Polyphenol Composition and in vitro Antioxidant Potential of Nigerian Canarium schweinfurthii Engl. Oil

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Abstract: Fruit mesocarp oil of Canarium schweinfurthii Engl used in the preparation of special delicacies by some ethnic nationalities in Nigeria and elsewhere in tropical west, central and east Africa, was analysed for its polyphenol composition using HPLC-UV, HPLC-MS and GC-MS techniques. Ten phenolic compounds, namely, catechol, p-hydroxybenzaldehyde, dihydroxyphenylacetic acid, tyrosol, p-hydroxybenzoic acid, dihydroxybenzoic acid, vanillic acid, phlorotic acid, pinoresinol, secoisolaricresinol and some other peaks of unknown identity were detected by GC-MS following derivatization of the polyphenol-enriched methanol extract of the oil with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (BSTFA). The methanol extract displayed very strong antioxidant and radical scavenging potential with IC₅₀ values of 56 and 104 μl, respectively, when tested with the hypoxanthine/xanthine and 2-deoxyguanosine assay models, presumably as a result of its rich content of antioxidant polyphenols. These results strongly suggest that consumption of Fruit mesocarp oil of Canarium schweinfurthii might play a significant role in the chemoprevention of cancer and other oxidative-damage-induced diseases.

Abbreviations: ROS Reactive oxygen species • RNS Reactive nitrogen species • BSTFA N-methyl-N-(trimethylsilyl)-trifluoroacetamide • LC-ESI Liquid-Chromatography Electrospray-Ionization Mass Spectrometry • HPLC High performance Liquid chromatography GC-MS Gas Chromatography-Mass Spectrometry

Key words: Canarium schweinfurthii • Polyphenol • Antioxidant potential • Chemoprevention • Vegetable oil

INTRODUCTION

Over the past few decades, an expanding body of evidence from epidemiological and laboratory studies suggest that some edible plants as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis [1-6]. One major group of antioxidants that have received attention in cancer chemopreventive studies in the recent past, are the polyphenols [7-10].

Polyphenols are diverse group of compounds generally characterized with the presence of several hydroxyl groups attached to a ring or multi-ring structures and are thus classified as phenolic acids, stilbenes, lignans and flavonoids [10]. Polyphenol with several cancer chemopreventive potentials have been detected in several foods [11], including soy products [12-14], tea [7, 11, 15-17] and vegetables [18, 19]. This decade, evidence has accumulated to suggest that olive oil, an unique component of the Mediterranean diet may play some significant roles in the low incidence of certain cancers in the region [20].

Although, information about cancer incidence in Nigeria is rather scanty [21, 22], it is generally believed that there is low incidence of cancer in Africa, due to the nature of the diets and herbal medicaments, which are rich in anticancer constituents, especially, antioxidants [23, 24]. Therefore we investigated the polyphenol content and the antioxidant potential of Canarium schweinfurthii Engl. pulp oil, which is used in the preparation of special delicacies by some ethnic nationalities in the middle belt of Nigeria.

Canarium schweinfurthii, Engl. (Family: Burseraceae), commonly referred to as 'African canarium' or 'African olive' is respectively called 'Atilé' and 'Odadá' by the Hausa and Igala ethnic groups of northern and
central Nigeria. The tree, which grows to a height of up to 150 ft with straight cylindrical bole to 90 ft, is widely distributed in the tropical west, east and central Africa. The fruits, stems and barks are used for treating coughs, venereal diseases and exudates. When parboiled, the mesocarp of the fruits are eaten and also used in the preparation of oil [25-28], which is utilized in the preparation of special delicacies by some ethnic groups in Nigeria and, probably, elsewhere in Africa.

The fruit mesocarp oil of *Canarium schweinfurthii* which constitutes 68% free-flowing lipid and 14% bound lipid [25] is reported to contain monoterpene hydrocarbons and linalool. The oil has an acid value of 0.68%, a saponification number of 196.35 and a peroxide value of 7.80 [27, 28]. Within this decade, a report appeared on phenolic metabolites from the seeds of the plant [29], but there are no reports on the polyphenol profile and antioxidant potential of its fruit oil and hence the focus of this investigation.

**Sample:** Fruit mesocarp oil of *Canarium schweinfurthii* Engl. was obtained from a local processor in Jos, Plateau State, Nigeria. It was stored at room temperature in glass bottle until transportation to laboratory of analysis in Heidelberg, Germany in August, 2004.

**Reagents:** Acetic acid, ethylene diaminetetraacetic acid (EDTA), hypoxanthine, methanol, xanthine and xanthine oxidase were obtained from Merck (Darmstadt, Germany). K$_2$HPO$_4$ and KH$_2$PO$_4$ were obtained from Serva (Heidelberg, Germany). Formic acid, salicylic acid and FeCl$_3$·6H$_2$O were obtained from Aldrich Chemie (Steinheim, Germany). N-methyl-N-(trimethylsilyl)-(trifluoroacetamide (BSTFA) was obtained from Fluka (Buchs, Switzerland), while tetrabutylammonium hydroxide was obtained from Sigma Chemie (Deisenhofen, Germany). Standard phenolic compounds were obtained from laboratory stock, acquired from commercial sources or isolated, purified and characterized from natural sources. All solutions were made in double-distilled water.

**Extraction of Phenolic Compounds:** The phenolic compounds in the oil were extracted from the oil, basically as described by Owen and co-workers [30], but with minor modifications. The oil (10g) was vortexed for 2 min. at maximum speed in 50 ml polyethylene bottles with 3 x 2 ml methanol. The mixture was centrifuged at 4000 rpm for 30 min and methanol layer collected into graduated 20 ml glass test tubes. Pooled methanol fractions were dried under nitrogen, taken up in 1 ml acetonitrile and lipid contaminants removed by vortexing with 3 ml hexane (3 times). The mixture was centrifuged at 3000 rpm for 15 minutes each and the hexane layer discarded. The acetonitrile layer was made up to 2.5 ml and used for subsequent analysis (Fig. 1).

**Analytical High Performance Liquid Chromatography (HPLC):** Analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 Liquid chromatograph fitted with a C-18, reverse phase (5um) column (25 cm X 4 mm ID) Latex, Bippelheim, Germany. For separation of individual compounds in the extract, 2% acetic acid in water (solvent A) and methanol (solvent B) were used as mobile phase when 20µl of the extract was injected.

The solvent gradient consisted of 95% A for 2 min, 75%A in 8 min, 60 % A in 10min, 50A in 10min and 0%A until completion of the run at 45 min[20, 30]. The flow rate of the mobile phase was maintained at 1ml/min and phenolic compounds in the eluate were detected with a UV dual-array detector (HP 1040M) set at 278 and 340 nm. Instrument control and data handling was by means of a HP Chemstation operating in the Microsoft Windows software environment.
Hypoxanthine/ Xanthine Oxidase Assay: To assess the total antioxidant potential due partly to the scavenging of reactive oxygen specie and the inhibition of the enzyme, xanthine oxidase, the Hypoxanthine/Xanthine oxidase assay system was utilized. In this assay, the extent of diphenol (2,5 dihydroxybenzoic acid and 2,3 dihydroxybenzoic acid) produced by hydroxyl radical (HO•) attack on salicylic acid was measured from standard curves of their respective diphenols [20,30]. The assay involves the re-suspension of different dried extract residues, prepared in duplicates (by drying 0-500 μL of extracts in vacuum evaporation) in 1ml phosphate buffer (pH6.6). After addition of 5μL xanthine oxidase 20μL of the mixture was analysed by HPLC using the mobile phase and gradient condition earlier mentioned. The hydroxylation of hypoxanthine was monitored at 278nm, while the hydroxylation of salicylic acid was monitored at A325nm. The end products of the enzyme or free radical reaction were quantified against standard curves measured at the same wavelength.

Deoxyguanosine Assay for Radical Scavenging Potential: To evaluate the radical scavenging capacity of the extract, the 2-deoxyguanosine-assay model was adopted. The buffer system is similar to that of the hypoxanthine/xanthine oxidase system, except that salicylic is replaced with 2-deoxyguanosine (2mM). The generation of ROS was initiated by addition of ascorbic acid (500 μM). Dried residues, prepared in duplicates (by drying 0-500 μL of extracts in vacuum evaporator) in 1ml phosphate buffer (pH6.6), were re-suspended in buffer and incubated at 37 °C for 24 hrs. The assay of the 8-oxo-2-deoxyguanosine resulting from the ROS attack on 2-deoxyguanosine was analysed using an isocratic system consisting of 5% methanol and 95% aqueous buffer (5mM tetrabutylammonium hydroxide, adjusted to pH 4.3 with 6% formic acid). The UV detector was set at A293nm [20,30].

Gas Chromatography-Mass Spectrometry: Analyses were performed on a HP 5973 mass spectrometer coupled to a HP 6890 gas chromatograph. Prior to GC-MS analysis, dried methanolic extracts (1μL) were derivatized by addition of BSTFA (100 μL) at 37°C for 30 min. Separation of the analytes was achieved using a HP 5MS capillary column, (30 m X 0.25 mm I.D., 0.25 um film thickness). Helium was used as the carrier gas with a linear velocity of 0.9 ml/s. The oven temperature program was: initial temperature 100 °C, 100-270°C at 4°C/min and maintained at 270°C for 20 min. The GC injector temperature was maintained at 250 °C, the transfer line temperature was held at 280 °C. The mass spectrometer parameters for EI mode were: ion source temperature: 230°C; electron energy: 70 eV; filament current: 34.6 μA; electron multiplier voltage: 1200V [20,30].

Liquid-Chromatography Electrospray-Ionization Mass Spectrometry (LC-ESI): LC-ESI was conducted on an Agilent 1100 HPLC coupled to an Agilent LC/MSD (HP1101). Chromatographic separation was conducted using a C-18, reversed phase (5 μm column (25 cm × 2mm I.D.; Latex Eppelheim, Germany) utilizing the same mobile phase and gradient as described for analytical HPLC, except that the flow rate was maintained at 0.5ml / min. The analyses were conducted in the negative ion mode under the following conditions: dry gas (nitrogen) flow rate 10l/min; nebulizer pressure= 30psi; drying gas temperature= 350°C; capillary voltage = 2500V; fragmenter voltage=100V; mass range=50-3000D.

RESULTS

The phenolic constituents of fruit mesocarp oil of Canarium schweinfurthii Engl are presented on Table 1, while the GC-MS chromatogram for the total ion count (TIC) and ion 179, one of the most characteristic fragments of phenolic compounds, are presented in Fig. 2. HPLC analysis revealed the presence of catechol, while GC-MS analysis following derivatization with BSTFA revealed the presence of ten phenolic compounds, including catechol, p-hydroxybenzaldehyde, dihydroxyphenylacetic acid, tyrosol, p-hydroxybenzoic acid, dihydroxybenzoic acid, vanillic acid, phlorotic acid, pinoresinol, secoisolariciresinol and some other peaks of unknown identity (Fig. 2). The structures of the identified phenolic compounds Canarium schweinfurthii oil are presented in Figure 3. The GC-MS data, including the molecular ions and the abundance of the characteristic fragment ions of these phenolic compounds are also presented on Table1.
Table 1: GC-MS data (EI mode) for TMS derivatives of identified phenolic constituents of fruit mesocarp oil of Canarium schweinfurthii

<table>
<thead>
<tr>
<th>S/No</th>
<th>Compound</th>
<th>Ret. Time (minutes)</th>
<th>TMS groups</th>
<th>M⁺ (calc.)</th>
<th>M⁺ obs.</th>
<th>Fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catechol</td>
<td>8.64</td>
<td>2</td>
<td>254.11</td>
<td>254(30)</td>
<td>239(9), 73(100), 45(10)</td>
</tr>
<tr>
<td>2</td>
<td>p-Hydroxybenzaldehyde</td>
<td>9.86</td>
<td>1</td>
<td>194.12</td>
<td>194(82)</td>
<td>179(100), 151(72), 73(57)</td>
</tr>
<tr>
<td>3</td>
<td>Tyrosol</td>
<td>15.24</td>
<td>2</td>
<td>282.17</td>
<td>282(23)</td>
<td>267(13), 193(12), 179(100), 180(18), 147(14), 103(15), 75(31), 73(74), 59(8), 44(14)</td>
</tr>
<tr>
<td>4</td>
<td>p-Hydroxybenzoic acid</td>
<td>16.72</td>
<td>2</td>
<td>282.12</td>
<td>282(25)</td>
<td>267(100), 268(23), 223(66), 193(51), 126(12), 73(44)</td>
</tr>
<tr>
<td>5</td>
<td>Dihydroxyphenylacetic acid</td>
<td>17.06</td>
<td>2</td>
<td>296.18</td>
<td>296(55)</td>
<td>281(56), 263(14), 252(58), 217(39), 179(100), 164(41), 149(21), 147(63), 133(27), 103(25), 295(10), 193(15), 192(76), 180(17), 179(100), 163(6), 147(9), 117(6), 73(62), 45(11)</td>
</tr>
<tr>
<td>6</td>
<td>Phloretic acid</td>
<td>20.24</td>
<td>2</td>
<td>310.18</td>
<td>310(28)</td>
<td>297(100), 282(27), 267(68), 253(43), 223(45), 193(18), 179(7), 147(9), 126(24), 75(15), 73(58)</td>
</tr>
<tr>
<td>7</td>
<td>Vanillic acid</td>
<td>20.42</td>
<td>2</td>
<td>312.15</td>
<td>312(66)</td>
<td>297(100), 282(27), 267(68), 253(43), 223(45), 193(18), 179(7), 147(9), 126(24), 75(15), 73(58)</td>
</tr>
<tr>
<td>8</td>
<td>Dihydroxybenzoic acid</td>
<td>21.97</td>
<td>3</td>
<td>370.12</td>
<td>370(65)</td>
<td>355(33), 311(22), 281(12), 267(7), 223(12), 193(100), 163(8), 147(6), 137(6), 73(65), 45(10)</td>
</tr>
<tr>
<td>9</td>
<td>Secoisolariciresinol</td>
<td>47.31</td>
<td>4</td>
<td>650.42</td>
<td>650(3)</td>
<td>560(7), 261(17), 209(50), 179(12), 173(16), 147(32), 131(11), 129(22), 103(19), 73(30), 73(100), 44(26)</td>
</tr>
<tr>
<td>10</td>
<td>Pinoresinol</td>
<td>58.07</td>
<td>2</td>
<td>402.39</td>
<td>502(86)</td>
<td>487(30), 277(16), 235(79), 223(100), 209(68), 194(45), 179(30), 166(19), 73(52), 44(70)</td>
</tr>
</tbody>
</table>

TMS groups (-Si(CH3)3, MW: 73) adds 72 to the original molecular weight for each hydroxyl proton it displaces.

*Abundance of molecular ions and fragment ions relative to base ions are provided in percentages (%).

*Base peaks are written in bold numbers.

Fig. 2: TIC (A) and Ion 179 chromatogram of fruit mesocarp oil of *Canarium schweinfurthii* following GC-MS of BSTFA-derivatized extract; The identified phenolic compounds are: 1, Catechol; 2, p-hydroxybenzaldehyde; 3, tyrosol; 4, hydroxybenzoic acid; 5, dihydroxyphenylacetic acid; 6, phloretic acid; 7, vanillic acid; 8, didroxybenzoic acid; 9, secoisolariciresinol; pinoresinol.
Fig. 3: Structure of identified Phenolic Compounds Detected in Fruit Mesocarp oil of *Canarium Schweinfurthii*

![Chemical structures of identified phenolic compounds](image)

The result for the antioxidant assay_RADICAL scavenging capacity is presented in Fig.4. The oil showed a very strong antioxidant potential (IC$_{50}$=56μl) and a very promising radical scavenging capacity (IC$_{50}$=104μl), when assayed by xanthine oxidase and 2-deoxyguanosine method, respectively.

**DISCUSSION**

Qualitatively, fruit mesocarp oil of *Canarium Schweinfurthii* is only similar to olive oil by containing tyrosol and pinoresinol. Apart from these, catechol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, phloretic acid, secoisolaricresinol which are present in canarium oil (Table 1, Fig. 2) are not present in olive oil, neither are hydroxytyrosol, acetoxyioresinol, secoridoid1 and secoridoid 2 and lignans found in olive oil [20, 30], detected in *Canarium oil* (Table 1; Fig.2).

The antioxidant activity compares very favourably with that of extra virgin olive oil [20, 30], which, perhaps, is the most celebrated cancer chemopreventive component of the Mediterranean diet [20, 30]. The high antioxidant and radical scavenging activity of *Canarium* oil (Fig. 3) is consistent with its polyphenol profile (Fig. 2; Table 1) and the fact that oils of other plant species, notably, olive [20, 30, 31] have been demonstrated to display high *in vitro* and to some extent, *in vivo* antioxidant activity.
Dietary phenolic may have physiological antioxidant properties, quenching reactive oxygen and nitrogen species (ROS and RNS, respectively) and hence potentially modifying pathological mechanisms relevant to cardiovascular diseases [32] and other diseases like gastrointestinal disorders and cancers [5, 9, 20, 30, 33-35]. The significantly lower incidence of breast and colorectal cancer in Mediterranean countries like Greece, Italy and Spain has been mainly attributed to the olive oil content of their diet. Like canarium oil, olive oil is known to contain tyrosol and pinocarotenol, among others [20, 30].

Tyrosol is reported to possess strong antioxidant activity with IC$_{50}$ value of 2.5 mM when tested with the hypoxanthine/xanthine oxidase system [20]. A derivative of tyrosol, hydroxytyrosol, the major representative phenolic compound of virgin olive oil is reported to protect human erythrocytes against oxidative damage [31]. Besides, both tyrosol and hydroxytyrosol have been shown to significantly inhibit autoxidation [31, 36, 37], production of isoprostanes and other markers of lipid oxidation [38] and oxidative stress induced by hydrogen peroxide and xanthine oxidation [31]. In addition, Owen and co-workers have demonstrated that tyrosol, secoisolariciresinol and pinocarotenol, which were also detected in Canarium oil, possess significantly higher antioxidant potential than the classical antioxidant, Trolox, the synthetic analogue of vitamin E [30].

The hydroxybenzoic acid content of edible plants is generally very low, with the exception of certain red fruits, black radish and onions, which have concentration of central tens of mg/Kg fresh weight [9]. p-Hydroxybenzoic acid, in combination with other phenolic acids have been reported to act synergistically with vitamin C to enhance human and hamster low density lipoprotein resistance to oxidation [39]; and with gallic acid, gentisic and /or coumaric acid it act synergistically to modulate phenylsulfotransferase activity. Individually, p-hydroxybenzoic and other phenolic acids inhibit the activities of the two forms of phenylsulfotransferase enzymes involved in sulfate conjugation, in a manner that reflects their respective antioxidant activity [40]. Isoforms of this enzyme are important in the detoxication of chemical carcinogens.

The presence of catechol in Canarium oil is also of major interest in cancer chemoprevention. According to Manach and colleagues [9], catechol and other polyphenols with catechol group may intervene in oxidation-precipitated diseases, such as cardiovascular diseases and cancer, because they competitively inhibit the catechol-O-methyltransferase-catalyzed O-methylation of endogenous catecholamines and catechol estrogens: Deregulation of the O-methylation metabolism of neurotransmitters and hormones in humans is an important risk factor for the development of some neurodegenerative diseases, cardiovascular disorders and hormone-dependent cancers [41,42].

Several studies have suggested that other phenolic acids detected in Canarium schweinfurthii oil may also play some role in the chemoprevention of cancer and ROS induced diseases. p-Hydroxybenzaldehyde, for instance, has been shown to significantly protect against lipid peroxidation [43], while phloretic acid, another component of Canarium oil, has been demonstrated to possess strong antioxidant activity with IC$_{50}$ value of 3.0mM [20]. Although some earlier investigators may have associated lagnins with certain cancers, recent evidence however suggest that, pinocarotenol, a common component of the lignin fraction of plants such as flaxseed, Forsythia specie and Sesamum indicum seeds [44-46], is believed to possess cancer chemopreventive properties [20, 30]. Similarly, detection of another lignan, secoisolariciresinol, whose major source is linseed, is also noteworthy. Different lagnins have been shown to inhibit mammary carcinogenesis, lung cancer cell growth, skin cancer and colon cancer [41, 46], partly, because lagnins, as phytoestrogens, exhibit structural similarity to the mammalian steroid hormone, 17 B-oestradiol [47]. Although, like matearinsol, secoisolariciresinol is not estrogenic by itself, it is readily converted by gut flora to the mammalian lignan, enterodiol and enterolactone which are estrogenic and have shown promise in reducing growth of cancerous tumors, especially hormone-sensitive ones such as those of the breast, endometrium and prostate [36, 48-50]. Lignans may also prevent cancer through their antioxidant and antiviral properties [36, 48-50].

Therefore, put together, these results indicate that fruit pulp oil of Canarium schweinfurthii is a rich source of cancer chemopreventive polyphenol compounds that possess strong in vitro antioxidant potential, as well as very potent radical scavenging capacity, suggesting that this oil might indeed play important roles in the chemoprevention of cancer and other diseases like diabetes, cardiovascular disorders, etc which are caused by oxidative damage. Studies are underway to identify the unknown phenolic components of the oil and demonstrate the antioxidant capacity of those components that have not been previously studied.
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