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Effect of seasonal variations on the secondary metabolites and antioxidant activities of *Bridelia ferruginea, Lippia multiflora,* and *Azadirachta indica* leaves

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The aim of the study was to investigate the effect of the annual seasonal variations in Ghana on the phytochemical composition and antioxidant properties of the leaves of Bridelia ferruginea (BF), Lippia multiflora (LM), and Azadirachta indica (AI). The medicinal plants were collected at the end of each quarter from September 2021 to June 2022, representing four seasons. The phytochemical constituents of the samples were screened and quantified. The antioxidant properties of the samples were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. Thin layer chromatography and infrared profiling of the extracts were also analyzed, serving as fingerprint. From the results, total phenolic content (TPC) of BF, LM, and AI leaves were all highest in season 1. The total flavonoid, alkaloid and saponin contents in BF leaves were highest in season 4. The total flavonoid and saponin contents were highest in season 1 and season 3, respectively for LM, but highest in season 2 and season 4, respectively for AI. The antioxidant activity of BF, LM and AI were strongest for samples collected in season 1(45.70 mg/ml AAE), season 3 (20.39 mg/ml AAE) and season 4 (62.67 mg/ml AAE), respectively. All three samples (BF, LM and AI leaves) collected in season 2 showed the lowest antioxidant activity: 54.07 ± 2.92, 60.23 ± 4.14, and 115.15 ± 13.55 mg/ml AAE, respectively. From the study, the rainy seasons (major or minor) appear to generally favor the accumulation of the phytochemical constituents screened in this study, thus recommended as the suitable harvesting period for high yield of the phytochemical constituents.

Key words: *Bridelia ferruginea*, *Lippia multiflora*, *Azadirachta indica*, seasonal variations, phytochemical composition.

INTRODUCTION

The use of medicinal plants for their therapeutic effects dates back to history, contributing significantly to health systems all over the world, especially in primary health care of developing countries (Jamshidi-Kia et al., 2018).

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In addition, the World Health Organization (WHO) has estimated the economic value of medicinal plants to increase from $14 billion to $5 trillion by the year 2050 (Ozioma and Chinwe, 2019; https://www.financialexpress.com). Plants are composed of various bioactive compounds called phytochemicals, and these are reported to be responsible for the various derived biological activities. Phytochemical compounds are naturally occurring secondary metabolites that serve as the plant's defensive mechanism, and present healing benefits to humans (Longbap et al., 2018). The presence or absence, and levels of these phytochemicals depend largely on factors affecting the photosynthetic activities occurring in the plant and these levels are highly influenced by among other factors, seasonal variations of the climate (Singh et al., 2018, 2017). Some earlier reports have confirmed traditional claims of effective harvesting time and demonstrated significant effects of seasonal variations on the bioactive compounds of plants (Ramasar et al., 2022).

At the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana, where innovative research on medicinal plants is translated into herbal products, *Bridelia ferruginea* Benth, *Lippia multiflora* Moldenke, and *Azadirachta indica* A. Juss, are examples of medicinal plants used in our flagship products; *Bridelia* tea, *Lippia* tea and Immunim tincture, respectively. *B. ferruginea* is a shrub belonging to the family Euphorbiaceae that grows to about 4 m high. It is mostly found in the savanna woodland. Traditionally, *B. ferruginea* is used for the management of diabetes, rheumatism, intestinal disorders, dysentery, infectious diseases, fever, anemia, dyspepsia, asthma, and amebiasis (Yeboah et al., 2022; Cimanga et al., 2001; Rhashid et al., 2000; Cimanga et al., 1999). A number of compounds have been isolated and characterized from *B. ferruginea*. These include gallocatechin-(4′-O-7)-epigallocatechin, quercetin, quercetin-3-neohesperidoside, rutin, myricetin-3-glucoside, myricetin-3-rhamnoside, 5-demethoxy-peltatin-5-O-D-glucopyranoside, peltatin-5-O-D-glucopyranoside, and caffeoyl esters. Previous pharmacological studies have demonstrated the antioxidant, antidiabetic, antiviral, anti-inflammatory, and antimicrobial properties of *B. ferruginea* (Yeboah et al., 2022; Ngueyem et al., 2009). At the clinic of CPMR, the dried leaves of *B. ferruginea* has been formulated into a tea prescribed for the management of diabetes.

L. multiflora is an aromatic shrub belonging to the Verbanaceae family. It is found throughout tropical Africa, South, and Central American countries. *L. multiflora* has been used traditionally in the treatment of respiratory and gastrointestinal disorders, malaria, hypertension, bronchial inflammations, conjunctivitis, fatigue, coughs, and cold (Koffi, 1985; Noamesi et al., 1985). Phytochemical screening of *L. multiflora* revealed the presence of catechin, gallic tannins, flavonoids, anthocyanins, leuco-anthocyanes, coumarins, and reducing compounds. In addition, studies have shown that the phytochemical composition of essential oil from *L. multiflora* include limonene, linalool, nerol, sabinene, β-carophyllene, myrcene, and 1,8-cineole. A wide range of pharmacological activities of *L. multiflora* have been reported including anti-hypertensive, antioxidant, antimicrobial, antimalaria, anti-inflammatory and analgesic, muscle relaxant, sedative, and anti-pyretic activities (Masunda et al., 2020; Tsiba et al., 2010; Pascual et al., 2001). At the clinic of CPMR, the dried leaves of *L. multiflora* have been formulated into a tea prescribed for the management of hypertension.

A. indica, commonly called "Neem", is a deciduous and fast-growing tree that grows to a height of 15 to 20 m. It belongs to the family Meliaceae. Although native to India, *A. indica* is now cultivated in the southern regions of Asia and Africa (Barstow and Deepu, 2018). Traditionally, *A. indica* is used in the treatment of various conditions such as malaria, stomach ulcers, jaundice, a variety of infectious and parasitic diseases, intestinal complaints, headache, and heartburns. Neem is reported to be rich in a wide range of phytochemical compounds to which several pharmacological properties are associated with. These include limonoids, flavonoids, catechin, gallic acid, saponins, tannins, azadirachtin, nimbins, and epoxy-azadiradione. Pharmacological studies have confirmed anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, anti-gastric ulcer, antifungal, antibacterial, and antitumor activities of *A. indica* (Saleem et al., 2018; Paul et al., 2011). At CPMR, a tincture has been formulated from the leaves prescribed for immunomodulation.

Currently, information on the effect of seasonal variations on the phytochemical constituents of the leaves of *B. ferruginea*, *L. multiflora* and *A. indica* are not available to advise on the best harvesting time for efficient and effective use of these medicinal plants. Thus, the present study aimed at examining the influence of the annual seasonal variations in Ghana on the quantities of phytochemicals in the leaves of *B. ferruginea, L. multiflora*, and *A. indica* in order to know the best season to harvest so as to obtain optimum activity of the products produced from these plants.

MATERIALS AND METHODS

Study area and study period

The study was carried out at the arboretum of the Centre for Plant Medicine Research (CPMR) located at Mampong-Akuapem in the Akuapem-North district of the Eastern region of Ghana (Figure 1). It lies between latitudes of 5.914752 N and longitude of 0.137005 E. The district falls under the Forest Savanah transition zone of Ghana (Issaka et al., 2012) with mountainous and hilly landscape ranging from 38 to 500 m above sea level. Temperatures in the district range from 24 to 30°C during the day and 13 to 24°C at night hours. There are two main seasons in Ghana, wet season marked with rains (April-November) and dry season marked with scarcity of rains (December-March). Rainfall pattern of the area is described as bimodal with the major between April and July and the minor

Figure 1. Map of Akuapem North District in the Eastern region of Ghana showing Mampong – Akuapem. Source: https://www.google.com/maps/@5.958496,-0.1524048,12z).

September and November. The mean rainfall value is 1,270 mm. On the other hand, there are two main dry seasons, the minor occurs in August and the major occurs from December to March (Owusu et al., 2015). For purposes of this study however, a year was segmented into quarters (January-March, April-June, July-September, October-December) and designated as seasons. The sample collection spanned from July, 2021 to June, 2022, where samples were collected at the end of each quarter, thus Season 1 (July-September, 2021), Season 2 (October-December, 2021), Season 3 (January-March, 2022), and Season 4 (April-June, 2022).

Plant authentication, selection and collection

The leaves of *B. ferruginea, L. multiflora* and *A. indica* aged 6.5, 1.5 and 4 years, respectively, were timely harvested from the arboretum of CPMR at Mampong - Akuapem, in the Eastern region of Ghana according to the guidelines for plant collection licensed by the Ghana Forestry Commission. The collection was done at an interval of a quarter (every three months) and four batches were obtained for the study, namely season 1 (July-September, 2021), season 2 (October-December, 2021), season 3 (January-March, 2022), and season 4 (April-June, 2022). The samples collected were identified and authenticated by Mr. Peter Atta-Adjei in the Plant Development Department of Centre for Plant Medicine Research, Mampong-Akuapem, Ghana and voucher specimens were deposited in the CPMR herbarium (*B. ferruginea* – CPMR 5154; *L. multiflora* - CPMR5153; *A. indica* – CPMR 5155). The different batches of the leaves of *L. multiflora*, *B. ferruginea*, and *A. indica* were dried for a week in a solar dryer facility.

Solvent extraction

The dried leaves of each batch of *B. ferruginea, L. multiflora*, and *A. indica* were separately pulverized. Two groups of each plant material (10 g per sample) were then macerated with 100 ml of 70% ethanol for 24 h or boiled with 100 ml of distilled water at 90°C for 60 min and filtered. The hydroethanolic extracts were concentrated using the rotary evaporator (EYELA SB-1200, CHINA). The resulting concentrates and the aqueous extracts were freeze dried and stored at 4°C. Stock solution (1.0 g/ml) of the samples were prepared from the aqueous and hydroethanolic freeze-dried samples for further analysis (Borquaye et al., 2020).

Phytochemical analysis of extracts

The phytochemical constituents present in aqueous and hydroethanolic extracts of *B. ferruginea, L. multiflora* and *A. indica* were comparatively analyzed qualitatively to inform the choice of solvent, and further quantified using established protocols. Total phenolic compounds, total flavonoids, total alkaloids and total saponins detected in the hydroethanolic extracts were quantified.

Total phenolic content

The total phenolic content (TPC) of the crude hydroethanolic extracts of the plant materials was determined using the Folin-Ciocalteu colorimetric method as described by María et al. (2018) with some modifications. To 25 µl of sample (2 mg/ml) in each well

of a 96-well microtiter plate was added 125 µl of Folin-Ciocalteu's phenol reagent and incubated for 8 min at room temperature (25-28°C). To the mixture in each well was added 100 µl of saturated sodium carbonate solution (7.5% w/v in water) and mixed gently. The mixture was then kept in the dark for 90 min at 23°C, after which the absorbance was read at 765 nm using Synergy HTX microplate reader against the reagent blank. The TPC was determined by extrapolating from a calibration curve prepared using gallic acid solution as the standard and expressed as milligrams of gallic acid equivalents (GAE) per 100 mg of dried sample. The estimation of the phenolic compounds was carried out in triplicate.

Total flavonoid content

The total flavonoid content of the crude hydroethanolic extracts of the plant materials was measured by a colorimetric assay as described by Bibi et al. (2022) with some modifications. To 50 µL of each sample (1 mg/ml) in each well of a 96-well microtiter plate was added 15 µl of 5% sodium nitrite. After 5 min of incubation at room temperature, 15 µl of 10% aluminium chloride was added to each well. In 6 min, 100 µl of 1 M sodium hydroxide was added to the mixture. The absorbance was read at 510 nm against a blank. Quercetin was used as standard for the calibration curve. Total flavonoid content of the extract was expressed as mg quercetin equivalents per 100 mg of dried sample (mg/100 mg). All determinations were carried out in triplicate.

Total alkaloid content

To 5 g of the dried milled leaves in a beaker was added 200 ml of 20% acetic acid in ethanol. The mixture was allowed to stand at room temperature in the dark for 4 h and then filtered. The resulting filtrate was concentrated by evaporating one quarter of the volume over a boiling water bath. Concentrated ammonium solution was then added dropwise to the extract until precipitation was completed. The solution was allowed to settle and the precipitates collected by filtration, dried, and then weighed. The resultant dry mass was expressed as a percentage of the mass of the starting plant material (Gololo et al., 2016).

Total saponins

To 5 g of the dried milled sample was added 100 ml of 20% ethanol. The mixture was heated with continuous stirring over a water bath (55°C) for 4 h. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined filtrate was reduced to 40 ml over water bath at about 90°C. The concentrated extract was then transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added, and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. To the recovered aqueous layer was added 30 ml of n-butanol and washed twice with 10 ml of 5% aqueous sodium chloride. The resultant solution was then evaporated over a boiling water bath, dried in an oven and then weighed. The resultant dry mass was expressed as a percentage of the mass of the starting plant material (Roghini and Vijayalakshmi, 2018).

Thin layer chromatography fingerprint

Dried hydroethanolic extract of each sample (2 mg) was reconstituted with chloroform and subjected to Thin Layer Chromatography (TLC) as per conventional one-dimensional ascending method using silica gel (Silica gel 60 F_{254}). Each sample (1 µl) was spotted on the TLC plate using glass capillaries at a distance of 1 cm. The solvent system, hexane, and diethyl ether at a ratio of 1:3 was used to run the plate. The bands were detected by staining the TLC plates with 10% sulphuric acid followed by heating over a hot plate (C-MAG HS 10) at 100°C to develop the chromatograms. The movement of bands was expressed by its retention factor (R_f) (Janakiraman and Jeyaprakash, 2015).

FTIR fingerprint

The hydroethanolic extract was placed in the diamond crystal of the PerkinElmer Fourier transform infrared spectrophotometer (spectrum 2, SR. No. 94133, UK). The force gauge was used to apply pressure to ensure maximum contact with extract. A spectrum was obtained between 4000 and 400 cm^{-1} (Ashokkumar and Ramaswamy, 2014).

DPPH free radical scavenging activity

The DPPH free radical scavenging assay was used to evaluate the antioxidant capacity of *B. ferruginea, L. multiflora* and *A. indica* extracts according to methods described previously by Ghadigaonkar et al. (2021) with slight modifications. Briefly, 100 µl of DPPH solution (0.2 mM) was added to 100 µl of serially diluted samples (0.0078 – 1.0 mg/ml) in a 96-well microtitre plate and incubated in the dark for 30 min at room temperature. After incubation, absorbance was read at 517 nm using Synergy HTX microplate reader. Ascorbic acid was used as positive standard. The negative (blank) was prepared by adding 100 µl of DPPH solution (0.2 mM) to 100 µl of methanol and treated under the same conditions as the samples. All tests were performed in triplicate. Concentration of samples resulting in 50% inhibition on DPPH (IC_{50} value) was calculated. The scavenging ability (%) was calculated as follows:

$$\%\ \text{inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of extract/standard} \times 100}{\text{Absorbance of blank}}$$

Statistical analysis

All data were analyzed using Microsoft Excel and GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, CA, USA). Differences in means of treatments were by one-way analysis of variance (ANOVA).

RESULTS

Phytochemical analysis of extracts

Phytochemical screening of the aqueous and hydroethanolic extracts of *B. ferruginea, L. multiflora*, and *A. indica* leaves harvested at different seasons of the year revealed the presence of the phytochemical constituents: phenolic compounds, reducing sugars, polyuronides, saponins, alkaloids, flavonoids, and phytosterols. The qualitative analyses data showed variations in the phytochemical compositions of both aqueous and hydroethanolic (70%) extracts of *B. ferruginea*, *L. multiflora*, and *A. indica* leaves harvested at different seasons of the year. The hydroethanolic extracts showed slightly higher number of the presence of the different phytochemicals tested than the aqueous fraction

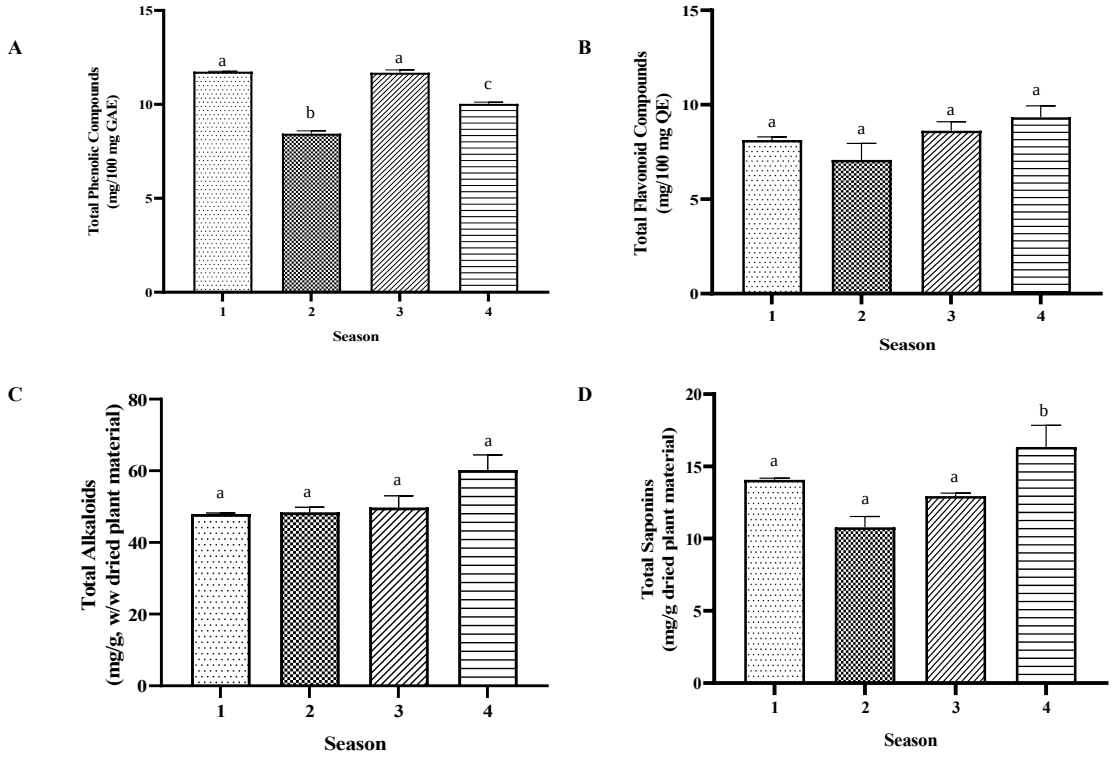


Figure 2. Effect of different seasons on the quantities of four phytochemicals in B. ferruginea leaves. The phytochemical constituents: (A) total phenolic compounds, (B) total flavonoid compounds, (C) total alkaloids, and (D) total saponins were quantified using standard protocols. Values are Mean ± SEM, n = 3. a-cMean values with same alphabet per phytochemical group (on the same graph) represent insignificant difference ($p>0.05$), while those with different alphabet per phytochemical group represent significant difference ($p < 0.05$).

(Supplementary data). The quantities of the phytochemicals detected in the hydroethanolic extracts of the respective plants are as shown in Figures 2 to 4.

Figure 2 shows the quantities of four groups of phytochemical constituents (phenolic compounds, flavonoids, alkaloids and saponins) in the hydroethanolic extract of *B. ferruginea* leaves harvested at different seasons. Generally, there were differences in the amount of each group of phytochemical constituents with seasonal changes. The highest quantity of phenolic compounds was recorded in season 1 although it was comparable ($p>0.05$) to the quantity in season 3 (Figure 2A). The highest quantities of flavonoids, alkaloids, and saponins were observed in season 4. Whereas the levels of saponins in season 4 was significantly different ($p<0.05$) from the other seasons (Figure 2D), the levels of flavonoids and alkaloids in season 4 were comparable ($p>0.05$) to the levels in seasons 1, 2 and 3 as shown in Figure 2B and C, respectively.

Figure 3 shows the quantities of three groups of phytochemicals (phenolic compounds, flavonoids and saponins) in the hydroethanolic extract of *L. multiflora* leaves harvested at different seasons. Generally, there were variations in the quantity of each group of phytochemical constituent with seasonal changes. Whereas the highest quantity of phenolic compounds and flavonoids were recorded in season 1 ($p<0.05$), the highest quantity of saponins was recorded in season 3 ($p>0.05$) as shown in Figure 3A, B and C, respectively.

The quantities of three groups of phytochemicals (phenolic compounds, flavonoids and saponins) in the hydroethanolic extract of *A. indica* leaves harvested at different seasons is as shown in Figure 4. From the results, the highest quantity of phenolic compounds was recorded in season 1($p<0.05$), flavonoids in season 2 ($p<0.05$), and saponins in season 4 ($p<0.05$) as shown in Figure 4A, B and C, respectively.

TLC fingerprint

The TLC profiling of hydroethanolic extracts of *B. ferruginea*, *L. multiflora*, and *A. indica* leaves harvested at four different seasons are as shown in Figure 5. A total of seven spots were observed for *B. ferruginea*, and nine spots each for *L. multiflora* and *A. indica* leaves. Apart from the TLC profile of *L. multiflora* that showed two spots (1 and 9) that run through all four seasons, most of

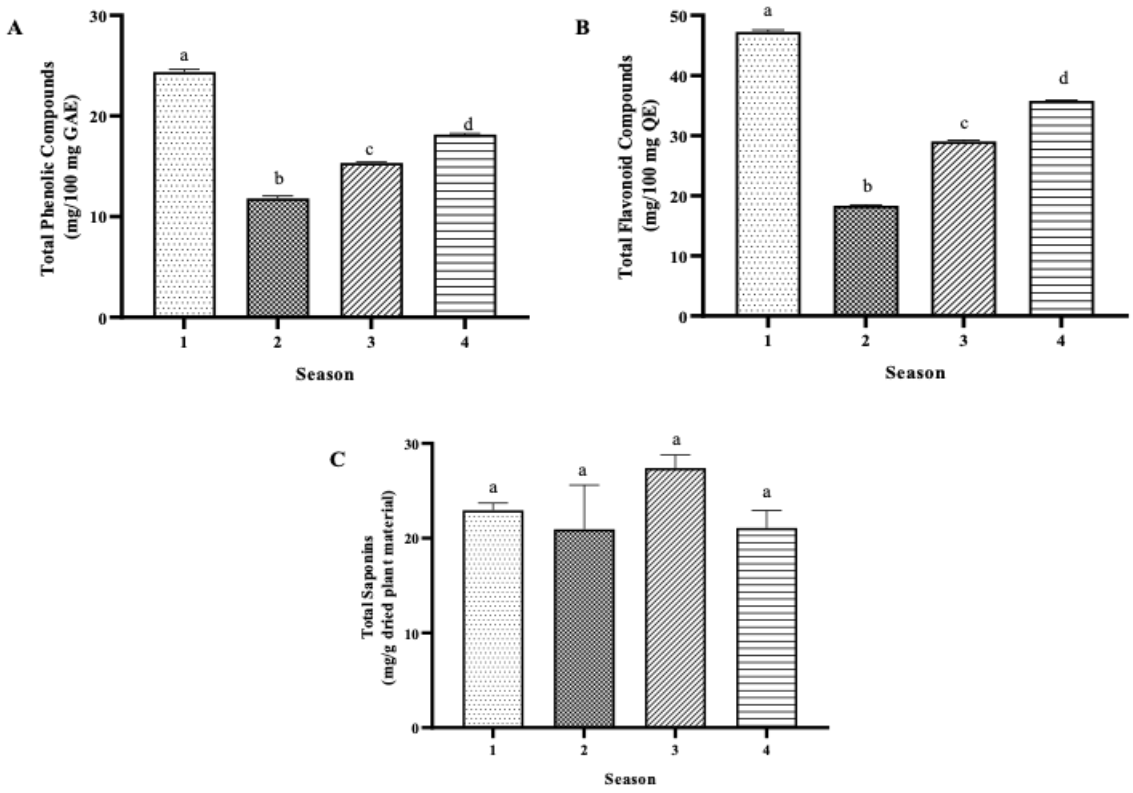


Figure 3. Effect of different seasons on the quantities of three phytochemicals in *L. multiflora* leaves. The phytochemical constituents: (**A**) Total Phenolic Compounds, (**B**) Total Flavonoid Compounds, (**C**) Total Saponins were quantified using standard protocols. Values are Mean ± SEM, n = 3. [a-d]Mean values with same alphabet per phytochemical group (on the same graph) represent insignificant difference ($p>0.05$), while those with different alphabet per phytochemical group represent significant difference ($p < 0.05$).

the spots in the TLC profile of the three plant materials were observed in at least two seasons. Few spots, such as spots 1 and 6 of *B. ferruginea* (season 4); spot 3 of *L.multiflora* (season 4); and spot 1 of *A. indica* (season 4) were not observed in other seasons.

FTIR functional group analysis and fingerprinting of extracts

The FTIR spectra of *B. ferruginea* leaves harvested at four different seasons is as shown in Figure 6. All four seasons showed similar FTIR spectra. The spectra revealed the presence of a broad band at 3279.52 cm^{-1} (O-H stretching vibration) indicating the possible presence of alcohols, phenols or flavonoids. A peak at 1597.87 cm^{-1} corresponding to the presence of a C=C bond stretch of aromatic compounds was also observed. In addition, the spectra revealed N-O (symmetric) bond stretch at 1347.40 to 1396.50 cm^{-1}, and C-O stretches of primary alcohol around 1026.41 $cm^{-1.}$

The FTIR spectra of *L. multiflora* leaves harvested at four different seasons is as shown in Figure 7. All the four batches of plant materials corresponding to the four seasons showed similar FTIR spectra. The spectra revealed the presence of a broad band at 3319.00 cm^{-1} (O-H stretching vibration) indicating the possible presence of alcohols, phenols or flavonoids. Also, the significant peak below the 3000 cm^{-1} corresponding to alkanes was observed at 2922.84 cm^{-1}. Further, another characteristic peak corresponding to the carbonyl (C=O) stretch was observed at 1690.92 cm^{-1}. A peak at 1598.97 cm^{-1} corresponding to the presence of a C=C bond stretch of aromatic compounds was also observed.

The FTIR spectra of *A. indica* leaves harvested at four different seasons is as shown in Figure 8. All the four batches of plant materials harvested showed comparable FTIR spectra. The spectra revealed the presence of a broad band at 3379.77 cm^{-1} (O-H stretching vibration) indicating the possible presence of alcohols, phenols or flavonoids. Also, the spectra showed peaks below the 3000 cm^{-1} corresponding to alkanes at 2926.15 cm^{-1}. Further, another characteristic peak corresponding to the carbonyl (C=O) stretch was observed around 1629.24 to 1707.86 cm^{-1}. A peak in the region of 1377.50 to 1438.20 cm^{-1} corresponding to the presence of C=C and/or N-0 bond stretch of aromatic/aliphatic nitro compounds was also observed.

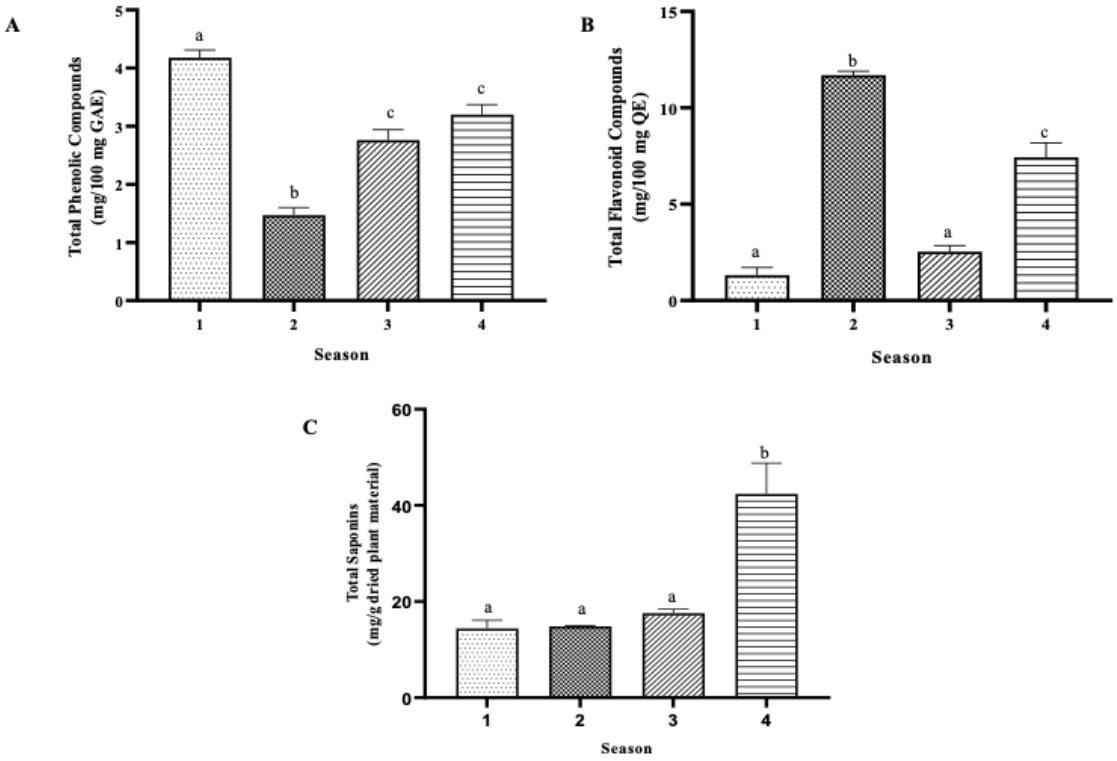


Figure 4. Effect of different seasons on the quantities of three phytochemicals in *A. indica* leaves. The phytochemical constituents: (A) total phenolic compounds, (B) total flavonoid compounds, and (C) total saponins were quantified using standard protocols. Values are Mean ± SEM, n = 3. [a-c]Mean values with same alphabet per phytochemical group (on the same graph) represent insignificant difference ($p>0.05$), while those with different alphabet per phytochemical group represent significant difference ($p < 0.05$).

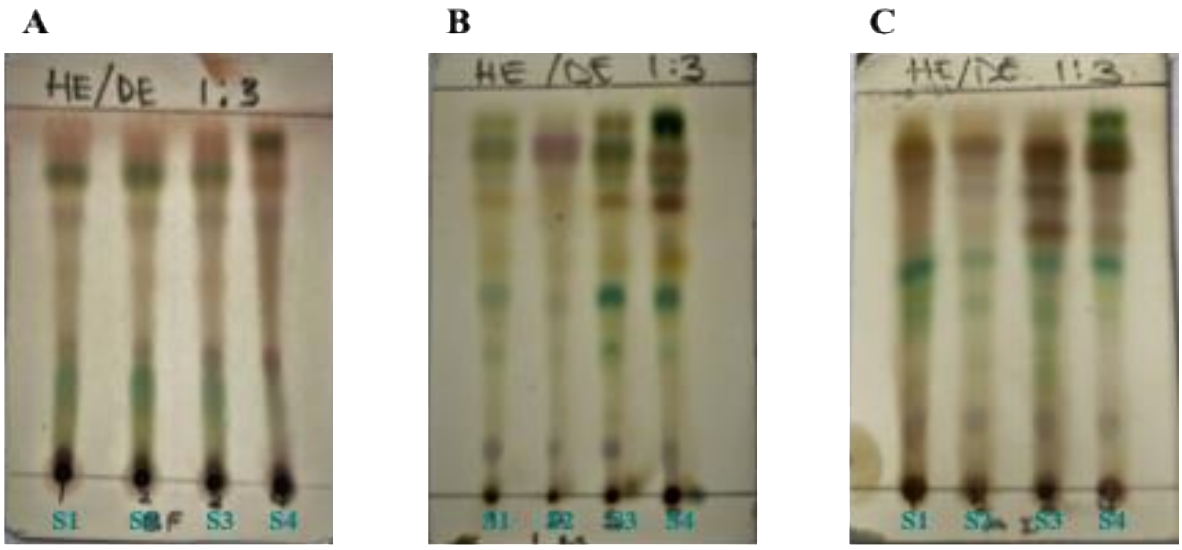
DPPH free radical scavenging activity

The DPPH free radical scavenging activity, a measure of the antioxidant property of the hydroethanolic extracts of *B. ferruginea*, *L. multiflora*, and *A. indica* leaves collected at four different seasons are shown in Table 1. The DPPH activities of *B. ferruginea* harvested over the four seasons were comparable, although the strongest activity was in season 1 (IC_{50} 45.703 mg/ml AAE) while the weakest activity was in season 2 (IC_{50} 54.07 mg/ml AAE). Similarly, the weakest DPPH activity of *L. multiflora* was in season 2 (60.227 mg/ml AAE) but the strongest activity was in season 3 (20.387 mg/ml AAE). The DPPH activity of *A. indica*, generally appeared weaker compared to that of *B. ferruginea* and *L. multiflora,* for all four seasons. The strongest activity was in season 4 (62.67 mg/ml AAE) and weakest activity in season 2 (115.15 mg/ml AAE).

DISCUSSION

Therapeutic property of a medicinal plant is a reflection of its phytochemical constituents. The key role of these phytochemicals is for defense against predators, but also contribute to the pharmacological properties of the medicinal plants (Jimoh et al., 2019). The phytochemical constituents initially screened in this study included reducing sugars, phenolic compounds, polyuronides, saponins, cyanogenic glycosides, alkaloids, flavonoids, triterpenes, phytosterols, and anthracenosides. The qualitative analyses data showed variations in the phytochemical compositions of both aqueous and hydroethanolic (70%) extracts of *B. ferruginea*, *L. multiflora*, and *A. indica* leaves harvested at different seasons of the year (Supplementary data). In the study, the preparation of both aqueous and hydroethanolic(70%) extracts was to ensure that a wider range of the phytochemical constituents (both polar and non-polar) were extracted into solution and screened while ascertaining the choice of solvent for further studies. Although both the aqueous and hydroethanolic extract of each plant of the various seasons showed comparable phytochemical composition, the hydroethanolic extracts showed the presence of slightly higher number of the different phytochemicals tested as compared to that of the aqueous fraction. This is because the 70% ethanol did not only extract the polar constituents, but also some non-polar constituents, example phytosterols.

The observed differences in the phytochemical



Spot	Retardation factor			
	S1	S2	S3	S4
1	-	-	-	0.914
2	0.843	0.843	0.857	0.829
3	0.729	0.714	0.714	0.743
4	0.571	0.571	0.543	0.557
5	0.314	0.314	0.314	0.343
6	-	-	-	0.286
7	0.243	0.229	0.229	0.229

Spot	Retardation factor			
	S1	S2	S3	S4
1	0.90	0.90	0.90	0.90
2	0.825	0.838	0.85	0.813
3	-	-	-	0.75
4	0.70	0.713	0.70	0.70
5	0.563	0.575	0.563	0.563
6	0.475	0.475	0.474	0.475
7	0.325	0.338	0.35	0.325
8	0.188	0.20	0.188	-
9	0.10	0.10	0.10	0.10

Spot	Retardation factor			
	S1	S2	S3	S4
1	-	-	-	0.875
2	0.80	0.813	0.813	0.825
3	0.725	0.75	0.75	0.775
4	0.675	0.675	0.675	0.70
5	0.55	0.575	0.55	0.55
6	0.475	0.475	0.463	0.475
7	0.388	0.325	0.325	0.375
8	0.30	0.20	0.188	-
9	0.125	0.10	0.1	0.125

Figure 5. TLC profile of three medicinal plants harvested at four different seasons with tabulated corresponding R_f values. The TLC plates were run with the solvent system hexane and diethyl ether (1:3). (A) *B. ferruginea*, (B) *Lippia multiflora,* and (C) *Azadirachta indica* harvested at four different seasons (S1 – season 1; S2 – season 2; S3 – season 3; S4 – season 4).

compositions after the qualitative analyses of the three medicinal plants provided a preliminary bases to suggest a possible effect of the seasonal changes on the group of phytochemicals expressed. However, the absence of a group of phytochemicals could also be due to the effect of quantity, where very low levels may be difficult to detect qualitatively. The variation in the phytochemical constituents was corroborated by the thin layer chromatography (TLC) profiling of the three medicinal plants. From the TLC profile, differences were observed in the number of spots and their respective retardation factors (Figure 5), where each spot may represent one or more class of compounds. The observation suggests that there are variations in the phytochemical constituents in the four different batches of the three medicinal plants arising from seasonal changes. Thus, a TLC profiling can serve as fingerprints for the standardization of the plants harvested at the respective seasons in the future.

FTIR analysis gives a much accurate and precise infrared spectra and has proven to be a useful tool for characterizing and identifying the functional groups of compounds (Eberhart et al., 2007; Hazra et al., 2007). The identification of functional groups of the phytochemical constituents is based on their peak value and pattern in the region of infrared radiation (Jamshidi-Kia et al., 2018). From the study, the hydroethanolic extracts of the four batches of each plant material showed similar IR spectra, irrespective of the harvest season. Notably among them was the presence of the O-H stretch, indicating the presence of alcohols, phenols and flavonoids (Figures 6 to 8). The presence of these group of phytochemicals was confirmed by the phytochemical analyses (Figures 2 to 4 and Supplementary data). Other functional groups deduced

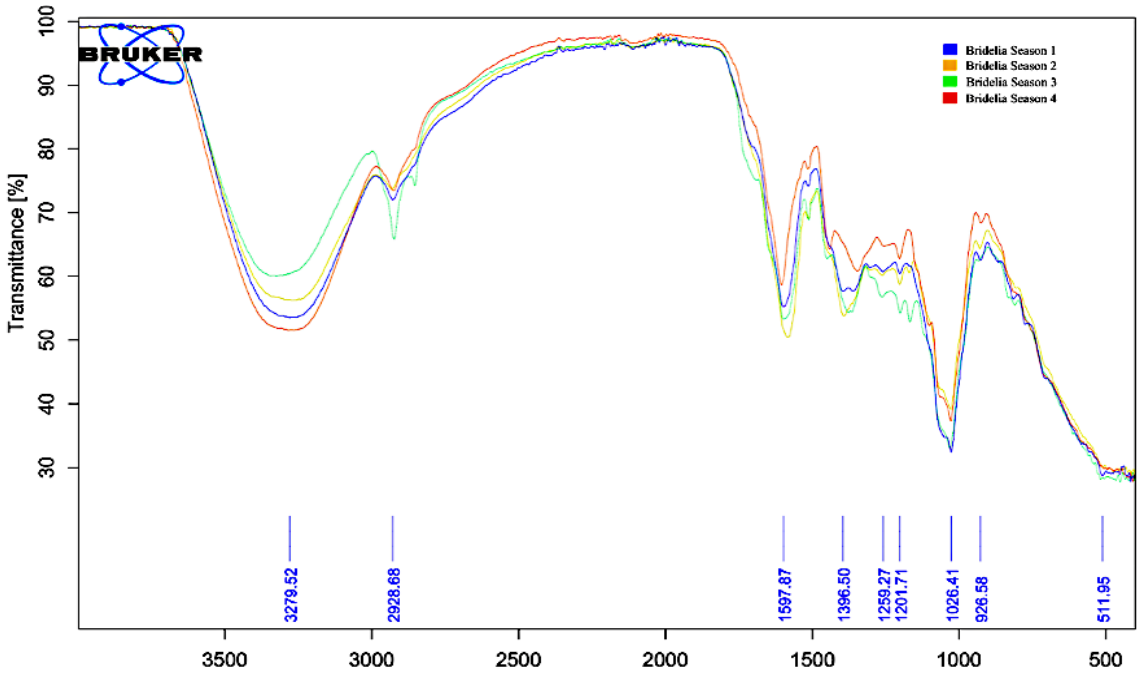


Figure 6. FTIR spectra of *B. ferruginea* leaves harvested at four different seasons. Hydroethanolic extracts of *B. ferruginea* leaves harvested at four different seasons were analyzed using FTIR to obtain their respective spectra between 4000 and 400 cm^{-1}.

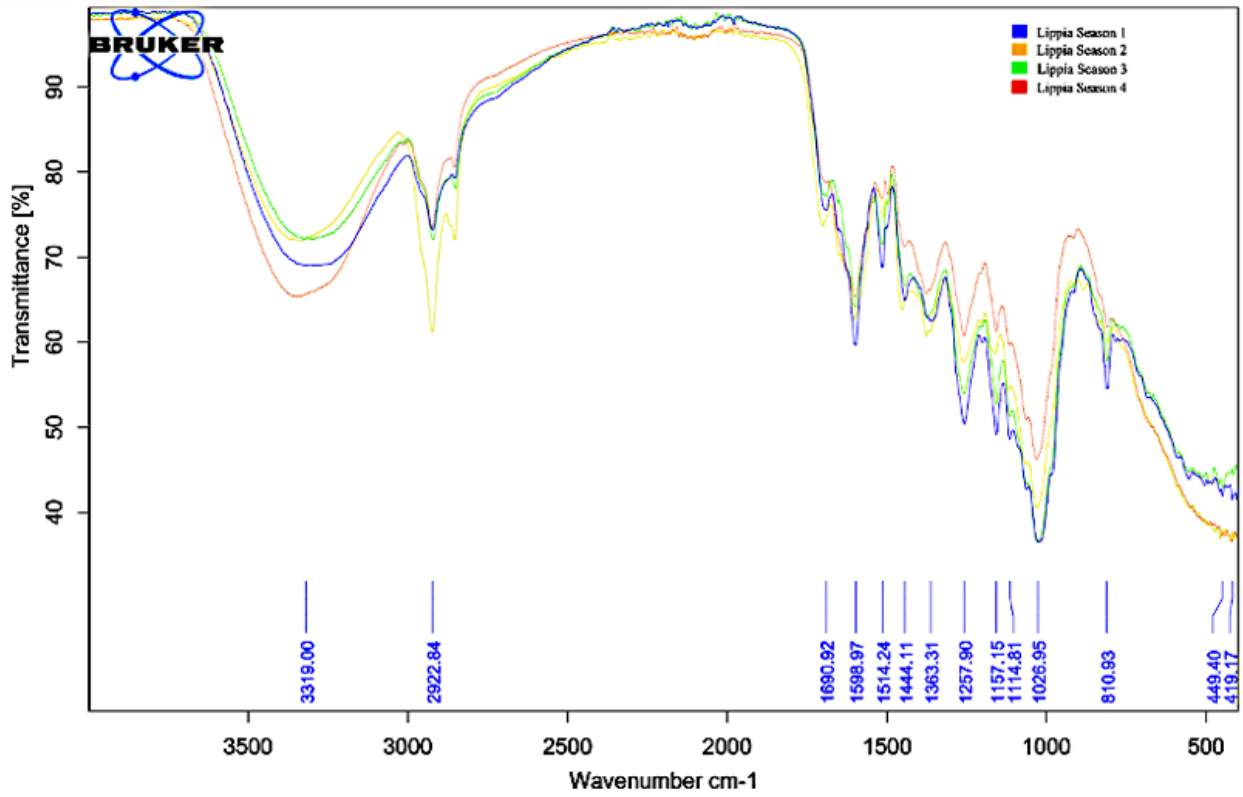


Figure 7. FTIR spectra of *L. multiflora* leaves harvested at four different seasons. Hydroethanolic extracts of *L. multiflora* leaves harvested at four different seasons were analyzed using FTIR to obtain their respective spectra between 4000 and 400 cm^{-1}.

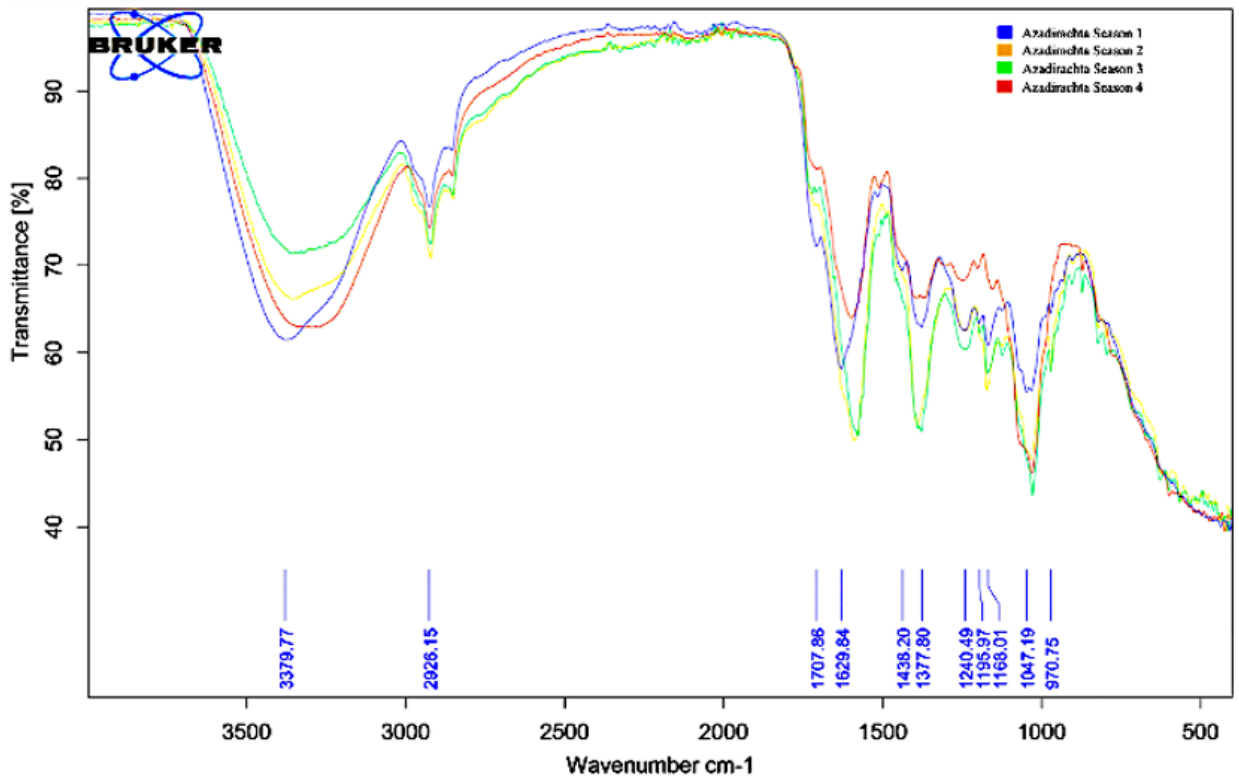


Figure 8. FTIR spectra of *A. indica* leaves harvested at four different seasons. Hydroethanolic extracts of *A. indica* leaves harvested at four different seasons were analyzed using FTIR to obtain their respective spectra between 4000 and 400 cm^{-1}

Table 1. DPPH Scavenging activity of three medicinal plants harvested at different seasons.

Season	IC_{50} mg/ml AAE		
	B. ferruginea	***L. multiflora***	***A. indica***
1	45.70	21.65	107.76
2	54.07	60.23	115.15
3	49.99	20.39	85.62
4	47.56	32.78	62.67

IC_{50} values are the concentrations of samples resulting in 50% inhibition on DPPH using the DPPH free radical scavenging assay.

from the FTIR spectra included alkane, carbonyl, and nitro compounds; a good indication of the medicinal value of these plants. Furthermore, the similarities of the FTIR spectra among the four batches of each medicinal plant represent a useful fingerprint tool for standardization of the three plants.

To further confirm the effect of the seasonal variations on the phytochemical composition of the three medicinal plants, selected phytochemicals detected in the hydroethanolic extract of *B. ferruginea*, *L. multiflora,* and *A. indica* leaves were analyzed quantitatively using standard protocols. From the results, varying amounts of phenolic compounds, saponins, flavonoids and alkaloids in the different seasons of the year were observed. Generally, the four seasons during which *B. ferruginea*, *L. multiflora*, and *A. indica* leaves were harvested are characterized by different environmental factors; mainly varying average temperatures and different rainfall patterns. These factors have been reported in previous studies to have significant effect on the quantities of phytochemical constituents in some studied medicinal plants (Usano-Alemanyet al., 2014; Kale, 2010). From the study, high phenolic content was recorded in season 1 for all the three medicinal plants 11.75 ± 0.02, 24.37 ± 0.27, 4.18 ± 0.13 mg/100 mg GAE for B. ferruginea, *L. multiflora*, and *A. indica*, respectively. According to the

study design, season 1 was defined by the third quarter of the year (July-September, 2021), which forms part of the rainy season in Ghana. Therefore, the findings corroborate with previous study which reported high total phenolic contents in *Datulametel* species during the rainy season (Kale, 2010). The high content of phenolic compounds during this season could be attributed to the high water and low temperature stress which can stimulate the accumulation of some phenolic compounds by hydrolysis of glycosides (Watoo et al., 2011).

Flavonoids were high in season 4 for *B. ferruginea*, but in the case of *L. multiflora* and *A. indica*, they were high in seasons 1 and 2 respectively. Season 1 (July-September, 2021) and Season 4 (April-June, 2022) largely form part of the rainy season in Ghana. Therefore, the high flavonoid content in the rainy season could be attributed to the same factors (high rains and low temperature stress) linked to the phenolic compounds. This is because flavonoids are one of the sub-groups of phenolic compounds. Moreover, the high flavonoid contents reported also corroborates with previous work, which reported high total flavonoids content in *Ocimum sanctum* during the rainy season (Mir et al., 2009). Contrarily, the high content of flavonoids in *A. indica* was in season 2 (October-December, 2021) which forms part of the minor rainfall season of the district (September-November) leading into the major dry season (December-February). The high flavonoids content despite the minor rains or scarcity of it, characteristic of the dry season, could be attributed to internal factors (genotype and physiological condition), and external factors (feeding of seasonal insects or herbivorous animals, interaction with seasonal pathogens and diseases, the availability of light, water and temperature) to *A. indica* (Isah, 2019; Narayani and Srivastava, 2018; Wurtzel and Kutchan, 2016; Chinnusamy et al., 2004).

Saponin content in both *B. ferruginea* and *A. indica* were high in season 4 (April-June), a period characterized by rains whereas *L. multiflora* exhibited high saponin content in season 3 (January-March) where there is scarcity of rains. Previous studies reported high content of saponins of *Nauclea latifolia* in the rainy season (Aderibigbe and Anowai, 2020). This suggests that rains and low temperatures possibly promote the accumulation of saponins. However, contrary to this observation, saponins were also reported to be in high amounts during warmer seasons (December) for *Grewia flava* and *Jatropha lagarinthoides* (Gololo et al., 2016). This probably also explains the high saponins content in season 3 for *L. multiflora.*

In addition to the phenolic compounds, flavonoids and saponins content in *B. ferruginea.* alkaloids were also detected and quantified. Relatively high content of the alkaloids was recorded in season 4 (April-June, 2022), a major part of the rainy season in Ghana. It is hypothesized that during the rainy season there is a decrease in biomass production or temperature stress which can result in changes in the biochemistry of the medicinal plant species. Furthermore, the decreased temperature stress influences the biosynthetic pathways in the development of alkaloids, by promoting precursors from the primary metabolism, thereby elevating the production of alkaloids. Also, most alkaloids are bitter and probably more of these are produced to prevent animals from feeding on the plants during the raining seasons when leaves are fresh and attractive.

Taken together, the study showed that the seasonal variations altered the quantities of phytochemical constituents in *B. ferruginea*, *L. multiflora*, and *A. indica* leaves. Changes in phytochemical constituents have also been implicated in possible variations in the pharmacological or biological properties of affected medicinal plants. Phenolic compounds, flavonoids, saponins and alkaloids have been reported to exhibit antioxidant properties, where they inhibit the oxidation of some molecules, such as DNA, by scavenging radicals and mitigating stress (Duangjai et al., 2018). Therefore, in this study, the antioxidant properties of the three medicinal plants harvested at the different seasons were analyzed using the DPPH free radical scavenging activity.

The results showed that DPPH scavenging activity of *B. ferruginea* was high in season 1 (IC_{50} 45.70 mg/ml AAE) followed by season 4 and 3 (IC_{50} 47.56 and 49.99 mg/ml AAE, respectively) with season 2 having the lowest DPPH radical scavenging activity (54.07 mg/ml AAE). The high antioxidant activities of *B. ferruginea* correlate to the high quantities of phenolic compounds in season 1, and flavonoids and alkaloids in season 4. The DPPH scavenging activity of *L. multiflora* was high in season 3 (IC_{50} 20.39 mg/ml AAE), although the phytochemicals (phenolic compounds and flavonoids but not saponins) to which the antioxidant property is attributed were high in season 1.

Conclusion

The study provides useful information on the effect of seasonal variations on the phytochemical composition of *B. ferruginea*, *L. multiflora*, and *A. indica*. From the study, the rainy seasons (major or minor) appears to generally favor the accumulation of the phytochemical constituents screened in this study. The variations in the phytochemical composition due to seasonal changes also correlated directly with changes in the antioxidant activities of the corresponding plants. The findings suggests that the rainy season (major or minor) is the recommended harvesting period suitable for high yield of phytochemical constituents and subsequently, activity. However, other advanced spectroscopic studies and pharmacological activities are recommended for identification and structural elucidation of active components from the leaves of *B. ferruginea*, *L. multiflora*, and *A. indica* harvested at various seasons, and their corresponding pharmacological activities.

ABBREVIATIONS

TLC, Thin layer chromatography; **GAE,** gallic acid equivalent; **AAE,** ascorbic acid equivalent; **QE,** quercetin equivalent; **CPMR,** centre for Plant Medicine research; **TPC,** total phenolic content; **R_f**, retention factor; **FTIR,** fourier-transform infrared; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **BF,** b*ridelia ferruginea;* **LM,** *Lippia multiflora;* **AI,** *azadirachta indica.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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