

## Phenolic Constituents and Antioxidant Potential of *Gossypium hirsutum* L. (Malvaceae)

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### Abstract

### Original Research Article

**Background:** In this study, the phenolic constituents and antioxidant potential of *Gossypium hirsutum* were evaluated. **Methods:** Dried and powdered leaves were subjected to extraction in ethanol by static maceration to obtain the ethanol extract (EEGH). From EEGH, hexane (HFGH), dichloromethane (DFGH), ethyl acetate (EAFGH) and butanol (BFGH) fractions were obtained by liquid/liquid partition. Total phenolic and flavonoids were quantified by spectrophotometric method using gallic acid and rutin as reference compounds, respectively. The antioxidant activity was determined by free radical scavenging of DPPH, ferric reducing antioxidant power (FRAP), co-oxidation of  $\beta$ -carotene/linoleic acid and thiobarbituric acid assays. **Results:** Total phenols ranged from 1.86 to 26.49 g/100g, while the flavonoid variation was 0.67 to 7.17 g/100g. The  $IC_{50}$  values ranged from  $24.99 \pm 0.16$  to  $215.48 \pm 0.11$   $\mu$ g/mL against the DPPH $^{\bullet}$  radical and FRAP produced  $IC_{50}$  between  $25.61 \pm 0.43$  and  $166.20 \pm 1.01$   $\mu$ g/mL. The inhibition of lipid peroxidation (%I) by beta-carotene/linoleic acid co-oxidation was between 42.97 and 82.18%. The  $IC_{50}$  values ranged from  $134.10 \pm 8.20$  to  $388.20 \pm 0.83$   $\mu$ g/mL when the inhibition of lipid peroxidation was investigated by thiobarbituric acid. **Conclusion:** The results indicate that *G. hirsutum* leaves are a promising source of compounds with antioxidant action that may justify their therapeutic properties.

**Keywords:** *Gossypium hirsutum*, total phenols, flavonoids, antioxidant activity.

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## INTRODUCTION

Oxidative stress results from an imbalance between the generation of oxidizing compounds and the action of antioxidant defense systems, which can culminate in metabolic and cellular disorders, including damage to DNA and RNA, increase in free intracellular  $Ca^{2+}$ , damage to the mechanism of ionic transport of the membrane or other specific proteins, oxidation of cysteine residues, oxidation of cytoplasmic proteins, and peroxidation of lipids [1]. Oxidative stress leads to the oxidation of biomolecules with consequent loss of their biological functions and/or homeostatic imbalance, manifesting as the potential oxidative damage against cells and tissues [1, 2]. The chronicity of this process has relevant implications for the etiological process of numerous chronic non-transmissible diseases, including atherosclerosis, diabetes, obesity, inflammatory diseases, aging, allergies, hemorrhages, neurodegenerative disorders, immune disorders, and cancer [3]. On the contrary, antioxidants are any compounds that, present in low concentrations when compared to the oxidizable substrate, effectively delays

or inhibits the oxidation of this substrate, and they can be found naturally in our body and in food. Antioxidants act by neutralizing free radicals, either by donating electrons or by preventing the formation of these and self-oxidizing chains, the ability to complex with metals, inhibiting pro-oxidant enzymes, and repairing damage caused [2, 4]. A strategy for controlling this organic imbalance is to use natural products obtained from medicinal plants containing phenolic compounds with reducing properties and chemical structure that inhibit oxidative processes [5]. Phenolic compounds play an important role in the neutralization or sequestration of free radicals and in the chelation of transition metals, acting both in the initiation and propagation stages of cellular damage [2, 5].

*Gossypium hirsutum* L., belonging to the family Malvaceae, popularly known as “cotton,” “cotton-herbaceous,” and “cotton-mocó,” is used mainly in the textile industry. In the ethnobotanical literature, the leaves of *G. hirsutum* L. are indicated for treating dysentery, uterine hemorrhage, scarring, and

internal inflammation [6]. Root tea is used to treat memory loss, amenorrhea, menopausal disorders, and sexual impotence, whereas flower and fruit extracts are used as a topical antimycotic agent [6]. The seed oil has purgative and deworming properties, as well as combats lice [7, 8]. Pharmacological actions of the plant are linked to its anti-inflammatory, healing, anti-infectious, memory and learning, antiepileptic, antidepressant, antidiabetic and hypolipidemic, diuretic, anthelmintic, antiprotozoal, insecticidal, antioxidant, and anticancer activities [6, 9-11].

Regarding chemical composition, terpenoids, fixed and volatile oils, terpenoid quinones, fatty acids, flavonoids, condensed tannins, and oligosaccharides have been isolated from *G. hirsutum* [6, 11-13]. However, gossypol, a dimer of aromatic sesquiterpenes, found in the leaves, stipules, sepals, stems, branches, fruits, and seeds of the plant, has antinutritive and toxic effects [6]. The most commonly identified flavonoids were quercetin, campferol, quercimeritrin, isoquercitrin, gossypitrin, gossypin, and herbacitrin [11, 13]. Subsequently, other flavonoids, which differ mainly by their glycosylation and methylation, were isolated and characterized from the *Gossypium* species [11, 13]. These chemical compounds had various biological properties, such as antitumor, analgesic, leishmanicidal, hypoglycemic properties, acetylcholinesterase inhibitor, angiotensin-converting-enzyme inhibitor, giardicide and in the prevention of osteoporosis and Parkinson's disease [6, 13].

Considering the chemical and pharmacological aspects, mainly the phenolic compounds and medicinal uses related to organic disorders that involve the generation of free radicals, such as inflammation, infections, cancer, and gastrointestinal disorders, this study was designed to determine the phenolic and flavonoid contents and to evaluate the antioxidant effects of the ethanol extract and fractions of *G. hirsutum*.

## MATERIAL AND METHODS

### Plant Material

*Gossypium hirsutum* L. was collected from the Medicinal Garden of the Faculty of Pharmacy of the Federal University of Juiz de Fora (UFJF). The species was identified by Dr. Fátima Regina Gonçalves Salimena and a voucher was deposited in the Herbarium Leopold Krieger/UFJF (CESJ n° 48.612). The leaves were placed in a drying oven with forced air circulation at 50 °C for loss of 90%–95% humidity. For extraction, the dried leaves were crushed in a mill with defined granulation, followed by spraying in tamise n° 20.

### Extraction and liquid/liquid fractionation

Powdered leaves (460 g) were submitted to extraction by static maceration in 95% ethanol at room temperature with renewal of the solvent 20 times in an interval of 2 days. After removing the solvent by

vacuum-evaporation method, 30 g of dry ethanol extract (EEGH) was dissolved in water:ethanol (9:1), followed by liquid/liquid partition with increasing organic solvent polarity: hexane, dichloromethane, ethyl acetate, and butanol. Thus, fractions of hexane (HFGH), dichloromethane (DFGH), ethyl acetate (EAFGH), and butanol (BFGH) were obtained [14].

### Determination of Total Phenolic Content

The total phenolic content was determined by the Folin–Ciocalteu method using gallic acid as the standard [15]. From a stock solution of gallic acid (1 mg/mL), six dilutions (10, 20, 30, 40, 50, and 60 µg/mL) were prepared to obtain the calibration curve. In triplicate, each dilution (1 mL), 10% Folin–Ciocalteu reagent (5 mL, v/v), and 7.5% sodium carbonate (4 mL, w/v) were transferred to test tubes and kept at room temperature for 2 h. The test blank was composed of deionized water (1 mL), 10% Folin–Ciocalteu reagent (5 mL, v/v), and 7.5% sodium carbonate (4 mL, w/v). From a scan in the range between 600 to 900 nm of gallic acid (40 µg/mL), an absorption spectrum with a maximum peak of 763.6 nm was obtained in a UV/visible double-beam spectrophotometer (Shimadzu®, UV-1800). At this wavelength (763.6 nm), in triplicate, the absorbances were measured to obtain the line equation and the coefficient of determination ( $R^2$ ) by using the least squares method.

The total phenolic content in the samples (EEGH and fractions) was quantified using the same analytical procedure. Each sample was solubilized in 4% dimethyl sulfoxide (v/v), to obtain a concentration of 500 µg/mL. The sample blanks were composed of the respective solutions in the absence of the Folin–Ciocalteu reagent. The total phenol content was determined by the line equation obtained from the standard.

### Determination of Total Flavonoid Content

The total flavonoid content was determined spectrophotometrically with rutin as a standard [16]. From a stock solution of rutin (0.5 mg/mL) in ethanol, concentrations of 2, 5, 10, 20, 30, 40, and 50 µg/mL, in triplicate, were prepared in test tubes. For this, 20, 50, 100, 200, 300, 400, and 500 µL of the stock solution, ethanol (400 µL), acetic acid (120 µL), 20% pyridine: ethanol (v/v, 2 mL), 8% aluminum chloride hexahydrate in ethanol (m/v, 500 µL), and distilled water (adjusting to 5 mL) were used. After 30 min at room temperature, a spectrophotometric scan in the range of 300–600 nm was performed using the rutin solution (20 µg/mL), which defined the peak of maximum absorption at 417.2 nm. In this wavelength (417.2 nm), in triplicate, the absorbance of the reaction mixture was measured with a double beam UV/Visible spectrophotometer (Shimadzu®, UV-1800). The blank of each concentration was prepared in the same fashion, but without the aluminum chloride solution. The data were subjected to linear regression analysis, using the

least squares method, which calculated the line equation and the coefficient of determination ( $R^2$ ).

EEGH and fractions were subjected to semi-purification using sample solution (5 mL), chloroform (2 mL), and distilled water (3 mL). After centrifugation at 3000 rpm for 2 min, the organic phase was discarded and aliquots of 400  $\mu$ L of each sample, in triplicate, were used for the analysis. Total flavonoids were quantified using the same analytical procedure with absorbance at 417.2 nm.

### Antioxidant Activity

#### DPPH Radical Sequestration Method

The antioxidant activity was determined spectrophotometrically using the free radical DPPH (2,2-diphenyl-1-picryl-hydrazil) as described by Mensor *et al.* (2001) [17]. Stock solutions at 1 mg/mL in methanol were prepared from the samples (extract and fractions) and rutin was used as the positive control. From these solutions, dilutions in methanol were made, obtaining different concentrations. Aliquots (2.5 mL) of the respective samples were transferred, in triplicate, to test tubes followed by the addition of 0.03-mM DPPH (1 mL). After 30 min of reaction, the absorbances were spectrophotometrically (Shimadzu®, UV-1800) measured, in triplicate, at 518 nm. The blanks consisted of the respective solutions (2.5 mL) in each concentration and methanol (1 mL). The negative control contained methanol (2.5 mL) and DPPH (1.0 mL), and the “auto-zero” was performed only with methanol.

The percentage of antioxidant activity (%AA) corresponded to the amount of reduced DPPH, using the following formula:

$$\%AA = 100 - [(Abs_{\text{sample}} - Abs_{\text{sample blank}})/Abs_{\text{control}} - Abs_{\text{control blank}}] \times 100,$$

Where  $Abs_{\text{sample}}$  is the absorbance of the samples or rutin for each concentration;  $Abs_{\text{sample blank}}$  is the absorbance of sample or rutin blank for each concentration;  $Abs_{\text{control}}$  is the absorbance of the negative control; and  $Abs_{\text{control blank}}$  is the absorbance of negative control blank.

After obtaining the %AA, by means of linear regression analysis, using the least squares method, the line equations were determined to calculate the 50% inhibitory concentration ( $IC_{50}$ ).

#### Ferric Reducing Antioxidant Power Method

The ferric reducing antioxidant power (FRAP) was determined by the method proposed by Oyaizu (1986) [18]. Stock solutions of EEGH and fractions, rutin (positive control) and ascorbic acid (positive control) were prepared in ethanol. From the stock solutions, dilutions, in triplicate, were made in test tubes with distilled water (adjusted to 1 mL). Then, 200  $\mu$ M potassium phosphate buffer (2.5 mL), pH 6.6, and

1% potassium ferrocyanide (2.5 mL, w/v) were added to the tubes. After incubation at 50°C for 20 min, 10% trichloroacetic acid (2.5 mL, w/v) was added to the tubes and centrifuged at 3000 rpm for 8 min. Then, the top layer (2.5 mL) of this mixture was transferred to a test tube followed by addition of distilled water (2.5 mL) and 0.1% ferric chloride (0.5 mL, w/v). The sample blank was made of distilled water (1 mL) and all reagents, except the sample solutions. In triplicate, the absorbances were measured using a spectrophotometer (Shimadzu®, UV-1800) at 700 nm. The concentration-versus-absorbance curves were prepared for linear regression analysis, using the least squares method, obtaining the line equations that determined the 50% inhibitory concentration ( $IC_{50}$ ).

#### $\beta$ -Carotene/Linoleic Acid Co-Oxidation Method

As described by Koleva *et al.*, [19], the  $\beta$ -carotene/linoleic acid co-oxidation method is used to measure the inhibition of lipid peroxidation from  $\beta$ -carotene (0.2 mg/mL) in chloroform. This solution (1 mL) was packed in a flask with linoleic acid (25  $\mu$ L) and Tween 40 (200 mg), followed by rotary evaporation at 40°C to remove chloroform. To this system, bubbled oxygenated distilled water (50 mL) was slowly added under constant agitation using an air pump to form an emulsion. Meanwhile, sample solutions (250  $\mu$ g/mL) and 3,5-di-tert-butyl-4-hydroxy toluene (BHT), rutin and quercetin (25  $\mu$ g/mL, positive controls) were prepared. In a microplate, aliquots of these solutions (30  $\mu$ L) were added, in triplicate, followed by emulsion (250  $\mu$ L). The negative control was performed in triplicate and consisted of ethanol (30  $\mu$ L) and emulsion (250  $\mu$ L). The test blank corresponded to ethanol (280  $\mu$ L).

For the test, readings of the microplate every 15 min after incubation in an oven at 50°C, the first reading ( $t_0$ ), was taken immediately after the addition of the emulsion. This procedure was repeated at times  $t_0$ ,  $t_{15}$ ,  $t_{30}$ ,  $t_{45}$ ,  $t_{60}$ ,  $t_{75}$ ,  $t_{90}$ , and  $t_{105}$  in a microplate reader (Thermoplate®, TP-Reader) at 492 nm to monitor the oxidation process of  $\beta$ -carotene. After obtaining the absorbances, the graph of decay as a function of time was drawn up and the percentage of inhibition of lipid peroxidation (% I) was calculated using the following formula:

$$\%I = [(Abs_{\text{control}} - Abs_{\text{sample}})/Abs_{\text{control}}] \times 100,$$

Where  $Abs_{\text{control}} = Abst_0 - Abst_{105}$ ;  $Abs_{\text{sample}} = Abst_0 - Abst_{105}$ ;  $Abs_{\text{control}}$ : negative control; and  $A_{\text{sample}}$ : EEGH, fractions, and positive controls (BHT, rutin and quercetin).

#### Lipid Peroxidation Method with Thiobarbituric Acid

As recommended by Buege and Aust [20], with minor modifications, lipid peroxidation was evaluated through the formation of reactive substances with thiobarbituric acid. Portions of ground meat (2 g),

which was low in fat, were homogenized in 3 volumes of 0.1 M phosphate-saline buffer (PBS), pH 7.4, containing 3,5-di-tert-butyl-4-hydroxy toluene (BHT) at 0.06% (w/v) and subjected to centrifugation at 4,000 g for 15 min. The supernatant was collected and the protein concentration determined using the method of Lowry *et al.*, [21]. Aliquots of the homogenate containing 2 mg of protein were placed in test tubes. Stock solutions of EEGH and fractions and rutin (positive control) were prepared in ethanol. From the stock solutions, dilutions were made and transferred, in triplicate, to test tubes, making up to 1 mL with PBS. The tubes were incubated at 37°C for 30 min, followed by the addition of 25% hydrochloric acid (500  $\mu$ L, v/v) and 1% thiobarbituric acid (500  $\mu$ L, w/v). The mixture was heated in boiling water bath for 15 min and cooled in an ice bath for 10 min. Subsequently, butanol (2 mL) was added, the tubes were vortexed vigorously, and centrifuged at 4,000 g for 15 min. The absorbances were measured using a spectrophotometer (Shimadzu<sup>®</sup>, UV-1800) at 535 nm, using malonaldehyde (MDA) as standard. The results were expressed as mmol MDA/mg protein. The blank for each concentration comprised the respective concentrations of the tested samples and other reagents. The negative control consisted of the reagents, without incubation with EEGH, fractions, or rutin.

The antioxidant activity was calculated as a percentage of lipid peroxidation inhibition (%I) according to the formula:

$$\%I = [(C_c - C_t)/C_c] \times 100,$$

Where  $C_c$  is the MDA concentration of the negative control and  $C_t$  is the concentration of samples incubated with EEGH and fractions or rutin.

After obtaining %I, the values were used to elaborate graphs of concentration versus inhibition (%I). Through the linear regression analysis, using the least squares method, the line equations were determined and the  $IC_{50}$  was calculated.

### Bioautography Method

The antioxidant activity of EEGH and fractions of *G. hirsutum* was also evaluated by the bioautography method using the free radical DPPH (2,2-diphenyl-1-picryl-hydrazil), as described by Cavin *et al.*, [22]. The samples were prepared in methanol (10 mg/mL). Sixty silica gel plates with fluorescence indicator (UV 254 nm) for TLC were applied 20  $\mu$ L of these solutions, separately, and eluted with the pre-determined mobile phases (FM) for each sample: HFGH [FM = hexane: ethyl acetate (7:3)]; DFGH [FM = dichloromethane: methanol (9.3:0.7)]; EAFGH [FM = ethyl acetate: formic acid: water (9:0.5:0.5)]; and BFGH [FM = butanol: water: acetic acid (8.5:1:0.5)]. To check for the presence of compounds, EEGH was applied to each TLC. After elution, the plates were dried or developed with ferric chloride ( $FeCl_3$ ) or DPPH to detect phenolic and antioxidant constituents. The flow ratio (FR) of the spots was also calculated.

### STATISTICAL ANALYSIS

The results are expressed as mean  $\pm$  standard deviation mean (S.D.M.). Analysis of variance followed by Tukey's honest significant difference test was used to measure the degree of significance for  $p < 0.05$ . The Graph Pad Prism<sup>®</sup> software was used for this analysis.

### RESULTS

#### Yield of the Extraction Process

From 460 g of dried and powdered leaves of *G. hirsutum*, 69.99 g of EEGH was obtained, amounting to an yielded of 15.21%. Using 30 g of EEGH, after fractionation by partition, we obtained 12.60 g (42.0%) of HFGH, 1.47 g (4.9%) of DFGH, 1.19 g (3.97%) of EAFGH, and 2.82 g (9.40%) of BFGH.

#### Total Phenolics and Flavonoids

To determine the total phenolic content of EEGH and fractions, the calibration curve of the gallic acid, with line equation ( $y = 0.1302x + 0.01007$ ) and determination coefficient ( $R^2 = 0.9989$ ), was calculated (Fig-1A). In addition, the calibration curve of rutin, with line equation ( $y = 0.02156x - 0.0966$ ) and determination coefficient ( $R^2 = 0.9992$ ), was obtained to measure the amount of total flavonoids (Fig-1B).

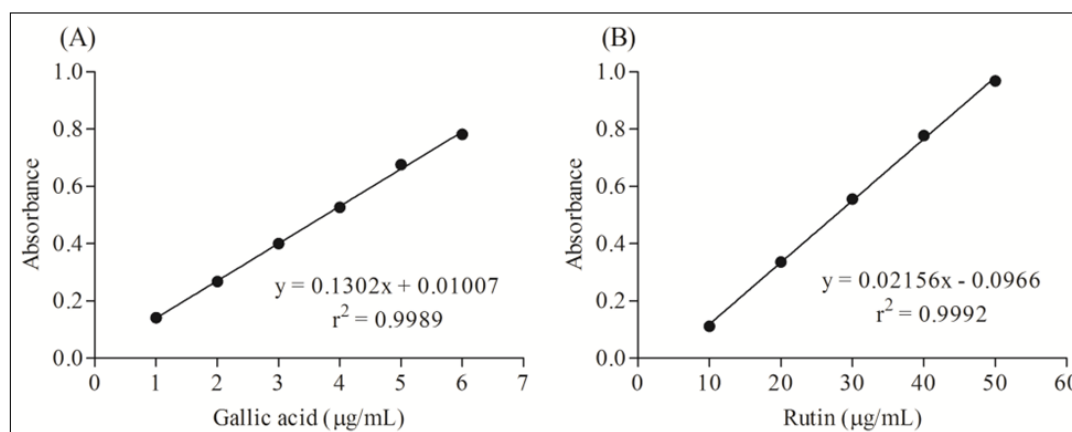


Fig-1: Standard curve of gallic acid (A) and rutin (B)

Total phenolic contents ranged from 1.86–26.49 g/100g in EEGH and fractions of *G. hirsutum* leaves (Table-1). EAFGH and BFGH had higher levels of total phenolics than did other samples. However, we

observed that total flavonoids varied from 0.67 to 7.17 g/100g in the tested samples (Table-1). As noted for total phenolics, flavonoids prevailed in EAFGH (7.17 ± 0.09 g/100g) and BFGH (4.53 ± 0.02).

**Table-1: Total phenolic and flavonoid contents in EEGH and fractions of *Gossypium hirsutum* leaves**

Sample	Total phenolic (g/100 g)	Flavonoids (g/100 g)
EEGH	6.88 ± 0.08	3.92 ± 0.02
HFGH	1.86 ± 0.06	0.67 ± 0.01
DFGH	5.21 ± 0.05	1.96 ± 0.19
EAFGH	26.49 ± 0.45	7.17 ± 0.09
BFGH	10.18 ± 0.05	4.53 ± 0.02

Each value in the table is presented as mean ± S.D.M. (n = 3). The means are different after analysis of variance was applied, followed by the Tukey test for p < 0.05.

**Antioxidant activity**

**Antioxidant activity by inhibiting free radical DPPH• and reducing power**

The antioxidant potential of EEGH and fractions of *G. hirsutum* against the DPPH• radical are shown in Table-2 as IC<sub>50</sub>. The IC<sub>50</sub> values ranged from 24.99 ± 0.16 to 215.48 ± 0.11 µg/mL and were significantly different from each other (p < 0.001). Of the tested samples, EAFGH was more effective in inhibiting the DPPH radical• with lower IC<sub>50</sub> (24.99 ± 0.16 µg/mL). Rutin produced IC<sub>50</sub> equal to 9.80 ± 0.09 µg/mL, justifying its use as a reference compound.

Regarding the FRAP method, the IC<sub>50</sub> values of EEGH and fractions are provided in Table-2, which ranged from 25.61 ± 0.43 to 166.20 ± 1.01 µg/mL. EAFGH was more active in neutralizing the oxidation of iron, because it had a lower IC<sub>50</sub> (25.61 ± 0.43 µg/mL). As a positive control, rutin produced the lowest IC<sub>50</sub> (8.10 ± 0.54 µg/mL), demonstrating its antioxidant potency.

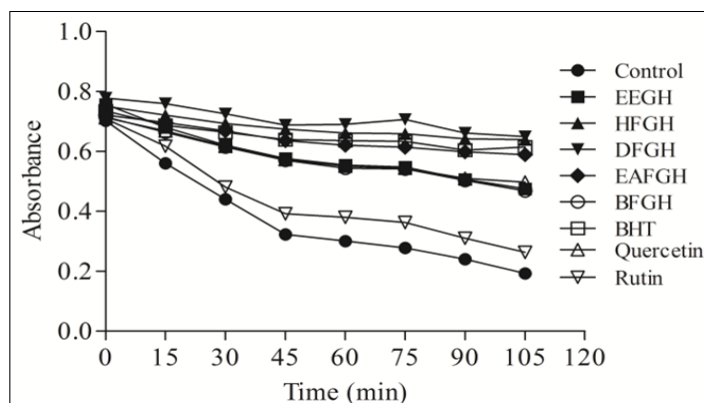
**Table-2: Antioxidant activity of EEGH and fraction of *Gossypium hirsutum* leaves by DPPH and FRAP methods**

Sample	IC <sub>50</sub> (µg/mL)	
	DPPH	FRAP
EEGH	71.76 ± 0.54	53.49 ± 0.52
HFGH	215.48 ± 0.11	166.20 ± 1.01
DFGH	81.78 ± 0.52	109.42 ± 0.51
EAFGH	24.99 ± 0.16	25.61 ± 0.43
BFGH	32.52 ± 0.50	33.57 ± 1.92
Rutin	9.80 ± 0.09	8.10 ± 0.54

Each value in the table is presented as mean ± S.D.M. (n = 3). The means are different after analysis of variance was applied, followed by the Tukey test for p < 0.05.

**Inhibition of Lipid Peroxidation by Co-Oxidation of β-Carotene/Linoleic Acid**

Figure-2 shows the decay of absorbances as a function time of EEGH and fractions at t<sub>0</sub>, t<sub>15</sub>, t<sub>30</sub>, t<sub>45</sub>, t<sub>60</sub>, t<sub>75</sub>, t<sub>90</sub>, and t<sub>105</sub> against the co-oxidation of β-carotene/linoleic acid (n = 3). In this figure, HFGH shows less variation in decay, indicating greater lipid peroxidation inhibition. Of the positive controls (BHT, rutin, and quercetin), BHT is the most active in response inhibition.



**Fig-2: Decay of absorbance as a function of time of EEGH and fractions of *Gossypium hirsutum* leaves by the co-oxidation β-carotene/linoleic acid method**

EEGH and fractions of *G. hirsutum* leaves (250 µg/mL), as well as positive controls (25 µg/mL), yielded values between 5.82% and 82.18% (Table-3). It was evidenced that HFGH (%I = 82.18) was the most

effective compound among the tested samples, being equal to BHT (%I = 81.41 ± 0.84) (p < 0.001). DFGH and EAFGH showed a significant inhibition, with %I equal to 71.21% and 71.79%, respectively.

**Table-3: Antioxidant activity of EEGH and fractions of *Gossypium hirsutum* leaves by the co-oxidation of  $\beta$ -carotene/linoleic acid method**

Sample	%I
EEGH	51.02 ± 1.92 <sup>a</sup>
HFGH	82.18 ± 1.62 <sup>b</sup>
DFGH	71.21 ± 1.07 <sup>c</sup>
EAFGH	71.79 ± 0.60 <sup>c</sup>
BFGH	42.97 ± 2.58
Rutin	5.82 ± 0.81
Quercetin	60.54 ± 2.13 <sup>a</sup>
BHT	81.41 ± 0.84 <sup>b</sup>

Each value in the table is presented as mean ± S.D.M. (n = 3). The means followed by the same letter do not differ after analysis of variance followed by the Tukey test for p < 0.05.

**Inhibition of Lipid Peroxidation by Thiobarbituric Acid**

The antioxidant activity of EEGH and fractions of *G. hirsutum* leaves was measured using the TBA method. The IC<sub>50</sub> values ranged from 134.10 ± 8.20 to 388.20 ± 0.83 µg/mL (Table-4). HFGH was more effective in inhibiting lipid peroxidation, because it presented a lower IC<sub>50</sub> (p < 0.001). Of the reference compounds, BHT was more active (IC<sub>50</sub> = 27.23 ± 0.84 µg/mL).

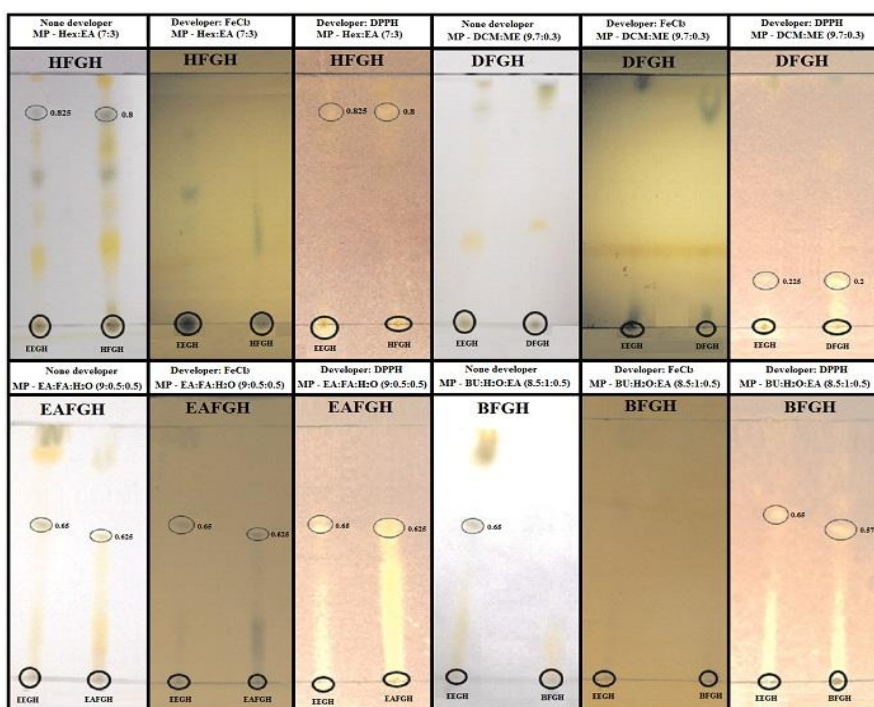
**Table-4: Antioxidant activity of EEGH and fractions of *Gossypium hirsutum* leaves by the thiobarbituric acid method**

Sample	IC <sub>50</sub> (µg/mL)
EEGH	243.28 ± 3.83
HFGH	134.10 ± 8.20
DFGH	388.20 ± 0.83
EAFGH	205.9 ± 0.55
BFGH	226.61 ± 2.67
Rutin	40.34 ± 0.09 <sup>a</sup>
Quercetin	31.78 ± 0.39 <sup>a,b</sup>
BHT	27.23 ± 0.84 <sup>b</sup>

Each value in the table is presented as mean ± S.D.M. (n = 3). The means followed by the same letter do not differ after analysis of variance followed by the Tukey test for p < 0.05.

EEGH and fractions of *G. hirsutum* leaves were applied to TLC plates and developed with 5% FeCl<sub>3</sub> and 2.5 mM DPPH. The dark spots revealed with FeCl<sub>3</sub> are indicative of the presence of phenolic compounds, whereas the yellowish-white spots revealed with DPPH are suggestive of compounds that have antioxidant potential because of their ability to scavenge free radicals (Fig-3).

Considering EEGH and EAFGH, phenolic constituents and presence of antioxidants were evidenced in these samples. EEGH produced FR of 0.650 and EAFGH produced an FR of 0.625, indicating that they are the same class of compounds. The presence of phenolic compounds and antioxidants were also detected in HFGH, DFGH, and BFGH (Fig--3).



**Fig-3: Chromatograms of EEGH and fractions of *Gossypium hirsutum* leaves revealed with ferric chloride and DPPH.**

## DISCUSSION

This study evaluated the antioxidant activity of EEGH and fractions of *Gossypium hirsutum* leaves, a plant of economic importance in the textile industry. Popularly, the species is used to treat inflammation, infections, wounds, uterine bleeding, amenorrhea, menopausal disorders, and sexual impotence [6]. *G. hirsutum* contains alkaloids, phenolic compounds, terpenoids, tannins, saponins, flavonoids, cardiac glycosides, and proteins [6,11].

Our results showed that EEGH and fractions of *G. hirsutum* leaves are rich in phenolic compounds, especially flavonoids, with emphasis on EAFGH and BFGH that produced expressive levels of these constituents (Table-1). A study by Ayeni et al. [12] showed that the leaves of *G. hirsutum* contain  $1.62 \pm 0.00$  mg/100 g of total phenols and  $11.90 \pm 0.14$  g/100 g of total flavonoids. However, these authors have not described the time of collection and the procedures for preparing the analyzed material, which makes it difficult to make a comparison between the results presented. It is worth mentioning that the differences in the contents of natural compounds can be based on biotic and abiotic factors, such as seasonality, water availability, ultraviolet radiation, and extraction processes, which can influence the chemical composition of plants [23].

On the contrary, the liquid-liquid partition fractionation process, using a solvent with increasing polarity (hexane, dichloromethane, ethyl acetate, and butanol), separates the constituents by chemical classes according to the solvent affinity. The low levels of total phenolics and flavonoids in HFGH (1.86 g/100 g and 0.67 g/100 g, respectively) are due to the lack of affinity of these constituents for the solvent, being suitable for extraction of steroids, terpenes, and acetophenones [14]. In DFGH, methoxylated flavonoids are extracted, whereas in EAFGH, free flavonoids are extracted. Glycosylated flavonoids are found in BFGH [14].

Considering the results of the antioxidant activity, EEGH and fractions of *G. hirsutum* leaves were effective in inhibiting free radicals via different mechanisms. In the DPPH and FRAP assays (Table-2), EAFGH, the fraction with higher content of total phenolics and flavonoids, produced lower  $IC_{50}$  (24.99 and 25.61  $\mu$ g/mL, respectively), showing its ability to sequester free radicals and reduce  $Fe^{3+}$  ions through electron donation. Flavonoids are oxidized by radicals, become more stable and less reactive [24]. Thus, the radicals are inactivated due to the high reactivity of the hydroxyl group of the flavonoids. Another proposed mechanism is the chelation of metal ions, which can initiate the production of hydroxyl radicals ( $OH^\bullet$ ) by the Fenton reaction [25]. This reaction consists of the interaction between  $Fe^{2+}$  and hydrogen peroxide ( $H_2O_2$ ) that generates  $OH^\bullet$  radicals with a high oxy-reduction potential, attacking species in the reactive medium. In

addition, as EAFGH contain free flavonoids, these compounds will have less steric impediment, leading to greater antioxidant activity. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical called peroxynitrite [25]. Epicatechin and rutin are also powerful radical scavengers. The scavenging ability of rutin may be due to its inhibitory activity on the enzyme xanthine oxidase. By scavenging radicals, flavonoids inhibit LDL oxidation in vitro. This action protects the LDL particles and, theoretically, flavonoids may have preventive action against atherosclerosis [24, 26].

Tests for assessing the lipid peroxidation inhibition using the  $\beta$ -carotene/linoleic acid co-oxidation system and determination of thiobarbituric acid reactive substances (TBARS) reproduce oxidative stress, such as oxidation of unsaturated lipids of cell membranes, which causes the destruction of structure, consequently altering homeostasis, which can cause cell death [27]. Our results showed that HFGH, EAFGH, and DFGH significantly delayed lipid peroxidation, especially HFGH (Tables 3 and 4). The stages of lipid peroxidation include initiation, propagation, and termination. Therefore, antioxidants act by inhibiting the initiation of the process by neutralizing reactive oxygen species, as well as inhibiting the spread of lipid peroxidation by neutralizing the peroxy radicals formed during oxidation [27]. The promising activity of HFGH is probably due to the affinity of lipophilic molecules for lipid interfaces that favors their antioxidant action, while the more hydrophilic substances can be dispersed in the aqueous medium, which reduces their activity [19]. Nonpolar antioxidants are more effective in emulsions because they are retained in the oil droplets and/or accumulated at the oil-water interface, where the interaction between hydroperoxides and pro-oxidizing substances in the aqueous phase occurs. By contrast, polar substances are more effective in large amounts of oil as they can accumulate at the air-oil interface or in reverse micelles within it, where lipid oxidation reactions occur more frequently due to the high concentration of oxygen [27, 28].

The results of the bioautography confirm the data demonstrated by the DPPH and FRAP methods. In addition, it appears that there is a relationship between phenolic components and antioxidant activity, because the FR values presented by EEGH and fractions showed antioxidant activity (Developer—DPPH) and phenolic compounds (Developer— $FeCl_3$ ).

## CONCLUSIONS

The results showed that the leaves of *G. hirsutum* contain expressive phenolic content, especially flavonoids, highlighting the ethyl acetate fraction. EEGH and fractions of *G. hirsutum* leaves showed antioxidant activity by scavenging the free radicals from DPPH, reducing iron, inhibiting lipid

peroxidation, and bioautography. Therefore, the results indicate that *G. hirsutum* leaves are a promising source of compounds with antioxidant action, which may justify their therapeutic properties in disorders associated with oxidative stress.

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