African Journal of Pure and Applied Chemistry Vol. 6(5), pp. 65-73, 10 March, 2012
Available online at http://www.academicjournals.org/AJPAC
DOI: 10.5897/AJPAC11.088
ISSN 1996 – 0840 © 2012 Academic Journals

Full Length Research Paper

Comparative study of two kalanchoe species: total flavonoid, phenolic contents and antioxidant properties.

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Accepted 4 January, 2012

The research was conducted to determine the antioxidant activity of two *Kalanchoe* species in Ghana which are used widely for the treatment of various ailments including stroke and ulcers. The leaves were subjected to methanol and aqueous extractions for each sample. Thin layer chromatography (TLC) was performed to determine the various components in the leaves extract. The concentration of phenol and flavonoid was determined using folin-ciocalteu Zishen et al. (2003) and Lee et al. (2005) method. The extract was also screened to determine the total flavonoid and phenolic content of the methanol and aqueous extracts of the leaves of *Kalanchoe pinnata* and *Kalanchoe integra* and also to assess the antioxidant activity. 1,1-Diphenyl-2-picrylhydryzyl [DPPH] radical scavenging activity of both aqueous and methanol extracts of *K. integra* and *K. pinnata* were determined by the Brand-William method (2003) with slight modification. The phytochemical analysis indicated that the leave extracts were rich in total flavonoid and phenolic compounds. However, the aqueous extract of both *K. pinnata* and *K. integra* shown excellent antioxidant activity whereas that of the methanol was less effective compared to the butylated hydroxytoluene (BHT) which was used as control. The reducing power increased with increasing concentration, and the scavenging effect of aqueous extract on the DPPH radical was excellent. In conclusion, n-butanol: chloroform: acetic acid: water (7:3:1:1) is most suitable solvent for TLC analysis of the aqueous and methanol extract of *K. pinnata and K. integra. Kalanchoe* species possess a strong antioxidant property that reacts with free radicals.

Key words: *Kalanchoe* species, antioxidant, total flavonoids, total phenolic content.

INTRODUCTION

Oxygen radicals, the products of some biochemical and physiological reactions, initiate cell signaling pathways, damage cellular lipids, proteins, and nucleic acids. Reactive oxygen species are pivotal for the onset of various conditions such as hypertension, atherosclerosis, cancer, and alzheimer's disease (Morris, 2005; Azadzoi et al., 2005).

During normal aerobic metabolism, activated oxygen popularly known as superoxides are formed in a stepwise reduction of oxygen to water. When these activated oxygen molecules diffuse into cells, they become reactive to damage these cells (Garrow, 1993) which continue till antioxidants are introduced to scavenge all the free radicals available.

Williams (1999) reported that the body handles this cell damage process by producing a number of antioxidant enzymes which can be found both in the human body and in plants (Garrow, 1993). Thus, antioxidants protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl

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A B

Figure 1. (A) K*alanchoe pinnata;* (B) *Kalanchoe integra.*

radicals, hydroxyl radicals and peroxynitrite.

The *Kalanchoe* genus is a succulent perennial plant belonging to the class *Bryophyllum*. It grows naturally throughout the temperate areas of the world. In Africa, over 200 species have being identified and many of these species have been used medicinally especially *Kalanchoe pinnata* and *Kalanchoe integra* (Figure 1A and B) which have been used traditionally for the treatment of many disease conditions like peptic ulcer, upper respiratory tract infections, coughs and as anti-infective in Ghana (Dokosi, 1998 and Torres-Santos et al., 2003). They grow widely along footpaths and forests in Ghana and is called by various local names (for example, the Ewes call it 'aflatoga', Fantes -'eporow', Twi and Ga - 'egoro' and 'tamiwu' respectively).

Kalanchoe is reported to contain considerable amounts of flavonoid and phenolic compounds (Gaind and Gupta, 1971; Adenike and Eretan, 2004).

Despite its rich flavonoid and phenolic content, available literature indicates that no study has been carried out to investigate the antioxidant properties of this medicinal plant. The aim of this study is to determine the total flavonoid and phenolic content of two *Kalanchoe* species, namely, *K. pinnata* and *K. integra* and also to assess their antioxidant activity.

MATERIALS AND METHODS

Chemicals and reagents

(1) Methanol;
(2) Butanol;
(3) Acetic acid;
(4) Chloroform;
(5) Hexane;
(6) 1,1-diphenyl-2-pycryhydrazyl;
(7) Aluminium chloride;
(8) Sodium hydroxide;
(9) Sodium nitrate;
(10) Pyrogallol.

Methanol, 1,1-diphenyl-2-pycryhydrazyl (DPPH), n-butanol, acetic acid, chloroform pyrogallol and hexane were obtained from sigma Chemical Company (St. Louis, MO).

Preparation, separation and concentration of plant extract

Fresh leaves of *K. pinnata* and *K. integra var crenata* were washed and divided into two parts. 400 ml of methanol was added to one part. The same volume of water was added to the other part. The leaf samples were then blended using Sanyo SM (G300) blender.

The leaf extracts were then strained separately using muslin cloth. The methanolic extract was concentrated under reduced pressure using the rotary evaporator at a temperature of 40°C. Aqueous extract together with the aqueous portion of the concentrated methanolic extract were lyophilized to dryness using freeze drier. The powdered samples were stored at 4°C and used within seven weeks after production.

TLC of *Kalanchoe* extracts

50 mg of each of the lyophilized samples were dissolved in 1 ml of the solvent used and mixed using Taiyo (S-5F) Vortex mixer. About 5 µl of each solution obtained were spotted near the bottom Merck TLC plates at a distance of 2cm apart and air-dried after spotting.

The TLC tanks were allowed to stand covered for an hour to ensure equilibration. The lid was removed and the TLC plates then placed vertically in the tank for the separation of the compounds using four different solvent systems, solvent A comprises n-butanol: acetic acid: water (8:1:1 v/v), solvent B comprise of chloroform: methanol: acetic acid: water (79:19:3:2 v/v, solvent C comprise of n-butanol: hexane: ethyl acetate: methanol: water (1:3:1:1:0.5 v/v), solvent D comprise of n-butanol: chloroform: acetic acid: water (7:3:1:1 v/v). when the solvent had reached 1 cm to the top of the plate, the TLC plates were removed from the developing chamber and dried. The separated components of each mixture were visualized under UV light with chromate-VUE cabinet using UV-chromato (CC-60 VUE).

The spots detected under the UV light were circled and their retardation factors (R_f) values calculated (Appendix 1).

Total flavonoid content

Total flavonoid concentration was measured using a colorimetric assay developed by Zishen et al. (2003). 0.2 g of each sample was weighed and dissolved in 5 ml of distilled water (ddH_2O) separately. 1 ml of each solution was added to 10 ml volumetric flask containing 4 ml of distilled water. At time zero, 0.3 ml of 5% $NaNO_2$ was added to each volumetric flask. At time 5 min, 0.3 ml of 10% A1CI was added and at 6 min, 2 ml of 1M NaOH was added. Each reaction in the flask was immediately diluted with 2.4 ml of ddH_2O and mixed. The mixtures were centrifuged for 5 min at 3000 run per minute using Sakuma (300-5-1) centrifuge. Absorbance of the mixtures was determined at 510 nm relative to a prepared blank using Shimadzu (UV-120-02) spectrophotometer. Quercetin was used as a standard in the determination of the flavonoid content.

Total phenolic content

The total phenolic concentration was measured using the Folin Ciocalteu method (Leek et al., 2003). 1 ml of the solution was added to 25 ml volumetric flask containing 9 ml of ddH_2O. A reagent blank using ddH_2O was prepared. 1 ml of folin-ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solutions was added with mixing. Each solution was immediately diluted to a volume of 25 ml with 4 ml ddH_2O and mixed thoroughly. The mixtures were then incubated for 90 min at room temperature. The absorbances of the mixtures relative to that of the blank were measured at 750 nm using Shimadzu (UV-120-02) spectrophotometer. The phenolic content determination, pyrogallol was used as the standard.

DPPH radicals scavenging activity

25 mg of the freeze-dried extract of *Kalanchoe* species were dissolved in 1ml of water and 50 µl of the mixture was added to 2.9 ml of DPPH (4.5 mg in 100 ml of 80% methanol). The mixture was then shaken vigorously and allowed to stand at room temperature in the dark for 30 min, at which time decrease in absorbance was measured at 517 nm using Shimadzu [UV-120-02] spectrophotometer. Butylated hydroxytoluene (BHT) was used as the standard. In order to determine the optimum concentration of the *Kalanchoe* extract showing the best radical scavenging activity, various concentrations of the extract were prepared and assayed (Brand-Williams et al., 1995). Statistical analyses were carried out using ANOVA.

RESULTS

TLC of *Kalanchoe* extracts

The results are presented in Table 1. Both the aqueous and methanol extract of *K. pinnata* gave seven resolved components in the n-butanol:chloroform: acetic acid: water (7:3:1:1; v/v). Regarding *K. integra*, the methanol extract gave seven resolved components in the n-butanol: chloroform: acetic acid: water (7:3:1:1 v/v) mixture but the aqueous extract gave 5 spots (Table 2).

Total flavonoid content

The results are shown in Figure 2A.The total flavonoid content for aqueous extract of *K. pinnata* was lower (32 mg/g) than aqueous extract of *K. integra* (42 mg/g, $p>0.05$). Similarly the total flavonoid content of the methanolic extract of K. *pinnata* (108 mg/g) was far lower than that for the methanolic extract of *K. integra* (178 mg/g, $p<0.05$).

Total phenolic content

As presented in Figure 2B, the total phenolic content of the aqueous extract of *K. integra* (340 mg/g) was significantly higher than aqueous extract of *K. pinnata* (242 mg/g, $p>0.05$). The methanol extract of *K. pinnata* also contained less phenolic compounds than the methanol extract of *K. integra*. The total phenolic content of the aqueous extract of *K. integra* was slightly higher (340 mg/g) than its methanol extract (315 mg/g).

Reduction of DPPH radical scavenging activity

The aqueous and methanol extracts of *K. integra* showed a more rapid free radical scavenging effect on DPPH compared to the standard -butylated hydroxytoluene. However, the rate of free radical scavenging effect of the aqueous extract of *K. integra* on DPPH was faster than that of the methanol extract. A similar observation was made for the aqueous and methanol extract of *K. pinnata.*

Effect of different concentrations of *K. pinnata* and *K. integra* on DPPH radical scavenging

The results are presented in Figures 3A and B. The free radical scavenging effect of *K. integra* extracts and BHT on DPPH increased with increased concentrations. Below 5 mg/ml, BHT showed a better antioxidant activity relative to the plant extract.

However, at concentrations higher than 10 mg/ml, the scavenging effect of BHT leveled off and remained lower than the *K. integra* extracts. The aqueous extract, however, showed much better scavenging effect on DPPH than the methanol extract.

Figure 3B shows that the scavenging effect of the extracts of *K. pinnata* on DPPH was far lower than BHT at concentrations lower than 12 mg/ml. As the concentration of the aqueous extract increased beyond 12 mg/ml, its scavenging effect became stronger than BHT. The scavenging effect of methanolic extract of *K. pinnata* on DPPH also improved considerably but remained slightly lower than that of BHT at all concentrations used in the study.

Table 1. TLC analysis of aqueous and methanolic extracts of *Kalanchoe pinnata.*

UV light detection			
Mobile phase	**Extraction method**	**Rf value**	**Colour**
Butanol: acetic acid: water (8:1:1 v/v)	Methanol	0.9	Orange
		0.55	Greenish yellow
		0.44	Light blue
		0.23	Light blue
		0.16	Light blue
		0.06	Light blue
Butanol: acetic acid: water (8:1:1 v/v)	Aqueous	0.92	Orange
		0.57	Greenish yellow
		0.21	Light blue
		0.14	Light blue
		0.07	Light blue
Chloroform:methanol: acetic acid: water (79:19:3:2 v/v)	Methanol	0.08	Greenish yellow
Chloroform: methanol: acetic acid: water (79:19:3:2 v/v)	Aqueous	0.12	Greenish yellow
Butanol: hexane: ethyl acetate: methanol: water (1:3:1:1:0:5 v/v)	Methanol	0.96	Light blue
		0.02	Orange
Butanol: hexane: ethyl acetate: methanol: water (1:3:1:1:0:5 v/v)	Aqueous	0.97	Light blue
		0.05	Orange
Butanol: chloroform: acetic acid: water (7:3:1:1 v/v)	Methanol	0.97	Orange
		0.33	Greenish yellow
		0.27	Brown
		0.15	Brown
		0.12	Greenish yellow
		0.09	Greenish yellow
		0.05	Light blue
Butanol: chloroform: acetic acid: water (7:3:1:1 v/v)	Aqueous	0.98	Orange
		0.29	Greenish yellow
		0.26	Brown
		0.17	Brown
		0.12	Greenish yellow
		0.08	Greenish yellow
		0.04	Light blue

DISCUSSION

In this study, TLC analysis of the extracts revealed that, the solvent system n-butanol: chloroform: acetic acid: water (7:3:1:1 v/v) was the most suitable solvent system for TLC analysis while chloroform: methanol: acetic acid: water (79:19:3:2) was the least suitable. The observation that the aqueous extract of *K. integra* was higher than the methanol extract, suggests that water is more suitable than methanol for extraction of phenolic compounds from this medicinal plant.

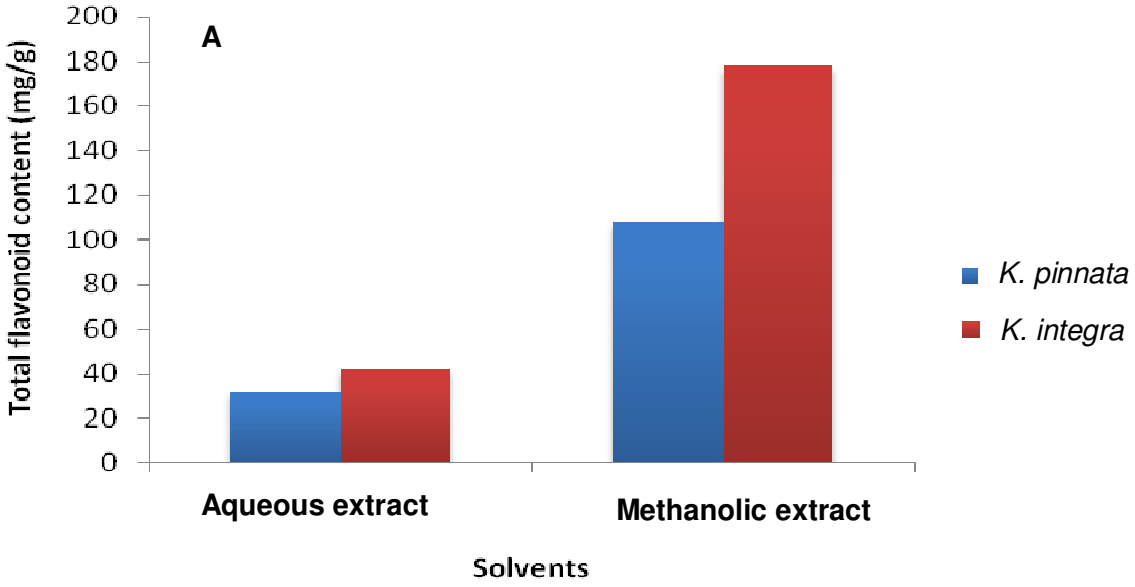
Further, the total flavonoid content of *K. integra* and *K. pinnata* also reveal that both species of *Kalanchoe* contain considerable amount of flavonoids but the

Table 2. TLC analysis of the aqueous and methanolic extract of *Kalanchoe integra*.

UV light detection			
Mobile phase	**Extraction method**	**Rf value**	**Colour**
Butanol: acetic acid: water (8:1:1 v/v)	Methanol	0.88	Orange
		0.60	Brown
		0.54	Greenish yellow
		0.47	Greenish yellow
		0.22	Light blue
		0.12	Light blue
Butanol: acetic acid: water (8:1:1 v/v)	Aqueous	0.33	Orange
		0.61	Light blue
		0.56	Greenish yellow
		0.46	Greenish yellow
		0.23	Light blue
		0.1	Light blue
Chloroform: methanol: acetic acid: water (79:19:3:2 v/v)	Methanol	0.61	Greenish yellow
Chloroform: methanol: acetic acid: water (79:19:3:2 v/v)	Aqueous	0.61	Greenish yellow
Butanol: hexane: ethyl acetate: methanol: water (1:3:1:1:0:5 v/v)	Methanol	0.04	Orange yellow
		0.02	
Butanol: hexane: ethyl acetate: methanol: water (1:3:1:1:0:5 v/v)	Aqueous	0.04	Yellow
Butanol: chloroform: acetic acid: water (7:3:1:1 v/v)	Methanol	0.96	Orange
		0.72	Light blue
		0.42	Light blue
		0.34	Light blue
		0.29	Light blue
		0.22	Light blue
		0.13	Light blue
Butanol: chloroform: acetic acid: water (7:3:1:1 v/v)	Aqueous	0.96	Orange
		0.40	Light blue
		0.34	Light blue
		0.28	Light blue
		0.13	Light blue

aqueous and methanol extracts of *K. integra* appeared to be richer. Comparatively, the methanol extracts of *K. integra* and *K. pinnata* is found to be richer in flavonoids than their corresponding aqueous extracts. Thus methanol appears to be more suitable solvent for flavonoid extraction as compared to water.

DPPH in solution produces stable free radicals whose odd electrons are paired off through electron capture from other electron donors (Blois, 1958). These reactions have been widely used to test the ability of compounds to act as free radical scavengers and to evaluate the antioxidant activity of various natural products (Zhu et al., 2002). In this study, DPPH was used to evaluate the antioxidant activity of the aqueous and methanolic extracts of *K. integra* and *K. pinnata*. The results show that the rate of free radical scavenging effect of the



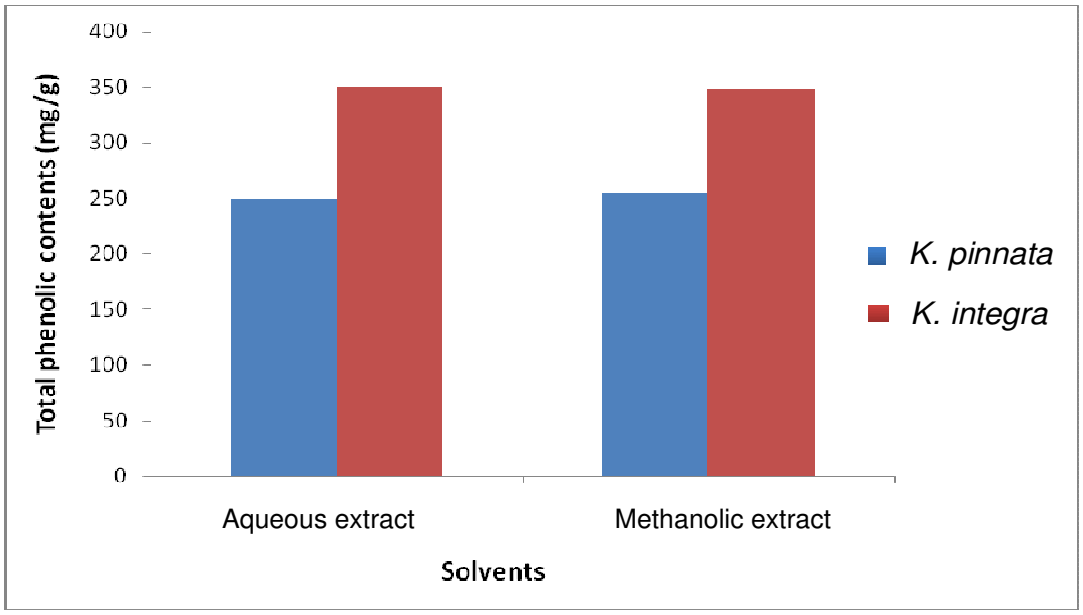


Figure 2. (A) Total flavonoid contents; (B) Total phenolic contents.

extracts of *K. integra* and *K. pinnata* on DPPH was good especially that of *K. integra* which was far better than BHT. A similar observation was made using different concentrations of the extract suggesting that the two species of *Kalanchoe* possess strong antioxidant properties.

The strong antioxidant activity demonstrated by the two species of *Kalanchoe* confirmed that they are free radical scavengers possibly as primary antioxidant that reacts with free radicals, particularly of the peroxy radicals, which are the major propagation of the autoxidation chain of fats, thereby terminating free radical chain reaction (Gordon, 1990; Frankel, 1991; Shahidi and Wanasesundara, 1992).

This can be attributed to the antioxidant activity of the extract provided by the chemical constituents of the plant (Bei et al., 2005). Thus the mechanism may be derived from the action of the chemical compounds, flavonoids and phenols in the plant medicine (Bei et al., 2005; Zhao, 2005). These findings therefore justify the use of these medicinal plants in the treatment of several ailments in Ghana.

Further research on isolation and identification of these flavonoid and phenolic compounds present in these

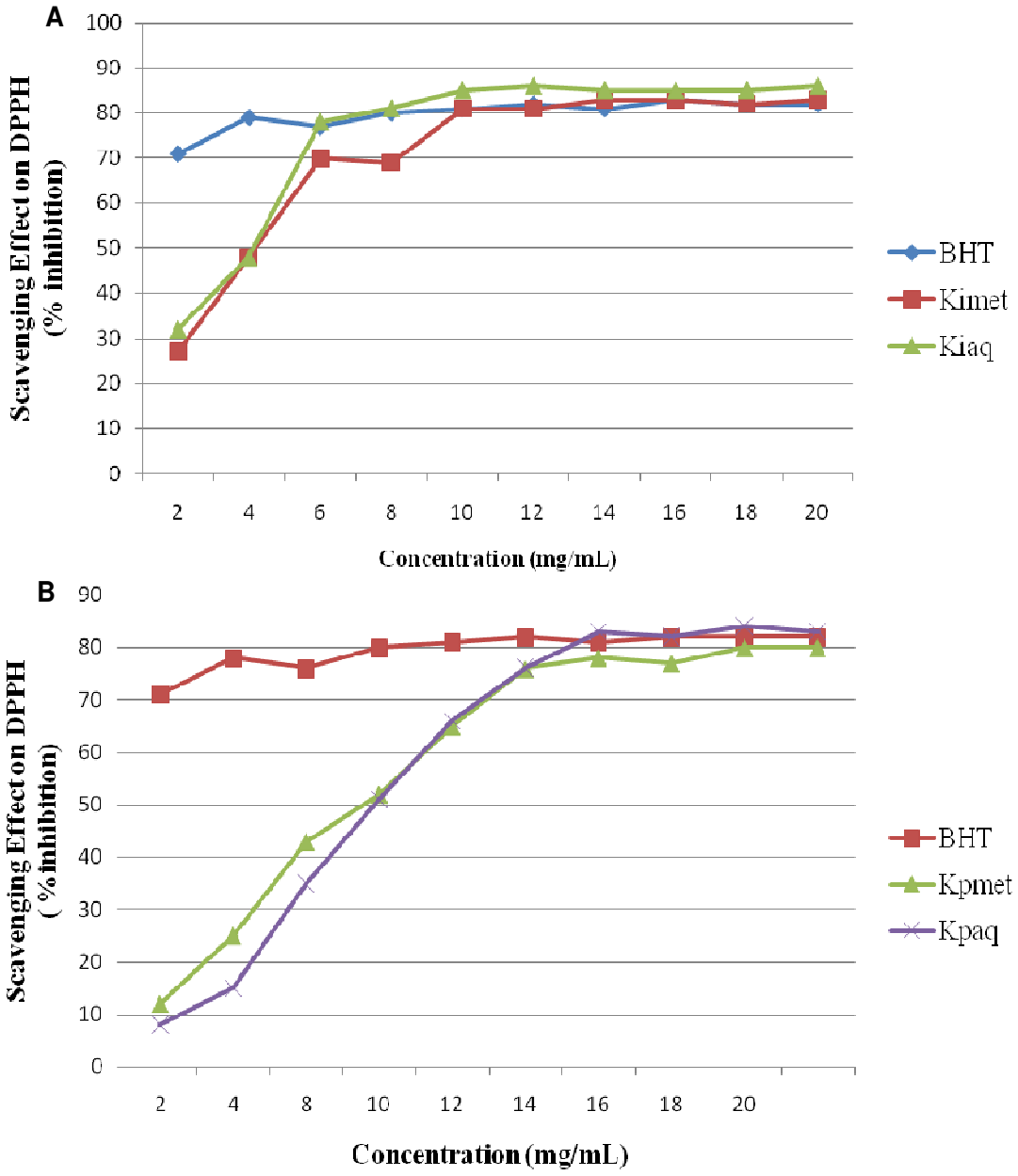


Figure 3. (A) Scavenging effect of *K. integra* leaf extracts and BHT on DPPH radicals; (B) Scavenging effect of *K. Pinnata* leaf extract and BHT on DPPH radicals.

plants is recommended.

A limitation to this work could be the lack of antioxidant activity of *Kalanchoe* species using ABTS and to assess its anti-peroxidative effect.

Conclusions

(1) n-butanol: chloroform: acetic acid: water (7:3:1:1) is most suitable solvent for TLC analysis of the aqueous and methanolic extract of *K. pinnata* and *K. integra.*

(2) Methanol extracts of *K. pinnata* and *K. integra* contain more flavonoids than their aqueous extracts but the methanol extracts of *K. integra* contained more flavonoid than the methanol extract of *K. pinnata.*

(3) Aqueous extracts of *K. pinnata* and *K. integra* contain more phenolic compounds than their aqueous extracts but the aqueous extracts of *K. integra* contained more flavonoid than the aqueous extract of *K. pinnata.*

(4) *K. pinnata* and *K. integra* possess a strong antioxidant

property that reacts with oxygen free radicals.

ACKNOWLEDGEMENT

The authors acknowledge Professor Alexander Nyarko, Director of Noguchi Memorial Institute for Medical Research for his immense support and advice during the study.

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Appendix 1

$$\text{Percentage of DPPH radical scavenged} = \frac{\text{Absorbance}_{517}\ \text{control} - \text{Absorbance}_{517}\ \text{Extract} \times 100}{\text{Absorbance}_{517}\ \text{Control}}$$

$$R_f = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$$

Chemicals and reagents

1. Methanol
2. Butanol
3. Acetic acid
4. Chloroform
5. Hexane
6. 1,1-diphenyl-2-pycryhydrazyl
7. Aluminium chloride
8. Sodium hydroxide
9. Sodium nitrate
10. Pyrogallol