Original Research

Phytochemical profile and a preliminary *in ovo* screening of the ethanolic extract of *Coffea arabica* green seeds

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Abstract

Aims. *Coffea arabica* has garnered significant attention for its potential therapeutic applications due to its rich phytochemical profile, including chlorogenic acids, flavonoids, and alkaloids like caffeine, which are associated with antioxidant, anti-inflammatory, and antimicrobial effects. This study aimed to (i) evaluate the phytochemical composition of an ethanolic extract derived from green seeds of *Coffea arabica* and (ii) perform a preliminary *in ovo* screening to determine its mucosal tolerability, irritant potential, and pro-angiogenic activity using the chick embryo chorioallantoic membrane (CAM) model.

Methods. An ethanolic extract was prepared from *C. arabica* green seeds and subjected to LC-MS analysis for the identification and quantification of polyphenols. The antioxidant capacity was assessed through the DPPH radical-scavenging assay, comparing extract performance to ascorbic acid as a standard. *In ovo* testing was conducted using the HET-CAM assay to observe potential irritation on the CAM surface and to evaluate angiogenic activity. Chick embryos were monitored for vascular changes, irritant signs (hemorrhage, lysis, coagulation), and overall tolerability post-application over six days.

Results. Phytochemical analysis confirmed the presence of chlorogenic acid and 4-O-caffeoylquinic acid in the extract. The extract exhibited substantial antioxidant activity (66.38%), though slightly lower than that of ascorbic acid (97.36%). In the HET-CAM assay, no signs of irritation or toxicity were observed, and the extract was well tolerated for up to six days post-application. Additionally, the extract promoted angiogenesis, with increased vascularization observed, suggesting a stimulatory effect on neovascularization without inducing tissue damage.

Conclusions. The ethanolic extract of *Coffea arabica* green seeds demonstrates promising antioxidant and pro-angiogenic properties, alongside high mucosal biocompatibility. These findings support its potential applications in therapeutic and cosmetic formulations, particularly those targeting tissue regeneration and skin repair. Further studies are recommended to explore the underlying mechanisms and to confirm efficacy in more advanced biological models.

Keywords: Arabica coffee, LC-MS investigation, FT-IR spectroscopy, HET-CAM assay, angiogenesis

Introduction

Coffea arabica, a coffee species recognized for its complex phytochemical profile, has recently attracted attention in both health and cosmetic applications. Extracts from the green seeds of *Coffea arabica* are valued for their high content of bioactive compounds, including chlorogenic acids, flavonoids, and caffeine, recognized for their antioxidant, anti-inflammatory, and anti-aging properties [1,2]. The antioxidant properties of *Coffea arabica* extracts are primarily attributed to their rich phytochemical composition. These compounds are crucial in neutralizing free radicals and mitigating oxidative stress, linked to numerous chronic diseases, such as cardiovascular disease, type 2 diabetes, and neurodegenerative disorders [1-3]. In addition, chlorogenic acid from *Coffea arabica* has been associated with increased insulin sensitivity and regulation of glycaemic levels, which are relevant for diabetes management [4].

In the cosmetic context, *Coffea arabica* is used for its benefits on the skin, such as inhibiting matrix metalloproteinase-1 (MMP-1), an enzyme involved in collagen degradation and skin aging, and stimulating collagen synthesis [5]. *Coffea arabica* extracts also exhibit the effects of reducing tyrosinase activity and inhibiting melanin synthesis, suggesting their use in products intended to even skin tone and reduce hyperpigmentation [5,6].

The anti-inflammatory properties of *Coffea arabica* have also been highlighted in several studies. Dewi and co-workers found that Arabica coffee leaf extracts significantly reduced tumor necrosis factor-alpha (TNF-α) levels in rats exposed to ultraviolet-B rays, indicating their potential to manage inflammatory responses [2]. In addition to the leaves, the roasted beans of *Coffea arabica* also contribute to its antioxidant profile. The roasting process not only enhances the flavor but also alters the chemical composition, potentially increasing the bioavailability of certain antioxidants [7]. The presence of flavonoids and other polyphenolic compounds in the extracts of *C. arabica* contributes to its anti-inflammatory effects, making it a candidate for treating inflammatory diseases [2,8]. Additionally, the extracts have shown promise in reducing lipid peroxidation, which is a key factor in the pathogenesis of various chronic diseases, including cardiovascular disorders [8].

The extraction method is essential for obtaining an optimal concentration of bioactive compounds in *Coffea arabica* extracts, thus influencing their cosmetic potential. Ethanol, a commonly used solvent for the extraction of phytochemical substances, allows efficient extraction of a wide range of polar and non-polar compounds [9]. Optimization of extraction parameters, such as time and temperature, is crucial for maximizing the concentrations of polyphenols and flavonoids, compounds involved in antioxidant and anti-inflammatory activity [10]. The volatile compounds of *Coffea arabica*, including 2,3-dimethylpyrazine and pentan-2-ol, contribute to the specific sensory profile of coffee and are influenced by the extraction method, geographical origin, and roasting process [11,12]. Therefore, *Coffea arabica* provides a valuable source of bioactive compounds with health and cosmetic applications, and optimization of extraction methods can significantly enhance the efficiency of these extracts, making *Coffea arabica* a versatile ingredient for therapeutic and personal care products.

The green seeds of *Coffea arabica* are an important source of phytochemical compounds, which give them multiple applications in dermato-cosmetic formulations. Their chemical composition includes a wide range of bioactive substances: carbohydrates (59-61%), lipids (11-17%), proteins (10-16%), phenolic compounds (6-10%) including chlorogenic acids, which are responsible for antioxidant and anti-inflammatory effects, minerals (4%) contributing to the optimal functioning of the skin and scalp, fatty acids (2%) which help maintain the integrity of the skin barrier, caffeine (1-2%) with stimulating effects on blood circulation and reducing inflammation, trigonelline (1%), with a role in protecting cells from oxidative stress [13].

The compounds contained by *Coffea arabica* green seeds are of particular interest in dermatocosmetics, where antioxidants may help prevent and reduce the negative effects of oxidative stress, a factor that accelerates skin aging and favors hyperpigmentation [5,14]. For instance, a study carried out by Sulaeman and colleagues demonstrated that topical application of Arabica coffee bean extract cream inhibited tyrosinase activity, thereby reducing melanin production in guinea pigs exposed to UVB radiation, which emphasizes the protective effect of this extract against photo-aging [15]. This is particularly relevant in the context of skin health, where antioxidants can play a crucial role in preventing photo-aging. Similarly, other research work has demonstrated that various components of the *Coffea arabica* plant, including the husk pulp, often considered as a by-product, possess high antioxidant activity and can be incorporated into cosmetic products to protect against the harmful effects of UV radiation [16]. Moreover, the phytochemical composition of *Coffea arabica* beans is influenced by factors such as the roasting process, which can significantly alter the polyphenol content, enhancing the antioxidant capacity of the final product [17,18]. Understanding the relationship between phytochemical composition and sensory properties is vital for the development of high-quality coffee products. Muchtaridi and co-workers highlighted that different extraction methods yield varying concentrations of bioactive compounds, which in turn affect the antioxidant activity [19].

In the context of the above, the present study proposes to examine the phytochemical profile of an ethanolic extract from green *Coffea arabica* seeds,

focusing on the identification and quantification of phenolic compounds and antioxidant capacity, thus contributing to the understanding of its potential value in health and skin care applications. In addition, to assess the *in vivo* safety of the ethanolic extract, the research includes the HET-CAM assay, a semi-quantitative method used to analyze the inflammatory responses of the chick embryo chorioallantoic membrane (CAM) suitable to evaluate the tissue response to various chemicals.

Methods

Chemicals and reagents

For obtaining the ethanolic extract of *Coffea arabica* green seeds, ethanol 95% was used, acquired from Girelli Alcool (Milano, Italy).

For LC-MS analysis, we used the following standards, purchased from Sigma-Aldrich (St. Louis, MO, USA), namely phenolic acids (chlorogenic acid and 4-O-caffeoylquinic acid). Methanol and acetic acid were purchased from Merck KGaA (Darmstadt, Germany) and ultrapure deionized water was supplied by a MiliQ system Milli-Q® Integral Water Purification System (Merck Millipore, Darmstadt, Germany). For the determination of total phenolic content, were used chlorogenic acid 99% from Sigma-Aldrich (St. Louis, MO, USA), and Na_2CO_3 99%, acquired from Roth (Dautphetal, Germany). The Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany).

To evaluate the antioxidant capacity, 2,2-diphenyl-1-picrylhydrazil (DPPH) acquired from Sigma Aldrich (Steinheim, Germany) was used, and for the comparison of the results, ascorbic acid (vitamin C), acquired from Lach-Ner Company (Prague, Czech Republic) was utilized as standard. All the chemicals used in the present research were of analytical purity.

For the hen's egg test–chorioallantoic membrane (HET-CAM) method, sodium lauryl sulfate (SLS) was used as the positive control, acquired from Merck (Darmstadt, Germany), and distilled water, was used as the negative control.

Extraction protocol

The raw material used to obtain this extract *(Coffea arabica*) was acquired from a local natural products store Ethiopia Sidamo-Hotspot. In brief, 300 g green seeds of *C. arabica* were weighed using the JT302N analytical balance and were triturated with the IKA A11 basic equipment. After trituration, 1000 mL of ethanol 95% (EtOH 95%) was added and the mixture was left to macerate for 24 h at 22 ± 2°C. After that, the mixture was sonicated for 30 min. using an ultrasonic water bath (ELMA S120 Elmasonic from Elma Schmidbauer GmbH, Singen, Germany), followed by a filtration procedure using the Whatman Grade 4 filter. The extract was subjected to concentration using a rotary evaporator (Laborata 4000eco from Heidolph Instruments, GmbH & Co. KG, Schwabach, Germany), at 97 mbar and 35°C. The initial parameters set were: water bath temperature at 35°C, 40 rpm, refrigerant water at 10°C, and starting pressure at 300 mbar. The separation operation was repeated twice, and it used 2 x 1000 mL ethanol 95% (EtOH 95%) respectively; for further reference, this crude extract was coded CA-EtOH. The extract obtained and tested in the current research (29.20 g), was stored in the refrigerator at 4°C. The extraction yield (9.73%) was calculated by dividing the amount of this crude extract by the initial amount of plant material, followed by multiplying by hundred. The schematic protocol is presented in figure 1.

Phytochemical profile

1. Liquid chromatography mass spectrometry (LC-MS) analysis

High-performance liquid chromatography was coupled with mass spectrometry (LC/MS) and was used to identify and quantify the polyphenols present in the ethanolic extract of *C. arabica* green seeds. The previously validated described method [20-22] was applied using an Agilent 1100 Series HPLC system coupled with an Agilent 1100 SL Series Ion Trap mass spectrometer from Agilent Technologies (Santa Clara, CA, USA). A Zorbax SB-C18 (100 x 3.0 mm, 3.5 µm particle size) reversed-phase analytical column was used for phase separation at a working temperature of 48°C.

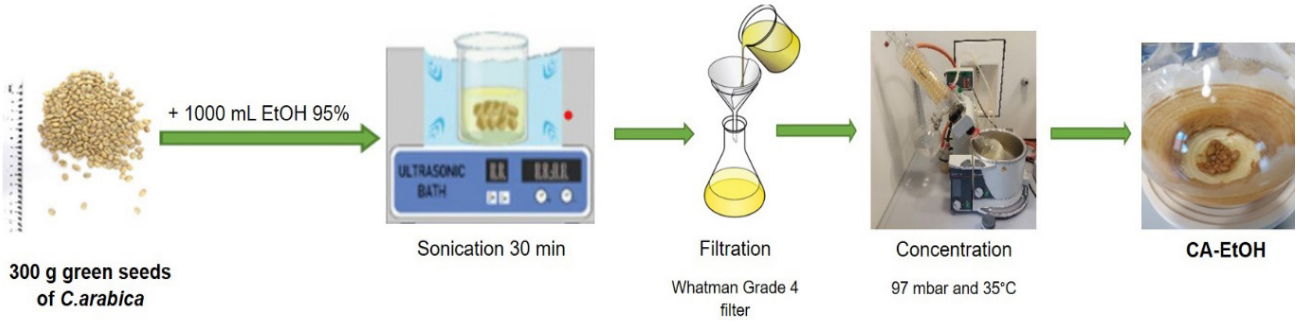

Figure 1. The main steps involved in the obtainment of the tested extract.

Methanol and 0.1% acetic acid form the binary gradient used as the mobile phase. The method uses a flow rate of 1 mL/min and an injection volume of 5 µL for 35 min. Detection is done by comparing retention times and UV and MS spectra with pure standards using UV and MS modes. Polyphenol concentration was determined according to a calibration curve of the standards, ranging from 0.1 to 50 µg/mL, with good linearity (R^2 = 0.999), and the results were represented as µg polyphenols/mL *C. arabica* extract.

2. Total polyphenolic content

The total phenolic content of green *Coffea arabica* seeds ethanolic extract was determined using the Folin-Ciocalteu reagent and chlorogenic acid as standards [23]. About 2 mg of the ethanolic extract was weighed in 15 mL plastic extraction tubes and vortexed with 1 mL of the extraction solvent (40 mL acetone : 40 mL methanol : 20 mL distilled water : 0.1 mL acetic acid). Then, the samples with the extraction solvent were heated at 60°C (water bath) for 1 h, allowed to cool to room temperature, and homogenized for 30 s with a sonicator. About 200 µL (three replicates) were introduced into screw-cap test tubes; 1.0 mL of Folin–Ciocalteu's reagent and 1.0 mL of sodium carbonate (7.5%) were added. The tubes were vortexed and allowed to stand for 2 h. The absorption at 726 nm was measured (Agilent BioTek Synergy H1 Hybrid Multi-Mode Reader) and the total phenolic content was expressed as mg of chlorogenic acid equivalents per g of dry material.

3. Fourier-transformed infrared spectroscopy (FT-IR)

The organic functional groups of the chemical molecules contained in *Coffea arabica* ethanolic extract were investigated through the qualitative method FT-IR spectroscopy, using the Prestige-21 spectrometer (Shimadzu, Duisburg, Germany). The extract was mixed with KBr until a pellet was obtained and further analyzed at room temperature (22 ± 2 °C) in the spectral region from 4000/cm to 400/cm, with a resolution of 4/cm.

The spectra were interpreted based on the match between the recorded absorption bands of the ethanolic extract of *Coffea arabica* green seeds at a specific wavenumber and the absorption band frequencies contained in the electronic library [24].

4. X-ray fluorescence (XRF) analysis

The mineral content in *C. arabica* ethanolic extracts was determined by XRF analysis, using the HHXRF spectrometer (XMET8000 series) from Hitachi, Chiyoda, Japan, equipped with a wavelength-dispersive X-ray fluorescence. The extract was measured with the portable stand to avoid scattered radiation against the user [25-27], and the elementary composition of the measured extract was calculated based on the principles of physics.

5. Antioxidant capacity

The ethanolic extract of *Coffea arabica* green seeds (Ca-EtOH) was investigated in terms of antioxidant capacity using DPPH free radical-scavenging assay [28]. Six different concentrations (1 mg/mL, 0.8 mg/mL, 0.5 mg/mL, 0.3 mg/mL, and 0.1 mg/mL) of this extract were prepared and tested. First, a 0.1 mM DPPH ethanolic solution was prepared and stored at 4°C until further use. As a standard, the ethanolic solution of ascorbic acid was used (1 mM in 95% EtOH). The analysis consisted of measuring a volume of 0.3 mL of each test sample, mixed with 2.7 mL of 0.1 mM DPPH ethanolic solution, and the entire mixture was analyzed spectrophotometrically at a wavelength of 517 nm in a quartz test cuvette (10 × 10 mm). By using the UviLine 9400 Spectrophotometer from SI Analytics (Mainz, Germany), were read in a continuous mode the absorbance, for 20 min.

We expressed the results obtained as the EC_{50} value, meaning the half-maximal inhibitory concentration of the antioxidants contained in the extract ethanolic of *Coffea arabica*, needed to scavenge the DPPH free radicals present in the test solutions with 50%. The antioxidant capacity(AOC%) was determined by using the following equation:

$$AOC\ [\%] = \left[\frac{A_{DPPH} - A_{ethanolic\ extract}}{A_{DPPH}}\right] \times 100$$

where: A_{DPPH} – the absorbance of the free radical DPPH (blank), measured at 517 nm; $A_{ethanolic\ extract}$ – the absorbance of the ethanolic extracts, measured at 517 nm, in the presence of DPPH radical.

The irritability factor of ethanolic extract of *Coffea arabica* green seeds by HET-CAM method

1. Chorioallantoic membrane testing

An *in vivo* evaluation was designed to investigate the potential irritability and tolerability on mucosa-like tissues as well as to assess a possible effect on the angiogenesis process of the chick embryo chorioallantoic membrane. To this end, the *in ovo* chorioallantoic membrane (CAM) test was used [29,30]. The basic protocol involves the use of fertilized chicken (*Gallus gallus domesticus*), following an adapted procedure [31,32]. Briefly, after incubation of the eggs in a humidified atmosphere at 37°C, on the fourth day of incubation, an opening is made in the upper shell to expose the developing CAMs to the test samples. All experimental procedures involving the use of stereomicroscopy (ZEISS SteREO Discovery.V8, Göttingen, Germany), and relevant captures were stored. Image acquisition and processing were performed by Axiocam 105 color, AxioVision SE64. Rel. 4.9.1 Software, (ZEISS, Göttingen, Germany), ImageJ (ImageJ Version 1.54i, https://imagej.nih.gov/ij/index.html, accessed 7 April 2024) and GIMP software (GIMP 2.10.36 revision 1, https://www.gimp.org/, accessed 7 April 2024). All images were captured at a resolution of 2560 × 1920 pixels. For more clarity, we also added scale bar details in the captions.

2. Irritation potential using the HET-CAM assay

Using the HET-CAM protocol [33] adapted to our laboratory conditions [31], we investigated the potential irritative effects of the ethanolic extract of *C. arabica*. Semi-quantitative evaluation allows the assessment of the irritation potential of the ethanolic extract by a protocol that requires stereomicroscopic observation of vascular changes, such as hyperemia, hemorrhage, and coagulation, during 300 seconds after application. For this purpose, a volume of 300 µL of extracts at a concentration of 400 µg/mL was applied to CAMs on day 10 of incubation. During the 5-minute monitoring of the treated CAM, the time when damages were first happening was noted in seconds, thus allowing to calculate the irritability score using the following equation:

$$IS = 5 \times \left[\frac{301 - \text{Sec H}}{300}\right] + 7 \times \left[\frac{301 - \text{Sec L}}{300}\right] + 9 \times \left[\frac{301 - \text{Sec C}}{300}\right]$$

where: Sec H (hemorrhage) = first appearance (in seconds) of bleeding reactions on the membrane, Sec L (lysis) = first appearance (in seconds) of vessel lysis on the membrane, and Sec C (coagulation) = first appearance (in seconds) of coagulation formation on the membrane. Sodium lauryl sulfate (SLS) was included as the positive control, while distilled water (H_2O) was the negative control, and a solvent control, DMSO at a concentration of 0.5%, was also included. Values were evaluated according to the scale established by Luepke [34].

3. Assessment of angiogenesis using the CAM assay

Ethanolic extract of *C. arabica* was also subjected to *in vivo* evaluation for possible effects on the angiogenesis process. We tested the extracts at a concentration of 400 µg/mL applied inside plastic rings pre-placed on top of developing CAMs, starting from day 8 of incubation [35]. We monitored the application areas daily using stereomicroscopy and relevant captures were recorded, allowing further investigation of the changes that occurred during treatment with *C. arabica* extract. DMSO in 0.5% concentration was used as the control solvent. The evaluation was performed daily for 72 hours – post-treatment. All experiments were performed in triplicate. Data were presented as mean ± standard deviation (SD).

Results and discussion

Phytochemical Profile

1. LC-MS analysis

For the total extract from *C. arabica* green seeds, the chromatogram revealed the presence of 3 peaks (Figure 2), of which the identity of chlorogenic acid and 4-O-caffeoylquinic acid could be confirmed in comparison with reference standards.

HPLC analysis of the ethanolic extract (CA-EtOH) of *Coffea arabica* green seeds yielded a low concentration of chlorogenic acid, below 4.6 µg/mL. Although it is a commonly used solvent in plant extraction due to its safety and ability to solubilize a variety of bioactive compounds, its medium polarity makes it more effective for more polar compounds and less effective for hydrophobic polyphenols such as chlorogenic acid.

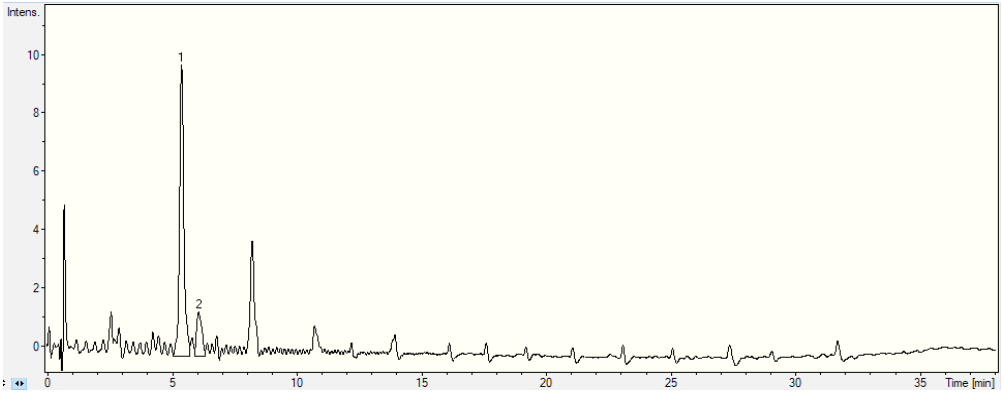

Figure 2. HPLC chromatogram of ethanolic extract from *C. arabica* green seeds.

Table I. Results of identification of compounds present in ethanolic extract by HPLC technique.

No. on the chromatogram	Compound	UV identifier	Qualitatively identified MS	Concentration in the extract (µg/mL)	Concentration in the extract (mg/g)
1	Chlorogenic acid	Yes	Yes	4.617±0.646	0.004617±0.646
2	4-O caffeoylquinic acid	Yes	Yes	0.813±0.032	0.000813±0.032

This can be explained by its low affinity for hydrophobic groups in the chlorogenic acid structure, which favors semi-polar solvents such as butanol and ethyl acetate. The main goal of the research was to obtain a crude extract aimed as a starting point for further bio-guided fractionations, containing compounds with a larger array of polarities.

The extraction methods used for the isolation of phytochemicals from *Coffea arabica* play a crucial role in determining the yield and bioactivity of extracts. Various solvents including ethanol, methanol, and dichloromethane have been used to extract bioactive compounds from coffee leaves and seeds [5]. Recent studies have demonstrated that ultrasound-assisted extraction techniques can optimize the extraction process, increasing the yield of phenolic compounds and improving the overall antioxidant activity of the extracts [11,12].

Studies on *Coffea arabica* green seed extracts emphasize the importance and versatility of chromatographic methods, in particular HPLC, for the determination of bioactive compounds such as chlorogenic acids, caffeine, flavonoids, isoflavones, and lignans. A study conducted by Monteiro and co-workers quantified chlorogenic acids in green seeds of *Coffea arabica* from Brazil using HPLC, reporting a range between 6.1 ± 0.7 and 6.6 ± 1.1 g/100 g of dry coffee. This analysis indicates a significant amount of chlorogenic acids, suggesting that chromatographic methods provide an accurate and reproducible estimation of these bioactive compounds in coffee. The study investigated the content of 5-CGA and chlorogenic acids in general, finding between 3.5% and 7.5% of dry mass chlorogenic acid content by origin [36]. Craig and co-workers further analyzed green coffee extracts obtained by two extraction methods: supercritical CO_2 extraction and ethanolic extraction, obtaining a total chlorogenic acid content of about 45% w/w. The ethanolic method, in this case, revealed a significant concentration of bioactive compounds, suggesting that the method is suitable for the efficient extraction of chlorogenic acids [37]. A new HPLC-MS/MS method was developed to simultaneously determine six polyphenolic compounds in green coffee beans from different geographical origins. The optimal extraction procedure involved acid hydrolysis at pH 2, an extraction temperature of 60°C, and a solvent of 70% ethanol. This method provided high recovery percentages and identified hyperoside as the most abundant compound [38]. Studies continued to explore other analytical and extraction methods. For example, two chromatographic methods, high-performance TLC (HPTLC) and HPLC, were compared for the separation and quantification of chlorogenic acid in green coffee seed extracts. Both methods showed no statistically significant differences in quantitative determination, indicating that either method could be effectively used for this purpose [39]. Alternative methods, such as microwave-assisted extraction (MAE), have also been studied for coffee oil extraction, showing advantages over the traditional Soxhlet method in higher yield and reduced extraction time [40].

2. Total polyphenolic content (TPC)

Analysis of polyphenols in ethanolic extract (CA-EtOH) of *Coffea arabica* green seeds showed a relatively low total phenolic content (TPC) of 122.06 mg chlorogenic acid equivalents (CAE)/g of extract, highlighting the limitations of ethanol as a solvent for efficient extraction of polyphenols from this matrix. Compared to semi-polar solvents such as ethyl acetate, ethanol demonstrates a lower extraction capacity for phenolic compounds, especially those with hydrophobic characteristics, such as chlorogenic acid. This observation is in agreement with the polarity profile of ethanol, a moderately polar solvent, which is more efficient for polar and slightly polar compounds. In conclusion, although ethanol provides a safe and versatile solution for the overall extraction of phytochemicals from *Coffea arabica*, for obtaining maximum polyphenol concentrations, the use of semi-polar solvents is more recommended.

Studies on the ethanolic extract from the green seeds of *Coffea arabica* emphasize the high content of polyphenols, in particular chlorogenic acid, which contributes to the increased antioxidant activity and potential health benefits of green coffee. These compounds are affected by several factors, including environmental conditions, such as altitude and light, and post-harvest processing methods, which can influence the chemical composition and stability of polyphenols in green coffee beans [41]. Cwiková et al. evaluated the impact of roasting on polyphenols and antioxidant activity in coffee, demonstrating by UV-VIS spectrometry that both green and lightly roasted coffee retained high levels of polyphenols, ranging from 49.19 ± 0.70 g to 74.05 ± 0.28 g for green coffee and from 38.34 ± 1.26 g to 59.79 ± 1.45 g for light roasting. Light roasting also retains relatively high antioxidant activity (up to 78.55 ± 0.89% in inhibition of DPPH radicals), in contrast to medium and intense roasting, which significantly reduces polyphenols [42]. Regarding processing methods, Cwiková's study found no significant differences in chlorogenic acid content between wet and dry processing methods, contrary to the findings of Balyaya et al. who reported decreases in wet processing [43]. Thus, the impact of processing on polyphenols may vary, but these differences could depend on regional and methodological variations. Studies on total polyphenol composition (TPC) also show conflicting results. For example, Cwiková et al. reported that roasting did not significantly influence TPC, while Król et al. showed that light and medium roasting increased the level of these compounds [17]. This variability may result from analyzing different matrices (silver husk in Bresciani's study *vs.* whole seeds in Cwiková's). In conclusion, the ethanolic extract of green *Coffea arabica* seeds remains rich in polyphenols and antioxidant active.

Although roasting is essential for flavor, it tends to reduce these benefits, except for light roasting.

3. FT-IR spectroscopy

By complementing the polyphenolic profile assessed by LC-MS analysis, FT-IR spectroscopy was used to express functional group fingerprints. Figure 3 depicts the FT-IR spectra of *C. arabica* extract (Ca-EtOH), resulting from the signals recorded by each molecule contained in this extract at a given wavenumber.

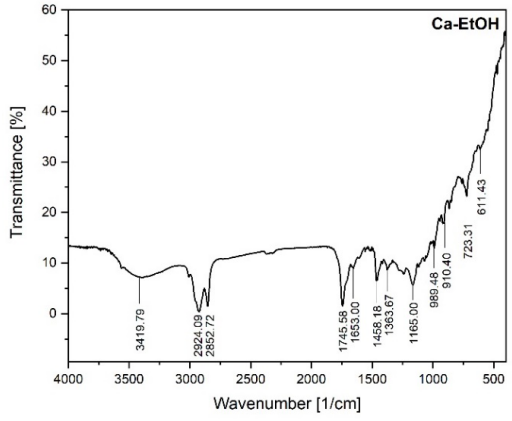

Figure 3. FT-IR spectra of *C. arabica* ethanolic extract.

Table II details the peak values and functional groups recorded in the FT-IR spectra.

One can observe that the most intense absorption bands are recorded at 3419/cm, 2924.09/cm, and 2852.72/cm, attributed to the O-H stretching vibration of functional groups in alcohols (intermolecular H-bonds), as well as to the C-H stretching vibration of functional groups in alkanes. Another intense absorption band is localized around 1745.58/cm and corresponds to the C=O stretching vibration of aldehyde, cyclopentanone, ester, or δ-lactone functional groups. The rest of the recorded bands are of medium intensity as follows: the alkene or conjugated alkene functional groups are emphasized by the presence of the C=C stretching vibration recorded at 1653.00/cm; the alkane functional groups are emphasized by the presence of the C-H bending vibration recorded at 1458.18/cm. The alcohols and phenols contained in the ethanolic extract are highlighted by the presence of O-H bending vibration functional groups recorded at 1363.67/cm. The band located at 1165.00/cm could be attributed to the C-O stretching vibrational functional groups in either esters or tertiary alcohols. At wavelengths 989.48/cm and 910.40/cm, the alkene functional groups, represented by C-H (monosubstituted) or C=C (monosubstituted or disubstituted) bending vibration, are located. At the same time, the peak recorded at wavenumber 910.40/cm could represent the O-H bending vibration functional groups in carboxylic acids. The peak recorded at 723.31/cm could be attributed to either the C-H bending vibrational functional groups in aromatic compounds or the C=C bending vibrational functional groups in (cis-disubstituted) alkene. The peak recorded at 611.43/cm is attributed to acetylenic C-H acetylenic C-H bending vibration functional groups in alkenes.

Table II. The peak values and functional groups recorded in the spectrum for ethanolic extract of *C. arabica.*

Ca-EtOH extract		
Wavenumber	**Functional groups**	**Bond**
3419.79	alcohol	OH stretch H bonded
2924.09	alkane/alcohol (acid)	C-H stretch/ O-H stretch
2852.72	alkane	C-H stretch
1745.58	aldehyde/ cyclopentanone/esters/δ-lactone	C=O stretch
1653.00	alkene/conjugated alkene	C=C stretch
1458.18	alkane	C-H bend
1363.67	alcohol/phenol	O-H bend
1165.00	esters/tertiary alcohol	C-O stretch
989.48	alkene	C-H bend (monosubstituted)/ C=C bend (monosubstituted)
910.40	alkene carboxylic acid	C-H bend (monosubstituted)/ C=C bend (disubstituted) O-H bend
723.31	aromatics/alkene	C-H bend/ C=C bend (disubstituted-cis)
611.43	alkynes	acetylenic C-H bend

Therefore, the results showed that the ethanolic e xtract from the green seeds of *Coffea arabica* possesses numerous functional groups such as O-H; C-O; C=O; C=C; C-H; attributed to different secondary metabolites contained in the extract such as polyphenols and alkaloids. According to LC-MS analysis, O-H functional groups are assigned to chlorogenic acids, which are a group of polyphenolic compounds. As reported in the literature, chlorogenic acids are known for their ability to scavenge free radicals, thereby reducing oxidative stress in biological systems [44]. Chlorogenic acid compounds, which include 3-, 4- and 5-caffeoylquinic acids, have been shown to exhibit significant antioxidant activity, which is essential for neutralizing free radicals and reducing oxidative stress in biological systems [44]. The presence of chlorogenic acids in coffee has been linked to various health benefits, including the potential reduction of chronic diseases such as type 2 diabetes and cardiovascular disease [3]. In addition, studies have indicated that the concentration of these phenolic acids may vary depending on the geographical origin of coffee beans, with some regions producing beans with higher chlorogenic acid content [45].

4. XRF analysis

To evaluate the mineral components present in *C. arabica* green seeds extract, X-ray fluorescence analysis (XRF) was employed. The results obtained are expressed in ppm and are presented in table III.

Table III. Mineral components of *C. arabica* green seeds (ppm).

Element detected in ppm	Value recorded
K	48897
Ca	8115
Fe	399
Ba	133
Mn	159
Cu	58
Rb	55
Ti	35
Zn	30
Ni	21
Cr	ND
Ta	25
Mo	6
Hg	8
Sb	ND
Sr	10
Zr	4
Th	5
U	ND
Tl	ND

One can observe that the *C. arabica* green seed extract contains no traces of minerals that could be harmful for medical use. The minerals found in the green seed extract of *C. arabica*, in insignificant quantities, could be due to the soil's component, where the plant has grown and developed. It is worth noting that potassium was found in large quantities followed by calcium, iron, manganese, and barium.

Despite that the XRF analysis showed that potassium and calcium are minerals present in high amounts in the extract of *C. arabica*, these two minerals have multiple benefits for the human body. For example, potassium is necessary for blood pressure regulation, normal functioning of the human body's cells, glycaemic management, and bone health. In addition, high amounts of potassium reduce cardiovascular disease and stroke [46]. On the other hand, calcium's benefits include: it assists blood vessels in moving blood throughout the body, facilitates the connection between the brain and other parts of the body, strengthens bone and tooth health, as well as reduces fractures and the risk of osteoporosis [47]. Therefore, XRF analysis showed that *C. arabica* green seed extract could be used in biomedical applications, as the analyzed extract does not contain trace metals that may be harmful to healthy living organisms.

5. Antioxidant capacity (AOC)

Figure 4 depicts the antioxidant capacity of the ethanolic extract of *C. arabica*, through the DPPH free radicals test, evaluated each at five different concentrations, as compared with the standard used – the ethanolic solution of ascorbic acid of 1 mM.

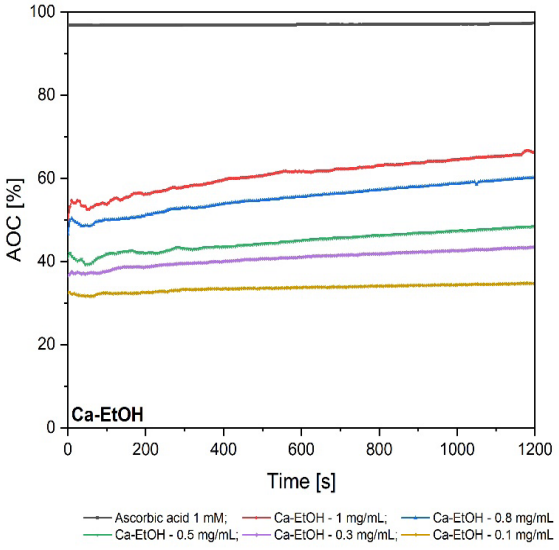

Figure 4. The time-dependent AOC of ethanol extract of *C. arabica* green seed.

As one can notice, the ethanolic extract provides a concentration-dependent AOC. At the maximum concentration tested (1 mg/mL), the antioxidant capacity of the ethanolic extract is 66.38%, indicating a strong antioxidant activity. At lower concentrations such as 0.8 mg/mL, 0.5 mg/mL, and 0.3 mg/mL, the ethanolic extract maintains high antioxidant capacity, suggesting an increased efficiency of the active compounds present in it even at moderate dilutions. This consistent efficiency at lower concentrations reflects the potential of the antioxidants in *Coffea arabica* ethanolic extract, which could be useful in applications requiring stable antioxidant protection at lower doses. The reaction kinetics with DPPH free radicals is another notable aspect. For concentrations of 1 mg/mL to 0.3 mg/mL, the antioxidant reaction does not reach equilibrium within 20 minutes of analysis, indicating a continuous and prolonged antioxidant action, a valuable feature for applications where long-term antioxidant protection is desired. In contrast, at 0.1 mg/mL, the reaction stabilizes rapidly, suggesting that at this low concentration, the antioxidant capacity is limited by the low amount of polyphenols. In conclusion, the ethanolic extract of *C. arabica* green seed provides effective and long-lasting antioxidant protection at moderate to high concentrations, but the efficacy decreases at very low concentrations. This characteristic makes it suitable for long-lasting antioxidant formulations.

The antioxidant capacity of *C. arabica* ethanolic extract compared with the antioxidant capacity of 1 mM ascorbic acid ethanolic solution is shown in table IV. The antioxidant capacity values, expressed as percentages, obtained for all five tested concentrations of *C. arabica* ethanolic extract are an average of three measurements ± standard deviation (SD).

The EC50 was calculated by linear regression analysis, taking into account the AOC values obtained and their concentrations. The EC_{50} of *C. arabica* ethanolic extract was 0.402 ± 0.06 mg/mL (R^2 = 0.91697).

The antioxidant capacity of *Coffea arabica* extracts has been extensively studied, revealing their ability to scavenge free radicals and reduce oxidative stress. Phan et al. demonstrated that *Coffea arabica* extracts exhibit high DPPH scavenging activity, suggesting their potential as natural antioxidants [1]. This antioxidant activity is primarily attributed to the high total phenolic content found in the extracts, which acts as a reducing agent and hydrogen donor, thereby mitigating oxidative damage [1]. A comparative study on the antioxidant activities of green and roasted *Coffea arabica* extracts showed that both forms possess significant antioxidant properties, although the roasting process may lead to a reduction of certain phenolic compounds [48]. The presence of chlorogenic acids, a group of phenolic compounds, has been identified as a major contributor to the antioxidant activity of *Coffea arabica*, as these compounds can act as reducing agents and metal chelators [44].

Evaluation of the irritability factor of the ethanolic extract by the HET-CAM method

Ethanolic extract of *C. arabica* was evaluated at a concentration of 400 µg/mL for potential irritative effect on epithelial tissues using the HET-CAM assay. This extract was well tolerated with no signs of toxicity to the chorioallantoic membrane in terms of hemorrhage, vascular lysis, and coagulability, compared to the positive control, SLS, which induced a strong irritative effect with intense damage to the developing CAM vasculature with visible hemorrhage and blood clotting. As indicated by the Luepke scale (Luepke scale: 0-0.9 - non-irritant, 1-4.9 mild irritant, 5-8.9 moderate irritant, 9-21 strong irritant) [34]. The degree of irritation induced by the positive control SLS was detected as a strong irritant with an IF of 16.90±0.15, while the ethanolic extract from the green seeds of *C. arabica* tested had no irritant effect, similar to the negative control (H_20), as shown in figure 5 and table V.

Table IV. The initial and final AOC values (%) of *C. arabica* ethanolic extract at five tested concentrations compared with ascorbic acid ethanolic solution of 1 mM (standard), and the corresponding EC_{50} values.

Sample tested	Concentration [mg/mL]	Initial AOC±SD [%] Time 0 seconds	Final AOC±SD [%] After 1200 seconds	EC_{50} value [mg/mL]
Ca-EtOH	1	52.57±2.31	66.38±0.12	0.402±0.06
	0.8	48.93±2.04	60.12±0.04	
	0.5	41.48±0.45	48.38±0.04	
	0.3	36.99±0.44	43.43±0.04	
	0.1	32.35±0.24	34.69±0.02	
Ascorbic acid	1 mM	96.83±0.008	97.36±0.04	—

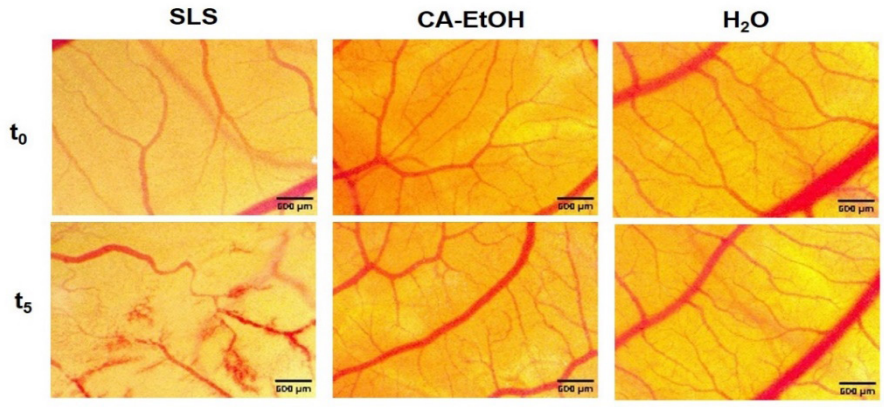

Figure 5. Stereomicroscopic images showing the effects induced by *C. arabica* green seed ethanolic extract on CAM using the HET-CAM anti-irritant assay. The images document the absence of hemorrhage, vascular lysis, and coagulability after the application of the tested extract. Scale bars represent 500 µm.

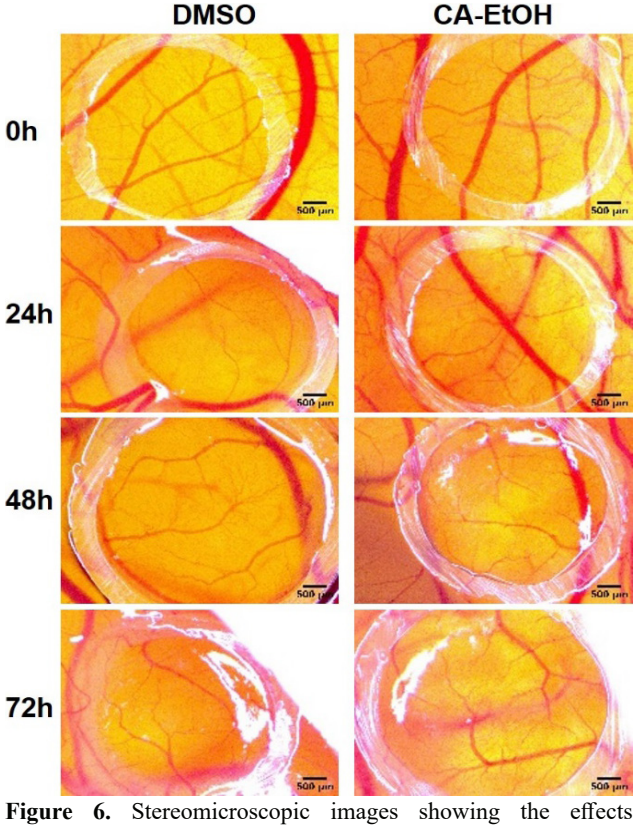

Figure 6. Stereomicroscopic images showing the effects produced by ethanolic extract of *C. arabica* ethanolic extract on CAM; images are shown initially at 0 h, 24 h, 48 h, and 72 h post-treatment, controlling for DMSO solvent and *C. arabica* ethanolic extract. Scale bars represent 500 µm.

Table V. Profile of ethanolic extract from *C. arabica* green seeds in correlation with their potential irritant effect.

Compound	Irritant score	Category
SLS	16.90±0.15	strong irritant
H_20	0±0	non-irritant
CA-EtOH	0±0	non-irritant

The images representing the angiogenic response of the ethanolic extract of *Coffea arabica* green seeds are depicted in figure 6.

One can observe an increased number of small vessels, particularly at 48 h post-treatment, with intense vascularisation visible at 72 h post-treatment. The ethanolic extract of *C. arabica* green seeds induced a normal blood vessel structure, showing an intense angiogenic response at 48 hours post-treatment. After 11 days of incubation, the ethanolic extract from *C. arabica* green seeds (CA-EtOH) showed a more pronounced effect in stimulating new vessel formation. No signs of toxicity were observed and the treatment was well tolerated at 6 days post-administration. These results suggest a promising potential of *Coffea arabica* extracts in therapeutic and cosmetic applications, especially in the context of promoting angiogenesis without compromising epithelial safety.

A limited number of experiments evaluate the irritant effects of this ethanolic extract of *C. arabica* evaluated *in vivo* using the HET-CAM assay. The literature shows that the 50% ethanolic extract of coffee seeds, rich in chlorogenic acid and caffeine exhibited lower toxicity than chlorogenic acid with high IC_{50} values around 3 mg/mL in human skin and hair cells [48]. Green coffee extract nanoemulsions did not induce any damage to the developing vascularisation and were non-irritant to the mucosa-like tissue of CAM [50]. The ethanolic extract from green seeds of *C. robusta*, intended for a nanostructured lipid transport

system, was also evaluated in the HET-CAM assay, with no signs of irritation [51]. The evaluated ethanolic extract obtained from green seeds of *C. arabica* did not interfere with blood vessel circulation or induce any damage on CAM, with good tolerability and biocompatibility with mucous membranes thus indicating their use in topical applications.

The study of ethanolic extract from the green seeds of *C. arabica* (CA-EtOH) shows a significant effect in stimulating angiogenesis – the new blood vessel formation process. Observations at 48, 72 hours, and after 11 days of incubation suggest a pronounced action of this extract on neovascularisation without compromising tissue integrity and without showing signs of toxicity. This indicates considerable therapeutic potential for medical and cosmetic applications, particularly in tissue regeneration and wound healing.

The absence of signs of toxicity 6 days after administration is a strong indicator of the safety of this extract, which is essential for clinical and dermal cosmetic applications. Thus, the use of CA-EtOH could be advantageous for treatments requiring vascular regeneration stimulation without the risk of inducing excessive vascular proliferation, which could lead to dysfunctional vascular structures. The high tolerability suggests that the extract may promote angiogenesis in a balanced manner, which is important for the safety and efficacy of the treatment, especially when applied to large areas or open wounds.

The ethanolic extract from the green seeds of *C. arabica* (CA-EtOH) shows remarkable pro-angiogenic potential and high tolerability, supporting its applicability in tissue regeneration and dermatocosmetics. Despite the promising results, future studies will be crucial to validate the long-term effects and to detail the molecular mechanisms contributing to the pro-angiogenic effect. These further evaluations will support the development of innovative and safe CA-EtOH-based treatments with diverse applications in medicine and cosmetology.

Conclusions

The present research study reveals the phytochemical profile and *in ovo* preliminary screening of the ethanolic extract obtained from green seeds of *Coffea arabica*. As pointed out by HPLC, the main phytochemicals present in the extract were chlorogenic acid and 4-O caffeoylquinic acid, reasonably motivating the most relevant biological effects and therapeutic potential. Furthermore, the FT-IR profile of the extract was showcased. The investigated extract proved to possess a significant antioxidant capacity. After application on the chorioallantoic membrane of chicken's egg, the extract did not induce irritation and no signs of toxicity were observed for 6 days post-administration.

As research continues to unveil the myriad benefits of *Coffea arabica*, it is evident that this natural material holds significant promise for the development of natural health products, therapeutic and cosmeceutical agents. If future research focuses on uncovering the mechanisms underlying these benefits, then *Coffea arabica* may play an increasingly important role in modern medicine, cosmetics, and health promotion.

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