Chemopreventive Effects of *Khaya senegalensis* Bark Extract on Human Colorectal Cancer

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**Abstract.** An extract of the bark of *Khaya senegalensis* is commonly used in African traditional medicine for pain and inflammation. *Khaya senegalensis* bark extract (KSBE) was hypothesized to contain inhibitors of the cyclooxygenase-2 (COX-2) gene and to be useful in the prevention and treatment of colorectal cancer. The diphenyl-2-picrylhydrazyl (DPPH)-free radical activity and the total phenolic content of KSBE were measured, followed by an investigation of cell growth inhibition, COX and prostaglandin E₂ (PGE₂) suppression, as well as apoptosis by Western blot analysis and ELISA. Our data clearly showed that KSBE displays anti-proliferative, anti-inflammatory and pro-apoptotic effects on HT-29, HCT-15 and HCA-7 cells. Since all three cell lines, irrespective of COX-2 status (HCT-15 is COX-2-deficient), were affected by the treatment, it can be concluded that both COX-dependent and COX-independent pathways are activated by KSBE.

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer deaths in the United States (1). An important strategy for combatting this deadly disease before tumors reach an invasive state is the use of a class of non-steroidal anti-inflammatory drugs (NSAIDs), which have shown great promise as chemopreventive agents (2). Epidemiological studies and clinical trials have demonstrated that NSAIDs reduce the incidence of CRC by 40%-50% (3, 4). The mechanistic action of NSAIDs includes abating the prostaglandin synthesis pathway and cyclooxygenase (COX)-independent pathways (5). COX-2, one of the COX isoforms, has been suggested as a promising chemopreventive target for CRC (6). Recently, The US Food and Drug Administration has placed severe limitations on the use of COX-2-specific inhibitors, such as celecoxib, rofecoxib and valdecoxib, due to cardiovascular toxicity. This emphasizes the importance of identifying safer COX inhibitors from natural sources, such as those which may be found in traditional medicinal plants.

*Khaya senegalensis* A. Juss., of the family Meliaceae, is a tall evergreen tree which grows in the Sahara savannah area from Senegal to Uganda. The extract from the bark of *Khaya senegalensis* is characterized by its bitter constituents, named "calicedrin" in West Africa. It is used extensively as a bitter tonic for the treatment of a variety of pro-inflammatory disease. In this study, the effect of *Khaya senegalensis* bark extract (KSBE) was investigated on three human colorectal tumor cell lines with different COX profiles. This herbal medicine, popular in West Africa, was found to have great potential as a natural chemopreventive agent for CRC.

**Materials and Methods**

**Cell lines and chemicals.** The HCT-15 and HT-29 cells were purchased from the American Type Culture Collection (ATCC Rockville, MD, USA). The HCA-7 cells were kindly provided by Dr. Susan Kirkland (University of London, UK). Both the HCT-15 and HT-29 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone, Logan, UT, USA). The HCA-7 cell lines were grown in Dulbecco’s modified Eagle’s
medium (DMEM, HyClone). The cells were grown as monolayers and were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.

DPPH and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BHT (99%) was purchased from ACROS (New Jersey, USA).

**Botanical collection.** The bark of *Khaya senegalensis* was collected in the Republic of Guinea, West Africa, in December of 2003 under the supervision of Dr. Youssouf Koita. The plant material was verified by Dr. Youssouf Koita at the Department of Pharmacy, Faculty of Medicine, University of Camel Abdel Nasser de Conakry, Guinea.

**Plant material preparation and extraction.** The dried and powdered bark of *Khaya senegalensis* (5 g) was sequentially extracted as follows: (a) hexane (150 ml) was used to extract the first batch of the most non-polar isolates; (b) extraction with ethyl acetate (150 ml) was used to obtain the non-polar isolate; (c) acetonitrile (150 ml) was used to acquire the moderate polar isolate; (d) absolute methanol was used to obtain the non-polar isolates; (e) extraction with 95% ethanol (150 ml) was used to extract the first batch of the most polar isolates. The extracts were incubated at 37°C in a humidified atmosphere of 5% medium (DMEM, HyClone). The cell lines were grown as monolayers and were incubated at 37°C for 48 h until needed. Isolating and identifying the most potent constituent from KSBE via a bio-assay-guided fractionation is currently under investigation in our laboratory.

**Measurement of DPPH free radical-scavenging activity.** The antioxidant activity of KSBE and BHT on scavenging diphenyl-2-picrylhydrazyl (DPPH) radicals was determined according to the method of Chung et al. (7). KSBE or BHT at various concentrations (total volume of 0.1 ml) was mixed with 0.5 ml of 250 μM DPPH, to reach a final concentration of 125 μM. The mixtures were shaken vigorously and left in the dark at room temperature for 30 min. The absorbances of the resulting solutions were measured and the anti-oxidant capacity to scavenge the DPPH radical was calculated.

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\text{Scavenging effect} \% = (1 - \frac{\text{absorbance of control at 517 nm}}{\text{absorbance of sample at 517 nm}}) \times 100
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**Determination of total phenolic content.** The content of total soluble phenolics was determined according to the modified Folin-Ciocalteu method (8). The reaction mixture was composed of 30 μl of extract, 270 μl of distilled water, 150 μl of the Folin-Ciocalteu reagent and 750 μl of 20% sodium carbonate solution. The test solution was mixed and incubated for 1 h and the absorbance was measured at 765 nm. The standard curve was prepared with gallic acid using known concentrations. The total phenolic content was expressed as μg of gallic acid equivalents (GAE)/mg extract.

**Cell viability analysis and ³H-thymidine incorporation.** Promega’s CellTiter 96® AQuios One Solution Assay (Madison, WI, USA) was used to determine the viability of the colon cancer cells. The cells (HCT-15, HT-29, HCA-7) were plated at 10,000 cells per well in a 96-well plate. The cells were treated with a series of concentrations of KSBE, 1 μCi/ml of ³H-thymidine was added to each well 3-16 h before the end of the treatment time. The cells were then washed twice with ice-cold PBS and once with 10% trichloroacetic acid. The extracts were analysed for radioactivity in a scintillation counter (Packard 1600 TR liquid scintillation counter, Shelton, CT, USA). The vehicle controls contained less than 0.05% DMSO.

**Protein extraction and Western immunoblotting.** Total protein was prepared and determined as previously described (9). After blocking with 5% milk in PBS containing 0.1% Tween 20, the membranes were incubated overnight at 4°C with anti-COX-2 (1:1000; Cayman Chemical, Co., Ann Arbor, MI, USA); anti-COX-1 (1:500); anti-PPARγ (1:1000) and anti-Cyclin-D1 (1:1000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-caspase-3 (1:1000), anti-caspase-9 (1:1000), anti-Bcl-2 (1:500) antibodies (MBL international Corporation, MA, USA). The blots were then incubated with horseradish peroxidase-conjugated antibody (1:5000) for 2 h at room temperature. The signals were detected using an ECL chemiluminescence kit (Amersham Bioscience, Piscataway, NJ, USA).

**Measurement of prostaglandin E2 production.** The cells (HCA-7, HT-29) were seeded in 24-well plates and were grown for 24 h to about 70-80% confluency. The cells were treated with KSBE at IC20 and IC50 for 3, 6, and 24 h (<0.05% DMSO contained in vehicle controls). The cells were then washed twice with PBS and stimulated with 20 mM arachidonic acid in fresh serum-free medium for 15 min at 37°C prior to the end of each treatment time. The levels of PGE2 released were measured in the culture medium by the enzyme immunoassay using a PGE2 EIA kit (Cayman Chemical Co.). Experiments were performed in triplicate each consisting of three independent assays per cell line.

**Caspase-3 activity assay.** After treatment with KSBE, the cells were collected and washed. The cell pellets were resuspended in 200 μl lysis buffer as described above and were incubated for 5 min on ice. The caspase-3 activities were determined following the instructions from the colorimetric Caspase Assay Kit from Assay Design, Inc.

**Statistical analysis.** The data were analyzed using SigmaStat statistical software (version 3.0, SPSS Inc., Chicago, IL, USA) and are expressed as mean±SD, the results were considered statistically significant if the p value was less than 0.05.
Results

**KSBE inhibits growth of colon cancer cells.** The effects of KSBE on cell viability were investigated in the HCA-7, HT-29 and HCT-15 cell lines (Figure 1). KSBE inhibited cellular growth in all three cell lines in a time and dose-dependent manner; the HT-29 cells exhibited the most resistance to KSBE treatment (IC\(_{50}\)=1.00 µg/µl), followed by HCT-15 (IC\(_{50}\)=0.30 µg/µl). The HCA-7 cells were the least resistant to the treatment with an IC\(_{50}\) of 0.22 µg/µl after 24 h of treatment. Our results also demonstrate that following 24-h treatment with KSBE, DNA synthesis was significantly reduced when compared with vehicle-treated cells (Figure 2). A dose-related reduction of ³H-thymidine incorporation was observed in all cells tested.

**KSBE displays strong anti-oxidant activity.** The total phenolic content of KSBE was 30.71±1.51 µg of GAE/mg extract. Furthermore, the DPPH-free radical scavenging activity of KSBE approached 90% at the concentration of 4.00 µg/µl, which was comparable to that of BHT, the anti-oxidant commonly used as a control (Figure 4A).

**KSBE blocks cell cycle progression in HCA-7 and HT-29 cell lines.** Treatment with KSBE caused an accumulation of cells in the G2-phase and inhibited S-phase DNA synthesis in HCA-7 cells. G1 arrest and stronger S-phase inhibition was observed in the HT-29 cells (Figure 5B).

**KSBE selectively inhibits COX-2 expression levels in both HT-29 and HCA-7 cell lines.** Pre- and post-treatment COX levels were evaluated by Western blot analysis. The HCT-15 cells were not found to express either COX-1 (not shown) or COX-2 (Figure 4B). KSBE inhibited COX-2 protein expression in both HCA-7 and HT-29 cell lines in a dose-dependent manner, whereas the COX-1 levels were unaffected; COX-2 protein expression was also decreased in the HT-29 cells in a time-dependent manner (Figure 3 A, B, C). The highest concentrations of KSBE used in Western blot analysis were the IC\(_{50}\) concentrations as determined by Promega’s CellTiter 96® AQueous One Solution Assay.

**Effects of KSBE on Cyclin-D\(_1\) and PPAR\(_\gamma\) expression in HCT-15 cells.** KSBE down-regulated the expression of Cyclin-D\(_1\), and up-regulated PPAR\(_\gamma\) expression in HCT-15, a COX-2 null cell line (Figure 4B). Two different concentrations (IC\(_{20}\) at 0.05 µg/µl and IC\(_{50}\) at 0.30 µg/µl) were used in the Western blot analysis.

**KSBE significantly reduced the production of PGE\(_2\).** Since KSBE strongly inhibited COX-2 protein expression levels in the HT-29 and HCA-7 cells, our attention was subsequently directed to whether the PGE\(_2\) levels were also altered. As
shown in Figure 5A, there were a 30% and 35% decrease of basal PGE2 production in the HCA-7 cells and a 30% and 46% decrease in the HT-29 cells following 24 h of treatment with KSBE at the IC20 and IC50 concentrations of each cell line.

KSBE induced apoptosis in all three cell lines. The inhibitory effect of KSBE observed in both COX-positive and -negative cells suggested that increased apoptosis may play a key role.

KSBE strongly reduced the protein expression levels of the anti-apoptotic protein Bel-2 in the HCT-15 cells. A similar effect was also evident in the HT-29 and HCA-7 cells (Figure 6). Caspase-3 and Caspase-9 Western blot analysis (Figure 6) demonstrated that pro-caspase-3 (the inactive form of caspase-3) levels decreased following 24 h of treatment; the increased amount of the cleaved form of caspase-3 was observed in all three cell lines, suggesting that caspase-3 is activated upon treatment. This effect was further examined by evaluating caspase-3 activity, which increased by 97%, 59% and 27% upon treatment with KSBE in the HCA-7, HCT-15 and HT-29 cells, respectively, as compared to the controls. In addition, our results revealed that pro-caspase-9 expression levels were decreased in all cells tested; the HCA-7 cells exhibited increases in the cleaved form of caspase-9, another important apoptosis-associated molecule (Figure 6).

Discussion

Extract of *Khaya senegalensis* is widely used in the treatment of fever, malaria, mucous diarrhea and as an antimicrobial agent in venereal diseases. It is also used as an anthelmintic and as a taeniacide remedy in West Africa (10, 11). The extract from the bark of *Khaya senegalensis* has been suggested to significantly lower the temperature of experimental hyperthermic animals when administrated via subcutaneous or intraperitoneal routes (12). These ethnopharmacological data were the basis for our investigations on identifying natural chemopreventive agents in KSBE.

COXs are the rate-limiting enzymes which catalyze arachidonic acid to form a variety of eicosanoids such as prostaglandins, thromboxanes and leukotrienes (13). COX-1 is generally considered to be expressed constitutively in most tissues and plays a role in maintaining normal cellular physiological states. COX-2 is inducible by various inflammatory stimuli (14). COX-2 levels are elevated in both human colorectal adenomatous polyps and colon cancers (15). In addition, knocking out COX-2 reduces the incidence of intestinal tumors in the *Apc*<sup>Min</sup> mouse model (16). Treatment of colon cancer cell lines with COX-2-specific inhibitors and COX-2-null fibroblasts with different NSAIDs inhibits tumor cell proliferation (17-19). Interestingly, sulindac sulfone, a NSAID metabolite that does not inhibit either COX isoform, exerts antiproliferative activity in *vitro* via apoptosis (20) and inhibits azoxymethane-induced colonic carcinogenesis in the rat (21). Our data obtained on COX expression levels and PGE2 production suggest that one of the pathways by which KSBE inhibits tumor cell growth is through the disruption of PGE2 synthesis. PGE2 production was lowered even when the COX-2 protein expression levels remained the same, indicating that KSBE may also inhibit the enzymatic activities of COX.
Since 20-50% of human sporadic colorectal adenomas do not express COX-2 (22), an investigation of KSBE in COX-null cell lines, such as HCT-15, was warranted. It was observed that KSBE suppressed the growth of HCT-15 cells, suggesting that KSBE may exert some of its effects through COX-independent mechanisms. The growth inhibition effect of KSBE was associated with an up-regulation of peroxisome proliferator-activated receptor gamma (PPARγ) and decreased expression of Cyclin D1. PPARs are important factors in controlling gene expression, critical to cell cycle regulation and cellular differentiation (23). PPARγ ligand activation has been reported to have anti-tumor effects in several models of cancer (24). Based on our results, PPARγ could be one of the downstream targets of KSBE in COX-null cell lines.

Dysregulation or inhibition of apoptosis has been reported to play an important role in a variety of cancers including CRC. Previous studies have suggested that

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**Figure 3.** Western blot analysis of COX-1 and COX-2 proteins in HCA-7 (A) and HT-29 (B, C) cell lines. c: control.
Figure 4. A) DPPH-free radical scavenging activity of KSBE was compared to that of BHT at concentrations ranging from 0 ~ 4.00 μg/μl. The results are expressed as mean±SD. B) Western blot analysis of COX-2, PPARγ, Cyclin D1 protein expression in the HCT-15 cell line. Lane 1, control; 2, 0.05 μg/μl; 3, 0.30 μg/μl; 4, control; 5, 0.05 μg/μl; 6, 0.30 μg/μl; 7, control; 8, 0.05 μg/μl; 9, 0.30 μg/μl. C) Results of caspase-3 activity assay. Significant differences in activities were observed compared to control cells following the treatment with KSBE at the IC_{50} of each cell line. C: control, 1, 0.07 μg/μl (IC_{20}), 2, 0.22 μg/μl (IC_{50}), 3, 0.50 μg/μl (IC_{50}), 4, 1.00 μg/μl (IC_{50}), 5, 0.05 μg/μl (IC_{20}), 6, 0.30 μg/μl (IC_{50}), * p<0.05.
apoptosis induction is essential to the action of many chemotherapeutic agents in tumor cells (25). There are two main apoptotic death pathways: the mitochondrial and the death receptor pathways. Both converge at the activation of caspase-3 via Bcl-2 mediation. Bcl-2 encodes an inner mitochondrial protein that antagonizes apoptosis (26). Our results clearly demonstrated that KSBE strongly inhibited anti-apoptotic Bcl-2 protein expression in all three cell lines evaluated. This effect was more significant in the HCT-15 cells, most probably due to the high basal levels of Bcl-2 in this COX-null cell line. Furthermore, the increase in caspase-3 protein expression and activity level suggests that caspase-dependent apoptosis is involved in the inhibition of tumor cell growth by the constituents of KSBE.

Decoctions of *Khaya senegalensis* prepared from the tree bark have been used in West African traditional medicine for centuries without overt toxicity. Interestingly, studies have shown that a total extract of *Khaya senegalensis* bark exerted as strong or even stronger anti-inflammatory effects than each of its fractions when applied as an anti-inflammatory ointment (27). It is very possible that the synergistic and additive effects of several constituents in the whole extract of KSBE will provide a wider safety margin and still remain efficacious for future chemopreventive regimens.

In conclusion, our *in vitro* data suggested that KSBE has potent antitumor effects and caused both cell cycle arrest and apoptosis, possibly via a COX-2-dependent pathway. Given the recent governmental removal and/or restriction from the market of selective COX-2 inhibitors of synthetic origin due to suspected cardiovascular toxicity, it is important to reassess natural products for a safer alternative. KSBE is one of the several anti-inflammatory plants used in West Africa that may have the potential to prevent colon cancer. This and other traditional medicinal plants are currently being systematically investigated in our laboratory for their chemopreventive potential.

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**References**

Figure 6. A) KSBE down-regulated the anti-apoptotic protein Bcl-2 in all three cell lines. B, C) KSBE decreased the amounts of pro-caspase-3 and pro-caspase-9 expression and induced activated caspase-3 formation in all three cell lines. C* Control; 1, 0.50 μg/μl, 2, 1.00 μg/μl, 3, 0.05 μg/μl, 4, 0.30 μg/μl, 5, 0.07 μg/μl, 6, 0.22 μg/μl.

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