ANTIOXIDANT AND ANTI-DENATURING ACTIVITES OF DEFATTED AND NON-DEFATTED METHANOLIC EXTRACTS OF THREE MEDICINAL PLANTS IN NIGERIA.

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ABSTRACT: The Phenolic content, antioxidant and anti-denaturing activities of deffated and non-deffated methanolic extract of *Acalypha wilkesiana*, *Cnidoscolus aconitifolius* and *Vernonia amygdalina* leaf were studied. The total phenolic content was determined using Folin-Ciocalteu's method while antioxidant activities were carried out using 2,2-diphenyl-1-picryl hydrazine (DPPH) free radical scavenging and total reducing power. The In-vitro anti-inflammatory activity was carried out by inhibition of protein denaturation method. The result of the study showed that defatted extracts of all the three plants showed higher phenolic content compared to the non-defatted extracts. The non-defatted extracts of all the three plants showed higher DPPH scavenging activity while the defatted extracts exhibited higher reducing power. Furthermore, the defatted extracts of all the plants showed higher anti- denaturing activity compared to the non-defatted. In conclusion, defatting the three plants in this study increased phenolic contents of the extracts, enhanced their reducing power and anti-denaturing activity.
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1.0 INTRODUCTION

Medicinal plants constitute one of the main sources of new pharmaceuticals and healthcare products. The role of these medicinal plants in disease prevention or control has been attributed to antioxidant properties of their constituents, usually associated to a wide range of amphipathic molecules, broadly termed polyphenolic compounds. This has led to the isolation of natural antioxidants, mainly of plant origin during the last decade (Demiray *et al*., 2009). Recently, extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative medicines for the treatment of many infectious diseases and also in preservation of food from the toxic effects of oxidants (Muthukumaran *et al*., 2011). Plant matrices including leaf, stem, root, and bark contain various solute molecules with more than one functional group (Kumoro et al., 2009). The natural bioactive compounds especially from plant sources have been investigated for their characteristics and therapeutic effects such as antibacterial, antiviral, anti-inflammatory and anticancer properties but their extraction presents specific challenges that must be addressed through the solvent extraction process (El-Hachi & Atik-Bekkaara, 2011; Ghasemzadeh *et al*., 2011). It was found that the polarity of the solvents seems to play an important role in the extraction of natural products and in a sequential extraction technique, chemical constituents are partially separated according to their polarity, the least polar components separates into the low polar solvents and this progressing through the separation of active components based on their polarity and the polarity of the solvent used. This partial separation of active components may be an advantage to reduce the antagonistic effects of chemical constituents because the compounds present in crude mixture may interfere with the action of the other (El-Hachi & Atik-Bekkaara, 2011; Jeyaseelan et al., 2011). Most of the bioactive components contained in the plant matrices are medium-sized molecules. Due to the presence of aromatic delocalized μ-electrons, the molecules are highly polarizable. Their high polarizability makes the molecules liable to a variety of specific interactions with polar solvents, e.g., protonation, hydrogen bonding, and specific salvation (Kumoro *et al*., 2009). Defatting plant extract with hexane or petroleum ether has been a pre-extraction procedure to remove undesirable components during Phytochemical and medicinal studies and the aim of this study is to determine its effectiveness in antioxidant and anti-denaturing activity of *Acalypha wilkesiana*, *Cnidoscolus aconitifolius* and *Vernonia amygdalina* leaves.

2. MATERIALS AND METHODS

COLLECTION OF PLANT MATERIALS

Fresh leaves of *Acalypha wilkesiana, Cnidoscolus aconitifolius* and *Vernonia amygdalina* were obtained from Babcock University, Ilishan-Remo, Ogun State in September, 2010.

EXTRACTION OF PLANT MATERIALS

Fresh leaves of the three plants were thoroughly rinsed and air dried. They were ground to fine powder and 50 g of each plant sample was defatted with hexane and then extracted with methanol for 72 hours. Another 50g of each plant sample was soaked in methanol for 72 hours. All the filtrates of each extract were concentrated using rotary evaporator at 40^{o}C. The crude extracts were weighed and stored at 4^{o}C till further use.

DETERMINATION OF TOTAL PHENOLIC CONTENT

This was estimated as described by Singleton and Rossi, 1965. The assay is based on the reduction of Folin-Ciocalteu reagent (Phosphomolybdate and phosphotungstate) by the phenolic compounds. The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer at 760nm.

PROCEDURE:

1 ml aliquot of extracts (0.1mg/ml) was added in a volumetric flask, containing 9 mls of water. One milliliter of Folin-Ciocalteu's reagent was added to the mixture and vortexed. After 5 min, 10 ml of 7% sodium carbonate was added to the mixture, and then incubated for 90 mins at room temperature. After incubation the absorbance against the reagent blank was determined at 750nm. A reagent blank was prepared using distilled water instead of the plant extract. The amount of phenolic compound in the extract was determined from the standard curve produced with varying concentration (10, 20, 30, 40, 50 µg/ml) of gallic acid. All samples were analyzed in triplicates.

ANTIOXIDANT ASSAY

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

A solution of DPPH mixed with that of a substance that can donate a hydrogen atom, gives rise to the reduced form with change in colour, from deep violet to pale yellow colour.

PROCEDURE:

This was carried out according to the DPPH spectrophotometric method of Mensor *et al.,* 2001. One ml of a 0.3 mM DPPH methanol solution was added to a 2.5ml solution of the extract or standard (20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml) and allowed to react at room temperature for 30 min. The absorbance of the resulting mixture was measured at 518nm and converted to percentage antioxidant activity (AA%), using the formula:

$$AA\% = \frac{[(Abs_{control} - Abs_{sample}) \times 100]}{Abs_{control}}$$

Methanol (1.0 ml) plus extract solution (2.5 ml) was used as blank. 1 ml of 0.3mM DPPH plus methanol (2.5 ml) was used as a negative control. Solutions of ascorbic acid and gallic acid served as positive controls. This assay was carried out in triplicates for each concentration. The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of radical formation, which was obtained by interpolation from linear regression analysis (Stoilova et al., 2007).

TOTAL REDUCING POWER

The total reducing power of the extracts were determined according to the procedure of Yen & Duh, 1993 as reported by Premanath & Lakshmidevi, 2010. Various extracts (20 - 100 µg/ml) were mixed with phosphate buffer (500 µl, 20 mM, pH 6.6) and 1% potassium ferricyanide (500 µl), and incubated at 50^{0}C for 20 min; 500 µl of 10% Trichloroacetic acid were added, and the mixture was centrifuged at 2500 rpm for 10 min. The supernatant was mixed with distilled water (1.5 ml) and 0.1% ferric chloride (300 µl) and the absorbance was read at 700 nm. The experiment was repeated thrice. Increase in the absorbance of the reactions mixture indicated increase in the reducing power. The extract concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration (Barros et al., 2007).

ANTIDENATURING ACTIVITY

The anti-denaturing activity of the plant extracts were determined according to the procedure described by Williams et al., (2002). A solution of 0.2% W/V of BSA was prepared in Tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. Stock solutions of 10,000µg/ml of all extracts were prepared by using methanol as a solvent. From these stock solutions 4 different concentrations of 10, 100, 200 and 500µg/ml were prepared by using methanol as a solvent. 50 µl of each extract was transferred to Eppendorf tubes using 1ml micro pipette. 5ml of 0.2% W/V BSA was added to all the above Eppendorf tubes. The control consists of 5ml 0.2% W/V BSA solution with 50 µl methanol. A concentration (100 µg/ml) of the standard (Diclofenac Sodium) was prepared in methanol with 5ml 0.2% W/V BSA solution. The test tubes were heated at 72° C for 5 minutes and then cooled for 10 minutes. The absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660nm. The % inhibition of precipitation (denaturation of the

protein) was determined on a % basis relative to the control using the following formula.
% inhibition of denaturation = (Abs of control-Abs of extract)/ (Abs of control) × 100

Statistical Analysis

Data were expressed as mean ± standard error (SE) after analysis using SPSS version 15.0. Student t-test was used to determine the difference between defatted and non defatted parameters. One way ANOVA was used to determine significant difference among the three plants.

3. RESULTS

3.1. Percentage Yield

The result of the extraction yield (figure 1) showed that the defatted methanolic extract gave a higher yield (15.04%) compared to the non defatted (13.82%) in *A. wilkesiana*. In *C. aconitifolius*, the non-defatted extract gave a higher yield (9.52%) compared to the defatted extract (5.06%). The non defatted extract in *V. amygdalina* gave a slightly higher yield (10.88%) compared to (10.64%).

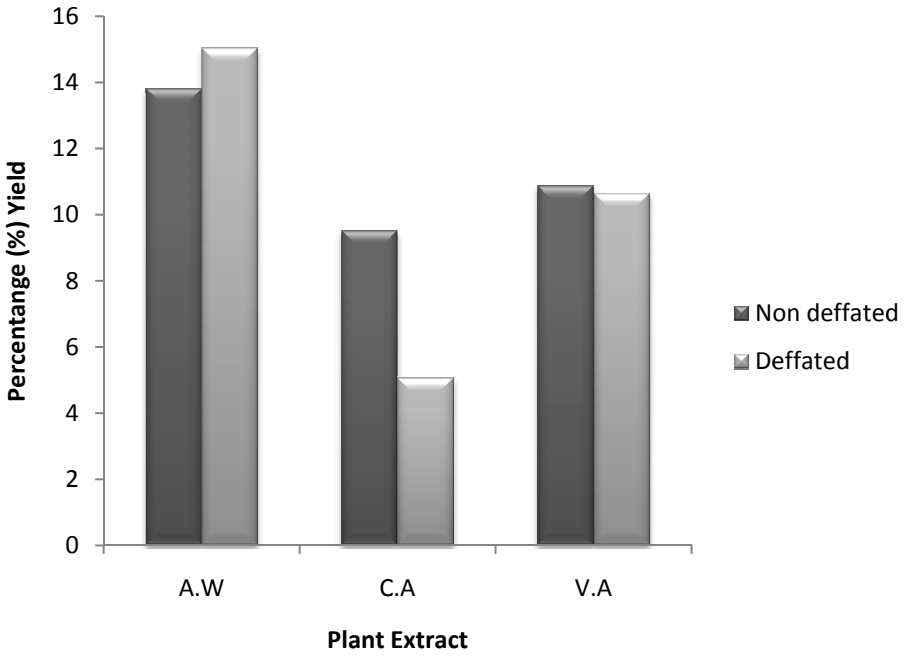

Figure 1: Percentage yield of methanolic extract of *A. wilkesiana, C. aconitifolius* and *V. amygdalina.*

3.2. Total Phenolic Content (TPC)

The total phenolic contents of *A. wilkesiana, C. aconitifolius* and *V. amygdalina* are shown in table 1. The total phenolic content was expressed in microgram Gallic Acid Equivalent per gram (mgGAE/g). The defatted extracts of *A. wilkesiana, C. aconitifolius* and *V. amygdalina* gave higher phenolic content which were significantly different than the non-defatted extracts of the plants ($p<0.05$). The phenolic contents of defatted and non-defatted extracts of *C. aconitifolius* were higher than *V. amygdalina* then *A. wilkesiana.*

Table 1: Phenolic content (mg GAE/g) of methanolic extract of *A. wilkesiana, C. aconitifolius* and *V. amygdalina.*

Extracts	*A. wilkesiana*	*C. aconitifolius*	*V. amygdalina.*
Non defatted	157.3±0.35^{a}	457.8±5.72^{c}	237.5±3.25^{e}
Defatted	285.9±1.92^{b}	924.2±3.68^{d}	574.2±0.55^{f}

Data are expressed as the average of three determinations ± S.E. Data with different lower case letters on each column and row are significantly different ($p<0.05$).

3.3. DPPH Scavenging Activity

The result of the percentage inhibiton of DPPH free radicals (see figure 2) showed that the scavenging activity of the non-defatted *A. wilkesiana, C. aconitifolius* and *V. amygdalina* methanolic extracts were higher than the defatted extracts

respectively. Furthermore, the magnitude of percentage Dpph inhibition of the non-defatted and defatted plant extracts is as follows: *A. wilkesiana* (96.2±0.03%; 95.04±0.05%) > *V. amygdalina* (75.6±0.4%; 43.9±0.6%) > *C. aconitifolius* (30.8±0.5%; 15.3±0.6%). All the plant extracts exhibited lower inhibition of Dpph compared to the gallic acid which was used as the standard.

3.4. Reducing Power

The result of the reducing power (see figure 3) showed that the defatted *C. aconitifolius* (0.35±0.01) and *V. amygdalina* (0.3±0.002) methanolic extracts exhibited significant higher reducing power compared to the non-defatted extracts (0.2±0.002), (0.2±0.001) respectively ($p<0.05$). However, the reducing power of the defatted methanolic extract of *A. wilkesiana* (0.49±0.004) was not significantly lower ($p<0.05$) than the non-defatted extract (0.5±0.002). The reducing power of the standard (1.23±0.02) was higher than all the extracts of all the plants used.

Table 2: IC_{50} values (µg/ml) of methanolic defatted and non defatted extracts of A. *wilkesiana*, C. *aconitifolius* and V. *amygdalina*.

Plant Extract	% DPPH Inhibition (µg/ml)	Reducing Power (µg/ml)
ACA	<1	114.68±2.69
ACAD	<1	93.75±0.48
CA	199.96±7.93	600±2.17
CAD	349.03±7.85	181.1±24.17
VA	72.97±0.18	479.34±16.98
VAD	120.28±3.51	264.33±3.93
Gallic Acid	3.30±0.54	1.50±2.16

Data are expressed as the average of three determinations ± S.E. ACA: Non-defatted *A. wilkesiana* extract; ACAD: Defatted *A. wilkesiana* extract. CA: Non-defatted *C. aconitifolius* extract; CAD: Defatted *C. aconitifolius* extract. VA: Non-defatted *V. amygdalina* extract; VAD: Defatted *V. amygdalina* extract.

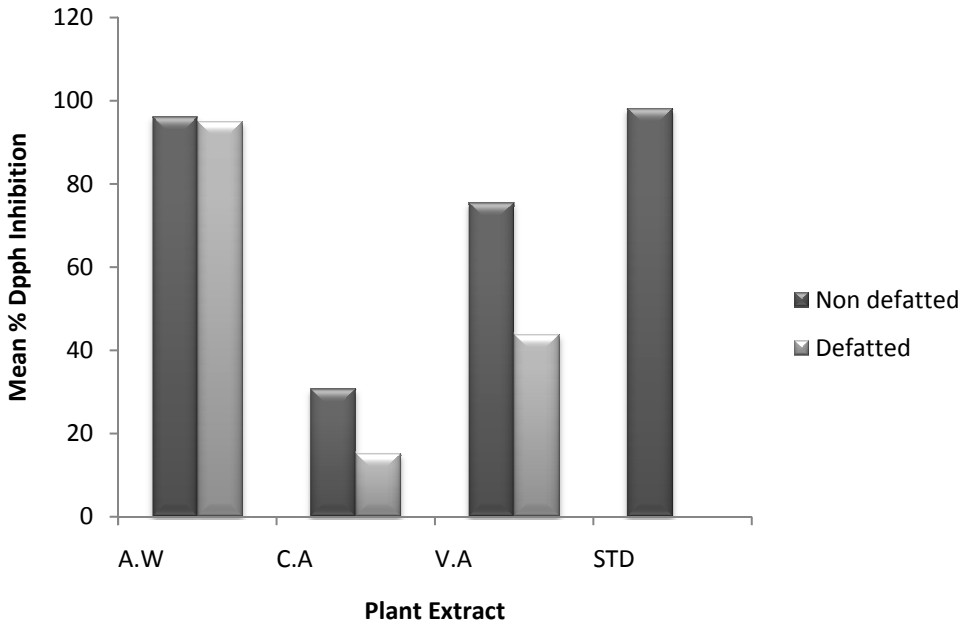

Figure 2: Percentage inhibition of Dpph radicals by methanolic extracts of *A. wilkesiana*, *C. aconitolius and V. amygdalina* leaf (100µg/ml). Standard (STD) antioxidant used was Gallic Acid (10µg/ml). The values represent the mean of triplicates ± SE of each extract.

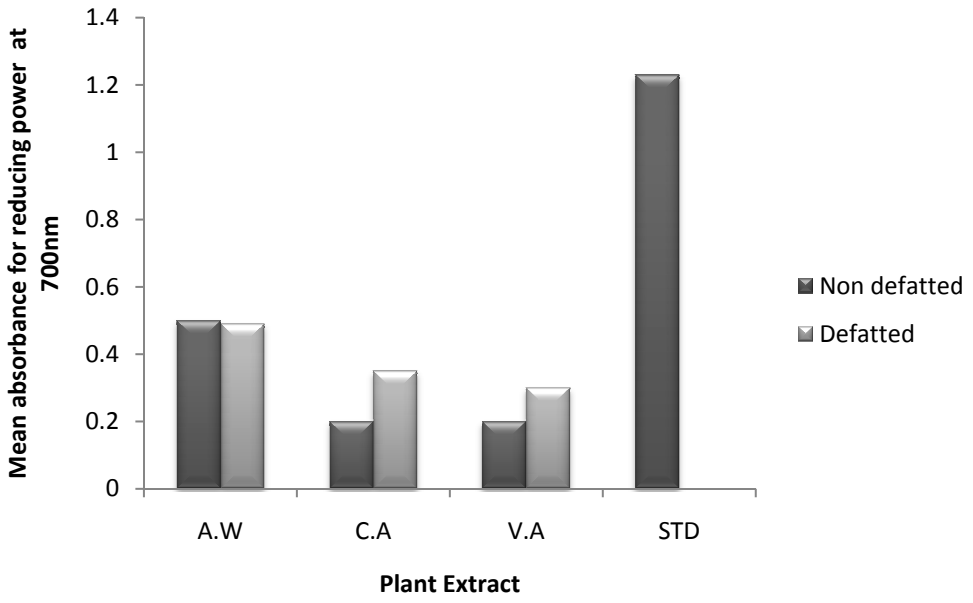

Figure 3: Reducing power of aqueous extracts of A. wilkesiana, C. aconitolius and V. amygdalina leaf at 100µg/ml. Standard (STD) antioxidant used was Gallic Acid at 100µg/ml. The values represent the mean of triplicates ± SE of each extract.

3.5. Anti-denaturation Activity

The result of the anti-denaturation of *A.wilkesiana, V. amygdalina,* and *C. aconitifolius* extracts showed that all the defatted extracts inhibited more denaturation of bovine serum albumin compared to the non-defatted extracts (see table 3). The percentage inhibitions of denaturation of A. wilkesiana extracts (non-defatted and defatted) were higher than all the extracts of *V. amygdalina,* and *C. aconitifolius*. At 100 µg/ml, only non-defatted, defatted *A*. wilkesiana and defatted *V*. amygdalina extracts inhibited more denaturation compared to diclofenac sodium (control). The result also showed that the percentage inhibition of denaturation decreased as the concentration of the extract increased.

Table 3: Percentage (%) inhibition of denaturation of BSA by A.*wilkesiana,* V. *amygdalina,* and C. *aconitifolius* aqueous extracts

Con(µg/ml)	A.*wilkesiana*		V. *amygdalina*		C. *aconitifolius*	
	Non-def.	Defatted	Non-def.	Defatted	Non-def	Defatted
10	61.28±1.0	73.52±0.1	50.05±0.2	53.67±0.1	29.32±0.1	54.08±0.3
100	50.87±0.3	58.06±0.9	46.11±0.1	52.64±0.7	28.21±0.2	45.7±0.2
200	48.38±0.8	53.83±0.1	44.93±0.5	46.8±0.2	17.23±0.1	29.9±0.5
500	12.38±2.0	36.12±0.3	20.29±0.3	36.68±0.7	2.65±0.6	4.8±0.2

Data are expressed as the average of three determinations ± S.E. Non-def. = Non-defatted. Standard used: diclofenac sodium (100µg/ml) = 49. BSA: Bovine Serum Albumin.

4. DISCUSSION

4.1.Extraction Yield.

Plant matrices including leaf, stem, root, and bark contain various solute molecules with more than one functional group. Therefore, it is difficult to predict the solubility of the solutes in a particular solvent. An alternative way of considering solubility is to use the concept of polarity (Kumoro *et al*., 2009). The result of the extraction yield showed that defatting *A. wilkesiana* will yield more extract while the reverse was the case in *C. aconitifolius*. There was no difference in the yields in *V. amygdalina.*

4.2. Phenolic Content

The higher phenolic content of the defatted A. wilkesiana, *C. aconitifolius* and *V. amygdalina* compared with the non defatted extracts showed that defatting the plant before extraction could enhance the availability of phenols in the plant (see table 1). The higher content of phenols in the defatted extracts of all

the plants compared to the non-defatted extracts could be as a result of the removal of fatty substances from the extracts making the phenolic compounds more polar. According to Ghasemzadeh *et al*., (2011), solvent with different polarity have significant effect on polyphenol content and antioxidant activity with higher content in more polar solvents. Moreso, phenolics compounds are often associated with other biomolecules (polysaccharides, proteins, terpenes, chlorophyll, inorganic compounds etc) and a solvent must be found that is suitable for extracting them. The result also showed that *C. aconitifolius* possed higher phenolic content compared to *A wilkesiana* and *V. amygdalina.* This is the first known report of the phenolic content of *C. aconitifolius* and the comparative phenolic content of the three plants under study.

4.3. Dpph Scavenging Activity

In this study, all the non-defatted extracted inhibited higher DPPH radicals compared to the defatted extracts. This suggests that the non-defatted extracts were able to donate more hydrogen atoms or electrons to quench the DPPH free radicals. The result also showed a reverse of the phenolic contents of the plants since the extracts with the highest phenolic content (defatted and non defatted *C. aconitifolius*) showed the least Dpph free radical scavenging activity, while the extracts with the lowest phenolic contents (Defatted and non-defatted *A. wilkesiana*) showed the highest Dpph radical scavenging activity. IC_{50} value is associated with a higher radical scavenging activity (Zhu et al., 2011). The IC_{50} values for Dpph scavenging activity of the extracts showed that *A. wilkesiana* had very strong free radical scavenging activity compared to the other plants (see table 4).

4.4. Reducing Power

Reducing power was determined using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the samples. The presence of antioxidants in the samples causes the reduction of the $Fe^{3+/}$ferricyanide complex to the Fe^{2+} form and Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. A higher absorbance at 700 nm indicates a higher reducing power (Barros et al., 2007).

Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary anti-oxidants (Yen and Chen, 1995).

Defatting the methanolic extracts of *C. aconitifolius* and *V. amygdalina* increased the reducing power of the extracts compared to the non-defatted extracts. There was no difference in the reducing power of the defatted and non-defatted *A. wilkesiana* extracts. The IC_{50} values showed that defatted extracts of each plant exhibited higher reducing power compared to the non-defatted extracts. However, the reducing potential of gallic acid was higher than all the extracts studied. Among the three plants, *A. wilkesiana* has shown to posses higher reducing power despite the low phenolic content. The result suggests that the removal of fatty substances in the plant extracts enhanced their reducing power.

4.5. Anti-denaturing Activity

Anti-denaturation study was performed by using Bovine Serum Albumin (BSA). BSA assay seeks to eliminate the use of live specimens as far as possible in the drug developmental process. When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Thus the assay applied for the discovery of those drugs which can stabilize the protein from denaturation process (Ramalingam et al., 2010). Denaturation of proteins is a well documented cause of inflammation (Sakat et al., 2010) and ability of extract to inhibit protein denaturationn has been studied. All the extracts in this study showed high percentage anti-denaturation activity at lowest concentration. This suggests that anti-denaturation of the extracts is more effective at low concentrations. Other authors have also reported similar trend (Duganath et al., 2010; Kumar et al., 2010; Ramalingam *et al*., 2010). This study has also shown that defatting the extract enhances the percentage inhibition of denaturation of BSA, suggesting that removing the fatty substances of the extracts enhances their bioactivity.

Conclusion

The results of this study showed that the removal of fatty substances from *A.wilkesiana, V. amygdalina,* and *C. aconitifolius* enhanced their yield, extraction of phenols, reducing power and anti-denaturing activity.

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