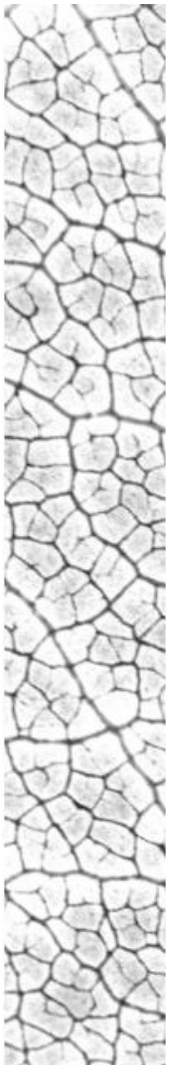

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Separation of four fatty acids and two phenolic compounds from *Camellia sinensis* using chromatographic techniques and evaluation of their antibacterial activity

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Abstract
The active components were separated of Camellia sinensis leaves, by column chromatography through solvent of (Petroleum ether - ethyl acetate) 5-1 for extract petroleum ether and (chloroform-Methanol) 10-1 of extract ethanol. The Saponification was performed on to isolated and identification of four fatty acids (Hiptanoic, Lauric, Oleic and Palmitic acids) by GLC-chromatography., some phenolic compounds were isolated with acidhydrolysis, like Thymol and Hydroquinone. These compounds was identified by HPLC-analysis. The active compounds separated in this study had multiple effects against some Gram-positive and Gram-negative bacteria, using disc diffusion method.

Keywords: GLC analysis, HPLC analysis, Camellia sinensis, fatty acid, antibacterial activity

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INTRODUCTION

Tea of *Camellia sinensis* L. is an a very useful plant because its contains bioactive substances and flavor. Green tea, especially its leaves are considered to be secondary source of natural compounds like flavonoids, alkaloids and polysaccharides. It grown and spreads in Asian countries, Latin America and Africa. Recently, increased demand for tea plant particularly for it health benefits as antioxidant, anticarcinogenic, effects against cardiovascular diseases and atherosclerosis (Zhou et al., 2017).

Antimicrobial activity of medicinal plants are widely used as sources of pharmacological compounds (Ushimaru et al., 2007; Tariq et al., 2013). The most important bioactive constituents of plants are, terpenoids, glycosides, tannins steroids alkaloids, flavonoids, and carotenoids, which have served as a valuable starting material for drug development (Akowuah et al., 2005). Tea (*Camellia sinensis*) is consumed worldwide and is the second after water in its popularity as a beverage and has many health benefits such as reduction of cholesterol, protection against cardio-vascular disease and cancer (Wang et al., 1992). Green tea has bactericidal activities against Gram positive *Staphylococcus aureus* and many other pathogenic organisms (Catherine 2007). Hence, consumption of tea has been associated with reduced risk of major diseases (Robinson et al., 1997). It has been stated that this antimicrobial activity is attributed to several substances like saponins, fatty acid, glycosides, phenolic compound, alkaloids and flavonoids (Padmini et al., 2011).

The prevalence of infection and highly pathogenic diseases increased recently by microorganisms. Antibiotics in treating diseases often produce side effects. Furthermore, the antimicrobial resistance is considerably increased worldwide and imposes change the attitude toward using natural alternative products. The present study aimed to evaluate the antibacterial activity of tea extracts against some bacteria. The study aimed to know the relationship between the active compounds extracted from the tea plant and its effect on bacterial.

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MATERIAL AND METHODS

Collection of Leaves

Camellia sinensis Leaves were collected during the period of 2018-2019 from the markets of Mosul, then classified according to the global sources by assistant professor of taxonomy.

Prepare Plant Extracts

50g of *Camellia* Leaves were weighed and then placed in a Soxhlet system and extracted for 6-12 hours using 500 ml of petroleum-ether , methanol and ethanol. The extracts were concentrated until 15 ml with a rotary evaporator of 45 °C, (Harborne 1998).

Fractionation of Petroleum Ether & Ethanol Extracts

200 mg of crude extract was prepared and mix it with a little silica gel 30 g, and, transferred to the prepared silica gel column (60 until 120 mesh). The column was eluted with (ethyl acetate- pet-ether; 1:5) V/V interval. One gm of *Camellia sinensis* extracted by using petroleum ether Cam_1 and ethanol Cam_2. Then, they were taken and used for separation. Using a column filled with silica gel the extract was separate (silica gel 60 A) solved with 25 g of gels with ethylacetate - petroleum ether (1:5 V/V). Two fractions of Cam_1F_2, Cam_1F_3 were obtained from crude petroleum ether extract .Ethanol crud it was filled by methanol-chloroform (1:10) V/V. by similarity in shape and location of spots in the TLC plate, of the same column solution system, we obtained several fractions were Cam_1F_2. The rotary evaporator was used to evaporated the parts under pressure (Al-Dulayymi 2014).

The Process of Saponification Plant Extracts

Using the petroleum ether extract of the (Cam_1) from (ethyl acetate - petroleum ether) (1-5) the saponification process was performed to get Cam_1F_2 and Cam_1F_3, the fraction is a result of the separation column. Well the extract Cam_1F_2 it has been saponified to get fatty. The ethanolic extract was fractionated by methanol-chloroform (1-10). In addition, acid hydrolysis performed for the crude ethanolic extract. The fraction Cam_1F_2 was subjected to saponification while the Cam_2 fraction was subjected to acid hydrolysis (Al-Kaisy et al., 1991; Anuonye et al, 2016).

Saponification Process to Get Fatty Acid

A mixture 5 g of each ethyl acetate - petroleum ether and also Pet-ether extracts from petroleum ether extracts (Cam_1F_2) and 50 ml of potassium hydroxide in Methanol : water 2:3 then refluxed for 90 min at 100°C. Putting the solution in the separating funnel and adding to it ether (2×50 ml). The mixture was acidified PH=2 by using H_2SO_4. Fatty acids were extracted by ether (2×50 ml) (Aruther 1972). One gram of fatty acid was obtained after evaporation of the ether. After that, the esterification process was performed on the fatty acids to make them less polar and more volatile when diagnosed with GLC analyses (Al-Kaisy et al., 1991; Bauer et al., 1966).

Acid Hydrolysis Process to Get Phenolic Compounds

A mixture 10 ml of extract fraction methanol – Chloroform (A_2F_2) from extracts of ethanol and 50 ml of (1N) Hydrogen chloride was refluxed for 1 hour. at 100°C. Put the solution after cooling in a separating funnel, after that, add (2×10 ml) of ethyl acetate. After the isolation of aqueous layer from organic layer, using magnesium sulfate dried the organic layer. Concentrated the ethyl acetate extract using rotary evaporate. Then, the sample of separated phenols was kept in glass bottles until the analysis by HPLC (Padmini et al., 2011; Harborne 1984).

The Biological Activity of *Camelia Sinensis* Extracts

The antibacterial activity of extracts separated from the plant under study was tested using the method Kirby-Bauer (Adomi 2006) 3-5 colonies of the bacteria under study were transferres to a nutrient broth then incubation at 37 °C for 24 hour. The bacterial suspension was diluted and then compared with the McFarland Standard. A little bacterial suspension was transferred to the nutrient agar.

The disc saturated with different concentration and antibiotics disc were placed in the nutrient agar and incubated with 37 °C for 14-16 hour. The diameter of inhibition zone using a graduated ruler, and the results are compared to the standard antibiotics and recorded (Amikacin 10 meg, Gentamicin 10 meg, ciprofloxacin 10 meg). In addition, different concentration (50-200) mg/cm^2 of plant extracts were used in this study (Asif 2011).

RESULTS AND DISCUSSION

During the previous studies and phytochemical screening (Wang et al., 2011; Setyopratomo 2014; Kopjar et al., 2015), many phenolic and fatty acid compounds were investigated in the Leaves of *Camellia sinensis*. **Table 1,** and **Figs. 1-2** shows four fatty acid in the extract fraction Cam_1F_2 including: Heptanoic (0.0184%), Lauric (0.0009%), Palmitic (0.0061%), (Cis & trans) and Oleic acid (0.0301%). In this species, found unsaturated fatty acids like oleic acid at high percentage. In addition, Oleic acid was also presented as a major compound in the extract Cam_1F_1. Oleic acid and Omega-3- are very beneficial compound for the health of the body, especially when mixed together (Wang et al., 2011). Other fatty acids were noticed in low concentrations. These fatty acids can be a valuable source of edible oil with suitable nutritional properties (George et al., 2016).

Table 1. The fatty acid compounds of the *Camellia sinensis* extracts which were analysis by GLC

Extracts of *Camellia sinensis*	Fatty acid compounds							
	Heptanoic		Lauric		Palimitic		Cis and trans Oleic acid	
	R_t min	Conc.	R_t min	Conc.	R_t min	Conc.	R_t min	Conc.
Fraction Cam_1F_2	6.63	0.0184	12.55	0.0009	15.717	0.0061	18.283	0.0096

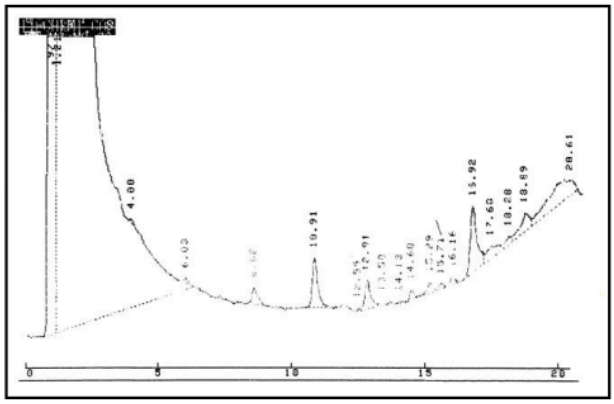


Fig. 1. GLC Chromatograms of fatty acid compounds presented in *Camellia sinensis*

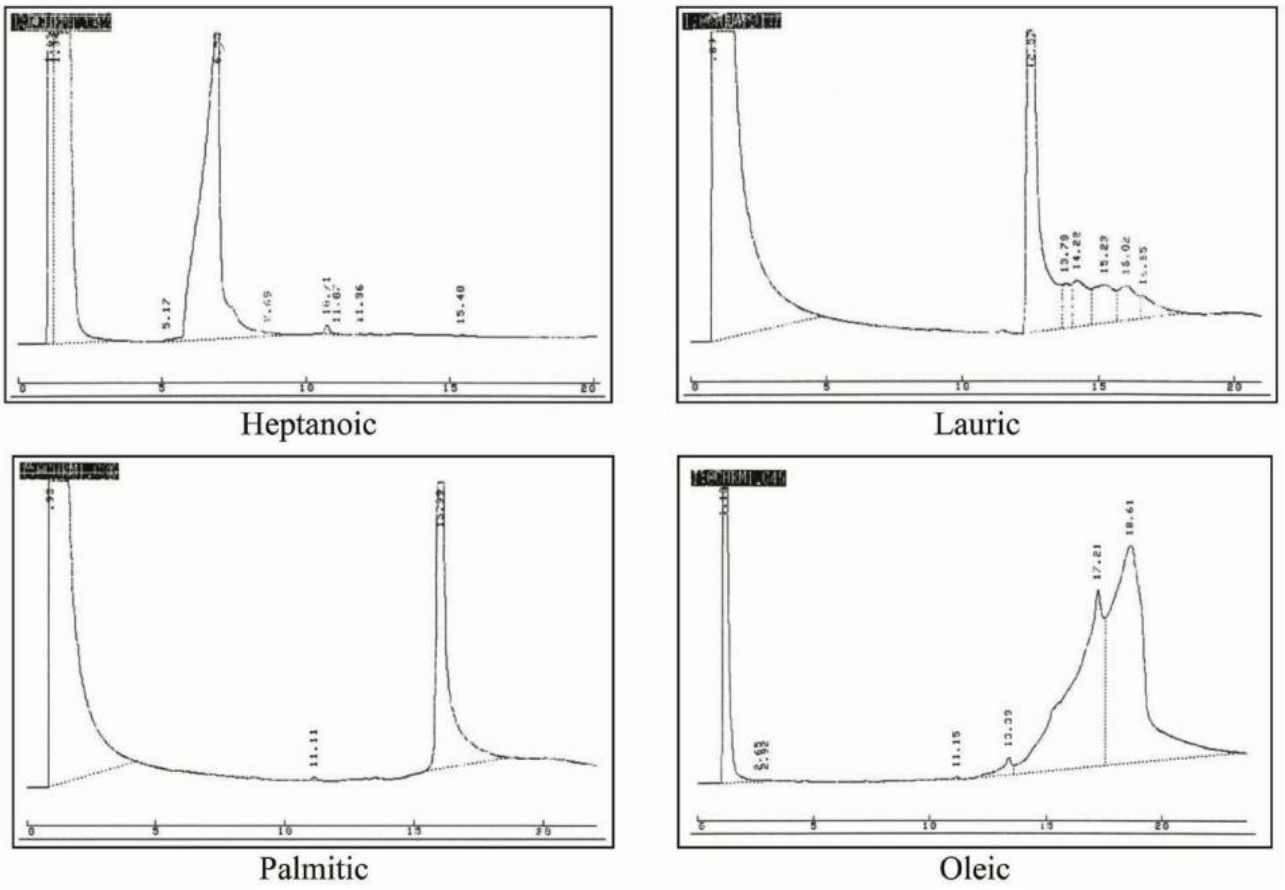
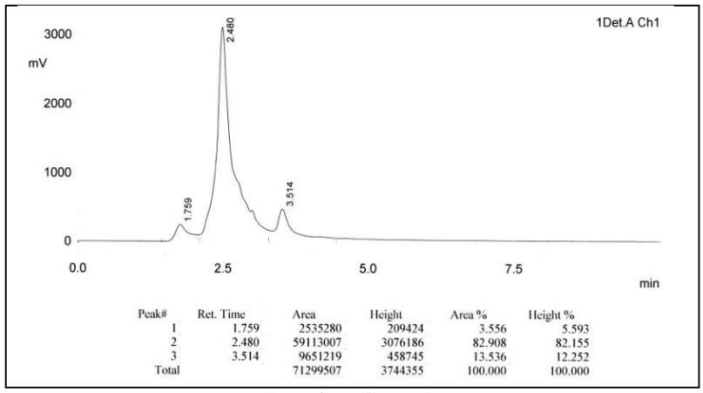


Fig. 2. GLC chromatograms of standard fatty acid compounds

The study conducted by Padmini et al. (2011) showed that *Camellia sinensis* extract contains fatty acid ester, palmitic acid and flavonoids when identified by GC-MS. In addition, two phenolic compounds were identified in the Leaves of *Camellia sinensis* **Table 2** and **Figs. 3**-**4**. The fraction Cam_2F_2 which was obtained by the column chromatography and acid hydrolysis was carried out to release free phenolic compounds. HPLC-analysis showed hydroquinone R_t (2.480 min.) and thymol R_t (3.514 min.). The current result is identical to the researchers study (Setyopratomo 2014; Kopjar et al., 2015).

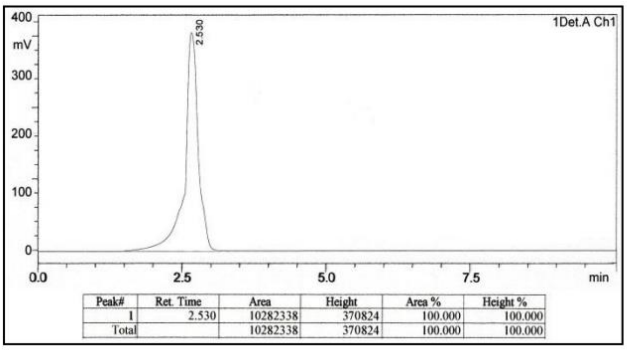
Table 2. The phenolic compounds of the *Camellia sinensis* extracts which were analysis by HPLC

The standard / Sample	The Phenolic compounds			
	Hydroquinone		Thymol	
	2.5530		3.512	
	R_t (min)	Area %	R_t (min)	Area %
Sample Cam_2F_2	2.480	82.908	3.514	13.536

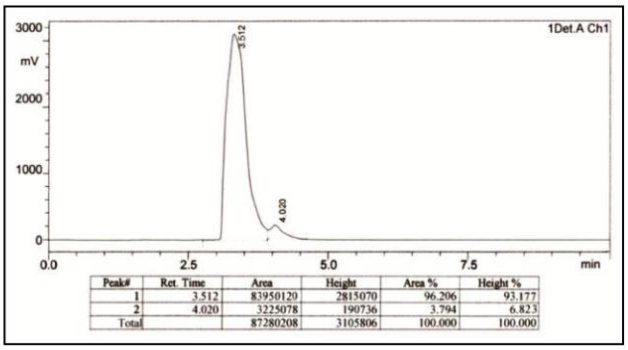


Fraction Cam_2F_2

Fig. 3. HPLC analysis of phenolic compounds in *Camellia sinensis* fraction



Hydroquinone



Thymol

Fig. 4. HPLC analysis of standard phenolic compounds

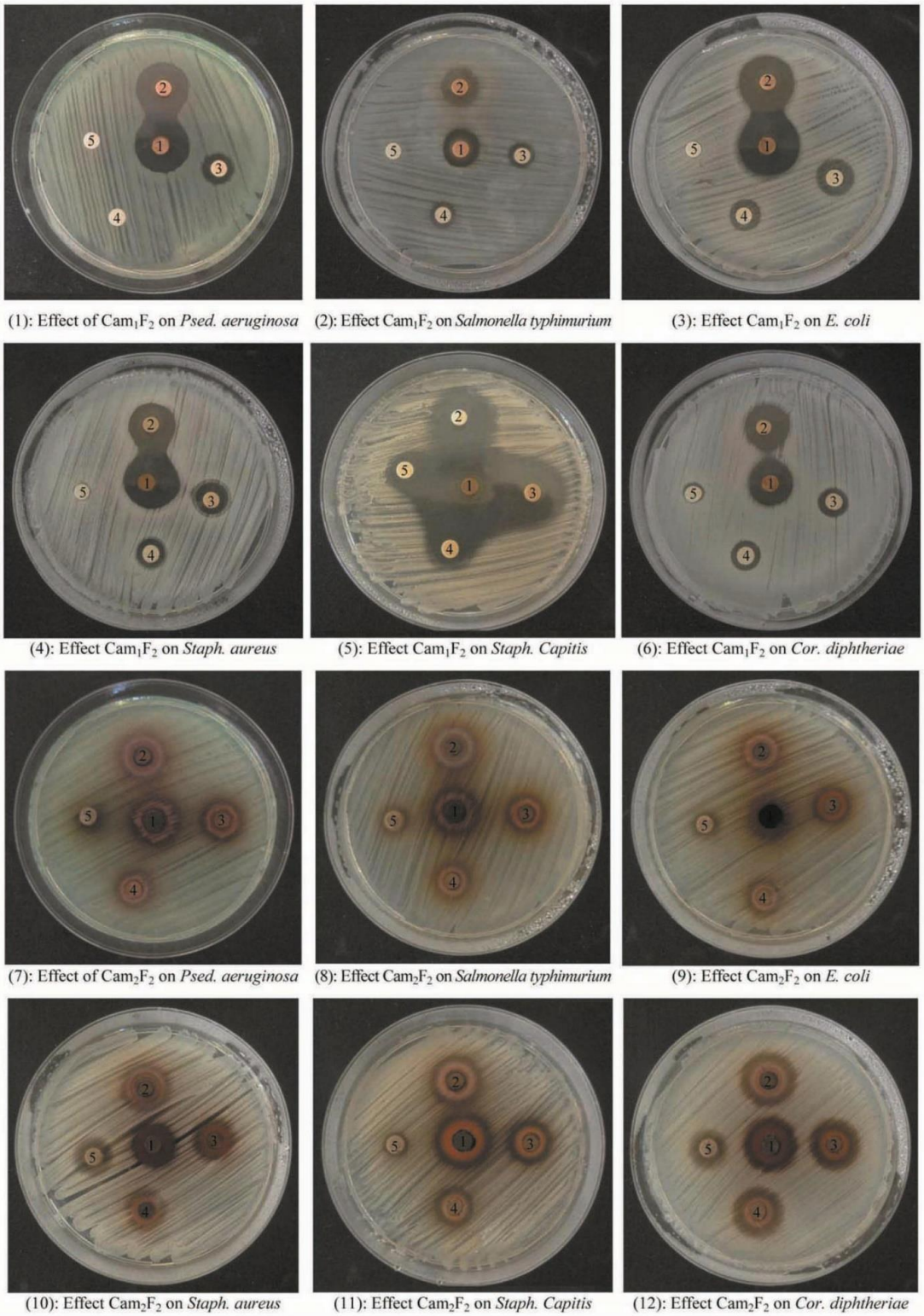
Table 3. Antibacterial effect of phenolic compounds and fatty acids of *Camellia sinensis* extract in several pathogenic bacteria (mm)

Microbial species	Fatty acids extract (Cam_1F_2) / Phenolic compounds (Cam_2F_2)	Concentration of extracts (mg/C³)				
		200	100	50	25	12.5
Psedumonas aeruginosa	Cam_1F_2	20	19	12	-	-
	Cam_2F_2	15	13	11	9	-
Salmonella typhimurium	Cam_1F_2	13	14	10	9	-
	Cam_2F_2	13	12	9	10	-
Escherichia Coli	Cam_1F_2	24	20	14	11	-
	Cam_2F_2	12	12	10	8	-
Staphylococcus aureus	Cam_1F_2	21	18	13	11	-
	Cam_2F_2	15	13	11	10	8
Staphylococcus capitis	Cam_1F_2	30	25	24	15	14
	Cam_2F_2	21	18	15	13	8
Corynebactrium diphtheriae	Cam_1F_2	16	17	11	10	8
	Cam_2F_2	20	18	17	16	12

Table 4. The inhibition zone (mm) of standard antibiotics against bacteria

Antibiotics / The Bacteria	*Staph aureus*	*S. typhimurium*	*E. coli*
(Ak)	15	15	16
(CN)	21	19	25
(Cip)	24	18	26

The chemical components of green tea chiefly include caffeine, polyphenols, flavonoids compounds and amino acids (Sharangi 2009). Results presented in **Tables 3**-**4**, also **Photos 1**-**12** showed the antibacterial effect of *Camellia sinensis* phenolic and Fatty acids compound against some gram positive pathogens: *Staphylococcus aureus*, *S. capitis*, and *Corynebactrium diphtheria*; and gram negative pathogens:



Photos 1-12. Antibacterial effect of some phenolic compounds and fatty acids in extracts of *Camellia sinensis* on bacteria

Pseudomonas aeroginosa, *Escherichia coli* and *Salmonella typhimurium.* All plant extract have high antibacterial effect. The fatty acid compounds in the extract of Cam_1F_2 showed a high antibacterial activity against *E. coli* and *S. capitis* when compared to standard antibiotics of Amikacin (AK) (10 meg/disc), Gentamicin (CN) (10 meg/disc) and Ciprofloxacin (Cip) (10 meg/disc) (Nitiema et al., 2012).

Previous studies have shown antibacterial activities of *Camellia sinensis* (Padmini et al., 2011; Nitiema et al.,

2012; Tariq et al., 2013; Keller and Ryan 2013). We focused in the current study on some phenolic compounds and their action against pathogens associated with gastroenteritis and diarrhea. These compound showed antibacterial effect against Gram-negative bacteria as *E. coli* and gram-positive bacteria such as *Bacillus subtilis, S aureus*. The highest antibacterial activity appeared in the extracts of *Camellia sinensis* and tested in vitro against cariogenic microorganisms (Anita et al., 2015; Kahlel and Sultan 2019).

Moreover, several studies have shown the effec of many plant extracts against many bacteria (Boran et al., 2005; Falleh et al., 2008). The antioxidant activity of the water extract of young shoot tea (*Camellia sinensis*) determined to have significant antioxidant effect as well as antimicrobial activity of water extract of this plant (Yildirim et al., 2000).

These results are similar to many researchers in that extract of *Camellia sinensis* showed high antibacterial, antifungal and antioxidant activities (Tariq and Reyaz 2013; Kopjar et al., 2015; Camargo et al., 2016; Kahlel and Sultan 2019).

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