Synergetic Effect of Green Tea Aqueous and Alcoholic Extracts with Ciprofloxacin Against some Standard bacterial Strains: *In vitro* study

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Abstract: Resistance to commonly-prescribed antibiotics is a global public health problem. Unfortunately resistance has been reported even to newer, potent and expensive antimicrobial agents thus, it necessary to find alternative treatment, such as medicinal plants either alone or in combination with antibiotics due to their fewer reported side effects moreover, their possible affordability and availability. Green Tea (GT) has potential benefits in many aspects most notably in cardiovascular diseases, cancer and obesity. The present study designed to investigate the antimicrobial activity of different concentrations of GT aqueous and alcoholic extracts against standard strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Moreover, this study provides novel findings about the synergetic activity of GT alcoholic extract and Ciprofloxacin against *Staphylococcus aureus* ATCC 25923. Two standard antimicrobial susceptibility procedures were used, well diffusion method and broth dilution technique. The clear inhibition zone diameter (IZD) of each extract was measured. The results obtained showed that the GT alcoholic extract had promising antibacterial activity against *S. aureus* ATCC 25923 only whereas, the two tested strains were resistant to all concentrations of GT aqueous extract. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the GT alcoholic extract against *S. aureus* ATCC 25923 were 225 mg/ml and 250 mg/ml respectively. Moreover, sub-MIC (200mg/ml) of GT alcoholic extract increased the IZD of Ciprofloxacin against *S. aureus* ATCC 25923 by 62.5 % which confirmed the synergistic effect. Finally, further studies in the GT with other organic solvents are of paramount importance and highly recommended.

Key words: Antibacterial, Ciprofloxacin, Green tea, Synergetic, *S. aureus* ATCC 25923

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1. Introduction

Antibacterial resistance is an increasingly serious threat to global public health and threatens the ability of successful treatment and control bacterial infection and ringing a bell to the end of golden era of antibiotics where antibiotics were truly wonder drugs that could be relied upon in treating wide range of life threatening infection and saving millions of lives (Mcewen and Collignon 2018).

Antibiotics resistance acquired by microorganisms, where the resistant microbe cease to be susceptible to the antimicrobial agent whom it was originally sensitive to moreover, they remain reproducing and thriving. This means the standard treatments no longer work, life-threatening infections are becoming hard to control and the risk of death is greater which entails high socioeconomic costs (Silva et al. 2020).

Regrettably the antimicrobial resistance has always existed, but the persistent overuse and misuse of antibiotics have elevated the rates of resistant strains for example, the enzyme penicillinase reported early in *Staphylococcus aureus* strains soon after the discovery of Penicillin (Silva et al. 2020).

The failure of the existing antibiotics to control infections caused by multi drug resistant (MDR) bacteria makes it necessary to find alternative treatment, one such novel therapeutic strategy involves the use of natural compounds such as spices, essential oils, herbal teas alone or in combination with antibiotics **(**Hacioglu et al. 2017). Furthermore medicinal plants have deep roots in man's history to treat and control various disorders (Shaheen et al. 2019).

While antibiotics use associated with serious side effects, including increasing reactive oxygen species (ROS) in the human body which are very dangerous and play important role in oxidative stress-related cancer, cytotoxicity and cardiovascular diseases while many of medicinal plants have an antioxidant, anticancer, anti-inflammatory beside their antimicrobial activities (Parham et al. 2020).

The present study designed to investigate the antimicrobial activity of GT aqueous and alcoholic extracts against standard strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Moreover, provides novel findings about the synergetic activity of GT alcoholic extract and Ciprofloxacin against *Staphylococcus aureus* ATCC 25923. According to the available literature there is no published work in the interaction of the sub_MIC of GT alcoholic extract with Ciprofloxacin against *S. aureus* ATCC 25923

where the most of previous studies were in the synergistic effect of GT and Ciprofloxacin were against both standard and clinical isolates of *E. coli*.

GT is obtained from dry unprocessed leaves of the *Camellia sinensis* plant which belongs to the family *theaceae* **(**Reygaert and Jusufi 2013).

The other kinds of tea include black tea, oolong tea and white tea. The black tea is obtained from fermented leaves while the oolong tea is semi-fermented**.** The white tea is minimally processed and obtained from the newest growth on the tea plant by harvesting both the younger leaves and buds that still covered with white hairs **(**Goswami et al. 2020).

GT has been a traditionally favored drink in Asian countries, many studies evaluated the potential benefits of GT in many aspects most notably in cardiovascular disease, cancer, diabetes, oral health, bone health, and cognitive function. GT also revealed some antimicrobial activity against wide range of microorganisms **(**Reygaert and Jusufi 2013). Moreover, GT play effective role on neurodegenerative diseases such as Alzheimer's and Parkinson's diseases which associated with oxidative damage and iron accumulation (Weinreb et al. 2004). GT is also used to fight obesity by increase burning of calories and reduce adipocytes differentiation, proliferation and lipogensis. Moreover, it acts by increase beta-oxidation and thermogensis (Jigisha et al. 2012).

Chemical composition of GT include carbohydrates, vitamins (A, C, E, K and trace amounts of B vitamins), metals and minerals such as zinc, iron, manganese and aluminium (Jigisha et al. 2012). Moreover GT is rich with lipids, theophylline, caffeine, carotenoids, proteins, chlorophyll and macroelements such as fluorine, iodine and phosphorus (Musial et al. 2020). But the most important active constituents in GT that responsible for several health effects are polyphenols such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) .the EGCG represent the highest amounts in the GT. Both EGCG and EGC are excreted in bile, but EGC is also excreted in the urine, suggesting the possibility for green tea having antimicrobial activity in urinary tract infection (UTI) **(**Reygaert 2017).

The antimicrobial activity of GT is mainly due the active constituent's catechins and polyphenols. Furthermore, there are several mechanisms suggested for the antimicrobial activity of GT such as Catechins induces production of cytokines and hydrogen peroxide generation. Also Catechins act by blocking the connection of conjugated R plasmid in *E. coli*

and interfere with the expression of b-lactamases in staphylococci. Moreover; Catechin-copper (II) complexes damage the cytoplasmic membrane of *E. coli*. EGC can inhibit the activity of the gyrase enzyme in bacteria. Polyphenols also play a role as anti-inflammatory agents and decreasing tumor necrosis factor-α gene expression, which is necessary in pathogensis of *E. coli* (Noormandi and Dabaghzadeh 2015).

The chemical composition of GT and the amount of its catechins depend on several factors such as the climate and cultivation conditions, age and position of the leafs, leafs processing and temperature so their antioxidant and antimicrobial properties may be uneven (Musial et al. 2020).

Despite the potential benefits of GT on health, several studies reported some harmful effects of GT overconsumption such as acute liver cytotoxicity and thyroid enlargement in rats and damage to pancreas and liver of a hamster. These adverse effects of GT mainly due to its caffeine content, the presence of aluminum, and the effects of tea polyphenols on iron bioavailability. Fortunately, these adverse effects are unlikely to occur in humans even with higher dietary intake (Chacko et al. 2010).

In USA 2013, *E. coli* isolates collected from urine samples of patient suffering from UTI between 2007 and 2008 and examined to assess their antimicrobial resistance to standard drugs. 80 of these isolates with variant susceptibility patterns were tested against 2.5 mg/ml, 3 mg/ml, 3.5 mg/ml, and 4.0 mg/ml of GT extract .the majority of the tested isolates (99%) had MICs of ≤ 4.0 mg/ml these results suggest that GT could have promising antimicrobial effects on UTIs caused by *E. coli* **(**Reygaert and Jusufi 2013).

In India a study was carried out to evaluate and compare the antibacterial activity of aqueous, ethanolic, methanolic and acetone extracts of GT and Black tea against standard ATCC bacterial strains by Agar well diffusion method. *S. aureus* ATCC 25923 was the most sensitive pathogen to the tea extracts followed by *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Of all tea extracts tested the aqueous extract of GT has shown the highest antimicrobial activity against *S. aureus* ATCC 25923. But all other extracts of GT has also shown a significant antibacterial activity against *S. aureus* except methanolic extract which exhibited poor or absent activity at lower concentrations. While assessing the antimicrobial activity of GT extracts against *E. coli* ATCC 25923 all of the four GT extracts were exhibited almost equal and significant activity at respective concentrations, but solvent extracts have shown poor or absent activity at low concentration of 12.5 mg/ml (Mehta et al., 2016).

In Sudan, 2018, a study designed to assess the *In vitro* antimicrobial effects of GT aqueous extract against uropathogenic *E. coli* isolates and to determine the MIC and MBC of GT aqueous extract. The results showed that the means of inhibition zone of bacteria to 25%, 50%, and 100% concentrations of GT extract were 10 mm, 18 mm, and 30 mm respectively. The MIC and MBC of GT extract for most isolates were 200 mg/ml and 400 mg/ml respectively (Ahmed et al. 2018).

In Iran 2007, A total of 18 *E. coli* isolates were collected from urine specimen, the MIC for water soluble GT extract of each isolate was 122.9 ± 40.3 mg/ml, to determine the synergetic effect of Ciprofloxacin and water soluble GT extract, a nutrient broth medium with GT extract (initial concentration equivalent to MIC of each isolate prepared by two-fold serial dilution, Ciprofloxacin was added to all tubes with sub-MIC concentration, after an overnight incubation at 37°C, MIC of GT in the presence of sub-MIC of Ciprofloxacin were determined, an obvious reduction of MIC of GT was detected in 93.7% (15 of 16) of bacterial isolates and this ensured the synergetic effect between water soluble GT extract and Ciprofloxacin (Jazani et al. 2007).

A study in Baghdad 2012, a total of 17 *E. coli* isolates were collected from urine specimens of patients with urinary tract infection. The MIC of the GT aqueous extract was 275 mg/ml and 250 mg/ml for two selected isolates. The interaction of Sub-MIC of GT aqueous extract with antibiotics showed that Green tea has synergistic effect with Ciprofloxacin and other antibiotics (Passat 2012).

A recent study in Kerbala city, Iraq of the antimicrobial effects of GT and lemon juice on a total of 10 *E. coli* isolates collected from patients with urinary tract infection in Al- Hussein medical city. The concentrations (25%), (50%), (75%), (90%) and (100%) were prepared from the stock for each aqueous and alcoholic for the GT and GT with lemon extracts. The results showed that the best antimicrobial activity found in concentration of 90% GT aqueous extract and at 90% of GT alcoholic extract with lemon. A pour plate method was used to measure the interaction between the extracts and the used antibiotics by pour plates with 0.1 ml of bacterial suspensions in sterile plates then mueller hinton's agar poured and mixed in infinity shape (∞) movement on the bench, after the agar dried 0.1 ml of the effective concentration of GT extracts were distributed over the plates then left to dry and then the antibiotics discs were distributed on the plates , then the plates were incubated for 48

hours at temperature 37ºC. After the incubation the IZD of bacterial growth measured and compared to control plate without the extracts to proof the synergism (Jaafar 2016).

2. Materials and Methods:

2.1. Preparation of GT Aqueous Extract:

GT was obtained from the Saudi Arabia markets and authenticated in National Centre for Research, Khartoum State, Sudan. Aqueous extract prepared by cold maceration by mixing 200 gm of non-fermented leaves of *Camellia sinensis* with 2000 ml distilled water in volumetric flask and left for an overnight, at room temperature, then the extract was filtered through several layers of sterilized cotton. After that the filtered extract poured in clean and sterile dishes and left to dry at room temperature for successive three days, after water evaporation, 50 grams of golden brown extract were collected and stored in sterile labeled bottle at 4°C until use (Mehta et al. 2016).

2.2. Preparation of GT Alcoholic Extract:

Alcoholic extract was prepared by cold maceration by mixing 100 gm of non-fermented leaves of *Camellia sinensis* with 1000 ml of (70%) ethanol in volumetric flask and left for an overnight, at room temperature, and then the extract filtered through several layers of sterilized cotton. The filtered extracts poured in clean and sterile dishes and left to dry at room temperature for successive three days. After water evaporation, 15 grams of golden brown extract were collected and stored in sterile labeled bottles at 4°C until use (Mehta et al. 2016).

2.3. Bacterial Identification:

S. aureus ATCC 25923 and *E. coli* ATCC 25922 were obtained from Medical Laboratory, University of Gezira, Sudan then re-identified using colonial morphology, Gram's stain and conventional biochemical tests (Ahmed et al. 2018).

2.4. Mueller Hinton's Agar Well Diffusion Method:

This method used to evaluate the inhibitory effect of different concentrations of the GT extracts. Bacterial suspensions were prepared from each isolate. 0.1ml of the suspension spread on Muller Hinton's agar using sterile cotton swab. Then pores with 5mm diameter of equal distribution in the plate was made using yellow tips and labeled appropriately. In each

plate three wells were filled with 0.5 ml of specific concentrations of GT extract (concentrations used were 500 mg/ml, 250 mg/ml and 100 mg/ml). Ciprofloxacin disc (5 mcg) used as positive control was placed in the fourth well while the fifth well was filled with water as negative control. Each plate incubated aerobically at 37°C for an overnight. The IZD of each isolate to different concentrations of both Aqueous and Alcoholic GT extracts were measured in (mm) using calibrated ruler. All tests were carried out in triplicate and the mean of IZD were calculated for each microorganism **(**Ahmed et al. 2018).

2.5. Broth Dilution Technique:

It used to determine the MIC and the MBC of GT extracts against the sensitive pathogen. First the bacterial culture was refreshed on nutrient broth and incubated for an overnight at temperature 37°C. Stock solution was prepared by distilled water then two fold serial dilutions of GT extract were prepared from the stock solution and diluted by broth as 1000, 500, 250, 125, 62.5 and 31.25 mg/ml respectively.

The inoculums were prepared by making a direct broth suspension of isolated colonies using peptone water medium, then the suspension adjusted to achieve turbidity equivalent to 0.5 McFarland's turbidity standards. Then 0.1 ml of the adjusted inoculums was added to each tube containing 1 ml of broth and 1 ml of GT extract in the dilution series. All tubes were mixed and incubated aerobically at 37°C for an overnight. MIC and MBC were measured. MIC was a first concentration in the tubes showed no visible growth (turbidity). MBC was detected by subculture of non-turbid tubes next to MIC tube in agar plates and incubated at 37°C for an overnight then MBC reported as first concentration has negative growth in subculture; all tests were carried out in triplicate. **(**Ahmed et al. 2018)

2.6. Interaction between GT Extracts and Ciprofloxacin:

After determination of MIC for GT extract using broth dilution method, the interaction between sub-MIC of GT and Ciprofloxacin (5 mcg) was investigated by taking 0.1 ml from the sub-MIC of GT extract and the inoculums were spread on nutrient agar, then the Ciprofloxacin disc was placed, the plates were incubated at 37°C for an overnight, then the IZD were measured in (mm) using calibrated ruler and compared to IZD of Ciprofloxacin alone All tests were carried out in triplicate and the mean of IZD was obtained for each microorganism (Passat 2012).

In order to determine that Sub-MIC of GT extract has synergetic effect with Ciprofloxacin it should increase it is IZD by 19 % or more, if the IZD increased by less than 19 % then we called it additive effect on the other hand antagonistic effect occurs when the IZD of control is greater than the IZD of the test (Esimone et al 2007).

3. Results:

The yield of 100grams of the dried leaves of *Camellia sinensis* extracted by 1000 ml distilled water (by cold maceration) was 25 grams of brown to gold extract. The yield percentage was 25%. While the yield of 100grams of dried leaves of *Camellia sinensis* extracted by one liter of 70% ethanol by cold maceration was 15 grams of black extract. The yield percentage was 15%.

Agar well diffusion method was used to determine the antibacterial activity of different concentrations (100 mg/ml, 250 mg/ml and 500 mg/ml) of both the aqueous and alcoholic GT extracts. This study demonstrated that only *S. aureus* ATCC 25923 was sensitive to all concentrations of the GT alcoholic extract (image 1,2,3) and both strains *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were resistant to all concentrations of GT aqueous extract.

Table 1: The susceptibility of different concentrations of GT alcoholic extract against *S. aureus* ATCC 25923.

GT alcoholic extract concentrations (mg/ml)	mean of IZD (mm)	Ciprofloxacin (positive control) mean of IZD (mm)
500	18.3	20
250	18	20
100	16.6	20

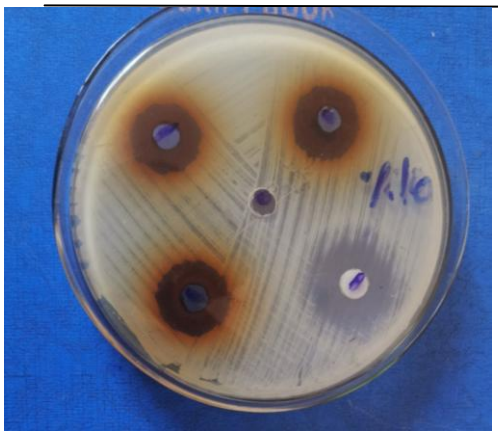

Image 1: 10% of GT alcoholic extract against *S. aureus* ATCC 25923 .

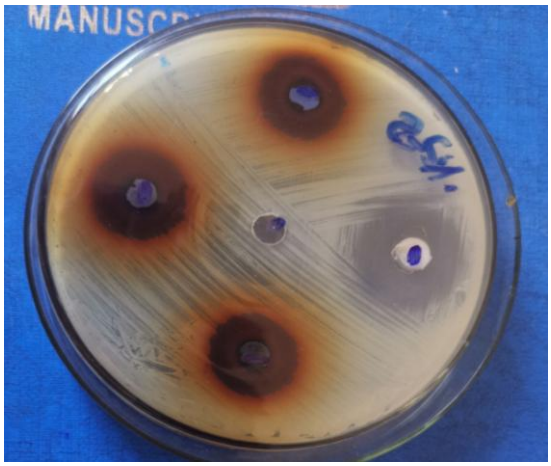

Image 2: 25% of GT alcoholic extract against *S. aureus* ATCC 25923 .

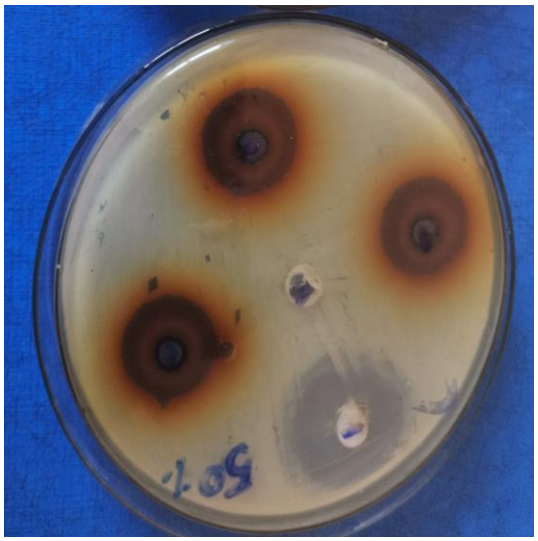

Image 3: 50% of GT alcoholic extract against *S. aureus* ATCC 25923 .

The broth dilution technique was conducted to determine the MIC of GT alcoholic extract against *S. aureus* ATCC 25923 where the results revealed that, the MIC was 225 mg/ml and the MBC was 250 mg/ml (image 4).

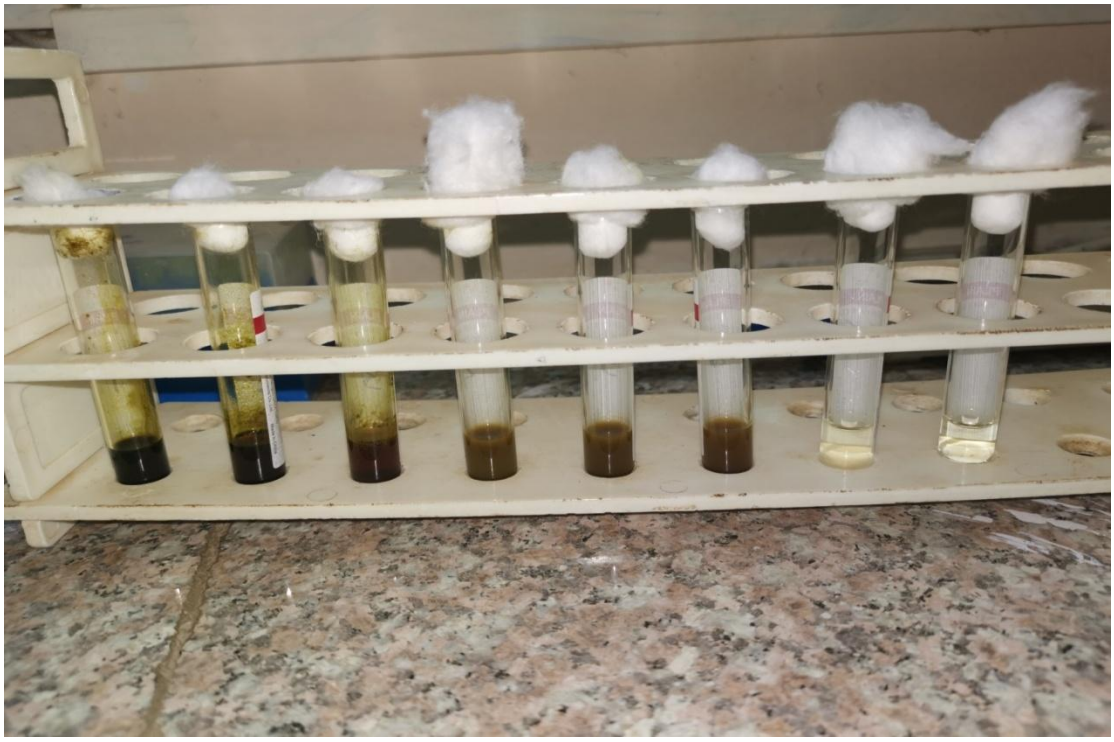

Image 4*:* determination of MIC of GT alcoholic extracts against *S. aureus* ATCC 25923 using broth dilution technique.

The first three tubes (1000 mg/ml, 500 mg/ml and 250 mg/ml) were transparent, then these tubes were sub cultured and they showed no growth so the 250 mg/ml tube was considered as MBC concentration.

For determination of the MIC the broth dilution technique was accomplished with the following concentration 225 mg/ml, 200 mg/ml, 175 mg/ml and 150 mg/ml. The first tube (225 mg/ml) was transparent so it was considered as the MIC concentration.

The interaction between the Sub-MIC of GT alcoholic extract (200 mg/ml) and Ciprofloxacin (5mcg) was investigated, the results revealed that Sub-MIC of GT alcoholic extract increased the IZD of Ciprofloxacin by 62.5 the mean of IZD of the combination was 32.5 mm while the IZD for the Ciprofloxacin alone was only 20 mm. Accordingly this means that GT alcoholic extract act synergistically with Ciprofloxacin against *S. aureus* ATCC 25923(image 5).

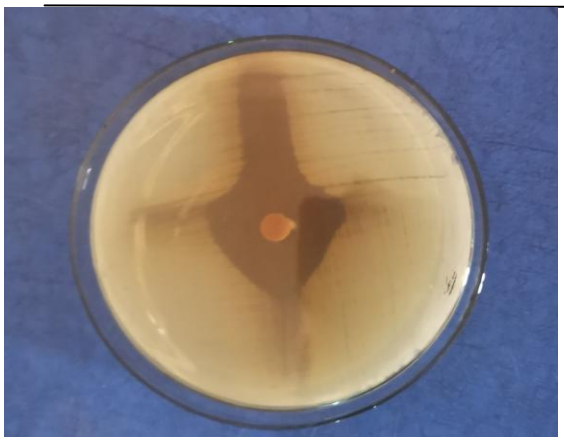

Image 5: Synergistic effect of Ciprofloxacin and sub_MIC of GT alcoholic extract against *S. aureus* ATCC 25923.

4. Discussion:

The present study revealed that the both tested strains were resistant to all concentrations (100 mg/ml, 250 mg/ml and 500 mg/ml) of the GT aqueous and alcoholic extract concentrations except *S. aureus* ATCC 25923 which was sensitive to all concentrations of the GT alcoholic extract comparing this result to a study carried in India by Abhishek Mehta and his colleagues in 2016, they found that the S. *aureus* ATCC 25923 susceptible to both GT aqueous and alcoholic extracts. Also Abhishek confirmed that both GT aqueous and alcoholic extracts has significant activity against *E. coli* ATCC 25922 with all tested concentrations (200, 100, 50, 25 and 12.5 mg/ml) this clearly conflicted with the results of the present study where *E. coli* ATCC 25922 was resistant to all of GT aqueous and alcoholic extracts different concentrations this may be due to the different brands of GT used in these studies as previously mentioned in (Musial et al. 2020) that cultivation and agro-technical conditions affected the GT content of catechins and polyphenols.

On the other hand in USA (2013), the concentrations of GT extract tested were 2.5, 3.0, 3.5, and 4.0 mg/ml where (99%) of the all 80 *E. coli* tested strains, had (MICs) of ≤4.0 mg/ml. In Sudan (2018) a study designed to assess the *in vitro* antimicrobial activity of GT extract against ten uropathogenic of *E. coli* isolates where the results demonstrated that the means of inhibition zone of bacteria to 25%, 50% and 100% concentrations of GT extract were 10 mm, 18 mm, and 30 mm respectively. In contrast in this study the ATCC *E. coli* isolate was resistant to all of GT aqueous and alcoholic extracts concentrations this may be due the different brands of GT used in these studies.

Most of the previous studies on the synergistic effect of GT and Ciprofloxacin were against *E. coli*, while the present study confirmed the synergistic effect of (GT) alcoholic extract and Ciprofloxacin against *S. aureus* ATCC 25923.

5. Conclusion:

This study has shown that GT alcoholic extract has promising antibacterial activity only against *S. aureus* ATCC 25923 where the MIC and the MBC were 225 mg/ml and 250 mg/ml respectively Furthermore, GT alcoholic extract act synergistically with Ciprofloxacin against *S. aureus* ATCC 25923. On the other hand, aqueous GT extract has no antibacterial activity against all tested pathogens.

Conflict of interest:

All authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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