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**Full Length Research Paper**

**Phytochemical screening, antioxidant and anticholinesterase effects of *Alangium salvifolium* (L.F) Wang root extracts**

Md. Nasrullah¹, Anamul Haque², Zerina Yasmin¹, Mohammad Ashraf Uddin¹, Kushal Biswas³ and Mohammed Saiful Islam³

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*Alangium salvifolium* *wang* is a medicinal plant of the Alanginaceae family which was used as a traditional medicine to cure or prevent a variety of ailments. The aim of the study was to investigate and compare the phytochemical profile, antioxidant and anticholinesterase effects of ethanol (EASR), dichloromethane (DASR), chloroform (CASR) and aqueous (AASR) extracts of *A. salvifolium* root. Phytochemical screening was done by using qualitative methods whereas total phenol content (TPC), total flavonoid content (TFC) and total flavonol content (TFlC) were determined by Folin-Ciocalteau reagent, aluminium trichloride and sodium acetate solution methods, respectively. Antioxidant activities were assessed by DPPH radical scavenging, ferric reducing antioxidant power (FRAP) and total antioxidant content (TAC) assay. Ellman's assay was applied to investigate acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme inhibitory effect. Preliminary phytochemical screening revealed the presence of valuable phytochemicals with significantly (*P*<0.05, *P*″<0.01, *P*‴<0.001) different content of TPC, TFC and TFlC. CASR, among the extracts, had shown the highest TPC (492.38±22.34 mg/g gallic acid), followed by TFC (276.25±17.23 mg/g quercetin) and TFlC (332.92±7.07 mg/g quercetin). Moreover, maximum antioxidant potential, including DPPH radical scavenging (*IC*₉₀: 11.26±1.29 µg/ml), FRAP (*EC*₉₀: 26.64±2.17 µg/ml) and TAC (639.55±10.51 mg/g ascorbic acid) was found in the CASR. Donepezil, a standard drug, showed maximum inhibitory effect of AChE (*IC*₉₀: 7.94±1.12 µg/ml) and BChE (*IC*₉₀: 12.58±2.15 µg/ml). CASR followed by DASR had potent inhibitory effects while AASR had mild and EASR practically had no inhibitory effects of the enzymes. The present study has demonstrated that the root extracts of the *A. salvifolium* have moderate to potent antioxidant and enzyme inhibitory effects.

**Key words:** *Alangium salvifolium*, antioxidant, Anticholinesterase effect, phenol content, flavonoid content, 2,2-diphenylpicrylhydrazyl (DPPH), reducing power.

**INTRODUCTION**

Free radical damage and oxidative stress are considered as important causative factors for generation as well as exacerbation of various ailments like cancer, diabetes, asthma, and the pathogenesis of alzheimer’s disease.
(AD) (Asmat et al., 2015). Oxidative stress, a potential source of damage to DNA, lipids, sugars and proteins, causes an imbalance between the intracellular production of free radicals/reactive oxygen species (ROS) and antioxidant defense mechanisms, resulting in cellular injury (Gjumrakch et al., 2008). The brain consumes a large proportion of the inhaled oxygen, and therefore produces a comparatively large quantity of free radical by-products (Yongxin et al., 2013). However, less quantity of the reactive oxygen (ROS) species are the precondition to keep the integrity of the neuronal cells and subsequently their normal functioning, since the elevated level of the radicals can lead to neuronal cell death (Yongxin et al., 2013). In contrast, antioxidants, being the defensive agents against the oxidative stress, have multiple functions in biological systems, including maintenance of cell integrity and cell signaling pathways (Kumar et al., 2008). One principal cellular function of antioxidants is to prevent damage caused by the ROS. Various studies have proved that an antioxidant may scavenge a highly reactive free radical or may inactivate it by donating a proton atom or by accepting an electron from the radical, and eventually prevents the free radical-induced diseases (Jiaojiao et al., 2012).

Alzheimer, the most common among the neurodegenerative disorders and dementia, is a major challenge of the modern era, and is a slowly progressive disease of the brain that is characterized by the impairment of memory (Rahmat et al., 2012). For normal functioning of brain, sufficient level of acetylcholine (Ach) is necessary which is essential for proper neurotransmission. Acetylcholinesterase (AChE) enzyme catalyzes hydrolysis reaction of the Ach and butyrylcholinesterase (BChE) potentiates the catalyzing activity of the AChE, resulting in a decreased level of Ach in the brain (Zeb et al., 2014). This condition leads to neurodegeneration and subsequently cognition. So, inhibition of AChE and BChE may be the most effective way of protecting the Ach to prevent or to improve dementia.

**Alangium salvifolium** wang belongs to the family of **Alanginaceae.** Ankola and Alangi are its common name in India, and Stone Mango in English. It is a small deciduous thorny tree or shrub (Uthiraselvam et al., 2012) which is distributed in tropical and subtropical region such as Bangladesh, India, China, Phillipines, Africa, Sri Lanka and Indochina (Ronok et al., 2013). An array of ailments including diabetes, jaundice, gastric disorders, protozoal diseases, rheumatic pain, burning sensation, haemorrhages, lung cancer, poisonings, leprosy and many inflammatory patches have been treated by using various parts of the plant (Meera et al., 2013). Many bioactive phytochemicals such as several flavanoids, phenolic compounds, irridoid glycosides and oxyglucosides have been isolated by phytochemical screening of it (Gopinath, 2013). Literature review of the plant indicates the presence of courmarins, triterpenoids, and some potent alkaloids in it (Savithramma et al., 2012). The aim of the present study was to evaluate antioxidant and anticholineesterase effects of various extracts of the **A. salvifolium** root.

**MATERIALS AND METHODS**

**Plant**

For the investigation, **A. salvifolium** wang root was collected from Rajshahi, Bangladesh between January and June, 2013 and identified by an expert of the Bangladesh National Herbarium, Dhaka, where a voucher specimen number was retained with an accession no. 40214. The collected plant part was cleaned, dried for one week and pulverized into a coarse powder using a suitable grinder. Powdered material was stored in an airtight container and kept in a cool, dark, and dry place until further analysis was taken.

**Extract preparation**

Approximately 500 g of powdered root was placed separately in four clean and flat-bottomed glass containers and soaked in ethanol, dichloromethane, chloroform and distilled water. All the containers with their contents were sealed and kept for 7 days. Then extraction was carried out using ultrasonic sound bath accompanied by sonicaction (40 min). The entire mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then the extract was filtered through Whatman filter paper and concentrated by using a rotatory evaporator at reduced pressure. The gummy extracts were then dried by using an electric oven, and finally obtained EASR (12.25 g), DASR (9.5 g), CASR (7.5 g) and AASR (14.17 g). The dried extracts were separately stored in air tight containers until completion of the analysis.

**Drugs and chemicals**

Enzymes including acetylcholinesterase (AChE) electric eel (type VI-S), butyrylcholinesterase (BChE) equine serum lyophilized, substrates acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BuTCI), chromogen 5, 5-dithio-bis (2-nitrobenzoic) acid (DTNB) and serine were purchased from Sigma-Aldrich, USA, 1,1-Diphenyl-2-picyrylhydrozyl (DPPH), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), quercetin, ascobic acid, gallic acid, ferric chloride, and glacial acetic acid were purchased from Merck.

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Phytochemical screening

Phytochemical screening of the extracts was done by applying some previously established methods. Alkaloids, saponins, terpenoids and steroids were detected by applying Harborne (Harborne, 1973) method. Flavonoids and tannins were examined by applying methods of Sofowara (Sofowara, 1993). Reducing sugar and resins were evaluated by following methods of Dipali et al. (2013). Coumarins, anthraquinones, cardiac glycosides and phlobatannins were detected by applying the methods of Trease and Evans (Trease and Evans, 1989).

Determination of total flavonoid content (TFC)

TFC of the extracts was determined by using the Folin-Ciocalteu method with slight modification (Gao et al., 2000). Briefly, the extracts and standard gallic acid solution (1 ml) was mixed with 2.58 ml of Folin-Ciocalteau’s phenol reagent. After 3 min, 0.3 ml of saturated sodium carbonate solution was added to the mixture and incubated at room temperature (25°C) for 20 min. Then, absorbance of each sample was measured at 760 nm with a spectrophotometer. TPC of the extracts was calculated from the regression equation \( r^2 = 0.958 \) of the standard gallic acid and the results were expressed as milligram per gram of gallic acid equivalent of the dried extracts.

Determination of total flavonoid content (TFC)

1 ml extract in methanol (200 mg/ml) was mixed with 1 ml aluminum trichloride in ethanol (20 mg/ml, and a drop of acetic acid), and then the mixture was diluted by the addition of ethanol up to its 25 ml volume. Blank samples were prepared by adding all the reagents with equal volume used in the sample, except the extract. The absorbance of the solution was read at 415 nm after 40 min of incubation at room temperature. Using the same procedure for absorbance of quercetin, standard compound of flavonoid was read and TFC of the extracts was calculated from the standard curve \( r^2 = 0.902 \) of the quercetin (12.5 to 200 mg/ml). Total flavonoid content was expressed as mg/g of quercetin equivalent (Kumaran and Karunakaran, 2007).

Estimation of total flavonol content (TFIC)

TFIC was determined by applying a method previously described by Mbaebie et al. with slight modification (Mbaebie et al., 2012). According to the method, 1 ml of the extracts (200 μg/ml) was taken separately in different test tubes. 2 ml ethanol solution of AlCl₃ and 3 ml of (50 g/l) sodium acetate solution were added in the test tubes. After gently mixing, all the test tubes were allowed to stand for 2.5 h at 20°C temperature. Then, absorbance was determined by using a spectrophotometer at a wavelength of 440 nm. Quercetin was used as standard flavonol compound. Following the aforementioned procedure, absorbance of the quercetin was taken at various concentrations (25 to 400 μg/ml) of series dilution. TFIC of the extracts was calculated from regression equation \( r^2 = 0.951 \) of the standard quercetin and the results were expressed as milligram per gram of quercetin equivalent of the dried extracts.

Antioxidant assay

Determination of total antioxidant content (TAC)

TAC of the extracts was evaluated by phosphomolybdenum complex method with slight modification, which was described by Prieto et al. (1999). Briefly, a reagent solution was prepared having 0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate in distilled water. 1 ml of each extract was combined with the reagent solution in separate test tubes. After shaking gently, the test tubes were incubated for 90 min at 95°C temperature. Then after cooling at room temperature, absorbance was measured at 695 nm wavelength using a spectrophotometer. Similarly, ascorbic acid, a standard antioxidant, was run through the process at different concentration gradient (25 to 400 μg/ml). Using this absorbance value, a standard calibration curve and a regression equation \( r^2 = 0.964 \) was derived, from which TAC of each of the extracts was determined and expressed as mg/g of ascorbic acid equivalent of the dried extracts.

Determination of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging activity was measured by an established method described by Braca et al. (2002). Briefly, 0.004% w/v of DPPH radical solution was prepared in methanol and then 900 μl of this solution was mixed with 100 μl of extract or standard ascorbic acid solution (12.5 to 200 μg/ml) and kept in a dark place for thirty minutes. Then, absorbance was measured at 517 nm. Scavenging capacity of DPPH radicals (% Inhibition) was measured by the following formula and finally the 50% inhibition concentration (IC₅₀) was calculated using MS-Excel software.

\[
\text{Inhibition} \% = \frac{A_0 - A_e}{A_0} \times 100
\]

Where \( A_0 = \) Absorbance of control group, \( A_e = \) Absorbance of sample.

Ferric reducing antioxidant power (FRAP) assay

The \( \text{Fe}^{3+} \) reducing power was determined by the method of Oyaizu (1986) with slight modifications. Shortly, 1 ml of extract or standard ascorbic acid solution was taken in a test tube and mixed with 2.5 ml of phosphate buffer solution (0.2 M, pH 6.6). Then 2.5 ml of potassium ferricyanide (1%) was added and incubated at 50°C for 30 min. After that, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged at 4000 rpm for 10 min. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.1 ml of FeCl₃ (0.1%) solution followed by incubation at 35°C for 10 min. The absorbance was measured at 700 nm and the reducing power of the extracts was compared with the standard ascorbic acid. From standard calibration curve, median effective concentration (EC₅₀) was calculated. The EC₅₀ value (μg/ml) is the effective concentration giving an absorbance of 0.5.

Anticholinesterase (AChE and BChE) assays

AChE from Electric eel and BChE from equine serum were used to explore the enzymes inhibitory potential of \( A. \) salivifolium root extracts by using Ellman's assay (Classics et al., 1961). The assay is based on the hydrolysis of acetylthiocholine iodide or...
butyrylthiocholine iodide by the respective enzymes and the formation of 5-thio-2-nitrobenzoate anion followed by complexation with DTNB to give a yellow colour compound which is detected with spectrophotometer beside the reaction time.

Preparation of solutions

A phosphate buffer solution (0.1 M and 8.0 ± 0.1 pH) was prepared by adding K₂HPO₄ (17.4 g/L) and KH₂PO₄ (13.6 g/L) in distilled water. Various concentrations (25, 50, 100, 200, 400, 800 μg/ml) of the extracts and standard drug Donepezil were prepared by series dilution, AChE (518 U/mg) and BChe (7 to 16 U/mg) were diluted by adding the freshly prepared buffer solution up to obtain 0.03 and 0.01 U/ml concentration of the enzymes, respectively. Solutions of DTNB (0.0002273 M), ATChl and BTChl (0.0005 M) were prepared in distilled water and were kept in eppendorf caps in the refrigerator at 8°C temperature.

Spectroscopic analysis

For these assays, 5 μl of AChE/BChE enzymes were taken in different cuvette followed by addition of 205 μl sample (extracts/standard solution) and 5 μl DTNB reagent solutions. The solution mixture in each cuvette was mixed gently and maintained at 30°C for 15 min using water bath with subsequent addition of 5 μl substrate solution (ATChl in AChE containing cuvette and BTChl in BChE containing cuvette). Absorbance was read against a blank solution using a UV-Visible spectrophotometer. The absorbance of each solution along with the reaction time was taken for four minutes at 30°C. The enzyme activity and enzyme inhibition by control and tested samples were calculated from the rate of absorbance change with time (V = ΔAbs / Δt) as follows: Enzyme inhibition (%) = 100 - percent enzyme activity. Enzyme activity (%) = 100 × V/Vmax. Where, V is the enzyme activity in the presence of standard drug or extracts and Vmax is the enzyme activity in the absence of extracts or standard drug. 50% inhibition concentration (IC₅₀) values were calculated by using MS-Excel software.

Determination of correlation (r²) between antioxidant activities and phytochemical assay

MS-excel program was used to determine the correlations between antioxidant activities and phytochemical contents. IC₅₀ values of DPPH, EC₅₀ values of FRAP and TAC were put against TPC, TFC and TFIC values of the extracts. In each set, pearson correlation (r² value) was determined from the regression equation.

Statistical analysis

All the data were presented as the mean value of triplicate experiment (n=3) along with standard deviation (Mean±SD). P < 0.05, P** < 0.01 and P*** < 0.001 were considered as significance level. ANOVA, followed by dunnett’s test was done in SPSS version 15.0 and 95% confidence of interval was calculated from it. IC₅₀ and EC₅₀ values were calculated by using the MS-excel program. TPC, TFC and TFIC were calculated from regression equation of each standard sample by using the program (MS-excel). All the figures were prepared by using Graph Pad Prism software, version 5.0.

RESULTS

Preliminary phytochemical screening

Preliminary phytochemical screening of the extracts revealed the important bioactive metabolites which are presented in Table 1.

Phytoconstituents

Total phenol content (TPC)

All the extracts showed phenolic content with significant (P**<0.01, P***<0.001) difference among them which are summarized in Figure 1A. CASR, among the extracts, showed the highest phenolic content followed by CASR. The order of TPC among the extracts was DASR > CASR > AASR > EASR. 95% confidence interval (CI) was 76.34 to 154.48 in EASR, 436.88 to 547.88 in DASR, 230.51 to 308.65 in CASR and 139.41 to 170.52 in AASR.

Total flavonoid content (TFC)

TFC was significantly (P*<0.05, P**<0.01, P***<0.001) different among the extracts. The CASR had the highest content while EASR had the lowest content, and the order of TFC was CASR > DASR > EASR > AASR (Figure 1B). The 95% confidence interval (CI) was 24.07 to 58.10 in EASR, 53.91 to 105.89 in DASR, 233.42 to 219.07 in CASR and 10.23 to 44.48 in AASR.

Total flavonol content (TFIC)

Both the EASR and AASR showed poor content, having 21.2 ± 4.63 and 78.29 ± 7.07 mg/g ascorbic acid equivalent of TFIC, respectively. On the other hand, significantly more content of the TFIC was found in DASR (294.35 ± 13.89 mg/g ascorbic acid equivalent) and CASR (332.92 ± 7.07 mg/g ascorbic acid equivalent). Here, the order of TFIC was CASR > DASR > AASR > EASR (Figure 1C). The 95% confidence interval (CI) was 9.69 to 72.70 in EASR, 259.84 to 328.85 in DASR, 315.34 to 350.49 in CASR and 60.72 to 95.86 in AASR.

In vitro antioxidant activity

DPHH free radical scavenging activity

All the extracts inhibited DPPH radicals at concentration gradient manner (more concentration more inhibition).
Table 1. Phytochemical screening of *A. salvifolium* root extracts.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Test</th>
<th>EASR</th>
<th>DASR</th>
<th>CASR</th>
<th>AASR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s test</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>Froth test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Salkowski tests</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ammonia test</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tannins</td>
<td>Ferric chloride test</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Liebermann-Burchard’s test</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>Benedict’s test</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Resins</td>
<td>Acetone, Distill water</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Borntrager’s test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Keller-Killiani test</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>NaOH</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>HCI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = low content, ++ = moderate content, +++ = high content, - = no response.

Ascorbic acid, the standard antioxidant compound, exhibited maximum inhibition such as: 40.14 ± 2.24% to 87.10 ± 2.15% at 6.25 to 200 µg/ml concentration range. CASR, among all the extracts have shown the highest inhibition which was 46.59 ± 2.71 to 79.39 ± 1.64% at 12.5 to 200 µg/ml concentration range. AA, CASR and DASR have shown potent antioxidant effect with the IC₅₀ value of 12.58 ± 1.45 µg/ml (5.88 to 15.05 CI), 11.26 ± 1.29 µg/ml (8.06 to 14.47 CI) and 16.48 ± 1.12 µg/ml (13.70 to 19.26 CI), respectively. The remaining extracts showed moderate antioxidant potential. The order of antioxidant effect was CASR > DASR > AASR > EASR (Table 2).

**Ferric reducing power assay**

Reducing power of all the extracts and the standard compound ascorbic acid was increased with the gradual increase of concentration. Ascorbic acid, a standard reducing agent, showed the highest absorbance (0.460 ± 0.01 to 2.13 ± 0.23) at concentrations ranging from 12.5 to 200 µg/ml. 50% effective concentration (EC₅₀) of it was 8.95 ± 1.03 µg/ml (5.04 to 12.86 CI). Among the extracts, CASR showed maximum reducing potential (0.350 ± 0.06 to 1.350 ± 0.13 absorbance value) at concentrations ranging from 12.5 to 200 µg/ml, and EC₅₀ value of it was 26.64 ± 2.17 µg/ml (22.83 to 30.45 CI). The reducing capability order of the extracts and the ascorbic acid was AA > CASR > DASR > AASR > EASR (Table 2).

**Total antioxidant content (TAC)**

The phosphomolybdate method, another quantitative method of antioxidant effect measurement, is based on the reduction of molybdenum (VI) to molybdenum (V) which takes place for the presence of antioxidant compound in the extracts. In the present study, all experimented samples had good TAC but in significantly (P* < 0.05, P** < 0.01 and P*** < 0.001) different extent. CASR had the highest (639.55 ± 10.51) while EASR had the lowest TAC (114.11 ± 12.83). The order of TAC among the extracts was CASR > DASR > AASR > EASR (Figure 1D). The 95% confidence interval (CI) was 82.21 to 146.00 in EASR, 452.34 to 489.74 in DASR, 613.44 to 665.66 in CASR and 159.19 to 184.70 in AASR.

**Anticholinesterase inhibitory effect**

All extracts, except EASR, and the standard drug donepezil showed AChE and BChE inhibitory effect in a concentration gradient manner. Among the extracts, CASR and DASR showed strong effect which displayed an IC₅₀ value of 152.73 ± 9.94 µg/ml (128.02 to 177.44 CI) and 192.28 ± 12.52 µg/ml (161.16 to 223.40 CI) in AChE inhibition, and 178.60 ± 20.53 µg/ml (127.60 to 229.60 CI) and 212.39 ± 12.23 µg/ml (182.00 to 242.78 CI) in BChE inhibition, respectively. The donepezil showed 7.94 ± 1.12 µg/ml (4.36 to 11.53 CI) and 12.58 ± 2.15 µg/ml (7.01 to 18.15 CI) IC₅₀ value in the AChE and BChE inhibiton, respectively (Table 3).

**Correlation between antioxidant effects and phytochemicals**

The correlation analysis was performed to investigate the
Table 2. DPPH radical scavenging and FRAP of A. salvifolium root extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH radical scavenging activity</th>
<th>Ferric reducing antioxidant power (FRAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrations (µg/ml)</td>
<td>Percent inhibition</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6.25</td>
<td>40.1±2.24</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>53.4±1.64</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>66.7±1.86</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>71.3±1.64</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>82.4±1.24</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>87.1±2.15</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>31.5±1.64</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>37.6±1.86</td>
</tr>
<tr>
<td>EASR</td>
<td>50</td>
<td>47.6±5.52</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>59.5±2.24</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>64.1±2.24</td>
</tr>
<tr>
<td>DASR</td>
<td>12.5</td>
<td>53.7±2.15</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>57.3±3.10</td>
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<td></td>
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<td>72.0±1.07</td>
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<tr>
<td></td>
<td>200</td>
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</tr>
<tr>
<td>CASR</td>
<td>12.5</td>
<td>46.5±2.71</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>65.2±2.24</td>
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<td>50</td>
<td>70.6±1.64</td>
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<td>100</td>
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</tr>
<tr>
<td></td>
<td>200</td>
<td>79.9±1.64</td>
</tr>
<tr>
<td>AASR</td>
<td>12.5</td>
<td>25.0±1.64</td>
</tr>
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<td>36.5±1.08</td>
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<td>60.9±1.64</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>65.9±2.24</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n = 3). P*< 0.05, P**<0.01 and P***<0.001 are considered as significant difference of IC50/EC50 value compared with the highest value.

The relationship between the phytochemicals and antioxidant activity of the extracts. Among the phytochemicals, TFlC showed strong positive correlation with DPPH ($r^2 = 0.913$), FRAP ($r^2 = 0.803$) and TAC ($r^2 = 0.782$). TFC had well positive correlation with TAC ($r^2 = 0.764$) while weak correlation with DPPH and FRAP effects. TPC showed weak correlation with FRAP and TAC but moderate correlation with the DPPH test (Table 4).

DISCUSSION

Oxidative stress plays a vital role for generation and progression of AD, where nerve cells or cellular components are oxidized by some free radicals that are considered as powerful oxidizing agents. Among these, the ROS ($\text{O}_{2}^·$, $\text{OH}$, $\text{H}_{2}\text{O}_{2}$, $\text{O}_{3}$) are very potential to induce lipid peroxidation and subsequently cell death. These are generated mostly by mitochondrial oxidation and moderately by the influence of environmental pollutants, smoking and harmful radiations (Lobo et al., 2010). We have a self protective mechanism against the radicals, namely antioxidant defense system, composed of some enzymatic antioxidants, main function of which is to protect our body from the oxidative stress. Here, antioxidants, enzymatic or non enzymatic, show their
Table 3. AChE and BChE inhibitory effect of A. salvifolium root extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>50% inhibitory concentration (IC_{50}) value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AChE inhibition</td>
</tr>
<tr>
<td>Donepezil</td>
<td>7.94±1.44***</td>
</tr>
<tr>
<td>EASR</td>
<td>No effect</td>
</tr>
<tr>
<td>DASR</td>
<td>192.28±12.52***</td>
</tr>
<tr>
<td>CASR</td>
<td>152.73±9.94***</td>
</tr>
<tr>
<td>AASR</td>
<td>1081.34±70.44</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n = 3). P<0.05, P<0.01 and P<0.001 are considered as significant difference of IC_{50} value compared with the highest value.

Table 4. Correlation of antioxidant activity with phytochemicals.

<table>
<thead>
<tr>
<th>Antioxidant test</th>
<th>Pearson correlation (r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPC</td>
</tr>
<tr>
<td>IC_{50} value of DPPH</td>
<td>0.634</td>
</tr>
<tr>
<td>EC_{50} value of FRAP</td>
<td>0.488</td>
</tr>
<tr>
<td>TAC</td>
<td>0.438</td>
</tr>
</tbody>
</table>

Acetylcholine, an organic molecule, acts as a neurotransmitter, and is associated with neuronal networking in central and peripheral nervous systems. Naturally, it is produced in some of our brain cells which are called cholinergic neurons. After a specific life span, ACh goes to break down by the AChE and BChE enzymes. In case of normal healthy people, the rate of synthesis and cleavage of the ACh remain steady to maintain its normal level. In this case, the AChE is 1.5-fold to 60-fold more active than that of BChE. But, in the case of AD, enzyme performance shifts towards the BChE, where its activity increases up to 120%. In contrast, AChE loses its effectiveness by 10 to 15% (Faiyaz et al., 2013). This abnormality, increased break down rate of Ach, leads to decrease the availability of the ACh than its normal physiological scale. Furthermore, the reduced level of ACh adversely affects the physiological functions of the brain. In addition, AChE and BChE potentiates neuronal degeneration by forming some protein complexes such as: neurofibrillary tangles (NFT) and neuritic plaques (NP) which are aggregates of hyperphosphorylated tau protein and extracellular neurotoxic deposits of Aβ, respectively (Dominik and Kamila, 2012). AChE/BChE bind with Aβ and a protein called ApoE protein, resulting in the formation of a highly stable complex (AChE/BChE-Ab-ApoE complex) in cerebrospinal fluid (CSF) of the brain. This stable complex directly interacts with ACh receptors and therefore, interferes with their signal transductions and potentiates ultrafast hydrolysis of ACh (Swetha et al., 2013). Researchers, for example, from...
postmortem studies of AD patients, have found strongly reduced number of ACh receptors and loss of basal forebrain and cortical cholinergic neurons (Taiwo et al., 2010). Therefore, inhibition of AChE and BChE is the most effective therapeutic approach to treat the symptoms of AD. Consequently, cholinesterase inhibitors are the only approved drugs for treating patients with mild to moderately severe Alzheimer's disease (Faiyaz et al., 2013).

Many phytochemicals have been reported to have satisfactory antioxidant and anticholinesterase effects. Among these phenolics and flavonoids, potent antioxidative compounds act as free radical scavengers (Fadwa et al., 2012). Majority of the phytochemicals having potent AChE and BChE inhibitory effects, are alkaloids followed by terpinoids, steroids, flavonoids, glycosides, saponins and essential oils (Seyed et al., 2014). Since most of the natural or synthetic products, having enzyme inhibitory effects are known to contain nitrogen atom, the promising effect of the medicinal plants could be due to their high alkaloidal contents (Seyed et al., 2014).

*Alangium salvifolium* is rich with biologically active phytochemicals where various types of alkaloids have

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**Figure 1.** (A) Total phenol content (TPC), (B) Total flavonoid content (TFC), (C) Total flavonol content (TFIC) and (D) Total antioxidant content (TAC) of *Alangium salvifolium* root extracts. P*< 0.05, P**<0.01 and P***<0.001 are considered as significance level compared with the lowest vale of a specific test.
been isolated and identified. Among these alangamaridine, mehyil-1H pyrimidine-2, 4-dione, alangine A and B, alangicine, markindine, lamarkcinine and emetine are important. Besides, phytochemical screening of it revealed the presence of flavonoids, phenolics, glycosides etc (Ashalatha and Gopinath, 2013; Ronok et al., 2013; Savithramma et al., 2013). So, these compounds may be considered for the antioxidant effect and enzyme (AChE and BChE) inhibitory activities of the extracts.

Conclusion

*A. salvifolium* Wang is extensively used as folk medicine. The present study showed that the plant is important for its phytochemical constituents. It has significant amount of phenolics, flavanoid and flavanol. Root extracts of the plant have shown moderate to potent antioxidant potential. These are also effective to inhibit AChE and BChE enzymes. So the plant is effective to protect from Alzheimer disease. However, further analysis is necessary to isolate the key compounds and to find out the actual mechanism of action.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


Antifungal activities of *Camellia sinensis* crude extract, mixture with milk, on selected pathogenic and mycotoxic fungi

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*Camellia sinensis* extracts have been documented to have antibacterial activity but little knowledge on their antifungal activity. The aqueous extracts of *C. sinensis* (tea) both green and black, mixed with milk in equal ratio parts, referred as mixture were investigated for their antifungal activity and minimum inhibitory concentrations (MICs) against seven fungal species; Green and black tea crude extracts (100 mgmL⁻¹) were evaluated for antifungal activities. Quantitative bioassay was done using disc diffusion method and MIC done using broth dilution methods. The fungal isolates used for bioactivity testing were yeasts. Green tea crude extract mixture showed stronger inhibitory effect against the fungal strains tested than black tea crude extract mixture. There was a significant difference in zone of inhibitions (T=4.09, P<0.05). Zone of inhibition exhibited by green tea crude extracts (8.33±0.87 mm) were higher than black tea crude extracts (6.75±0.66 mm). The pattern of activity by tea crude extracts mixture against ATCC standard fungal strains and clinical isolates strains were similar. *Candida tropicalis, Candida lusitaniae, Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* ATCC 66031 and *Candida famata* were inhibited by green tea crude extract mixture (IZD≥15±0.50 mm). Clinical isolates of *Candida albicans* (strain 5) showed susceptibility to *C. sinensis* green crude extracts mixture. The MIC of *C. sinensis* crude extracts mixture against fungal isolates tested ranged from 50 to 1.6 mg mL⁻¹, with green tea crude extract mixture showing highest MIC on clinical fungal isolates. The studies on *C. sinensis* have shown remarkable antifungal activity and highlighted its significance as potential health products.

Key words: *Camellia sinensis*, crude tea extracts, fungal species.

INTRODUCTION

Fungal infections in human pose serious medical issues. There is a general consensus among researchers, clinicians and pharmaceutical companies that new, potent, effective and safe antifungal drugs are needed. *C.
sinensis (Tea) is one of the most consumed drinks worldwide where green tea accounts for about 20% of the total tea consumption. In recent years, several studies have shown that green tea consumption can protect against diseases that are associated with free radical damage including atherosclerosis, coronary heart disease and cancer (Leenen et al., 2000). Kenyan black tea has between 7 and 27% more polyphenols when compared with tea from China, Japan and Taiwan (Wachira and Kamunya, 2005).

The Kenyan tea germ plasma has also been observed to be diverse in its polyphenol composition and contents and therefore provides raw material for production of different types of tea products including health drinks (Magoma et al., 2000). However, the state of research on tea regarding its pharmacological properties to fungi is limited and the majority of work has been conducted on green tea with very little on black and white tea against bacteria. Beneficial effects of tea have been attributed to the strong antioxidative activity of the tea phenolic compounds known as catechins (Fernando et al., 2005).

Tea catechins possess strong antioxidants properties, which protects the body from damage caused by free radical induced oxidative stress. In addition, many reports have presented data on the antimicrobial activity of different types of tea extracts on various pathogenic microorganisms (Luczaj and Skrzyliewska, 2005). Green tea elicits strong antibacterial activity including potential to inhibit gram positive cocci; gram negative bacilli. Studies have also shown that tea can inhibit and kill a wide range of pathogenic bacteria at or slightly below typical concentrations found in brewed tea (Rechner et al., 2002).

Various studies have shown significant suppressive effects of green tea polyphenols against many microorganisms. Black tea, a major source of phenolic, including theaflavins and thearubigins, has also been shown to have antimicrobial properties both in vivo and in vitro (Bandyopadhyah et al., 2005). Screening for antifungal properties of tea products is an important strategy for development of novel drugs or rational ways of managing fungal resistance to azoles group of compounds. This study attempts to facilitate to unravel the potentiality of C. sinensis plant product as novel modalities in the line of new drug discoveries.

MATERIALS AND METHODS

Samples of Camellia sinensis

Processed Commercial C. sinensis (black and Green tea) produced and packed by James Finlay (K) Ltd were purchased off shelf in retail outlet at the factory in Kericho County, Kenya.

Test fungal organisms

The standard test fungi of American Type Culture Collection (ATCC) was sourced from Kenya Medical Research Institute (KEMRI) and included: Cryptococcus neoformans ATCC66031, Candida albicans ATCC 90028, Candida krusei ATCC6258, Candida glabrata ATCC24433, Candida tropicalis ATCC750, and Candida parapsilosis ATCC22019 as standard organisms. Clinical isolates included: Cryptococcus neoformans, Candida albicans, Candida famata, Candida lusitaniae, Trichophyton mentagrophytes, Micosporum gypseum. Mycotoxigenic fungi included: Environmental pathogenic isolates Fusarium moniliforme, Aspergillus niger and Penicillium chrysogenum. The selection of test strains was based on their significance as opportunistic pathogens and their resistance to conventional drugs.

Experimental design

Preparation of fungal strains

Viability tests were carried out by picking the organism from the stock using sterile loop and inoculating into RPMI 1640 media and then incubated at 35 and 30°C for yeast and moulds respectively, for a period of 3 h. They were then sub-cultured onto sterile Sabouraud Dextrose Agar (SDA) and incubated for 72 h at 35 and 30°C for yeast and moulds respectively. Distinct pure colonies were picked and used for bioactivity testing. The test fungi were confirmed using macro and micro morphological characteristics with up to date identification keys (Lorene et al., 2002).

Preparation of McFarland standard

McFarland standard is used as a reference to adjust the turbidity of fungal suspension so that fungal organisms will be within a given range. Exactly 0.5 McFarland equivalent turbidity standards was prepared by adding 0.6 mL of 1% barium chloride solution (BaCl2·2H2O) to 99.4 Ml of 1% sulphuric acid (H2SO4) and mixed thoroughly. A small volume of the turbid solution was transferred to cap tube of the same type that was used to prepare the test and control inocula. It was then stored in the dark at room temperature (25°C). Exactly 0.5 McFarland gives an equivalent approximate density of fungi 1.5 × 10⁶ Colony Forming Units per ml (CFU) mL⁻¹ (Stein et al., 2005).

Crude extraction of C. sinensis (Tea)

The prepared soluble granules of both black and green tea samples sealed in silver lined sachets stored at room temperature were obtained. The mixture of aqueous crude extract for each tea was prepared by mixing (50 mL) fresh milk with (50 mL) water in the ratio of 1:1, in a 250 mL conical flask. 10 g of tea sample was then weighed and added to the contained conical flask and boiled for 20 min. The aqueous mixture tea extract obtained was approximately more in the strength of normal “cup of tea”. The extracts were then filtered using sterile Whatman filter paper No.1 to exclude any granules and filtrate of 100 mg/mL allowed to cool, then transferred to sterile screw cap bottles, labeled and stored under refrigerated condition (4°C) until use. Only fresh extracts was used in the experiment, as marked chemical changes occurred when tea was allowed to stand (Yam et al., 1997).

Preparation of tea extracts stock and working solutions

A twofold dilutions were obtained (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 mg/mL) concentrations. Antifungal activities of the above concentrations were determined.
Preparations of antifungal compounds stock and working solutions

The antifungal compounds were removed from storage (-20°C) and allowed to come to room temperature. Each 250 μg of antifungal compound (Fluconazole) was weighed and dissolved in sterile distilled water to make a final 10 mL solution. The stock solutions of azole group of compounds (Fluconazole) used was usually kept at -20°C until used. Doubling dilutions of stock solutions were made to obtain working solution.

Antimicrobial assay

The antimicrobial activities of the extracts were evaluated by the disc diffusion method (Muanza et al., 1994). The use of agar disc diffusion method to screen for antimicrobial activities of the crude tea extracts was done according to the National clinical and laboratory standards institute (NCLSI, 2007) now CLSI. The fungal inoculums for susceptibility test were standardized using barium sulphate standard equivalent to McFarland No 0.5, giving a cell density of 1.5 × 10^8 Colony Forming Units per ml (CFU/mL). Circular chromatographic paper discs (6mm diameter) were prepared with the aid of an office paper perforator. The discs were placed in a Petri dish and sterilized in an autoclave. Dilutions of several concentrations of the crude tea extracts and azole group of compounds, Fluconazole, were then made in a test tube using sterile distilled water. Positive and negative standard controls were used.

Blank sterile paper discs measuring 6mm were impregnated with 20 μL of test concentration of crude tea extract mixture. The discs were air dried and aseptically transferred into respective inoculated plates (Esimone et al., 2006). Briefly, approximately 1.5 × 10^5 cells of freshly grown fungal suspension were uniformly spread in the sterile Muller-Hinton agar dishes using sterile cotton swabs. The discs with respective crude tea extract mixture concentrations were aseptically placed on a Muller-Hinton agar plates to which the test fungi had been inoculated. The inoculated plates were incubated at 4ºC for at least 24 h to allow the tea leaves liquor to diffuse into the media. The cultures were then incubated for 72 h at 35 and 30ºC for yeast and mould respectively, before the activity was determined. The activities of the tea crude extracts mixture were established by the presence zones of inhibition which were measured in mm. Fluconazole discs containing (25μg) were used as antifungal reference standards. Similarly the sterile distilled water was set as negative controls.

Extracts with activity were serially diluted and re-tested to determine the minimum inhibitory concentrations (MIC). All the assays were carried out in triplicates, average result calculated and recorded against corresponding concentrations as described by Elgyayyar et al. (2001). Assays were subjected to quality control procedures recommended by clinical laboratory standard institute (CLSI). Fluconazole disc was prepared as described by Klevay et al. (2005). Minimum inhibitory concentrations were determined by Broth micro dilution method for the active crude extracts mixture against test fungal organisms. The procedures were done as recommended by the National Clinical Laboratory Standards institute (NCLSI) now Clinical Laboratory Standard Institute (CLSI) (Ferraro, 2003).

The tests were performed in 96 well-micro-titer plates. Crude tea extracts were transferred into micro-titer plates to make serial dilutions ranging from 10^-1, 10^-2, 10^-3 up to 10^-10. The final volume in each well was 100μL. The wells were inoculated with 5μL of microbial suspension. The yeasts were incubated at 35°C for 24h while molds were incubated 30°C for 3 to 7 days in ambient air. The MIC was recorded as the lowest extract concentration demonstrating no visible growth as compared to the control broth turbidity (Michael et al., 2003). Wells that were not inoculated were set to act as control. All the experiments were done in triplicates and average results were recorded.

The experimental work flow was as shown in Figure 1. The samples were analyzed using paired sample T-test to establish the differences in zones of inhibition caused by black tea crude extracts mixture from green tea crude extracts mixture.

RESULTS

The antifungal activities of green and black tea (C. sinensis) crude extracts having a concentration of 100 mg/mL of extracting solvent (sterile distilled water) and from the same tea mixture with milk (with ratio of milk to extracting solvent is 1:1) are presented in the tables below. Their inhibitory effects against selected pathogenic and mycotoxic fungi were then compared.

From Table 1, yeasts C. albicans ATCC 90028, C. glabrata ATCC 24433, C. krusei ATCC 6258 and moulds. Penicillium chrysogenum as well as Aspergillus niger showed no inhibition (6 mm) in either of C. sinensis green and black crude extraction. Green tea crude extracts mixture, showed maximum antifungal activity for yeasts C. tropicalis ATCC 750, followed by C. lusitaniae, C. parapsilosis ATCC 22019, C. famata and Cryptococcus neoformans ATCC 66031 respectively. None of the mould cultured showed inhibitory activity above the cut-off point of IZD ≥ 15±0.5 mm. However, black tea crude extract mixture on the other hand showed slightly moderate inhibition of 10±0.50 mm for yeasts C. lusitaniae, C. famata and mould Fusarium moniliforme with IZD of 12±0.62 mm as compared to break point of IZD ≥ 15±0.5 mm.

The results revealed that there was a significant difference in zones of inhibitions (T = 4.09, P < 0.05). Zones of inhibition caused by green tea crude extracts mixture (8.33 ± 0.87 mm) were higher than inhibition by black tea crude extracts mixture (6.75 ± 0.66 mm).

Among the clinical isolates tested, C. neoformans strain 5, C. albicans strain 4 and strain 5, showed susceptibility to antifungal activity of green tea extracts mixture with inhibition zone diameter ≥ 10.0±0.5 mm each. This is moderately active as considered highest at 15.0mm and least at6.0mm. This conform to earlier studies that extracts of green tea have been reported to be more effective in inhibiting bacterial growth than black tea (Tiwari et al., 2005).

Minimum inhibitory concentration (MIC) to standard fungal test strains

Minimum inhibitory concentrations (MIC) of tea crude extracts mixture to fungal strains were established. Tested at 15±0.5 mm diameter of inhibitory zone diameter, the MICs of mixture tea crude extracts were recorded in mg/mL. Black tea crude extract was only tested against C. famata strain because it was the only one which showed inhibition activity (Table 2).
Figure 1. Experimental work flow chart.

Table 1. Zones of inhibitions (mm) by crude tea extracts mixture on the selected pathogenic and mycotoxic fungi.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Mixture extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black tea</td>
</tr>
<tr>
<td>Candida albicans ATCC 90028</td>
<td>6</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>10</td>
</tr>
<tr>
<td>Candida parapsilosis ATCC 22019</td>
<td>6</td>
</tr>
<tr>
<td>Candida glabrata ATCC 24433</td>
<td>6</td>
</tr>
<tr>
<td>Candida famata</td>
<td>10</td>
</tr>
<tr>
<td>Candida tropicalis ATCC 750</td>
<td>7</td>
</tr>
<tr>
<td>Candida krusei ATCC 6258</td>
<td>6</td>
</tr>
<tr>
<td>Cryptococcus neoformans ATCC 66031</td>
<td>6</td>
</tr>
<tr>
<td>Microsporogypseum (clinical isolate)</td>
<td>7</td>
</tr>
<tr>
<td>Aspergillusniger (clinical isolate)</td>
<td>6</td>
</tr>
<tr>
<td>Fusarium moniliforme (clinical isolate)</td>
<td>12</td>
</tr>
<tr>
<td>Penicillium chrysogenum (Clinical isolate)</td>
<td>6</td>
</tr>
<tr>
<td>Trychophyton mentagrophytes (Clinical isolate)</td>
<td>6</td>
</tr>
</tbody>
</table>

The MIC of the C. sinensis crude extracts mixture which had inhibition diameters of 15±0.5mm and above (significance activity) was determined. Green tea crude extracts mixture had the least minimum inhibition concentration at 1.6 mg/mL against C. neoformans ATCC 66031 and C. famata; and highest MIC against yeast C.
Table 2. The minimum inhibition concentration of tea crude extracts to the standard fungal test strains.

<table>
<thead>
<tr>
<th>Fungal test strain</th>
<th>Tea crude extract</th>
<th>Black tea mixture (mg/mL)</th>
<th>Green tea mixture (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lusitaniae</td>
<td></td>
<td></td>
<td>6.250</td>
</tr>
<tr>
<td>C. famata</td>
<td></td>
<td>50.00</td>
<td>1.600</td>
</tr>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td></td>
<td></td>
<td>50.00</td>
</tr>
<tr>
<td>C. tropicalis ATCC 750</td>
<td></td>
<td></td>
<td>8.250</td>
</tr>
<tr>
<td>C. neoformans ATCC 66031</td>
<td></td>
<td></td>
<td>1.600</td>
</tr>
<tr>
<td>M. gypseum</td>
<td></td>
<td></td>
<td>50.00</td>
</tr>
<tr>
<td>T. mentagrophytes</td>
<td></td>
<td></td>
<td>6.250</td>
</tr>
</tbody>
</table>

The black crude extracts mixture tea showed no detectable inhibitory activity. -, No inhibition activity.

parapsilosis ATCC 22019 and mould M. gypseum. Green tea mixture minimum inhibition concentration of 1.6 mg/mL was adequate to inhibit growth of C. famata. However, at a concentration of 6.25 mg/mL of mixture green tea, 50% of the tested fungi were inhibited in growth; this gives the MIC of the test fungi when using green tea crude extract mixture.

Green tea crude extract mixture minimum inhibition concentration of 50 mg/mL was adequate to inhibit growth of M. gypseum. At this concentration of 50 mg/mL of mixture green tea, 90% of the tested fungi were inhibited, that is all fungi tested were inhibited.

Synergism/antagonism between crude extracts of Kenyan tea and conventional antifungal drugs on azoles resistant fungi

To establish synergism effect, fluconazole was mixed with tea crude extracts mixture (blended with milk) and zone of inhibition recorded. The findings show that there were no significant difference in zones of inhibitions (F = 0.90, df = 3, P = 0.455). However, fluconazole alone (mean inhibition zone 20.00 ±1.29 mm) was greater than tea crude extracts mixture inhibition zones.

Using a combination of green tea crude extract mixture (blended with milk) to Fluconazole mainly inhibited growth of C. neoformans 5, C. tropicalis ATCC 750 and C. albicans15. The C. sinensis crude extracts exhibited diminished activity when combined with Fluconazole (lesser inhibition zone diameters as compared to fluconazole IZD) as compared to activity by Fluconazole alone. This shows antagonism between the crude extracts mixture and conventional antifungal drug, Fluconazole.

Effect of temperature and addition of milk to crude extracts

To test for effect of temperature on MIC of green and black tea crude extract mixture, a pair sample T-test was used to compare the MIC values. The result showed that there was no significant difference in MIC (t = 1.51, P = 0.182). Mean MIC of green tea crude extract mixture (mean 0.017 ± 0.008mm) was higher than black tea (0.0143 ± 0.007mm). Minimum fungicidal concentration (MFC) of green tea crude extract mixture was tested on C. tropicalis ATCC 750, C. neoformans ATCC 66031, C. lusitaniae, C. famata and C. parapsilosis ATCC 22019.

Green tea crude extract mixture at 3.12 mg/mL was effective enough to kill C. lusitaniae while at 6.25 mg/mL, C. tropicalis ATCC 750 and C. neoformans ATCC 66031 was made static and could not grow. At a concentration of 8.25 mg/mL it was fungicidal to C. parapsilosis ATCC 22019. When the concentration reached 50 mg/mL all the tested fungi including C. famata were killed by the tea crude extract mixture.

DISCUSSION

According to World Health Organizations, WHO (2000) report of infectious diseases, overcoming antimicrobial resistance is the major issue of W.H.O for the next millennium. Hence, the last decade witnessed an increase in the investigation of plants as a source of human disease management. In the present study, the crude extracts of C. sinensis (green and black) blended with milk, giving a mixture produced inhibitory activity against pathogenic and mycotoxigenic fungi. The water crude extraction produced yields enough for the experimental study and is the most commonly used and cost effective method of tea preparation.

The choice for water extraction was due to the fact that water is very polar than organic solvents hence it is able to extract more polar compounds from a plant material. Kigondu et al. (2009) also found that water extracts was blended with milk as normal home-made tea giving strength of “normal cup of tea”. The results obtained in this study indicate a considerable difference in antifungal activity of antmycotic activity of C. sinensis green and black crude extracts mixture. For all the yeasts tested, Candida tropicalis ATCC750 was the most sensitive
fungus to all the crude extracts (Table 1). This conform to
earlier studies that extracts of green tea have been
reported to be more effective in inhibiting bacterial growth
than black tea (Tiwari et al., 2005).
The results from the present study revealed that there
was a significant difference in zones of inhibitions
(T=4.09, P<0.05). Zone of inhibition caused by green tea
crude extracts (8.33 ± 0.87 mm) were higher than
inhibition by black tea crude extracts (6.75 ± 0.66 mm,
Figure 2). C. albicans strain 4 and strain 5 among all the
clinical isolates had greatest susceptibility to antifungal
activity of green tea extract with IZD of 15±0.5 mm
(Figure 3). C. neoformans strain 3, strain 5 and strain 12;
showed susceptibility to antifungal activity of green tea
extracts mixture with IZD ≥ 10.0±0.5mm each. This is
moderately active as considered highest at 15.0mm and
least 6.0 mm. These findings are in line with Bii et al.
(2010) which indicates that the lowest activity was at
7.0±0.5 mm and the highest was at 18.0±0.5mm in
diameter.
The green tea crude extract mixture has shown higher
antifungal activity than black tea (Figure 6). This
difference in results is probably due to presence of
different contents of active substances in the teas.
Several studies have shown that the antimicrobial
property is due to presence of polyphenols. Specific
antioxidant polyphenols called catechins play an
important role in green tea’s inhibition of microbial
growth. Several significant catechins include: EGCG,
EGC, ECG, EC and GCG (Isogai et al., 2001).
Antimicrobial activities of tea extracts are very selective. This difference in their activity depends upon the concentration and type of the extracts. These effects may also differ depending on (microbe) fungal species so that they may be either growth inhibitory or stimulatory (Tiwari et al., 2005).

Green tea and black tea crude extracts mixture tested in current study have also shown varying activities against fungal organisms. Hirasawa et al. (2003) showed that the actions of catechins ECGG, EGC were fungicidal. Studies of the antibacterial activity of catechins against phytopathogenic bacteria showed similar results to those against C. albicans. Catechines are known to have an affinity for proteins; this is clearly shown by a decrease in antibacterial activity of tea. This property is referred to as “astringency” contributes to the sensation known as “mouth feel” experienced when drinking tea. The mode of action involves inducing rapid leakage of small molecules entrapped in the intraliposomal space and aggregation of the liposomes. Thus, a number of membrane dependent cellular processes, such as cell signaling and cell cycle, arachidonic acid metabolism and cell proliferation, and apoptosis and mitochondrial functionality may be influenced by interaction of catechines with cellular phospholipid palisade (Caturia et al., 2003).

The resistance of fungal strains (least susceptible) of clinical isolate C. neoformans strain 3, strain 5, strain 12, strain 97, strain j076 and strain 065 (Figure 3) was most probably because of the presence of mucopolysaccharide capsule. The polysaccharide capsular material in some of the pathogenic microorganisms is responsible for virulence and antimicrobial resistance (Hooper, 2001). The Candida species such as C. albicans ATCC 90028, C. glabrata ATCC 24433, C. parapsilosis ATCC 22019 that showed less susceptibility to antymycotics of C. sinensis crude extract mixture could be due to their outer membrane consisting of chitin binding proteins and thus able to regulate the access of antifungal properties into the underlying structures. Candida species expresses multidrug efflux transporter (MET), which mediates the efflux of broad range of compounds including antifungal agents (Marchetti et al., 2000). But in this study, we found contradicting results among the Candida strains of clinical isolates. C. albicans strain 3, 6 and 20 showed least or no activity whereas strain 4 and 5 had activity (Figure 3). The disparity in findings could be due to differences in strains of fungi used and their susceptibility to antifungal drugs. The preliminary screening assays for antifungal activity can largely be considered as qualitative assays and are used for identifying the presence or absence of bioactive constituents in the extracts. However, these methods of assays offer little information on these compounds. The minimum inhibition concentration (MIC) is a quantitative assay and provides more information on the potency of the compounds present in the extracts. Thus, the MIC values of the crude extracts of C. sinensis which had inhibition zone diameter of 15±0.5 mm and above was determined so as to demonstrate the potency of the extracts against the selected strains of fungi.

The least the MIC the better the C. sinensis crude extract against the isolate in question. The green tea crude extract mixture had the least minimum inhibition of 1.6 mgmL⁻¹ against yeast Candida famata, and C. neoformans ATCC 66031 and the highest MIC against yeast C. parapsilosis ATCC 22019 of 50 mgmL⁻¹ and mould M. gypseum (Table 2). When the green tea crude extract was mixed with milk in the ratio of 1:1, the MIC was established to be 1.6 mgmL⁻¹ against C. famata which also formed the MIC₅₀ (Figure 4); whereas, at a concentration of 50 mgmL⁻¹, 90 % of the fungal isolates tested were inhibited. At this concentration of mixture green tea crude extract, all the fungi tested were inhibited as shown in Figure 5.

Generally, the MIC of the C. sinensis crude extracts mixture was as high as 50 mgmL⁻¹ as compared to the standard drugs which is 0.5 mgmL⁻¹ for yeasts and 1.0 mgmL⁻¹ for dermatophytes at 95% confidence interval (P=0.05 level of significance). Although this was significantly lower than that of Fluconazole (P<0.01), the extracts are promising since they are crude extracts compared to pure compound of Fluconazole. This is a clear indication that the active ingredient is present in low quantities which necessitate the use of large amounts of crude extracts to gain the desired therapeutic effects. The difference in bioactivities of green and black tea crude extracts mixture could be attributed to the differential processing methods of green and black tea and the blending with milk as well as boiling which affect/alter their composition (cold and hot water extraction).

Absence of bioactivity does not warrant disapproval of ethno botanical utilization of the C. sinensis, simply because it may suggest that the extracts are acting in an indirect way where active ingredient exists as a precursor requiring activation in vivo. The present study also showed antagonistic antifungal activity of the combination of tea crude extracts mixture and antymycotic, Fluconazole against tested fungal isolates (Table 3). This is in contrary to earlier studies, since the arrival of azole antifungal agents as first-line drugs; Fluconazole-resistant C. albicans has begun to appear. Similar studies have been reported by Hirasa et al. (2003), on the combined use of EGCG and Fluconazole effective against Fluconazole resistant C. albicans.

More detailed studies by Hirasa and Takada (2004) revealed that EGCG enhanced the antifungal activity of the drug Amphotericin B; and the combined use of ECG and antifungal drug Fluconazole inhibited Fluconazole-resistant strains of this fungus. It is suggestive to have converted Fluconazole resistant phenotypes to sensitive ones. Earlier studies showed that ECG converted a Methicillin-resistant phenotype to a Methicillin-sensitive one (Zhao et al., 2001). EGCG synergizes the activity of β-lactam antibiotics against Staphylococcus aureus by
binding to the peptidoglycan component of the bacterial cell wall (Zhao et al., 2001). The wide ranging effects that catechins gallates have on modulation of drug resistance has recently been emphasized by the novel observation that sub-inhibitory concentrations of EGCG are able to reverse resistance by inhibition of efflux pump, in addition to further sensitizing susceptible isolates to antibiotic (Roccaro et al., 2004).

However, findings of the present study established that using a mixture of tea crude extracts to Fluconazole mainly diminished inhibitory effect to fungal species. In terms of effects of inhibition as a result of difference in extraction temperatures, the present study revealed that higher temperatures reduces the polarity of water, thus increasing its extraction efficiency and capability to dissolve polar compounds (Hassas-Roudsari et al., 2009). Raising the temperature of water also reduces its surface tension and viscosity, which increases the diffusion rate and the rate of mass transfer during extraction. The mean MIC of green tea crude extract mixture (mean 0.017±0.008 mm) was higher than black tea (0.0143±0.007 mm). When green tea crude extract was mixed with milk, the mixture crude extracts at a concentration of 3.12 mgmL-1 was fungicidal to C. lusitaniae but fungi static to other fungal isolates tested (Table 4). But at concentration of 6.25 mgmL-1 was fungicidal to C. tropicalis ATCC 750 and C. neoformans ATCC 66031; while at 8.25 mgmL-1 was fungicidal to C. parapsilosis ATCC 22019 but fungi static to C. famata. The MFC of C. famata was 50 mgmL-1. These results are
Table 3. Synergism/antagonism between *C. sinensis* crude extracts mixture and Fluconazole.

<table>
<thead>
<tr>
<th>Fungal test strain</th>
<th>Mixture extract + Fluconazole (mm)</th>
<th>Fluconazole alone (mm) (positive control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> 4</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td><em>Candida albicans</em> 15</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td><em>C. tropicalis</em> ATCC 750</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> 3</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td><em>C. neoformans</em> 5</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td><em>F. moniliforme</em></td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td><strong>Mean ± SE</strong></td>
<td><strong>17.86 ± 2.10</strong></td>
<td><strong>20.00 ± 1.29</strong></td>
</tr>
</tbody>
</table>

Table 4. Minimum fungicidal concentration of green tea crude extract mixture.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Green tea crude extract mixture (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. tropicalis</em> ATCC 750</td>
<td>6.25</td>
</tr>
<tr>
<td><em>C. neoformans</em> ATCC 66031</td>
<td>6.25</td>
</tr>
<tr>
<td><em>C. lusitaniae</em></td>
<td>3.12</td>
</tr>
<tr>
<td><em>C. famata</em></td>
<td>50.00</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> ATCC 22019</td>
<td>8.25</td>
</tr>
</tbody>
</table>

Figure 6. MICs of green and black tea crude extracts mixture on clinical fungal isolates.

*C.n = Cryptococcus neoformans; C.a = candida albicans*

suggestive that addition of milk to blend the crude extracts altered the bioactive ingredients resulting in higher concentration for its MFC as compared to crude extracts alone. These results conform to previous studies by Wachira et al. (2011) that milk decreases antioxidant activity of *C. sinensis*.

The mechanistic aspect of fungicidal brought about by tea crude extracts is suggestive to be due to catechins...
and gallates. The bioactive ingredients in crude tea extracts binds to ergosterol, one of the cell membrane sterols, and damages the cell membrane directly, leading to fungicidal activity against fungi. Catechins regulate expression of the gene(s) coding for Cytochrome P450 (Muto et al., 2001; Yang and Raner, 2005). Detailed physiochemical studies suggest that fungicidal activities of galloylated tea catechins at the cell membrane level may be due to their specific perturbations of ordered structure of chitin binding proteins, a nitrogen containing polysaccharide constituting fungal cell wall. Differential effects of catechins on fungal cell walls compared to membrane of human cells may be due to differences in structures of the respective walls (membranes). The fungicidal action of EGCG may depend on hydrogen peroxide derived from the reaction EGCG with oxygen (Prooxidative activity) (Arakawa et al., 2004). These observations suggest that antifungal activity of antymycotic effect seem to arise from the interactions of catechins in crude extract with oxygen, genes, cell membranes and enzymes. This aspect merits further study. This predominantly in vitro information has ramifications for Mycotic disease prevention in humans.

CONCLUSION AND RECOMMENDATIONS

The C. sinensis crude extracts possess antifungal activity. In the present study, the crude extracts of C. sinensis (green and black) produces inhibitory actions against the fungal test strains. The Minimum Fungicidal Concentration (MFC) of the C. sinensis crude extracts mixture with milk was slightly higher as compared to that of fluconazole drug. Therefore, addition of milk to the crude extracts alters the bioactive ingredients resulting in higher concentration for its MFC as compared to azole drug alone (it diminishes fungicidal activity). The plant based crude extracts represents unlimited sources of modern therapies therefore; a continued and regular exploration of C. sinensis for antifungal agent is required. Tea is an infusion of the leaves of C. sinensis plant, and is one of the most widely consumed beverages in the world. For potential antifungal beneficial effects, the green tea should be consumed in preference to black tea. The green tea as beverage should also be consumed purely without blending with milk so as to achieve maximum health benefits. The fractionation of crude extracts and purification of active compounds is needed to isolate these bioactive compounds to establish their mechanistic aspect of action against the fungal isolates and elucidate mechanism of synergism/antagonism. Assayed antifungal were tested in vitro, but practically in human aspect both antifungal and polyphenolic compounds of C. sinensis undergo metabolic processes in the body; there is no information on the interaction of the related metabolites. This needs further studies to examine directly in human populations under carefully controlled conditions to get positive results.

Conflict of Interests

Authors declare that the present work was done by the authors and there is no any financial support from any agency and there are no conflicts of interest.

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REFERENCES


National Clinical and Laboratory Standards Institute NCLSI (2007). M100-S17, Performance Standards for antimicrobial susceptibility testing, seventeenth informational supplement Wayne, P. A.


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