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. The aqueous tea extract 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 100-150mgml^{-1} having comparable diameters of zones of inhibition of 10.0±0.0 20.0±0.0, 4±0.2- 8.0 ±0.0 and 6.5±0.0, 7.4 ±0.2 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. Only black tea inhibition the growth *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 *E. 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}. Phytoscreening of the three extracts of tea showed the presence of cardiacglycosides, alkaloids, saponins, flavanoids, terpenes and tannins.

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

1.0 Introduction

Tea is one of the most widely consumed beverage. Its worldwide prominence is attributed to its pleasant flavor combined with it stimulating effects and health benefits. 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) theaca and its varieties by different manufacturing processes. The continued use of tea as a beverage has gained worldwide prominence due to the quality of its phytochemicals and other related tea extracts such as polyphenols and catechins. 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) andtheir 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). Immunosuppression and lowering oxidative stress (Kaliyor *et al*., 2003), antidiabetis including hyperglycemia (Vinson *et al*., 2001) lowering levels of cholesterol, triglycerides and decreasing fat tissue accumulation (Tokimitsu *et al.*, 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 e.g., in 2003 China exported 800 tons of tea polyphenols,300 tons of tea pigments (thaflavins and thearubigins), 10 tons of L-theanin and appreciable amount of tea saponins (Wan *et al*., 2004).

2.0 Materials and Methods

Processed tea samples (green, orthodox and black tea) collected in Murang'a Kenya in March 2012, were used. The micro-organism *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Streptococcus faecalis, Candida albiccans* 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, Flavinoids and Cardiac glycosides. The phytochemical screening was carried out using standard procedures (Martnez, 2003, Jigna *et al*., 2007, Herborne 1973, Trease and Evans, 1989). The description is as follows:

2.1.1 Anthraquinones

One gram 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 pipette into test tubes and 2ml each of ammonia sulphate added. An observation of a delicate pink rose indicated the presence of anthraquinones.

2.1.2 Cardenolides

Four grams of each sample was extracted in the test tube with 80% ethanol, and appropriately labeled. They were divided into two portions for Kedde's test and Keler-Killian's test. For Kedde's test, few drops of 10% lead acetate were added to each of the tubes, followed by few 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 dinitrobenzene acid. For Keller-Killian's test, few 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 a few drops of concentrated sulphulic acid were added. For Keller-Keillan's test, a brown ring indicated the presence of cardenolides, while for Keddi's test a brown to purple colour was indicative of cardenolides.

2.1.3 Phenolics

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

2.1.4 Flavinoids

2g tea was extracted in 10ml alcohol or water. To 2ml filtrate few drops of concentrated hydrochloric acid (HCl) followed by 0.5g zinc or magnesium turnings was added. After 3min magenta or pink colour formation indicated the presence of flavonoids.

2.1.5 Terpenes

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

2.1.6 Cardiac Glycosides

To 2ml alcoholic filtrate, 1ml glacial acetic acid and 1-2 drops of ferric chloride were added followed by 1ml of concentrated sulphulic acid. A presence of brown ring at the interface indicated the presence of cardiac glycosides. A violet ring might also appear below the brown ring.

2.1.7 Saponin

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

2.1.8 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 a few drops of Dragendoff's Mayer's Wagner's reagents, alkaloids were recorded as present in the sample if turbidity or brownish precipitate was observed.

2.2 Antimicrobial Screening

Four human pathogenic bacteria made 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, JKUAT, Kenya. Three to five identical colonies from stored slopes of microorganisms were lifted with a sterile wire loop and transferred into a single strength nutrient broth(sigma) contained in a 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 72hrs for fungi. The agar well diffusion method was used to test the tea extract for antimicrobial activity. Briefly 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 gar in each plate solidified, 6 wells of 5mm each were bunched in each plate using aseptic pipette tip. 0.1ml of tea extracts at varying concentrations (50mgml^{-1}, 100mgml^{-1}, 200mgml$^{-1,}$and 400mgml^{-1}) as well as the standard antibiotic solution was loaded into the wells. Control experiments were set up using streptomycin and cefadroxil (4mgml^{-1}) for the bacteria 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 triplicate. With the aid of a transparent ruler 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-Baur technique. The minimum inhibitory concentration (MIC) of tea extracts was determined using the broth dilution method as described by Salon and Washington (1990). Briefly 1ml of the 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, 100mgml^{-1}50mgml^{-1}) in different test tubes. The 1ml of bacterial and fungal suspension and 1ml of 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.

2.3 Statistical Analysis

Data obtained from diameter zones of inhibition were subjected to ANOVA. Further analysis of the tea extracts to assess variation on disc diameter showed that the crude tea extracts had an activity had great variation on inhibition of the microorganisms (Table 3). There was significant difference ($P<0.05$). Those that did not show activity had a disc diameter of less than 3mm. Thus in inhibition activity, table 3 gives the variation inhibitory activity on the microorganisms since they show susceptibility to the tea extracts except for *Salmonella typhimurium* and *Candida albicans* which were not susceptible to orthodox tea.

3.0 Results

The results of the phytochemical screening of the tea samples is presented in Table 2. The secondary metabolites tested wereAlkaloids, Saponin, Phenolics, Tannins, Anthraquinones, Cardenolides, Terpenes, Flavinoids and Cardiac glycosides. The results shows that Alkaloids, Saponin, Phenolics, Tannins, Anthraquinones, Cardenolides, Terpenes ,Flavinoids and Cardiac glycosides are present in all extracts except in green tea. Cardenolides are present in green and black tea but absent in orthodox tea. Phenolics are 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 3, while the minimum inhibitory concentration (MIC) of each extract are shown in table 4. 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 200 and 400mgml^{-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 4.02mm and the MIC of 100mgml^{-1}.

Table 1: Sample information of tea studied

Name	Locality of Collection	Fermentation Status
Green tea	Kangaita	Non-fermented
Orthodox tea	Kangaita	Semi- fermented
Black tea	Murang'a	Fermented

Table 2: Phytochemical constituents of crude extract of tea samples

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

+ Presence of secondary metabolite
- Absence of secondary metabolite

Table 3: Antimicrobial activity of crude tea extracts of Green tea, Orthodox tea and Black tea

Tea extract	Conc. $mgml^{-1}$	*Staph aureus*	*Escherichia Coli*	*Salmonella typhim.*	*Streptococcus faecolis*	*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±.002
	200	6.5± 0.0	3.5 ±0.0	3± 0.0	5±0.0	6±0.01
	400	7.4± 0.2	14 ±0.0	4 ±0.0	6±0.2	9±0.01
Streptomycin	4.0mg/ ml	20±0.0	10±0.0	15±0.0	10±0.0	25±0.0
Cefadoxil	4.0mg/ ml	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

Table 4: Minimum inhibitory concentrations of aqueous extracts of tea on selected micro-organisms

Tea extract	Conc. mgml^{-1}	*Staph aureus*	*Escherichia Coli*	*Salmonella typhimurium*	*Streptococcus faecolis*	*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, tannins, flavonoids, cardiac glycosides, cardenolides anthroquinones and phenols) in tea partly enhances the antimicrobial and anti-parasitic activity of the green, black and orthodox tea. 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 polphenols brought about by the different processing of manufacture. Owing to the presence of these five secondary metabolites and similarly 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 (Gessier *et al*., 1995) or vise 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. Therefore the presence of anthraquinones in these samples could be due to environmental conditions. These substances of anthroquinones can exist both in the free form and as glycosides. Natural anthroquinones are synthesized either via acetate mevolanate 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 K^+ for specific enzyme receptor suite in a cell. They act in competition in the

membrane of cardiac muscle when there is influx of 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. The tea extracts also contains saponins which is used to stop bleeding and treating wounds and ulcers as it helps in red blood cell coagulation (Okwu and Josiah, 2006). Other secondary metabolite constituent detected in tea are alkaloids. Alkaloids have numerous functions and among the foremost are their analgesic, antispasmodic and bacterial effects (Okwu and Josiah, 2006). 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* (Sakanaka *et al.,* 1989b). One of the bacterial responsible for causing dental carries (Hamada and Slade, 1980), 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 *Streptococcus aureus, Escherichia coli, 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 of note. Green tea had low MIC of $100mgml^{-1}$,$150mgml^{-1}$, $200mgml^{-1}$, $100mgml^{-1}$ for *Streptococcus 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 and it contains polyphenol compounds which include flavanols, flavaniods, Flavinoids and Phenolic acids account for 30% of the dry weight of green tea leaves. In general, antimicrobial activities decrease 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 these studies, 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 and purify pure compounds of these polyphenols and phytochemicals and identifying specific drug targets in the microorganism of interest. This study will prove 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 herb with greater quantity and quality of medically and therapeutic important phytochemicals.

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