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Phytochemical Screening, Antioxidant Activity, Functional Groups and Chemical Element Characterization Analysis of (-)-Epigallocatechin-3-Gallate (EGCG) in East Javanese Green Tea Methanolic Extract: An Experimental *In Vitro* Study

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ABSTRACT

Background: East Javanese green tea or *Camellia sinensis* is one of the biggest commodities with abundant health benefits. In Indonesia, people drink about 0.35 kg/capita/year green tea with approximately 2,781 tons produced in East Java. (-)-Epigallocatechin-3-gallate (EGCG), one of the main catechins in East Javanese green tea, has anti-cancer, anti-inflammatory, antioxidant, and anti-microbial activities
Aim: To analyze EGCG in East Javanese green tea methanolic extract (GTME) using phytochemical analysis, antioxidant activity, screening functional groups, and chemical element characterization analysis.
Materials and Methods: The maceration method was conducted to obtain the extract of green tea (*C. sinensis*). Isolation of EGCG was performed using high-performance liquid chromatography (HPLC) and verified with thin-layer chromatography (TLC). Phytochemical liquid chromatography analysis was done using alkaloid, saponin, flavonoid, triterpenoid, and steroid tests. The antioxidant activity test was evaluated with a UV-Vis spectrometer. Furthermore, Chemical element characterization analysis was evaluated using Fourier-transform infrared spectroscopy (FTIR) to determine functional groups and energy dispersive X-ray (EDX) to characterize the chemistry element.
Results and Discussion: There is 4.41% of EGCG isolated from East Javanese green tea. During the phytochemical analysis, discoloration appears in alkaloid, flavonoid, triterpenoid, and steroid. Meanwhile, saponin test shows an intense and consistent forth for all indicating positive samples. As EGCG has strong antioxidant activity with IC_{50} value of 36.71, this can restrain the reactive oxidative species activity and advanced glycation end products creation which are connected to antioxidant and anti-inflammatory properties. FTIR and EDX analysis reveals that EGCG has hydroxyl bonds, double bonds C=O, single bond C-O, with carbon and oxygen as mostly the elemental composition, and no toxic substances.
Conclusion: EGCG in East Java GTME has flavonoids, saponins, steroids, triterpenoids, and alkaloids compounds contained with strong antioxidant activity.
Keywords: Antioxidant, *Camelia sinensis*, Epigallocatechin-3-Gallate, Green Tea, Phytochemistry.
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INTRODUCTION

Tea (*Camellia sinensis*) is the most popular caffeine-containing beverage in the world that originally comes from China[1]. In Indonesia, tea was first introduced in the early 1600 and began mass cultivated in 1830 by Dutch Colonies[2]. Jamus tea plantation located in Jember Regency is one of the oldest tea plantations in East Java, Indonesia. Van Rappard, a Dutch businessman, has grown tea in 478.20 hectares in the area since 1866, then in 1973, the management was handed over by Candi Loka Company, Indonesia[3].

Tea becomes an important commodity with economic and health benefits. Drinking tea is part of the culture in several countries like Japan, China, Britain, and Indonesia. Every household in Indonesia has tea and become their favorite beverage besides coffee. Tea consumption per capita in Indonesia approximately 0.35 kg/capita/year[6]. Furthermore, the global tea production in 2013 significantly has increased from 6% to 5.07 million tons.

Meanwhile, Indonesia is the seventh largest tea producer in the world with 152.700 tons of production in 2013[4]. East Java is one of the provinces which has the biggest tea production in Indonesia, with the total tea production around 2,781 tons in 2017 with about 2,060-hectare production area[5].

Moreover, Indonesian tea is known for its high catechins content and approved by The International Society of Antioxidant in Health and Nutrition (ISAHN) as the highest tea catechin content in the world. Catechin, a compound that determines tea quality, is known for polyphenol derivatives that have high antioxidant properties. Catechin levels are affected by the tea variety, the elevation of plantation, the age of tea leaves, the harvesting time, and the production method[7,8]. If compared to other types of tea, green tea has a higher amount of catechin because it is processed after harvesting[9]. East Javanese green tea is believed to have a plentiful amount of catechin. Likewise, green tea has four main catechins; they are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG). EGCG is the most studied catechin derivate and it has an abundant amount compared to another catechins[9].

Green tea is well-known to have great health benefits which put EGCG to play important role. EGCG in green tea has been studied to have an anti-cancer effect, it has angiogenesis inhibition, protects DNA from carcinogenic agents, and promotes cancer cell apoptosis[10,11]. Consuming green tea regularly helps avoiding cardiovascular disease by increasing anti-inflammatory activity, while antioxidant substances in green tea help reducing the amount of oxidant in the body,

inhibiting pro-thrombotic contents that prevent vessel occlusion, and reducing the oxidation of low-density lipoprotein (LDL)[11]. Antimicrobial properties in green tea prevent cell membrane damage, inhibit enzyme and bacterial fatty acid synthesis, restrict the virus binding on host cells, and have synergism effects with antibiotics and antifungal[9]. Furthermore, EGCG is beneficial to maintain the bone homeostasis, not only to enhance Runt-related transcription factor 2 (RUNX2) and osterix expressions but also to decrease Nuclear Factors Associated T Cell-1 (NFATc1) and Sclerostin expression in the bone metabolism to boost the bone remodeling and reduce the bone resorption[12,13].

Thus, the aim of this study is to analyze EGCG in East Javanese green tea (*Camellia sinensis*) methanolic extract (GTME) *in vitro* study through phytochemical screening, antioxidant activity, functional groups, and element characterization analysis.

MATERIALS AND METHOD

Ethical clearance and study design

This research used a quasi-experimental laboratory with a descriptive observational design and an *in vitro* study in nature. The study ethical clearance was obtained from the ethical research committee of the Faculty of Dental Medicine, Universitas Airlangga, located in East Java, Indonesia with appointment number of 086/HRECC.FODM/III/2019.

Sample Preparation of East Javanese Green Tea (C. sinensis)

The sample of Grade I East Javanese green tea or *Camellia sinensis* (*C. sinensis*) was obtained from Perseroan Terbatas Perkebunan Nusantara XII (PTPN XII), located in Wisata Kebun Wonosari, Joyo Marto Street, Toyomarto, Singosari, Bodean Putuk, Toyomarto, Singosari, Malang, Jawa Timur 65208 in a dry condition, then weighed, washed, dried, and cut into small pieces (simplicia).

Extraction Process

This study was performed using a maceration method to obtain EGCG in East Javanese green tea extract. Three hundred and fifty grams of *C. sinensis* simplicia was macerated with methanol 1:4 (w/v) for 3x24 hours at room temperature until the filtrate appeared almost clear. The maceration results were filtered with filter paper to produce the filtrate and residue. The filtrate was evaporated with a vacuum rotary evaporator at 40℃. The thick extract from the evaporation results were put in a desalting process using the decantation technique by mixing the thick extract with methanol and then the salt would settle. Finally, this process was repeated until the white color that indicated the presence of salt in the solvent disappeared. The extract was then evaporated again using a vacuum rotary evaporator at 40℃ (see Figure 1).

TLC and HPLC Examination on EGCG from C. sinensis extract

The isolation of EGCG from *C. sinensis* extract was validated by using thin-layer chromatography (TLC) method by comparing (-)-Epigallocatechin Gallate Hydrate (Cat no. E0694, Tokyo Chemical Co., Ltd., Japan) for verification. Furthermore, the identification of phenolic compounds was performed using a high-performance liquid chromatography (HPLC) system (Agilent 1260, Agilent Technologies, German) consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar[12].

Figure 1: Production of EGCG in East Javanese green tea using maceration method.

Phytochemical Analysis

Flavonoid Test

The sample was weighed then extracted with methanol, filtered with cotton and transferred to another tube (methanol extract). Afterward, a test using concentrated HCl was performed by applying the methanol extract of the sample and added with 2 drops of concentrated HCl. The extract was shaken strongly added with Mg powder and then shaken it again strongly. The positive samples were confirmed when they contained flavonoids with concentrated HCl reagents and if there were froths and the color of solution turned orange. Moreover, for testing by using H_2SO_4 2N reagent, methanol extract of the sample was added with 2 drops of 2N H_2SO_4, then shaken strongly afterward. The samples were confirmed positive when they contained flavonoids with H_2SO_4 2N reagents and if the color of solution changed into yellow, red or brown significantly.

Alkaloid Test

First, the sample was weighed and then extracted with ammoniacal chloroform. Then, it was filtered with cotton and transferred to tubes A and B. Tube A was added with Dragendorff's reagents, while tube B with Wagner's reagents. Samples in tube A contained with positive alkaloids if reddish deposits occurred and in tube B, if there were brownish deposits.

Saponin Test

The sample was weighed and then extracted using ammoniacal chloroform. Next, it was strained with cotton and transferred to another tube. The tube was shaken strongly, left for 2 minutes, then added with 2 drops of HCl 2N. It was shaken well and examined whether any foam formed after being settled for 10 minutes. The sample was confirmed positive and contained with saponins if there were froths with a lot of intensity and they were consistent for 10 minutes.

Triterpenoid and Steroid Test

The sample was weighed and extracted with ethanol. Then, it was strained using cotton and then heated to dry. Next, the sample was extracted again with chloroform and water (1:1). Two drops of chloroform were dropped to the extract on the spotted plat and let it dry. Furthermore, 1 drop of concentrated sulfuric acid and 1 drop of anhydrous acetic acid were added into the extract. The positive samples contained with triterpenoids were confirmed if there was red or brown discoloration, while the positive samples contained steroids were confirmed if there was blue, purple or green discoloration.

Antioxidant Activity Test

For antioxidant activity test, it needed a DPPH $4x10^{-4}$M solution and was put it in vials and close tightly. The vial surface needed to be covered with aluminum foil to avoid light. Then, it needed a 1000 ppm sample solution by dissolving the sample with methanol as a solvent. For sample testing, the stock and methanol solutions had to be inserted in accordance with the volumes listed in table 1. In each test tube (A – H), 1 ml DPPH solution was added to each of these test tubes then left for 30 minutes. As a final step, it was then measured using a UV-Vis spectrometer with a wavelength of 517 nm.

Functional Group Analysis and Chemistry Element Characterization

EGCG functional group analysis in East Javanese GTME was carried out with the help of fourier-transform infrared spectroscopy (FTIR). This instrument was equipped to predict any active organic compounds by identifying the functional groups through infrared ray absorption[14]. To start this analysis, 1 mg sample was crushed with 100 mg KBr homogeneously, then measured at infrared absorption at 4000–400 cm^{-1} then analyzed. An energy dispersive x-ray (EDX) analysis was performed to characterize the chemistry element within EGCG in East Javanese GTME. EDX and FTIR examinations were then conducted at the Faculty of Industrial Engineering, Institute of Technology Sepuluh November Surabaya.

RESULTS

In this study, we successfully isolate 4.41% of EGCG from the East Java green tea by using HPLC and verified it by applying TLC with 276 wavelengths (see Figure 2). Phytochemical screening of East Javanese GTME Extract reveals that EGCG has alkaloid, saponin, flavonoid, triterpenoid and steroid properties (see Table 1).

EGCG in East Javanese GTME has strong antioxidant activity with IC_{50} value as much as 36.71 (see Figure 3). The FTIR results of this study exhibits the function and structure of EGCG. In EGCG, the tip of peak 3220 cm-1 shows the presence of hydroxyl bonds of EGCG. Peaks at 1517 cm-1, 1448 cm-1, 1335 cm-1, and 1188 cm-1 indicate the presence of double bonds C=O and single bond C-O (Figure 4). EDX is used to examine the elemental composition and heavy metals in EGCG. Element composition (wt%) based on EDX analysis presents that EGCG has both carbon and oxygen as the most elemental composition (see Figure 5 and Table 2).

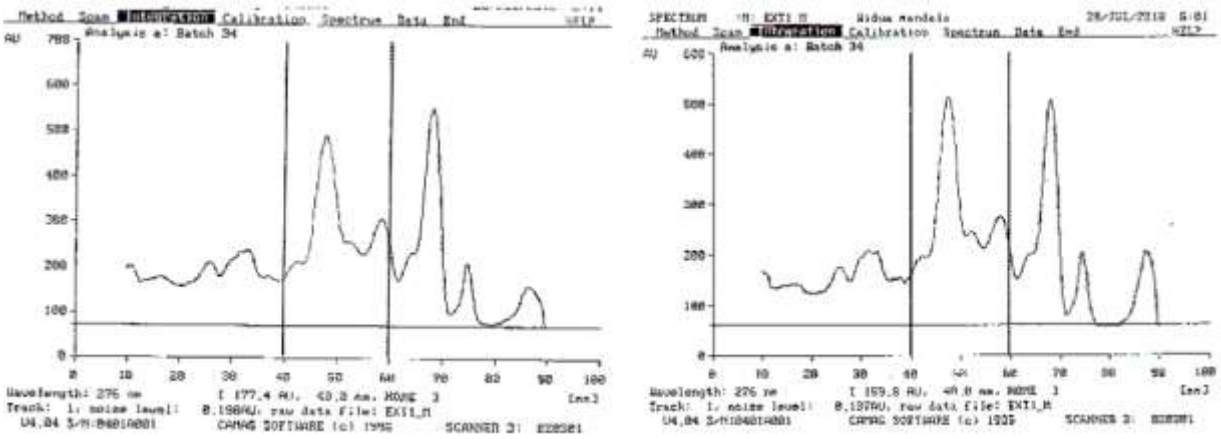


Figure 2: TLC examination verified the EGCG from the East Javanese GTME.

Table 1: The phytochemical screening result of the East Javanese GTME.

Extract	Secondary Metabolite				
	Alkaloid	Saponin	Flavonoid	Triterpenoid	Steroid
Green Tea (East Java)	+	+	+	+	+

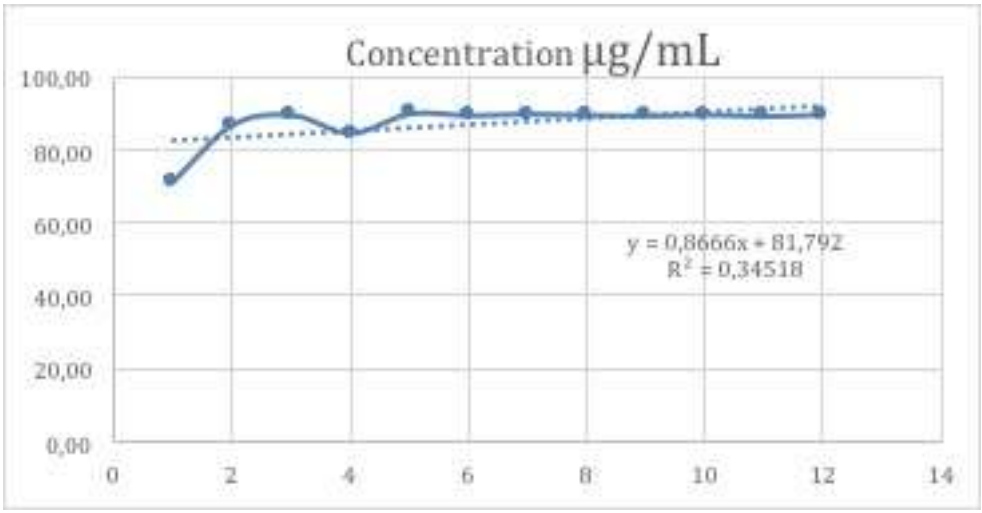
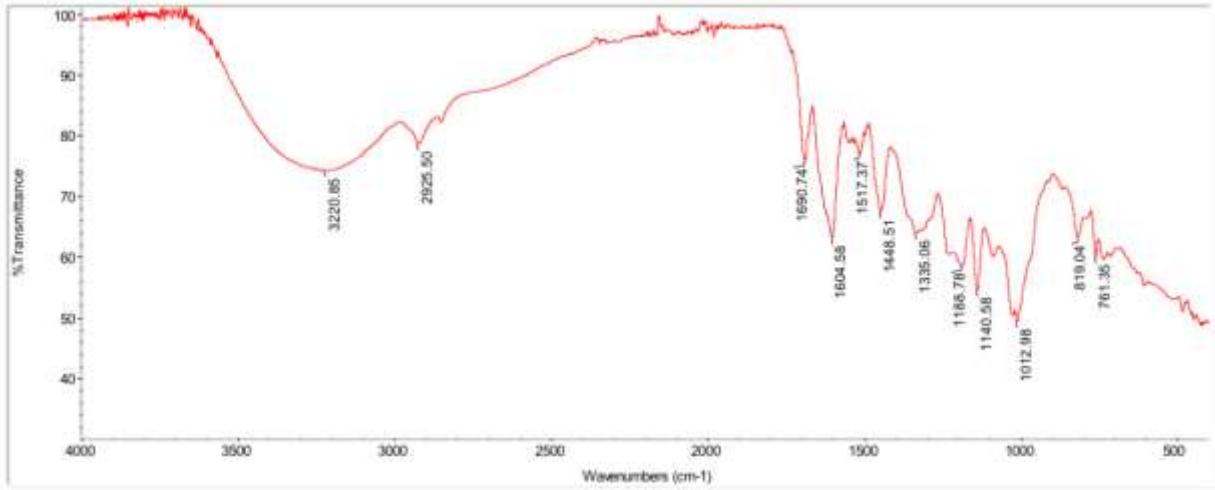


Figure 3: Antioxidant activity of EGCG in East Javanese GTME.



Collection time: Mon Oct 21 13:29:31 2019 (GMT+07:

Mon Oct 21 13:30:23 2019 (GMT+07:00)
FIND PEAKS:
Spectrum: FTIR
Region: 4000.00 400.00
Absolute threshold: 86.469
Sensitivity: 50
Peak list:

Position:	761.35	Intensity:	59.787
Position:	819.04	Intensity:	62.948
Position:	1012.98	Intensity:	49.144
Position:	1140.58	Intensity:	54.240
Position:	1188.78	Intensity:	58.208
Position:	1335.06	Intensity:	63.690
Position:	1448.51	Intensity:	67.255
Position:	1517.37	Intensity:	76.706
Position:	1604.58	Intensity:	62.951
Position:	1690.74	Intensity:	75.489
Position:	2925.50	Intensity:	78.425
Position:	3220.85	Intensity:	73.977

Spectrum: FTIR
Region: 3495.26-455.13
Search type: Correlation
Hit List:

Index	Match	Compound name	Library
15479	42.53	2-Butyn-1-ol, 98%	HR Aldrich FT-IR Collection Edition II
51	37.03	A,B-METHYLENEADENOSINE 5'-DIPHOSP	Sigma Biological Sample Library
29	34.44	SUBSTANCE P FRAGMENT 7-11 ACETATE	Sigma Biological Sample Library
3	33.93	2-IODOPROPANE, 97%	Aldrich Condensed Phase Sample Library
67	33.48	QUININE SULFATE IN KBR	Georgia State Crime Lab Sample Library
7244	33.35	Olivetol, 95%	HR Aldrich FT-IR Collection Edition II
11002	33.33	Methyl 3-hydroxybenzoate, 99%	HR Aldrich FT-IR Collection Edition II
2900	32.87	trans-2-Nonenal, 97%	HR Aldrich FT-IR Collection Edition II
9567	32.80	2-Hydroxy-9-fluorenone	HR Aldrich FT-IR Collection Edition II
81	32.23	PHENYLPHOSPHONIC ACID, 98%	Aldrich Condensed Phase Sample Library

Figure 4: The FTIR results of this study reveals the function and structure of EGCG in East Javanese GTME.

Table 2: Average corresponding atomic of EGCG based on EDX Spectrum

	C K	O K	CoL	P K	ClK	K K
At% (1)	60.11	35.96	2.35	0.45	0.45	0.68
At% (1)	60.97	34.98	1.67	0.48	0.75	1.15
At% (1)	60.91	36.66	0.78	0.46	0.48	0.71
Average	60.7	35.87	1.6	0.46	0.6	0.85

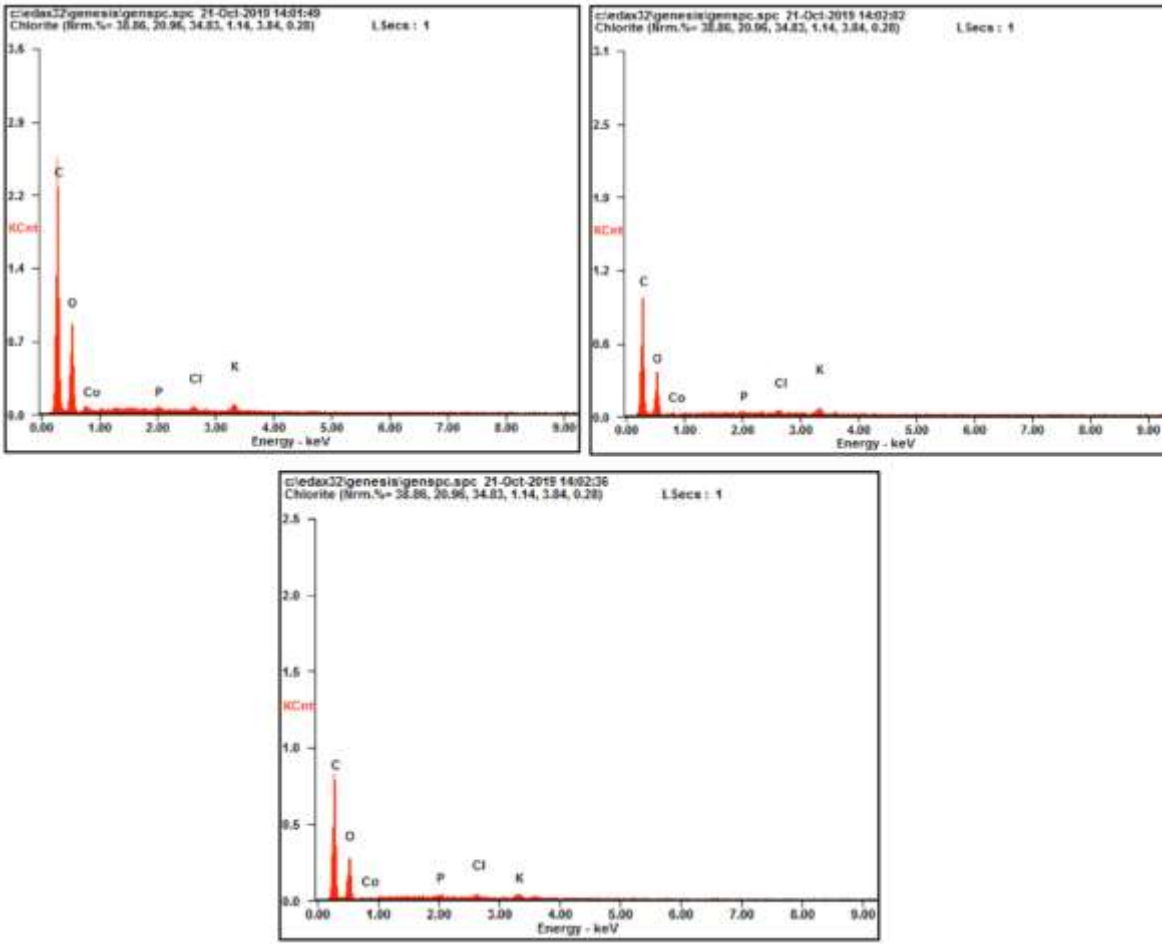


Figure 5: EDX result shows the chemical element in the EGCG from the East Javanese GTME.

DISCUSSION

Phytochemicals are naturally chemical compounds which occur in plants that elicit various biochemical activities and provide health benefits for humans[15]. The phytochemical screening of this study reveals that the presence of flavonoid, alkaloid, saponin, triterpenoid, and steroid in East Javanese GTME. The presence of these phytochemicals is responsible for green tea significant health benefits that have been recognized for decades[16]. Meanwhile, Flavonoids are a large group of phenolic compounds characterized by the C6-C12-C6 skeleton and found ubiquitously in plants. The primary class of flavonoids is catechin (comprising 80-90% of the flavonoids) and the main component of catechin is EGCG (60%)[9,17]. Furthermore, flavonoids possess a wide array of biochemical properties, such as antioxidant, antibacterial, hepatoprotective, anti-inflammatory, anticancer, and antiviral properties[18]. These different capacities of flavonoids are influenced by their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization[19,20].

Moreover, one of the most prominent functions of flavonoids is their antioxidant capacities. Flavonoids are able to exert antioxidant activity by suppressing the formation of reactive oxygen species (ROS) by inhibiting enzymatic activity, scavenging ROS, or upregulating the host of antioxidants defense systems. Flavonoids also have been proposed to be an alternative in antibiotic treatment due to their potent antimicrobial capacity, either by combination with antibiotics or as a singular treatment[20,21]. A previous study by Tamba *et al*. reports that a combination of ceftazidime and apigenin (class of flavonoids) causes the damage of ceftazidime-resistant *Enterobacter cloacae* cytoplasmic membrane[22]. Meanwhile, a study by Bai *et al.* also states that the mechanisms of EGCG antibacterial properties towards *Streptococcus mutans* are done by inhibiting growth and biofilm formation and by damaging the cell membrane of the bacteria[23]. Furthermore, a previous review also states that flavonoids elicit their antibacterial properties through several mechanisms, such as nucleic acid synthesis inhibition, alteration of the cell membrane, attenuation of the bacteria pathogenicity, and others[21]

Meanwhile, saponins are phytochemicals consisting of triterpenoid and steroidal aglycones linked to oligosaccharide moieties. The main characteristic of these phytochemicals is their amphiphilic properties[24,25]. This amphiphilic activity induces pore formation and interferes with membrane cell permeability; thus, it mediates their bioactive function in antifungal, anticancer, and hemolytic properties. Other medicinal effects of saponin are hypocholesterolemic activity, immune system modulation, specific antibacterial properties (mostly gram-negative bacteria)[26]. Furthermore, triterpenoids and steroids are the most abundant group of phytochemicals that possess a plethora of biological activities, such as anti-inflammatory,

antidiabetic, antioxidant, antimicrobial, antimycotic, immunomodulatory, and anticancer properties[27].

Likewise, alkaloids are phytochemicals that contain heterocyclic nitrogen atoms. Naturally, alkaloids, as any other phytochemicals act as the defenses system for plants mainly through their antibacterial and antifungal activities. Other biological activities of alkaloids include anticonvulsant, analgesic, antihypertensive, antiarrhythmic effect, antimalarial, and anticancer effects[15,28].

An antioxidant assay using DPPH radicals was performed in this study and it shows the high antioxidant activity with IC_{50} under a concentration of 36.71 µg/mL. Phongpaichit *et al.* study in 2007 previously states that the IC_{50} threshold of strong antioxidant activity is between 10-50 µg/mL with lower IC_{50} value which implies a stronger activity[29]. This result emphasizes the scavenging ability of EGCG to half the concentration of the DPPH that may come from the contained phytochemicals by providing hydrogen atoms and electrons leading to a stable radical[30]. This ability further leads to an effective inhibition of ROS formation and the possible hazard of oxidative stress [31]. A previous study also reported that EGCG cell protection ability from hydrogen peroxide (H_2O_2) induces cell damage and ROS production with DPPH scavenging rate around 77.2%[32]. It was also reported that EGCG has an antiglycation ability through methylglyoxal trapping by inhibiting advanced glycation end products (AGEs) production and inflammation[33]. These antioxidant properties manage cellular response towards stress and prevent its overexpression.

Moreover, a previous study reported that EGCG administration not only significantly decreases the high mobility group box 1 (HMGB1) and heat shock protein 70 (HSP70) and intensifies Fibroblast Growth Factors (FGF-2) and Vascular Endothelial Growth Factor (VEGF) expression on animal models induced with orthodontic stress[34,35]. The reduction can come from the ROS scavenging ability of EGCG, whereas, the two proteins are expressed as a cellular response of oxidative stress. An excessive ROS production is also known to induce the various inflammatory pathways and messengers. Likewise, EGCG stimulation is reported to inhibit Nuclear Factor- κB (NF-κB) and AP-1 activity that would further lead to reduce Tumor Necrosis Factor-α (TNF-α), Nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions along with massive scavenging of nitric oxide (NO)[31,36]. Additionally, these properties can elaborate the ability of EGCG as an antioxidant and anti-inflammatory agent that act as a possible effective therapy of related diseases and pathological conditions.

Furthermore, FTIR spectroscopy has been proven to be a valuable apparatus in identifying the structure and functional groups of active substances, including phytochemicals. Characteristic bands detected at 3220 cm-1/2990 cm-1 correspond to the presence of hydroxyl bonds (O-H). The absence of absorption in the 2220-2260 cm-1 region indicates no cyanide or toxic substances contained in the extract[37]. While, the absorbance region marked by peaks at 1517 cm-1, 1448 cm-1, 1335 cm-1, and 1188 cm-1 shows the presence of double bonds C=O and single bond C-O. These functional groups are responsible for various pharmacotherapeutic effects exerted by EGCG[38].

In addition, EDX analysis was performed to examine the elemental composition of the EGCG and confirms that it is composed mainly of carbon and oxygen atoms with the weight percentages of 60.66% and 35.87% respectively. We also confirm a low weight percentage of heavy metal such as cobalt for 1.6% in EGCG. The heavy metals commonly are found in plants as a consequence of industrial and agriculture activity, in fact, some of them in high concentrations are reported affecting phytochemical constituents in plants. Various studies report the reduced secondary metabolites production such as flavonoid which mainly constitutes in EGCG and phytotoxicity leading to irritations and disorders caused by high concentration heavy metals exposure[12,39,40]. Thus, a low weight percentage of cobalt might emphasize the biocompatibility of EGCG without altering its effects in physiological concentration.

CONCLUSION

To sum up, the group of compounds contained in the East Javanese GTME are flavonoids, saponins, steroids, triterpenoids, and alkaloids. EGCG as a bioactive ingredient contained in the East Javanese GTME has strong antioxidant activity. Finally, further study needs to be done to manufacture EGCG East Javanese Green Tea as an herbal medicine for health benefits.

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