Measurement of anti-proliferative activity experiment

Human cancer cell lines A549 (Lung carcinoma), MCF-7 (Breast adenocarcinoma), DU 145 (Prostate carcinoma), DLD-1 (Colorectal adenocarcinoma), FaDu (squamous cell carcinoma of pharynx) were obtained from American Type Culture Collection (ATCC), USA. These cells were cultured in DMEM supplemented with 10% FBS and antibiotic combinations in 5% CO_2 humidified atmosphere at 37 ^{0} C.

A colorimetric sulforhodamine B (SRB) assay was used for the measurement of anti-proliferative activity as described before (Adaramoye et al., 2011; Fricker and Buckley, 1995; Keepers et al., 1991; Skehan et al., 1990). It is the second major technique for testing and is the more preferred. This basically depends on the incur of the negatively charged pink amino xanthine dye, sulphorhodamine B (SRB) through basic amino acids in the cells. The released dye will give a more intense colour and more absorbance, when the number of cells and amount of dye is taken up is greater, after fixing, when the cells are lysed, (Skehan et al., 1990). The SRB assay is sensitive, simple, reproducible and more rapid than the formazan-based assays and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement, as do the MTT or XTT assays (Fricker and Buckley, 1995; Keepers et al., 1991).

Ten thousand cells were seeded to each well of 96-well plate, grown overnight and exposed to test samples at 100 µg/ml concentration for 48 h. Cells were then fixed with ice-cold tri-chloro acetic acid (50% w/v, 50µl/well), stained with SRB (0. 4% w/v in 1% acetic acid, 50µl/well), washed and air

dried. Bound dye was dissolved in 150 µL of 10mM Tris base and plates were read at 510 nm absorbance (Epoch Microplate Reader, Biotek, USA).

Anti-proliferative activity of test samples was calculated as:

% inhibition in cell growth = [100-(Absorbance of compound treated cells/ Absorbance of untreated cells)] x100.

1. *Principal component analysis*

PCA was carried out based on the contents of eighteen bioactive compounds in fruits and leaves of five *Cassia* species, using STATISTICA 7. 0 software. When the contents of investigated compounds were below the quantitation limit or not detected in the samples, the values of such elements were considered to be zero.

2. Results and discussion
    1. *Optimization of chromatographic and MS/MS conditions*

Complete separation of proximate analytes is certainly not required for MS/MS detection. In this study, chrysophanic acid and emodin are having same product ion, while catechin and epicatechin are having same precursor and product ion. Therefore, mobile phase was optimized using different compositions of solvents and adjusting their gradient elution for separation of all the compounds. Acetonitrilepossesses stronger elution ability in comparison to methanol, which shortens the elution time and thus selected for this method. On the basis of the polarity of anthraquinones, phenolics, flavonoids and terpenoids in the extracts of *Cassia* species samples, an Acquity UPLC BEH C18 (2. 1 mm × 50 mm, 1. 7µm; Waters, Milford, MA)

column was selected for their separation, which was more suitable for acidic mobile phasewith smoother baseline in the separation as compared to other tested columns. Compared with acetic acid, formic acid was found more effective for ionization of compounds detected in the negative ESI mode. Thus, different concentration strengths (0. 05%, 0. 1% and 0. 2%) of formic acid were investigated, and finally 0. 1% formic acid concentration was selected for analysis. Therefore, optimized gradient elution with 0. 1% formic acid in water and acetonitrile at a flow rate of 0. 4 mL/min with the column temperature of 30°C resulted in separation of the 18 compounds in less than 8 min chromatographic run time.

All the compound dependent MS parameters (precursor ion, product ion, declustering potential (DP) and collision energy (CE) were carefully optimized for each targeted compound in negative ESI mode, which was performed by flow injection analysis (FIA). The chemical structures of 18 components were characterized based on their retention behaviour and MS information such as quasimolecular ions $[M-H]^-$, fragment ions $[M-H-COO]^-$, $[M-H-COO-CH3]^-$, [M- CO-H2O] compared to related standards and literatures (Pandey et al., 2014; Wei et al., 2013; Xia et al., 2011; Yu et al., 2009). MRM parameters: DP, EP, CE and CXP were optimized to achieve the most abundant, specific and stable MRM transition for each compound as shown inTable 1. MRM extracted ion chromatogram of analytes are shown inFig. 1.

2. *Analytical Method Validation*

The proposed UPLC-MRM method for quantitative analysis was validated according to the guidelines of international conference on harmonization

(ICH, Q2R1) by linearity, LOQs and LODs, precision, solution stability, and recovery.

1. *Linearity, LOD and LOQ*

The internal standard method was employed to calculate the contents of eighteen analytes in *Cassia* species. The stock solution was diluted with methanol to different working concentrations for the construction of calibration curves. The linearity of calibration was performed by the analytes-to-IS peak area ratios versus the nominal concentration and the calibration curves were constructed with a weight (1/x2) factor by least-squares linear regression. The applied calibration model for all curves was y = a x + b, where y = peak area ratio (analyte/IS), x = concentration of the analyte, a = slope of the curve and b = intercept. The LODs and LOQs were measured with S/N of 3 and 10, respectively as criteria. The results were listed inTable 1. All the calibration curves indicated good linearity with correlation coefficients (r^2) from 0. 9990 to 0. 9999 within the test ranges. The LODs for each analyte varied from 0. 02-1. 34 ng/mL and LOQs from 0. 06-3. 88 ng/ml and were much lower than those obtained with previous HPLC methods (Chewchinda et al., 2012; Chewchinda et al., 2014; Chewchinda et al., 2013; Ni et al., 2009; Prakash et al., 2007).

2. *Precision, Stability and Recovery*

The intra-day and inter-day variations, for the determination of precision of the developed method, were evaluated by determining the eighteen analytes in six replicates on a single day and by duplicating the experiments over three successive days. The overall intra-day and inter-day precision

were not more than 3. 37 %. Stability of sample solutions stored at room temperature was evaluated by replicate injections at 0, 2, 4, 8, 12 and 24 h. The RSDs value of stability of the eighteen analytes ≤ 3. 19 %. A recovery test was applied to evaluate the accuracy of this method. Three different concentration levels (high, middle and low) of the analytical standards were added into the samples. Three replicates were performed at each level. The percentage recoveries were calculated according to the following equation: (detected amount–original amount) × 100% / added amount. The analytical method developed had good accuracy with overall recovery in the range from 97. 75-105. 09 % (RSD ≤ 2. 42 %) for all analytes (Table 1).