoa butter (Joanes Industrial S/A Chemicals and Plants) and commercial soy lecithin (Bunge Alimentos S/A) were added. The chocolate was carried out for tempering process in a shaking platform brand until reaching temperature of 42° C to form stable crystals of cocoa butter. The chocolate was molded in a polyethylene former, producing a 5g bars. The chocolates were cooled, packaged and remained at 18° C.

**Extraction of phenolic compounds**

Methanolic extracts were obtained according to Fantozzi and Montedoro (1978). Ten grams of cocoa mass and chocolate were weight and defatted with petroleum ether, under shacking during 30 minutes for three times. From defatted samples, five grams were weight and 80% methanol in water (v/v) solution were added and stirring during one hour. After filtration, the methanolic extracts were stored in dark flask under nitrogen atmosphere at low temperature.

**Determination of total phenols**

The spectrophotometric method was used to determine the total phenolic content according to the method described by Gutfinger (1981) using Folin-Ciocalteu reagent. The phenolic content was calculated based on the catechin calibration curve.

**Determination of monomeric phenols and methylxanthines by HPLC**

The determination of monomeric phenolic compounds (galic acid, catechin, caffeic acid and epicatechin) and methylxantines (caffeine and theobromine) was performed according to the method described by Elwers et al. (2009). Ten microlitres of each sample solution was analyzed by HPLC system (Perkin Elmer Model Flexar) equipped with VI Flow injector, C 18 column (100mm x 4.6mm O.D.S.-2, 3µ) and using the solvents (A): 2% acetic acid in water and (B): a mixture of acetonitrile, water and acetic acid (400:90:10 v/v/v). Elution was performed with a linear gradient showed in table 1. The compounds were monitored by UV detection at 280 nm wavelength. The total run time was 20 min and the temperature was 26° C. All standards used for quantitative determinations were from Sigma-Aldrich, St. Louis, MO.

Table 1 – HPLC gradient used for separation of phenolic compounds and methylxanthines in cocoa mass and chocolates.

| Time (min) | Flow rate (ml $min^{-1}$) | A (%) | B (%) |
|---|---|---|---|
| 2 | 0.4 | 90 | 10 |
| 3 | 0.3 | 88 | 12 |
| 3 | 0.4 | 86 | 14 |
| 2 | 0.4 | 84 | 16 |
| 2 | 0.5 | 82 | 18 |
| 10 | 0.5 | 90 | 10 |

**Antioxidant Activity of Cocoa Mass and Chocolates Extracts**

The antioxidant activity of the extracts was determined using the DPPH (1,1-difenil-2-picrilidrazil) test as described by Vinson et al. (2001). For evaluating the antioxidant activity, the methanolic extracts at 2.5mg $ml^{-1}$ were submitted to the reaction with DPPH, in which 0.5 ml of the sample was mixed with 4 mL of a 0.004% (m/v) DPPH solution. The radical DPPH reduction was measured through the continuous monitoring of the absorbance decline at 517 nm against ethanol in 4 ml cuvettes using a UV spectrophotometer for 30 min at room temperature in the dark. The absorbance decrease of samples was calculated in comparison to the blank sample and expressed as percentage of the antioxidant activity. The antioxidant activity of each sample ($IC_{50}$) was then reported as the final concentration in µg $ml^{-1}$ of the extract present in the cuvette required for the initial DPPH concentration decrease to 50%.

**Statistical analysis**

Data were subjected to analysis of variance (one-way ANOVA) using the Tukey test at 5% significance for means comparison .

**RESULTS AND DISCUSSION**

The total phenolic content in cocoa mass did not vary among cultivars ranging from 23.95mg $g^{-1}$ to 25.03mg $g^{-1}$ and expressed as catechin equivalent (Table 2). When comparing these compounds in chocolate, non resistant cultivars of blend chocolate showed higher phenolic content than the other ones, SR162 and PH16. This is expected due to the fungal infection, since the synthesis of phenolic compounds is enhanced in the presence of phytopathogens (SOARES, 2002). When a plant is attacked by a potential pathogen, there is activation of the defense responses complex, with expression of various genes, resulting in synthesis and accumulation of secondary

Table 2 – Total phenolics (mg $g^{-1}$) in cocoa mass and chocolates (mean±sd).

| | Cocoa mass | Chocolate |
|---|---|---|
| Non resistant | $25.78 \pm 1.40^{a*}$ | $19.11 \pm 0.14^{a}$ |
| SR162 | $25.03 \pm 0.76^{a}$ | $16.08 \pm 0.79^{b}$ |
| PH16 | $23.95 \pm 1.58^{a}$ | $15.46 \pm 1.02^{b}$ |

* Means with same letter in the columns are not significantly different according to the ANOVA with Tukey test ($p<0.05$)

metabolites such as phenolic compounds (SOUZA et al., 1999).

Another important point to be considered is the pre-processing (fermentation, drying and roasting) of cocoa, which according to some authors reduces the concentration of these kind of compounds (ADAMSON et al., 1999; STAHL et al., 2009). Furthermore, the concentration of phenolic compounds varies according to cultivar employed and also the type of chocolate produced (MILLER et al., 2006; PAYNE et al., 2010).

The results obtained in this work were lower than those found by Vinson et al. (2006) in USA, with 23.80mg $g^{-1}$ of phenolics in dark chocolate and Cooper et al. (2008) in Europe that found 23.40mg $g^{-1}$, but on the other hand, Meng et al. (2009) found 5.78mg $g^{-1}$ in Malaysia, which suggest that different regions with specific soil and climatic characteristics can result in fruits with their own characteristics for chocolate production.

The phenolic composition of cocoa mass samples is reported in table 3. Caffeic acid was the phenolic compound with lower content detected in the samples, showing no statistical differences between them. Gallic acid was not detected in the SR162 sample, but found in non resistant cocoa and PH16, also with no statistical difference between them. This acid is the basic constituent of hydrolysable tannins, being widely found in lignified plants. Besides the antioxidant potential, gallic acid is responsible for the astringent sensation in the mouth, which can promote different sensory characteristics to these cultivars. Epicatechin had higher content than catechin and, the SR162 showed higher values than the other cultivars, either for epicatechin or cathechin. Although no differences in total phenolic content had been observed among the three samples, the SR162 had higher content of monomeric phenols, thus having greater proportion of phenolic polymers, such as procyanidins in the other cultivars (PH16 and non resistant).

It is expected tccchat these facts contribute to chocolate flavour, since those compounds promote different sensory characteristics to the chocolates. The monomeric phenolic is responsible for the bitter taste and the polymeric forms for adstringency (AFOAKWA et al., 2008).

Concerning to methylxanthines in cocoa mass, there were significant differences among samples for caffeine. Cocoa mass from PH16 showed higher concentration, followed by the non resistant cocoa and the lowest concentration was found in SR162. No statistical difference ($p < 0.05$) was found among samples for theobromine.

Nevertheless, changes can occur in polyphenols and methylxanthines content during chocolate production, mostly during refining and conching, where high temperatures are achieved and the air is present. However, knowledge of these changes is limited (WOLLGAST; ANKLAM, 2000).

As occurred in cocoa mass, in chocolates, caffeine contents showed statistically significant difference between samples, SR162 showed lower values than the other cultivars (Table 4). Theobromine levels were higher than caffeine ones and also the SR162 showed higher values than the other cultivars.

This fact is very important, considering the health aspects, since caffeine acts as stimulant and vasoconstrictor, while theobromine acts as vasodilator, reducing the blood pressure. Moreover, theobromine acts as muscle relaxant and diuretic (BRUINSMA; TAREN, 1999; VAN DEN BOGAARD et al., 2010).

Table 3 – Phenolic and methylxanthines composition in cocoa mass samples (mean±sd).

| | Non resistant | SR162 | PH16 |
|---|---|---|---|
| | | **Phenolics compounds (μg $g^{-1}$)** | |
| Galic acid | 124.26 ± 6.88[a*] | - | 132.32 ± 9.81[a] |
| Catechin | 1,095.44 ± 23.73[a] | 1,734.45 ± 33.40[b] | 949.90 ± 11.92[a] |
| Caffeic acid | 24.8 ± 17.54[a] | 34.44 ± 6.39[a] | 43.63 ± 5.85[a] |
| Epicatechin | 1,618.74 ± 31.30[a] | 1,852.07 ± 29.89[b] | 1,551.90 ± 14.44[a] |
| | | **Methylxanthines (μg $g^{-1}$)** | |
| Caffeine | 1.98 ± 0.01[a] | 1.01 ± 0.20[b] | 3.02 ± 0.20[c] |
| Theobromine | 6.57 ± 0.03[a] | 7.23 ± 1.32[a] | 6.67 ± 0.30[a] |

* Means with same letter in the rows are not significantly different by the ANOVA with Tukey test ($p<0.05$).
(-) Not Detected.

Table 4 – Phenolic and methylxanthines composition in chocolate samples (mean±sd).

| | Non resistant | SR162 | PH16 |
|---|---|---|---|
| | | **Phenolic compounds (μg $g^{-1}$)** | |
| Galic acid | 30.03 ± 0.33[a*] | - | 14.83 ± 10.20[a] |
| Catechin | 845.37 ± 15.83[a] | 928.60 ± 18.75[a] | 767.83 ± 43.83[a] |
| Caffeic acid | - | - | 26.3 ± 1.68 |
| Epicatechin | 1,107.73 ± 30.60[a] | 1,161.66 ± 36.94[a] | 991.14 ± 60.65[a] |
| | | **Methylxanthines (μg $g^{-1}$)** | |
| Caffeine | 0.90 ± 0.36[a] | 0.62 ± 0.07[b] | 0.84 ± 0.05[a] |
| Theobromine | 2.21 ± 0.11[a] | 3.00 ± 0.27[b] | 2.01 ± 0.10[a] |

* Means with same letter in the rows are not significantly different according to the ANOVA with Tukey test ($p<0.05$).
(-) Not Detected.

Concerning phenolic compounds in chocolate, their content was reduced for all samples, since other ingredients are combined in their production, reducing the phenolic content. Nevertheless, in chocolate made from SR162, caffeic acid and gallic acid were not detected, though this sample had the highest content of monomeric compounds (2,090.26 $\mu g.g^{-1}$), which represents 13.0% of total phenolics. In chocolate made with non resistant cultivars, caffeic acid was not detected and the total monomers identified was 1,983.13 $\mu g.g^{-1}$ representing 10.4% of total phenolic compounds, while the chocolate from the PH16 variety had all monomers detected, with a total of 1,800,10 $\mu g.g^{-1}$ : equivalent to 11.64% of total phenolics for chocolate.

SR162 showed higher levels of monomeric phenolics in chocolate, which can help its flavor composition, since the phenolic compounds are responsible for the bitter characteristic. Besides, the healthy aspects of these compounds should be considered, since the monomeric form of phenolic compounds is ready to be absorbed and used by human body as exogenous antioxidants (RICHELLE et al., 1999; HOLT; LAZARUS; SULLARDS, 2002).

The assay with DPPH (the stable radical 2,2-diphenyl-1-picryl hydrazyl) has been frequently used to assess the ability of natural antioxidants to "mop up" free radicals (ROESLER et al., 2007). The capacity of the different extracts from chocolates of reducing free radicals was expressed as the extract final concentration necessary to inhibit 50% of the DPPH radical oxidation, which represents $IC_{50}$ (Table 5).

The chocolate sample with the highest antioxidant activity is SR162, followed by the non resistant and PH16, with the lowest antioxidant activity among the three varieties. The DPPH free radical scavenging activity is strongly correlated with the total flavonoid content (ABU BAKAR et al., 2009). Indeed, according to Oliveira et al. (2011), the relationship between the antioxidant activity of cocoa extracts and the concentration of total phenols seems to be quite significant, since the extracts with the highest concentrations of monomeric phenols are those with highest antioxidant activity. Besides, the SR162 extract showed the highest content of catechin and epicathechin, flavonoids with excellent antioxidant activity. However, the result did not show a relationship between the antioxidant activity and the total phenolics, since the extract with the highest concentration of total phenolics (non resistant) had the second highest antioxidant activity. It can be explained by the flavonoid-flavonoid interaction that reduces or increases the total antioxidant activity (HIDALGO; SÁNCHEZ-MORENO; PASCUAL-TERESA, 2010) and probably the higher levels of polymeric phenols. Therefore, epicatechin and catechin seem to be the main compounds responsible for the antioxidant activity of chocolate extract. These results suggest a major correlation between antioxidant capacity and monomeric phenolic content instead of the total phenolic content (MACIEL et al., 2011).

Table 5 – Antioxidant activity (μg $ml^{-1}$) of chocolates produced from non resistant and resistant cultivars of cocoa to "witch broom disease" in Brazil.

| Sample | $IC_{50}$ (μg $ml^{-1}$)* |
|---|---|
| Non resistant | 19.20 ± 0.55[b**] |
| SR162 | 15.15 ± 1.88[c] |
| PH16 | 20.47 ± 1.86[a] |

* The $IC_{50}$ value was obtained by using three replicates from four different extract concentrations. This covered the range from low to high inhibition of the DPPH radical.
** Means with same letter in the rows are not significantly different according to ANOVA with Tukey test ($p < 0.05$).

## CONCLUSIONS

The chocolates from SR162 cultivar, which is resistant to "witches' broom disease", showed higher antioxidant activity as well as higher concentration of monomeric phenolic compounds (catechin and epicatechin) than the other cultivars studied. Besides, the SR162 has higher levels of theobromine and lower caffeine. These results provide a new perspective of using the SR162 and other new cultivars resistant to "witches' broom disease" to produce chocolate with desirable sensory attributes, and great potential for use as functional food.

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