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Original Article

Seasonal Variation of Phytochemicals in Four Selected Medicinal Plants

Bonface Mwamatope[1], David Tembo[2,*], Elizabeth Kampira[3], Cecilia Maliwichi-Nyirenda[4], Victoria Ndolo[5]

ABSTRACT

Background: The study aimed at assessing total alkaloids content (TAC), total flavonoids content (TFC), total phenolic content (TPC), antioxidant activity (AA) and predominant individual phenolic compounds in methanol extracts of *M. oleifera, S.singueana, M.azedarach* leaves and stem barks of *L. discolor* collected during different seasons of the year. **Materials and Methods:** TPC, TFC and TAC were analyzed using the Folin Ciocalteau assay, aluminum chloride assay and bromocresol green assay, respectively. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) techniques were used to analyse AA. Phenolic compounds; Vanillic acid, (+)-catechin, quercetin, rutin, sinapic acid, 2,4-hydroxybenzoic acid and ferulic acid were analyzed using high performance liquid chromatography diode array detector (HPLC - DAD). **Results:** Barks of *L. discolour* exhibited high levels of TPC, TFC, TAC and AA during hot-dry season (summer) as compared to *S. singueana, M. oleifera* and *M. azedarach* leaves which showed significantly ($p< 0.05$) higher levels in hot-wet season (rainy). Levels of TPC, TFC and TAC were significantly ($p< 0.05$) influenced by seasonal variations and correlated with levels of AA. Profiles of all phenolic compounds analyzed were not similar in terms of chemical composition and concentration during different seasons. Sinapic acid and 2,4-hydroxybenzoic acid were predominant in *S. singueana* while sinapic acid and ferulic acid were predominant in *M. oleifera*. Vanillic acid and 2,4-hydroxybenzoic acid were predominant in *L. discolor* and *M. azedarach* respectively. **Conclusion:** The phytochemical constituents in medicinal plants are subject to seasonal variations which in turn influence the optimum season of harvesting.

Keywords: Alkaloids, Antioxidant activity, Flavonoids, Medicinal plants, Phenolic compounds, Seasonal variations.

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INTRODUCTION

There is increase in the use of plants as medicines world-wide, due to lack of access to conventional drugs, financial challenges and the ineffectiveness and side-effects of some conventional drugs.[1-3] Plant parts such as roots, leaves and barks are usually used as medicinal agents and sources of natural bioactive compounds that work for treatment of different ailments and as natural antioxidants.[4,5] These bioactive compounds such as flavonoids, anthraquinones, stilbenes, terpenes, alkaloids, tannins, saponins and phenolic compounds are synthesized in plants via different pathways, such as shikimic acid, salonic acid, mevalonic acid and non-mevalonate.[6,7] In many African countries including Malawi, some of these medicinal plants (e.g. *S. singueana, L. discolor, M. oleifera* and *M. azedarach*) are used for treatment of different diseases.[8-13]

Senna singueana also known as wild *Cassia* whose family is Fabaceae is used a lot in Africa for treating various ailments.[8,10] It has been claimed that this plant is effective in managing skin cancer,[8,14] diabetes, constipation, stomach pains, sexually transmitted infections, inflammation, fever and malaria.[15-17] *Melia azedarach* is a species of medicinal plant in Meliaceae family.[18,19] Some of reported pharmacological activities of this species include hepatoprotection, anti-fertility, antioxidant, anti-viral, anti-bacterial, wound healing, analgesic, insecticidal and anti-cancer.[18,20,21] *Moringa oleifera* belongs to a family of Moringaceae and is used to treat different ailments and prevent malnutrition.[9,11] The plant parts of *M. oleifera* possess some biological activities such as anti-cancer,[22] cardiac and circulatory stimulation, cholesterol lowering, antitumor, antioxidant, anti-bacterial and anti-fungal activity.[11,23] *Lannea discolor* belongs to the family of Anacardiaceae and this is also used for treatment of different ailments.[10,12] Some of biological activities of this plant include anthelmintic, antiplasmodial, anti-cancer, nematicidal and antioxidant activity.[13,24]

The availability and concentration of phytochemicals in plants are affected by seasonal fluctuations.[25,26] These changes in phytochemicals have been widely attributed to variations in environmental variables

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(abiotic factors) such as soil composition, responses against a variety of season specific pathogens and changes in temperature and rainfall.[27,28] Other factors (biotic factors) such as development of the plant and plant genotype also change rapidly and these too may affect the composition of the plant parts.[29,30] These variations seem to influence the harvest time which ensure optimum quality and yield of medicinal plant species.[31] It is thus not surprising that the World Health Organization (WHO) recommended that parts of medicinal plants should be harvested during the appropriate season so that they should be of high quality (both raw materials and finished products).[32]

Studies have been published on the effects of seasonal variations on phytochemical composition and content of numerous medicinal plants,[25-30] but little is known about *M. azedarach, M.oleifera, S. singueana* leaves, and *L. discolor* stem barks of Malawi. In addition, no comprehensive data representing optimized harvesting season for these medicinal plants exists. The present study uses HPLC to fingerprint and identify individual phenolic compounds. Variations in levels of antioxidant activity, phenolic compounds and alkaloids at different seasons of the year were investigated in plant species under study. Specifically, ferulic acid, vanillic acid, sinapic acid, (+)-catechin, rutin, quercetin, 2,4-dihydroxybenzoic acid, phenolic content, alkaloids content, flavonoids content and antioxidant activity were analyzed at different seasons of the year. The data obtained in this study provides important information that will help to determine the optimum harvesting seasons for these medicinal plants.

MATERIALS AND METHODS

Chemicals

Chemicals of HPLC and analytical grade used in this study included methanol, formic acid, acetonitrile, folin-ciocalteu phenol reagent, standards (vanillic acid, (+)-catechin, quercetin, rutin, sinapic acid, 2,4-hydroxybenzoic acid and ferulic acid) from Sigma-Aldrich Chemical Co. (USA), Gallic acid from EMD Millipore Corporation (USA), 2, 2-Diphenyl-1-Picrylhydrazyl Radical (DPPH) and 2, 4, 6-tris-2-pyridyl-s-triazine (TPTZ) from Sisco Research (India) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) from Acros Oganics (USA).

Plant materials and preparation

Stem barks of *L. discolor* and *S. singueana, M. oleifera, M. azedarach* leaves were collected from northern part of Malawi, Karonga district. The samples were collected during three different seasons between the year 2019 and 2020; cool-dry (July), hot-dry (October) and hot-wet (January). The three seasons have the following temperature and rainfall patterns: cool-dry (winter), temperature 15 to 23°C and rainfall 1.6 mm; hot-dry (summer), temperature 21 to 29°C and rainfall 3.1 mm; hot-wet (rainy), temperature 21 to 27°C and rainfall 147.1mm (World weather online.com). National Herbarium and Botanic Gardens (NHBG) taxonomist identified and verified medicinal plant species. The stem barks and leaves were dried by air under shed for three weeks. Domestic blender (Kenwood) was used to ground the samples to fine powder (0.4 mm).

Sample extraction

Extraction of phenolic compounds was achieved as previously described.[33-36] The powdered samples (25g) were soaked in 80% v/v methanol (300mL) for 72 hr while intermittently stirring and then filtered thereafter. Rotor evaporator (Buchi R-215, Switzerland) was used to concentrate the filtrates at 40°C and low pressure. Alkaloids were extracted as previously described.[37] Semi-solid extracts (0.1g) were dissolved in hydrochloric acid (2M, 5.0mL), filtered and analysed.[36]

Total phenolic content determination

Total phenolic content was analysed using Folin-Ciocalteu assay.[36,38] The effectiveness of this method is on ability of phenolic compounds to transfer electrons in a basic environment to complexes of phosphomolybdic or phosphotungstic acid which, when reduced, produce a blue colour. This colour is measured spectrophotometrically at 765 nm and absorption is proportional to concentration of phenolic compounds.[38] UV-Vis Spectrophotometer was used to measure absorbance. Total phenolic content was quantified as milligram of gallic acid equivalents per gram of dry weight.

Total flavonoid content determination

Total flavonoids was analysed using aluminum chloride colorimetric assay [36,39] A stable acid complex is produced when aluminium chloride reacts with keto groups or hydroxyl groups of flavonoids. This reaction produces a yellow color which can be measured spectrophotometrically at 415 nm.[39] Total flavonoids content was quantified as milligram of quercetin equivalents per gram of dry weight.

Total alkaloids content determination

Total alkaloids contents were estimated spectrophotometrically using bromocresol green assay.[37] A yellow colored complex develops when bromocresol green (BCG) and alkaloids reacts and this color is readily soluble in chloroform at pH 4.7.[37] The filtrates were mixed with Wagner's reagent and development of brown/reddish precipitate indicated availability of alkaloids. The filtrates (1.0 mL) were transferred into falcon tubes in which phosphate buffer (pH 4.9, 5 mL) was added, followed by bromocresol green solution (10-4 M, 5 mL) and vortexed. The complex formed was extracted using chloroform (5 mL). UV-Vis Spectrophotometer was used to measure absorbance at 450 nm. Total alkaloids content was quantified as milligram of caffeine equivalent per gram of dried weight.

Antioxidant activity determination (DPPH, FRAP)

The ability of extracts to scavenge radicals was evaluated using DPPH assay.[36,40] DPPH which is a free radical and dissolves in methanol or ethanol. The color of DPPH solution becomes lighter when antioxidant donates hydrogen atom or electron and scavenges the radical. The absorbance as a result of a change in color is measured at 517 nm.[40] Results were quantified as % DPPH.

The ability of extracts to chelate metal cations was evaluated using FRAP assay.[36,41] This method measures the potential of antioxidants to release electrons and reduce iron(III) tripyridyl-triazine complex to Iron(II) complex in acidic medium.[41] When antioxidants reduce ferric complex, a blue colour develops which is measured at 593nm.[41] Ferric reducing antioxidant capacity was quantified as milligram of trolox equivalent antioxidant capacity per gram of dry weight.

HPLC-DAD assay

High performance liquid chromatography Diode Array Detector (HPLC-DAD) assay was done as previously described.[42] With some modifications; HPLC instrument (1260 HPLC Agilent Technologies, Manchester, UK) equipped with a moel G1311C binary gradient pump, G1316A column oven and G1315D diode array detector (DAD) was used. Each standard (0.1 mg) (vanillic acid, (+)-catechin, quercetin, rutin, sinapic acid, 2,4-hydroxybenzoic acid and ferulic acid) was dissolved in 80% methanol (100 mL) to make 1 mg/L and used as stock solutions. Diluted extracts were filtered using 0.45µm filter membranes (Cronus PTFE). The sample (10.0µL) was injected in a 100 mm × 4.6 mm Eclipse plus 3.5µm C_{18} column (Agilent Technologies, Manchester, UK) for achievement of separations. This operation was conducted at 30°C

using a binary mobile phase of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Separation of phenolic compounds was done using a 22-min programme with the following solvent gradient: 0.01 min 5.0% B; 5 min 20% B; 20 min 40% B; 22 min 90% B; and 30 min 90% B. The flow rate was 0.5 mL/min. Identification of phenolic compounds was done by comparing UV spectra and retention time (R_T) on chromatograms which were recorded at 280 and 302 nm. Standard calibration curves were constructed and used for quantifying phenolic compounds in samples. Limit of detection (LOD) was calculated as LOD = $y + 3SD$ and Limit of quantification (LOQ) as LOQ = $y + 10SD$, where y is signal of blank and SD is the standard deviation of least concentrated analyte. Recoveries of phenolic compounds were calculated after spiking the blank of the medicinal plant extracts with the reference standard. Repeatability and intermediate precision of the HPLC method was also calculated.

Data Management and Analysis

Statistical analysis was done using Paleontological Statistics Software version 3.26 (2019) and GraphPad prism version 8.3.0 (San Diego, USA). Data was presented as means ± standard deviations (*SD*) of analyses in triplicates. The significance between means was done at confidence level of 95%.

RESULTS AND DISCUSSION

Total phenolic content

These are aromatic secondary plant metabolites with aromatic ring(s) possessing hydroxyl group(s).[43] They exhibit different biological actions including antioxidant, anticancer, and antimicrobial, through interactions with key enzymes or antioxidant systems.[5,6,44] The analysis of total phenolic content in medicinal plants was carried out using Folin-Ciocalteu method because the assay is both simple and sensitive.[38] Results for total phenolic content are presented in Table 1. It was observed that total phenolic content varied significantly ($p< 0.05$) with season in all the samples. Overall TPC values ranged from 4.25 ± 0.13 mg GAE/g DW to 59.92 ± 2.32 mg GAE/g DW. *Senna singueana, M. oleifera* and *M. azedarach* leaves exhibited highest TPC in hot-wet (rainy), followed by the cool-dry (winter) and lowest in the hot-dry (summer) season. Stem barks of *L. discolor* showed exceptionally highest TPC in summer followed by rainy season and lowest in the winter (Table 1). High contents of total phenolic compounds during hot-wet season could be a result of high water and low temperature stress which can stimulate the accumulation of some phenolic compounds by hydrolysis of glycosides.[45] These results agree with the findings of Kale,[46] who reported high total phenolic contents in *Datulametel* during the rainy season.[46] The biosynthesis and accumulation of phytochemicals including phenolic compounds can be greatly influenced by internal factors such as genotype and physiological condition of the plant species.[47,48] However, external factors including the biotic and abiotic that occur during the plant growing period may also influence the chemical composition. These external factors include feeding of seasonal insects or herbivorous animals, interaction with seasonal pathogens and diseases, the availability of light, water and temperature.[26,30,48-51] Changes in the levels of TPC among plants may also be due to genetic responses to edaphic factors such as soil composition and nutritional stress during production of secondary metabolites.[52,53]

Total flavonoids content

Flavonoids are phytochemicals with a polyphenolic structure.[54] They have been reported to exhibit broad biological activities including antitumor, antimicrobial, antioxidant and anticancer activities.[6,13,20] An aluminium chloride assay was used to analyse TFC in plants due to its sensitivity and reliability.[39] The flavonoids contents of each plant species varied according to seasonal changes with all the samples exhibiting high contents in the hot-wet season as presented in Table 1. *S. singueana* leaves showed high contents of TFC (16.97 ± 0.23 mg QE/g DW). TFC decreased significantly ($p< 0.05$) in the following order: *S. singueana* >*L. discolor* > *M. oleifera* > *M. azedarach*. *Lannea discolor* stem barks and *M. azedarach* leaves contained low flavonoid content during cool-dry season while *S. singueana* and *M. oleifera* leaves contained low content during hot-dry season. *Lannea discolor* stem barks showed low amounts of flavonoids (0.15 ± 0.01 mg QE/g DW) despite exhibiting high level of TPC in cool-dry season (Table 1). The changes in amounts of TFC might be because of the same variables that have been linked to affect TPC, because flavonoids are one of the subgroups of phenolic compounds.[26,30,48-51] These high flavonoid contents reported here during the hot-wet season are in agreement with previous work, which indicated that total flavonoids content were high during the rainy season in *Ocimum sanctum*.[55]

Total alkaloids content

Alkaloids are complex heterocyclic nitrogen compounds of natural origin with a basic character found in plant species.[56] The biological actions of some alkaloids has resulted to investigations as pharmaceuticals, which have proven to be effective.[57,58] A Bromocresol green assay was used to analyse TAC in plants as the method is both simple and efficient.[37] Total alkaloids content varied significantly ($p< 0.05$) and values ranged from 98.2 ± 2.2 mg CE/g DW to 295.1 ± 3.8 mg CE/g DW. The contents of alkaloids, as did the total phenolic content and flavonoids, varied widely according to season (Table 1). *Lannea discolor* contained high amounts of alkaloids in summer and low in winter. Leaves of *S. singueana, M. oleifera* and *M. azedarach* contained high alkaloids contents during the rainy season and low in the winter. High alkaloids contents in the rainy season in *L. discolor* could result from a decrease in biomass production or temperature stress which can result in changes in the biochemistry of medicinal plant species.[49] Temperature stress affects biosynthetic pathways in the development of alkaloids, by providing precursors from the primary metabolism which elevate production of alkaloids to counteract oxidative stress.[28,49] Similar observations of high alkaloid contents during the rainy season in medicinal plants have been previously reported.[46] The results are also in agreement with findings of other researchers in different plant species. For example, the contents of alkaloids in *B. prionitis*, *B. diffusa*, *C. colocynthis* and *G. tenax* were higher in summer compared to winter and the rainy season.[27] High contents of phytochemicals during the hot-wet season in leaves of *S. singueana, M. oleifera* and *M. azedarach* may be associated with an increase in soil salinity due to anthropogenic activities or transfer of minerals from rocks into soils from runoff. In plant cells, these phytochemicals serve as an osmolyte through decreasing stresses that arise from the salinity stress in growth conditions of plant species by increasing their amounts of production.[49,59] Plant samples were collected in three different seasons which are characterized by variable average temperatures and rainfall patterns. These abiotic factors have previously been suggested to affect the availability and quantity of phytochemicals in other plants.[45,46,50]

Antioxidant Activity

Antioxidants are phytochemicals that prevent or inhibit the oxidation of some molecules, such as DNA, by scavenging radicals and mitigating stress.[44,54,57] Antioxidant activity was analyzed using easy and rapid methods (FRAP and DPPH).[40,41] Results showed significant variation of antioxidant activity with season (Table 1). For barks of *L. discolor*, maximum values were observed in summer. For *S. singueana, M. oleifera and M. Azedarach* leaves, highest antioxidant values were found in samples from the rainy season. Antioxidant activity results were proportional to the TPC and TAC described earlier. The antioxidant actions

Table 1: Total phenolic content (mg GAE/g DW), Total flavonoids content (mg QE/g DW), Total alkaloids content (mg CE/g DW) and antioxidant activity (FRAP (mg TEAC/g DW); DPPH (%)) of medicinal plant species.

	Cool-Dry Season (winter)					Hot-Dry Season (summer)					Hot-Wet Season (rainy)				
	TPC*	TFC*	TAC	FRAP*	DPPH*	TPC	TFC	TAC	FARP	DPPH	TPC	TFC	TAC	FRAP	DPPH
Ss	15.82 ± 1.01^{b}	7.37 ± 0.1^{a}	106.2 ± 4.61^{d}	124.4 ± 0.91^{b}	67.2 ± 1.42^{b}	7.81 ± 1.22^{b}	4.46 ± 1.85^{a}	112.1 ± 7.04^{c}	101.3 ± 2.11^{b}	56.5 ± 1.12^{b}	44.51 ± 2.43^{b}	16.97 ± 1.23^{a}	212.1 ± 6.89^{a}	166.8 ± 4.23^{b}	69.5 ± 3.14^{b}
Ld	27.64 ± 2.5^{a}	0.15 ± 0.01^{d}	176 ± 2.23^{a}	166.3 ± 1.65^{a}	89.1 ± 1.18^{a}	59.92 ± 3.21^{a}	4.12 ± 0.65^{a}	295.1 ± 9.33^{a}	186.4 ± 3.37^{a}	95.22 ± 3.44^{a}	53.78 ± 5.13^{a}	16.68 ± 0.11^{a}	215.1 ± 12.45^{a}	174.7 ± 3.94^{a}	90.2 ± 5.43^{a}
Mo	12.33 ± 1.32^{c}	4.83 ± 0.11^{b}	115 ± 7.33^{c}	85.5 ± 0.96^{c}	57.9 ± 2.81^{c}	5.14 ± 1.32^{c}	3.49 ± 0.32^{b}	98.22 ± 5.53^{c}	80.31 ± 6.49^{c}	43.62 ± 2.14^{c}	26.06 ± 1.32^{c}	12.34 ± 1.32^{b}	128.2 ± 3.42^{c}	126.4 ± 5.61^{c}	65.2 ± 2.58^{c}
Ma	7.83 ± 0.01^{d}	0.53 ± 0.02^{c}	120 ± 9.33^{b}	68.5 ± 1.72^{d}	42.7 ± 0.18^{d}	4.25 ± 0.11^{c}	1.87 ± 0.01^{c}	126.3 ± 10.3^{b}	79.92 ± 2.31^{c}	48.91 ± 4.59^{c}	22.73 ± 2.32^{d}	9.23 ± 0.01^{c}	148.3 ± 17.55^{b}	115.3 ± 2.37^{d}	54.9 ± 1.17^{d}

Abbreviation: *Ss; Senna singueana*, *Ld*; *Lannea discolor*, *Mo*; *Moringa oleifera*, *Ma*; *Melia azedarach*; TPC; total phenolic content, TFC; total flavonoids content, TAC; total alkaloids content, FRAP; ferric reducing antioxidant power, DPPH; 2, 2-diphenyl-1-picrylhydrazyl radicle, mg GAE/g DW; milligram of gallic acid equivalents per gram of dry weight, mg QE/g DW; milligram of quercetin equivalents per gram of dry weight, mg CE/g DW; milligram of caffeine equivalents per gram of dry weight, mg TEAC/g DW; milligram of Trolox equivalent antioxidant capacity per gram of dry weight. Each value is expressed as mean ± standard deviation (n = 3). Mean values within a column with different letters are significantly different (p< 0.05, Tukey's test). *Results (cool-dry) from previous publication included to complement three seasons.[36]

Table 2: Correlation coefficients between antioxidant activity and variables.

	Cool-Dry Season (winter)		Hot-Dry Season (summer)		Hot-Wet-Season (rainy)	
	FRAP	DPPH	FRAP	DPPH	FRAP	DPPH
TPC	0.9764*	0.9882*	0.9897*	0.9824*	0.9873*	0.9136
TFC	-0.0896	-0.0803	0.5043	0.4565	0.9501*	0.7544
TAC	0.7313	0.7490	0.9728*	0.9778*	0.9389	0.7207
FRAP	1*	0.9818*	1*	0.9956*	1*	0.8636
		0.9640**		0.9913**		0.7458**

Abbreviations: TPC; totoal phenolic content, TFC; totoal flavonoids content, TAC; totoal alkaloids content, DPPH; 2, 2-diphenyl-1-picrylhydrazyl radical, FRAP; ferric reducing antioxidant power. p< 0.05 (*), linear regression coefficients (**).

of these medicinal plant species could be as a result of the presence of phytochemicals such as phenolic compounds and alkaloids.[53-56] In contrast, the effect of seasonal changes on antioxidant activity of *Ginkgo biloba* was highest in the autumn.[60] In another study, seasonal changes on antioxidant activity of *Nothapodytes nimmoniana* were found to be high in winter.[61] Production of phytochemicals and antioxidant activity, in respect to environmental stress during different seasons, have been linked to defense mechanisms.[6,7,29-32,49,54]

Correlation between antioxidant activity and TPC, TFC and TAP

Linear regression and Pearson's correlation analyses were performed to validate the two assays used to analyze total antioxidant activity of four plant species under study. The Pearson's r and linearity (r^2) are presented in Table 2. FRAP and DPPH were significantly (p< 0.05) and positively correlated. The findings demonstrate the suitability and reliability of the two assay methods as vehicles for evaluating total antioxidant capabilities of plant extracts. Amounts of phenolic compounds and alkaloids of all the medicinal plant species under study exhibited positive correlations with antioxidant capacity (FRAP and DPPH) throughout the seasons (Table 2). Positive correlations which were significant (p< 0.05) were also observed between TPC and FRAP. Flavonoids showed a positive correlation with antioxidant activity except during cool-dry season, in which it exhibited a non-significant negative correlation (-0.0896 and -0.0803 for FRAP and DPPH respectively). Most flavonoids are generally present as glycosides in plants of which they are usually conjugated with glucose. Previous studies indicate that glycosylated flavonoids have lower antioxidant capacity than their corresponding aglycones.[62,63] Generally, the two assays showed consistent results with the seasonal variations of total phenolic compounds and total alkaloids. This demonstrates that higher antioxidant activity of samples, especially those collected in summer and rainy seasons, maybe attributed to higher total phenolic and alkaloids contents. Phytochemicals such as phenolic compounds and alkaloids significantly contribute to antioxidant activity of selected medicinal plants.[64,65] This stronger correlation between antioxidant activity and phytochemicals observed in the present study supports the findings of literature.[64-69]

Phenolic compounds identification and quantification

HPLC fingerprints of standard phenolic compounds can be used as a baseline for comparison when analyzing unknown compounds responsible for therapeutic activities in plants.[34,40] Therefore, to identify individual phenolic compounds responsible for antioxidant properties, HPLC was used because the technique is extremely quick and efficient.[42] Seven standard phenolic compounds were used for peak identification and the chromatography parameters are presented in Table 3. Except quercetin, values for compounds in all samples were satisfactory. Five (5) of phenolic compounds including (+)-catechin, sinapic acid, 2,4-dihydroxybenzoic acid, quercetin and ferulic acid were observed in leaves of *S. singueana* extracts using HPLC– DAD (Figure 1, Table 4). Similar observations have been reported about the availability of phenolic compounds in *S. singueana* extracts.[70,71] The presence of (+)-catechin, quercetin, 2,4-dihydroxybenzoic acid, ferulic acid, and sinapic acid in S. *singueana*

Table 3: Chromatography parameters of standard compounds used for peak identification.

Standard	R_T (min)	Wavelength (nm)	LOD (µg/m)	LOQ (µg/mL)	Linearity (r^2)	Recovery (%)	Repeatability				Intermediate Precision		
							Concentration (µg/mL)	X (n = 3)	SD	RSD %	X (n = 3)	SD	RSD %
(+) – catechin	8.16	280	1.99	2.00	0.9965	95.01 ± 5.1	40	39.32	0.324	0.8240	41.22	1.4521	1.7542
							50	46.82	0.4933	1.053	48.3	0.7365	1.114
							100	97.72	1.083	1.108	98.88	2.167	0.2191
							150	146.9	0.4195	0.2854	145.21	0.1132	0.1761
Rutin	9.23	280	0.03	0.10	0.9940	82.10 ± 0.4	40	34.54	0.1957	0.5666	38.76	1.5343	0.2534
							50	52.69	0.1596	0.3029	51.55	2.8632	0.6521
							100	103.4	3.218	3.113	104.23	4.3327	1.3425
							150	144.1	3.406	2.365	147.32	0.2763	0.4628
Vanillic acid	9.77	302	0.38	1.28	0.9972	89.97 ± 7.2	40	38.44	0.1218	0.3169	42.31	2.7132	1.6362
							50	49.79	0.0223	0.0449	52.56	3.127	2.3287
							100	103.8	1.346	1.296	99.21	0.0721	3.0517
							150	147.9	0.2021	0.1366	153.63	1.7632	0.1001
2,4-dihydroxy benzoic acid	10.62	302	0.24	0.80	0.9909	115.0 ± 3.5	40	38.7001	0.05766	0.14898	43. 911	1.4381	4.0618
							50	47.8631	0.0656	0.13705	49.041	0.1298	2.9172
							100	107.1	0.4916	0.4589	110.14	3.854	0.6652
							150	146.3	0.2107	0.144	152.12	2.016	1.635
Sinapic acid	11.92	280	0.26	0.86	0.9944	88.21 ± 4.2	40	37.9	0.0342	0.0902	42.142	1.436	1.3141
							50	50.61	2.106	4.16	51.717	4.014	3.615
							100	105.3	0.182	0.1728	99.612	1.7351	1.631
							150	146.8	4.402	2.999	153.72	3.128	1.0301
Ferulic acid	12.47	302	0.08	2.86	1.0000	73.59 ± 1.6	40	40.04	0.8992	2.246	41.021	2.873	1.2342
							50	-	-	-	-	-	-
							100	-	-	-	101.32	2.605	7.862
							150	149.9	0.7654	0.5106	147.16	0.2431	1.4352
Quercetin Dehydrate	18.05	302	-	-	0.9989	71.40 ± 0.1	40	38.61	0.0342	0.0887	44.12	1.23	2.103
							50	50.46	0.0401	0.0794	48.53	3.651	1.641
							100	102.1	0.0837	0.0819	96.53	0.725	0.171
							150	148.8	0.058	0.039	154.51	1.634	0.01

Abbreviations: X; mean concentration, R_T; Retention time, SD; Standard deviation, LOQ; Limit of quantification, RSD%; Relative standard deviation, n; number of replicates, LOD; Limit of detection,

varied significantly ($p< 0.05$) with season (Figure 1, Table 4). It was observed that ferulic acid and (+)-catechin were present in only two seasons; winter and rainy seasons, while quercetin was present only during the rainy season. Vanilic acid and rutin were not detected throughout seasons. Sinapic acid and 2, 4-dihydroxybenzoic acid were the predominant compounds observed across seasons and their concentrations increased significantly ($p< 0.05$) in the following order: summer < winter < rainy season. Watoo *et al.*[45] hypothesized that water stress during the rainy season can stimulate the accumulation of some phenolic compounds through hydrolysis of phenolic glycosides.[45] In another study, quercetin concentration increased significantly as a result of temperature and water stress response in *H. brasiliense.*[72] In contrast, Gray *et al.*[73] Observed an increase in concentration of quercetin in *H. perforatum* plants which were subjected to drought stress.[73] These findings suggest that plants have developed different defense systems to mitigate the oxidative effects caused by different stresses including overproduction of antioxidant compounds which terminates oxidative chain reactions.[59,74,75]

In the barks of *L. discolor* extracts, four phenolic compounds, ferulic acid, vanillic acid, 2,4-dihydroxybenzoic acid and (+)-catechin were identified (Table 4). Seasonal variations affected the presence of phenolic compounds in extracts of *L. discolor*. For example, vanilic acid was a predominant compound as it was detected in all seasons, while ferulic acid and 2,4-dihydroxybenzoic acid were detected during winter only. (+)-catechin was present in summer and rainy seasons. On the other hand, sinapic acid and rutin were not detected throughout the seasons. Such variations may be due to differences in the control mechanisms of the compound biosynthetic pathway, alteration of substrate availability and degradation of the compounds at different seasons.[76] In the findings of another study, variation in the development and accumulation of

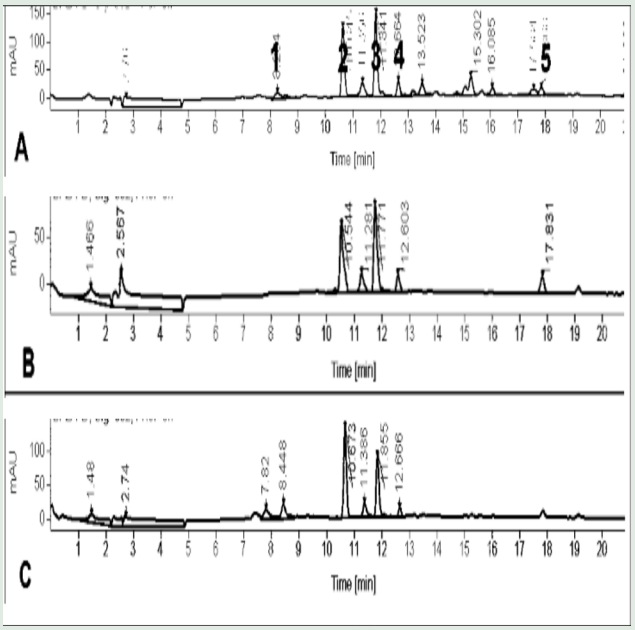

Figure 1: HPLC-DAD chromatograms of phenolic compounds present in *senna singueana* extracts during different seasons. (A) hot-wet season (B) hot-dry season (C) cool-dry season. Peaks: 1: (+)-catechin; 2: 2,4-dihydroxybenzoic acid; 3: Sinapic acid; 4: Ferulic acid; 5: quercetin.

phenolic compounds were associated with defense processes of the plants.[49,50] It has been reported that ferulic acid increases the strength of plant cell walls by covalently linking with lignin and other biopolymers,[77] while benzoic acids provide defenses against antimicrobial activities of reproductive tissues.[78] Catechins contribute to defence in plants by scavenging reactive oxygen species and diminishing oxidative stress.[79]

In *M. azedarach* extracts, four phenolic compounds including 2,4-dihydroxybenzoic acid, (+)-catechin, vanillic acid and sinapic acid were observed (Table 4). Similar compounds in leaves of *M. azedarach* have been reported by other researchers.[80-82] The presence and levels of observed phenolic compounds varied significantly with season just like the other three medicinal plants described earlier. In contrast to leaves of *Moringa oleifera* and barks of *L. discolor,* a different phenolic compound, 2,4-dihydroxybenzoic acid was the predominant compound in *M. azedarach*. The concentration of 2,4-dihydroxybenzoic acid increased significantly ($p< 0.05$) with season in the following order; summer < winter < rainy season. (+)-Catechin was present in two seasons only: summer and winter seasons. The concentration of (+)-catechin was significantly higher ($p< 0.05$) in summer than in winter (Table 4). Vanillic acid was detected during winter while Sinapic acid was present across all seasons. Ferulic acid, rutin and quercetin were not detected throughout the seasons. The observations are in line with those suggested by Cezarotto *et al.*[83] Who observed variation of phenolic compounds in *Vacciniumashei* leaves due to seasonal changes.[83]

Finally, in the leaf extract of *M. oleifera,* five phenolic compounds including vanillic acid, ferulic acid, 2,4-dihydroxybenzoic acid, (+)

Table 4: Amounts of phenolic compounds in medicinal plants extracts using HPLC –DAD (mg/100 g DW dry weight).

Compound/Herb	Hot-Wet Season (rainy)				Hot-Dry Season (summer)				Cool-Dry Season (winter)			
	Ss	*Ld*	*Mo*	*Ma*	*Ss*	*Ld*	*Mo*	*Ma*	*Ss*	*Ld*	*Mo*	*Ma*
(+) –Catechin,	22.76 ± 0.01^{e}	27.95 ± 6.21^{a}	nd	nd	nd	31.16 ± 0.24^{b}	202.55 ± 20.54^{a}	141.70 ± 29.04^{a}	33.28 ± 10.10^{a}	nd	39.70 ± 5.96^{a}	80.36 ± 13.63^{a}
Rutin	nd	nd	nd	nd	nd	19.15 ± 0.81^{a}	nd	nd	nd	nd	nd	nd
Vanillic acid	nd	36.56 ± 0.55^{b}	24.56 ± 0.03^{c}	nd	nd	23.69 ± 0.01^{c}	nd	nd	nd	49.26 ± 0.02^{c}	nd	6.86 ± 0.01^{c}
2,4 dihydroxy benzoic acid,	66.72 ± 0.32^{c}	nd	nd	93.34 ± 0.13^{b}	49.29 ± 1.28^{a}	nd	18.24 ± 0.09^{d}	26.25 ± 1.85^{b}	64.73 ± 0.10^{b}	36.03 ± 0.07^{b}	13.53 ± 1.08^{b}	41.53 ± 0.07^{b}
Sinapic acid	7.12 ± 0.03^{d}	nd	4.53 ± 0.16^{a}	5.16 ± 0.17^{a}	4.62 ± 0.02^{b}	uq	7.56 ± 0.19^{c}	ud	5.24 ± 0.01^{d}	nd	8.34 ± 0.00^{d}	1.59 ± 0.01^{c}
Ferulic acid	264.0 ± 2.84^{a}	nd	104.61 ± 0.14^{b}	nd	nd	nd	91.25 ± 0.52^{b}	nd	140.34 ± 0.06^{c}	1498.6 ± 0.11^{a}	140.02 ± 0.72^{c}	nd
Quercetin	16.69 ± 0.81^{b}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Abbreviations: DW: Dry weight; *Ss: Senna singueana; Ld: Lannea discolor; Mo: Moringa oleifera; Ma: Melia azedarach*; nd: not detected; ud: under the limit of detection; uq: under limit of quantification. Data are means ± standard deviation ($n = 3$). The values which do not share superscript letters within a column indicate significant differences ($p< 0.05$, Tukey's test)

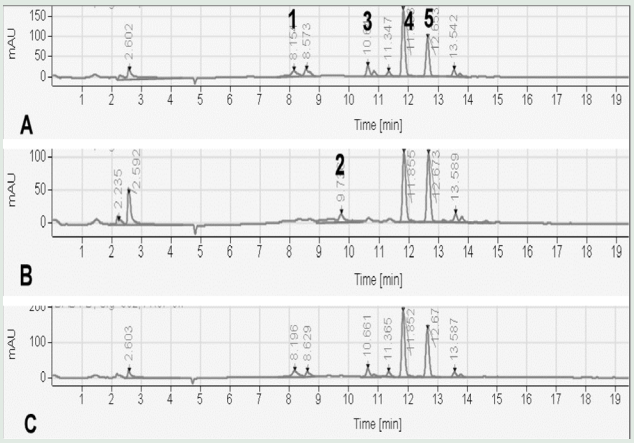

Figure 2: HPLC-DAD chromatograms of phenolic compounds present in *Moringa oleifera* extracts during different seasons. (A) hot-dry season (B) hot-wet season (C) cool-dry season. Peaks: 1: (+)-catechin; 2: Vanillic acid; 3: 2,4-dihydroxybenzoic acid; 4: Sinapic acid; 5: Ferulic acid.

-catechin, sinapic acid and were observed (Figure 2, Table 4). The availability of phenolic compounds in *M. oleifera* has been reported by several other researchers.[84-87] These phenolic compounds also varied during different seasons. (+)-Catechin was present in two seasons only: summer and winter. The concentration of (+)-catechin was significantly higher ($p< 0.05$) in summer as compared to winter (Table 4). Vanilic acid was detected in the rainy season only, while 2,4-dihydroxybenzoic acid was present in summer and in winter. Ferulic acid and sinapic acid were predominant compounds in extracts of *M. oleifera*. The concentrations significantly increased ($p< 0.05$) in the following order: rainy season <summer< winter for sinapic acid; and summer < rainy season < winter for ferulic acid. Different environmental conditions, leaf maturity stage and reactions against seasonal pests may have contributed to the variations of phenolic compounds in the leaves of *M. oleifera extracts,* as observed by Ndhlala *et al.*[88] Similarly, seasonal variations of phenolic profiles in extracts of *Juglanssigillata* Dode were also observed by Binbin *et al.*[89] Changes in environmental conditions during different seasons and the genetic predisposition of plant species under study may contribute to the variations of compounds.[90] The present findings indicate that plant chemical composition is influenced by seasonal changes. A specific compound may be present in one or two seasons and not available in the other. Thus, the quality of fresh or derived products is likely to be dependent upon harvesting season which in turn affects pharmacological properties, as described by Costa *et al.*[91]

CONCLUSION

The present study has revealed some important phytochemicals in Malawian medicinal plants that may be useful in the management of several disease conditions, including cancer. In addition, it has shown that the availability and levels of phytochemicals are largely influenced by season. Barks of *L. discolor* exhibited high levels of (+)-catechin, rutin, amounts of total phenolic compounds, total flavonoids, total alkaloids and antioxidant activity during hot-dry season (summer). Leaves of *S. singueana* showed high levels of sinapic acid, ferulic acid and 2,4-dihydroxybenzoic acid during hot-wet season. *S. singueana*, *M. oleifera* and *M. azedarach* leaves showed significantly ($p< 0.05$) high amounts of total phenolic compounds, total flavonoids, total alkaloids and antioxidant activity in the hot-wet season (rainy). Based on the results we conclude that the hot-dry season is optimum for harvesting barks of *L. discolor,* while the hot-wet season is optimum for harvesting, *M. oleifera*, *M. azedarach* and *S. singueana* leaves for pharmacological use. Further studies including *in vivo* antioxidant activity and characterization of bioactive compounds are recommended for therapeutic use.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ABBREVIATIONS

DPPH: 2,2-diphenyl-1-picrylhydrazyl; **TFC:** Total Flavonoids Content; **TAC:** Total Alkaloids Content; **FRAP:** Ferric Reducing Antioxidant Power; **HPLC – DAD:**High performance liquid chromatography diode array detector; **TPC:** Total Phenolic Content; **AA:** Antioxidant Activity.

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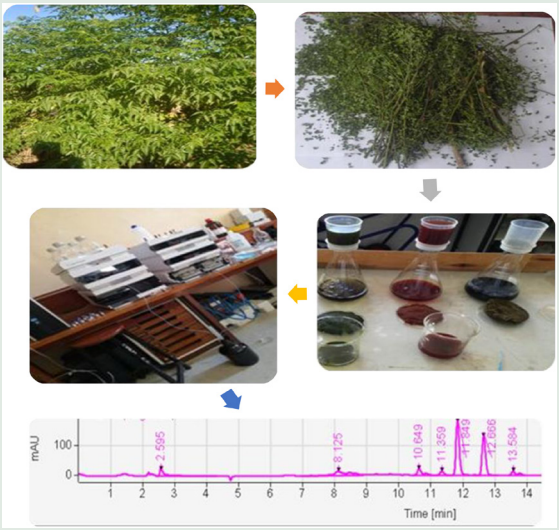
GRAPHICAL ABSTRACT



SUMMARY

- The phytochemical constituents in medicinal plants are subject to seasonal variations which in turn influence the optimum season of harvesting.
- Hot-dry season is the optimum time for harvesting stem barks of *L. discolor* while hot-wet season represents the optimum harvest time of *M. oleifera, S. singueana* and *M. azedarach* leaves.

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