PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL STUDIES OF GREEN, ORTHODOX AND BLACK KENYAN TEA

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Abstract
This study evaluated the phytochemical and antimicrobial activities of green, orthodox and black Kenyan tea on five microorganisms with the possible purpose of determining their pharmacological significance/ medicinal value. The *in vitro* antimicrobial activities of three extracts of tea was done using humanly isolated strains of *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Streptococcus faecalis,* and*, Candida albicans*. The assays were carried out by agar well diffusion. Streptomycin and cefadroxil served as the control drugs. Aqueous tea extracts were used for the assays. The aqueous tea extracts were found to be more effective against the tested bacteria than fungi at high concentration. Orthodox tea had no antimicrobial activity against *Salmonella typhimurium* and *Candida albicans*. Extracts of green tea, orthodox and black tea showed activity on *Staphylococcus aureus* at concentrations ranging from 100-200mgml^{-1} having comparable diameters of zones of inhibition of 10.0±0.0, 4±0.2 and 6.5±0.0 respectively. The first two tea extracts demonstrated activities on *Escherichia coli* and *Streptococcus faecalis* at concentrations ranging from 100-400mgml^{-1} with relatively close diameters of zones of inhibition of 14mm and 12mm respectively. Only black tea inhibited the growth of *Candida albicans* at the MIC of 100mgml^{-1} whereas, *Salmonella typhimurium* was inhibited by green tea and black tea extracts at the MIC of 200mgml^{-1}. Black tea also inhibited growth of *Escherichia coli*, but at concentration ranging from 200-400mgml^{-1} with diameter zones of inhibition from 3.5±0.0- 4.0±0.0 and a MIC of 150mgml^{-1}. Phytochemical screening of the three extracts of tea showed the presence of cardiac glycosides, alkaloids, saponins, flavanoids, terpenes and tannins. Green tea lacked anthraquinones while orthodox tea lacked cardenolides. Results were interpreted according to Kirby-Bauer technique. The results obtained in this study provide preliminary evidence of the significance of secondary metabolites of tea and and their pharmacological effects.

Key words: Green tea, orthodox tea, black tea, phytochemical screening, antimicrobial studies.

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1.0 Introduction

Tea is one of the most widely consumed beverages. Its world wide prominence is attributed to its pleasant flavor combined with its stimulating effects and health benefits (Benjamin *et al*., 1991). Scientific data from pharmacological and physiological studies continue to show that tea has beneficial effects on human health. There are many types of tea, including green tea, black tea and oolong tea and each has several sub-classifications (Benerjee, 1992). All of them are prepared from *Camellia sinensis* (L) theaceae but they vary according to different manufacturing processes. The continued use of tea as a beverage has gained world wide prominence due to the quality of its phytochemicals and other related tea extracts such as polyphenols and catechins (Onishi *et al*., 1981a, 1981b). These pharmacological aspects had been perceived to be more in green tea than in black tea hence the tendency to influence market trends. Tea polyphenols (flavonoids) and their oxidative products are being identified with a number of diverse phamarcotherapeutic effects such as reduction of heart diseases and cancer in humans (Venesa and Williams, 2009), immunosuppresion and lowering oxidative stress (Kaliyar *et al*., 2003), antidiabetis including hyperglyceamia (Vinson *et al*., 2001), lowering levels of cholesterol, triglycerides and decreasing fat tissue accumulation (Tokimitsu, 2004), and the potential improvement in special cognitive learning abilities (Hague *et al*., 2004). These pharmacological roles of tea tend to affect the consumption with tea trade now thriving due to medicinal value associated with catechins. In 2003 China exported 800 tons of tea polyphenols, 300 tons of tea pigments (theaflavins and thearubigins), 10 tons of L- theanin and appreciable amount of tea saponins (Wan, 2004). In this study, green, orthodox and black teas were extracted in water and where necessary ethanol. The presence of phytochemicals was determined calorimetrically by carrying out various chemical tests. The antimicrobial assays were done using agar well diffusion method. Antimicrobial assays were interpreted according to Kirby-Bauer technique (Bauer *et al.,* 1989), by comparing the zones of inhibition produced round tea extract wells with the test org**a**nism and those of the standard control. A venier slide calipher was used to measure inhibition zone diameters in mm to imply activity. However a diameter zone that was more than 6mm implied moderately sensitive, 4mm or less implied resistant, same as or larger than that of the control implied sensitive.

2.0 Materials and Methods

Processed tea samples namely; green (non oxidized), orthodox (partially oxidized) and black (completely oxidized) teas collected from Ngere in Murang'a county Kenya in March 2012, were used. The micro-organisms *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Streptococcus faecalis, Candida albicans* were obtained from the microbial bank maintained in Medical Microbiology Department of JKUAT. The laboratory reagents used in these experiments were of analytical grade obtained from Sigma, Oxoid, Aldrich, Merck, Biochemical and BDH through local dealer Kobian.

2.1 Phytochemical screening

The aqueous extracts of tea were subjected to phytochemical analysis to screen for the presence of secondary metabolites such as alkaloids, saponin, phenolics, tannins, anthraquinones, cardenolides, terpenes, flavonoids and cardiac glycosides. The phytochemical screening was carried out using standard procedures (Martnez and Valencia, 2003, Jigna *et al*., 2007, Herborne, 1973, Trease and Evans, 1989, Ajaiyeoba *et al.,* 2003). Brief description is as follows:

Anthraquinones: 1g of each tea sample was shaken with 10ml of ferric chloride solution with 5ml of hydrochloric acid (HCl). Each mixture was heated in a water bath for 10-15min, filtered and allowed to cool. The filtrate was extracted with chloroform and shaken gently. The clear layer at the base was pipetted into test tubes and 2ml of ammonia sulphate added. An observation of a delicate pink rose indicated the presence of anthraquinones.

Cardenolides: 4g of each tea sample was extracted in the test tube with 80% ethanol, and appropriately labeled. They were divided into two portions for Kedde's test and Keller-Killian's test. For Kedde's test, five drops of 10% lead acetate were added to each of the tubes, followed by five drops of distilled water and chloroform. The contents were evaporated to dryness in a water bath. 5% sodium hydroxide was added to each residue and then 2% of 3,5 dinitrobenzoic acid. For Keller-Killian's test, six drops of 10% lead acetate, water and chloroform were added to each test sample. The mixture was evaporated to dryness in the water bath and subsequently six drops of concentrated sulphuric acid were added. For Keller-Keillan's test, a brown ring indicated the presence of cardenolides, while for Kedde's test a brown to purple colour was indicative of cardenolides.

Phenolics: To 2ml of aqueous tea extract, 1ml of 1% ferric chloride solution was added. Blue or green colour formation was an indication of phenols.

Flavonoids: 2g tea was extracted in 10ml water. To 2ml filtrate four drops of concentrated hydrochloric acid (HCl) followed by 0.5g magnesium turnings was added. After 3min magenta or pink colour formation indicated the presence of flavonoids.The test was repeated by using 2g of tea extracted in 10ml ethanol.

Terpenes: To 2ml of aqueous extract, 5mg chloroform, 2ml acetic anhydride, concentrated HCI were added carefully to form a layer. Redish brown colour at the interface was an indication of terpenes.

Cardiac glycosides: To 2ml of ethanoic filtrate, 1ml glacial acetic acid and 1-2 drops of ferric chloride were added followed by 1ml of concentrated sulphuric acid. Presence of brown ring at the interface indicated the presence of cardiac glycosides.

Saponin: 5ml of each aqueous tea extract was placed into a test tube and diluted with 5ml of distilled water. The mixture was shaken vigorously for 2min, persistence appearance of foam lasting for 5min or the forming of emulsion when olive oil was added confirmed the presence of sponins.

Alkaloids: The tea extracted (2g) was hydrolyzed with 2ml hydrochloric acid (HCl) solution by heating in water bath for 10 min, allowed to cool and 5ml of filtrate was reacted with five drops of Dragendoff's Mayer's Wagner's reagents (18mM I_2, 18mM KI). Alkaloids were recorded as present in the sample if turbidity or brownish precipitate was observed.

2.2 Antimicrobial screening

Four human pathogenic bacteria consisted of two gram positive *Staphylococcus aureus* and *Streptococcus faecalis* and two gram negative *Salmonella typhimurium* and *Escherichia coli* were used for antibacterial assay. One yeast *Candida albicans* was used for antifungal assay. All the organisms were local isolates from the laboratory bacterial stock of the Medical Microbiology Department of JKUAT, Kenya. Three to five identical colonies from stored slopes of microorganisms were lifted with a sterile wire loop and transferred into single strength nutrient broth(sigma) contained in well labeled screw cap bottles for each bacterium and fungus respectively. The bottles were well shaken and incubated at room temperature for 18-24 hrs for bacteria and 72 hrs for fungi. The agar well diffusion method was used to test the tea extract for antimicrobial activity. In a duration of 5 minutes, 15ml of melted and cooled nutrient agar (Sigma Laboratories, USA) and potato dextrose agar (Sigma Laboratories, USA) were added to 0.2ml in 100 dilutions of bacteria and fungal cultures respectively in sterile petri dishes. The contents were mixed after the agar in each plate solidified. Six wells of 5mm each were bunched in each plate using aseptic pipette tip. 0.1ml of aqueous tea extracts at varying concentrations (50mgml^{-1}, 100mgml^{-1}, 200mgml$^{-1,}$ and 400mgml^{-1}) as well as the 4.0 mgml^{-1} standard antibiotic solutions were loaded into the wells as references. Control experiments were set up using streptomycin and cefadroxil (4mgml^{-1}) as positive control, and water as negative control for the bacterial and fungal assays. The plates were incubated at 37^{o}C for 24hrs for bacteria and 48hr for fungi.

All inoculation procedures were carried out under aseptic conditions. The antimicrobial studies were done in three replicates. With the aid of a venier slide calipher the diameter of zones of inhibition around the wells were measured in mm for all the three replicates and the average of the three measurements were calculated as an indication of activity. The results were interpreted according to the modified Kirby-Bauer technique (Bauer *et al.,* 1989). The minimum inhibitory concentration (MIC) of tea extracts was determined using the broth dilution method as described by Salon and Washington (1990). In a duration of 5 minutes, 1ml of the tea extract solution at the concentration of 400mgml^{-1} was added to1 ml of nutrient broth and subsequently transferred to make solution of varying concentration (400mgml^{-1}, 200mgml^{-1}, 100mgml^{-1}, 50mgml^{-1}) in different test tubes. Then 1ml of bacterial and fungal suspension and 1ml of tea extracts at different concentrations was added to each test tube and incubated at 37^{0}C for 24 hrs for bacteria and 48hr for fungi. The test tube with the concentration of tea extract at which no detectable growth was observed was considered as the MIC.

3.0 RESULTS

The results of the phytochemical screening of the tea samples are presented in table 1. The secondary metabolites tested were alkaloids, saponin, phenolics, anthraquinones, cardenolides, terpenes, flavonoids and cardiac glycosides. The results showed that alkaloids, saponin, phenolics, anthraquinones, cardenolides, terpenes, flavonoids and cardiac glycosides were present in all extracts except in green tea which lacked anthraquinones. Cardenolides were present in green and black tea but absent in orthodox tea. The test method used showed that phenolics were absent in orthodox tea but present in green and black tea. The results of the antimicrobial screening of the aqueous tea extracts are presented in table 2, while the minimum inhibitory concentration (MIC) of each extract are shown in table 3. The tea extracts were found to be more effective in the tested bacteria than they were on fungi. Green and orthodox tea extracts showed important inhibition of *Salmonella typhimurium* and *Escherichia coli* gram positive bacteria, at the concentrations of 200mgml^{-1} and 400mgml^{-1}. Orthodox tea extract did not show activity against *Salmonella typhyimurium* but had activity against *Escherichia coli* at a concentration of 200mgml^{-1} and 400 mgml^{-1}. All the tea extracts had activity against *Staphylococcus aureus* and *Streptococcus faecalis* gram negative bacteria and only the extract of black tea was active against *Candida albicans* (a fungus) with the diameter of the zone of inhibition of 6±0.01mm and the MIC of 200mgml^{-1}.

Further analysis of the tea extracts to assess variation on disc diameter showed that the crude tea extracts had activities that had great variation on inhibition of the micro organisms (table 2). There was significant difference ($P<0.05$) in inhibition diameter variation and thus activity between and within various tea treatments. The tea extract that did show activity had an inhibition zone diameter of more than 4mm for example, green tea inhibited *Escherichia coli* at a concentration of 200mgml^{-1} and had an inhibition diameter of 14mm. The tea extract that did not show activity had a disc diameter of less than 4mm, for example orthodox tea had no activity against Salmonella *typhimurium* and *Candida albicans* (table 2).

Table 1: Phytochemical constituents of crude extract of tea samples

	Green tea	**Orthodox tea**	**Black tea**
Phenolics	+	-	+
Flavonoids	+	+	+
Terpenes	+	+	+
Cardiac glycosides	+	+	+
Cardenolides	+	-	+
Anthraquinones	-	+	+
Alkaloids	+	+	+
Saponins	+	+	+

+ Presence of secondary metabolite;
- Absence of secondary metabolite

Table 2: Antimicrobial activity of aqueous crude tea extracts of green tea, orthodox tea and black tea, zones of inhibition diameter (mm).

Tea extract	Conc. $mgml^{-1}$	*Staphylococcus aureus*	*Escherichia Coli*	*Salmonella typhimurium*	*Streptococcus faecalis*	*Cadida albicans*
Green tea	50	-	-	-	-	-
	100	10± 0.0	-	-	12.0±00	-
	200	15± 0.0	14± 0.0	5± 0.0	14.0±00	1±0.0
	400	20± 0.0	18 ±0.0	18± 0.0	15.0±00	1±0.4
Orthodox tea	50	-	-	-	-	-
	100	-	-	-	-	-
	200	4 ±0.2	6± 0.0	-	10± 0.0	-
	400	8 ± 0.0	14± 0.0	-	12 ±0.0	-
Black tea (Murang·a)	50	-	-	-	-	-
	100	-	-	1± 0.0	2±0.0	4±.00
	200	6.5± 0.0	3.5 ±0.0	3± 0.0	5±0.0	6±0.0
	400	7.4± 0.2	14 ±0.0	4 ±0.0	6±0.2	9±0.0
Streptomycin	4.0	20±0.0	10±0.0	15±0.0	10±0.0	25±0.0
Cefadoxil	4.0	20±0.0	20±0.0	20±0.0	20±0.0	10±0.0

Absence of antimicrobial activity
Arabic numerals – inhibition diameters in mm.

The antimicrobial assays were done in three replicates and diameters of zones of inhibition measured in mm as indication of activity. Results are means ± SD of the three replicates diameters of zones of inhibition of assays.

Table 3: Minimum inhibitory concentrations of tea aqueous extracts on selected micro-organisms.

Tea extract	Conc. $mgml^{-1}$	*Staphylococcus aureus*	*Escherichia Coli*	*Salmonella typhimurium*	*Streptococcus faecalis*	*Cadida albicans*
Green tea	400	-	-	-	-	-
	200	-	-	-	-	-
	150	-	-	+	-	-
	100	-	+	+	-	+
	50	+	+	+	+	+
	25	+	+	+	+	+
Orthodox tea	400	-	-	-	-	+
	200	-	-	-	-	+
	150	-	-	+	-	+
	100	-	+	+	-	+
	50	+	+	+	+	+
	25	+	+	+	+	+

Black tea (Murang'a)	400	-	-	+	-	-
	200	-	-	+	-	-
	150	-	-	+	-	-
	100	-	+	+	-	-
	50	+	+	+	+	+
	25	+	+	+	+	+

- No growth observed
+ Growth observed

4.0 Discussion and Conclusion

The presence of the secondary metabolites (alkaloids, terpenes, saponins, flavonoids, cardiac glycosides, cardenolides anthroquinones and phenols) in tea partly enhances the antimicrobial and anti parasitic activity of the green, black and orthodox tea . The antimicrobial and antiparastic activities shown by tea metabolites are in line with the previous work of antifungal, antioxidant and larvicidal activities of compounds isolated from the heartwood of *Mansonia gagei* (Tiew *et al*., 2003). Although the presence of similar secondary metabolites may necessarily justify the closeness of the three types of tea, it is a noteworthy observation that the three types of tea differ in terms of the oxidization in their polyphenols brought about by the different processing of manufacture. Owing to the presence of these five secondary metabolites and similarity of their occurrence it is however worth to note that their presence depends on many factors; season, rain, collection time, part collected and other agronomic factors.

The lack of an *in-vitro* antimicrobial activity may not necessarily imply the same *in-vivo* since compounds may either act as pro-drug which must undergo metabolic change to achieve the required activity. Whereas, some plants cannot display *in vitro* activity they may display *in vivo* activity (Gassier *et al*., 1995) or vice versa. This claim could be strengthened with a further evaluation of the active principles responsible for the antimicrobial activities observed in these tea extracts. The environment is known to potentially influence the monopoly and expression of compounds in plants (Folkers *et al*., 2008, Tsukaya *et al*., 2007). This might be the case with orthodox and black tea samples used; they showed the presence of anthraquinones. Normally those plants found in the forest posses this compound other than the ones domesticated in their habitat (Shen *et al*., 2008, Brag *et al*., 2006). Therefore, the presence of anthraquinones in these samples could be due to environmental conditions (Cybulskill *et al*., 2000) or due to the omission of aeration step during processing of green tea, since the basis of aeration are to bring oxygen and substrates together by rupturing the membrane so that polyphenols can diffuse into the cytoplasm. The enzyme polyphenol oxidase oxidizes the polyphenolic bodies to anthraquinones. These substances of anthroquinones can exist both in the free form and as glycosides. Natural anthraquinones are synthesized either via acetate mevalonate pathway or from shikimate and mevalonate pathway. The

medicinary important purgative anthroquinone are formed by the latter pathway and all have 1,8-dihydroxyl substitution. Purgatives are known to cause excessive gastrointestinal muscle contractions. The absence of cardenolides and phenolics in orthodox tea is an unusual occurrence; more research is needed to make a meaningful deduction of this condition. Cardiac glycoside is known to act in competition with Potassium (K^+) for specific enzyme receptor suite in a cell. They act in competition in the membrane of cardiac muscle when there is influx of Sodium (Na^+) ions. The aglycones of cardiac glycoside are derived from the melonic acid but the final molecules arise from the condensation of C-21 steroid with a C-2 chrut (Kren *et el*., 2001). The tea extracts also contains saponins which are used to stop bleeding and treating wounds and ulcers as they help in red blood cell coagulation (Okwu and Josiah, 2006). Other secondary metabolite constituents detected in tea were alkaloids. Alkaloids have numerous functions and among the foremost are their analgesic, antispasmodic and bacterial effects (Okwu and Josiah, 2006., Kisangau *et al*., 2007). Tea is also rich in tannins and contributes property of astigency i.e faster healing of wounds and inflamed mucus membrane ((Okwu and Josiah, 2006).

The antimicrobial activities shown by green tea on *Streptococcus faecalis* are in line with the previous antimicrobial works in the species of *Streptococcus mutans* and other work linking antimicrobial activities with presence of secondary metabolites (Sakanaka *et al*., 1989b., Ebana *et al*., 1991.,Kisangau *et al*. ,2007., Kubnamwa *et al*., 2007., Rheid *et al*., 2005). One of the bacterial responsible for causing dental carries (Hamada and Slade, 1980., Onishi 1981a., Onishi, 1981b), where green tea was found to exhibit important inhibitory activities against the bacteria. The crude aqueous extract of green, orthodox and black tea showed important activity against *Staphylococcus aureus, Escherichia coli,* and *Streptococcus faecalis*. These extracts could be a source of new antibiotic compounds. Further work is needed to isolate the secondary metabolites from the extracts studied in order to test specific antimicrobial activity. Further to that, the minimum inhibitory concentration (MIC) of tea yielded promising results that are worthy to note. Green tea had low MIC of $100mgml^{-1}$,$150mgml^{-1}$, $100mgml^{-1}$, and $200mgml^{-1}$ for *Staphylococcus aureus, Escherichia coli, Streptococcus faecalis* and *Salmonella typhimurium,* respectively. This suggests that they can be gainfully employed in the production of antibiotics as low MIC mean that only a small quantity of the extract will be required to impair bacteria growth. The average MIC of black tea on *Candida albicans* was $100mgml^{-1}$, a value which is still low enough to be of great antimicrobial advantage. The closeness observed in antimicrobial activities demonstrated by green tea and orthodox tea as revealed by values obtained for the MIC could also indicate a close relationship. In conclusion, the chemical composition of green tea is similar to that of the leaf since it is not oxidized and it contains polyphenolic compounds which include flavones, flavonols, flavonoids among others and phenolic acids account for 30% of the dry weight of green tea leaves. In general, antimicrobial activities decreased when the extents of tea fermentation increased. The antimicrobial

activities of various tea extracts with different extent of fermentation varied with test organisms. Green tea exerted the strongest antimicrobial activity followed by the partially fermented orthodox tea and lastly by black tea. From the results obtained in this study, tea polyphenols and the other metabolites could serve as models for the rational design of synthetic antibiotic analogues with higher *in vitro* and i*n vivo* activities and more favorable properties. More scientific research is also required to fractionate tea extracts using ethyl acetone or methyl acetone (Ajayeioba anb Sama, 2006) and purify pure compounds of these polyphenols and phytochemicals and identifying specific drug targets in the microrganism of interest. This study can be useful in the comparative studies of the presence of bioactive principles present in tea with its other clones and population, belonging to different climatic conditions. This data can also help us to choose the superior race of this valuable shrub with greater quantity and quality of medically and therapeutic important phytochemicals.

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