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ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIALS OF *BAUHINIA VARIEGATA* SEEDS
AGAINST FERRIC CHLORIDE-INDUCED LIPID PEROXIDATION IN CHICKEN LIVER
HOMOGENATE

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Abstract

Background: Iron accumulation in the liver is customary in pathological conditions associated with oxidative stress. Iron is also an essential element for organisms at its physiological levels. This study was aimed at investigating the antioxidant and hepatoprotective potential of acetone and methanol defatted seed extracts of *Bauhinia variegata* Linn (Leguminosae) against ferric chloride (FeCl₃)-induced lipid peroxidation in chicken liver *in vitro*.

Materials and Methods: Antioxidant capacity and hepatoprotective potential of acetone and methanol defatted seed extracts of *B. variegata* were evaluated using the established *in vitro* models, FeCl₃-induced lipid peroxidation in chicken liver homogenate and thin layer chromatography-2,2-diphenyl-1-picrylhydrazyl (TLC-DPPH). Ascorbic acid, and acetone and methanol seed extracts of milk thistle (*Silybum marianum*) were used as antioxidant and hepatoprotective controls.

Results: An overall of 80% acetone and absolute methanol defatted seed extracts of *B. variegata* [*B. variegata* acetone extract (BVAc) and *B. variegata* methanol extract (BVMe)] revealed to possess antioxidant and free radical scavenging capacity, which can protect the liver from FeCl₃-induced lipid peroxidation in a concentration-dependent manner when compared with controls, ascorbic acid and extracts of milk thistle. At concentrations of 250 µg/mL and 300 µg/mL, thiobarbituric acid reactive species (TBARS) inhibitions were 80.5% and 97.6% for BVAc; and 84% and 98.7% for milk thistle acetone extract (MSAc). At concentration of 300 µg/mL, BVMe and milk thistle methanol extract (MSMe) TBARS inhibitions were 89.0% and 93.8%, respectively. These findings may confirm the presence of antioxidant compounds with hepatoprotective potential in both defatted seed extracts of *B. variegata*.

Conclusion: The findings of this study suggest acetone and methanol extracts of *B. variegata* defatted seeds may serve as good sources of natural antioxidant and hepatoprotective agents.

Keywords: Antioxidant, *Bauhinia variegata*, hepatoprotective, lipid peroxidation, seeds.

Introduction

Oxidative processes are essential in several living organisms, producing energy to activate the biological systems in the body. These oxidation systems produce reactive oxygen species (ROS). The most common ROS, are superoxide anion (O²⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[·]), resulting from the cellular redox systems. All ROS are beneficial at low concentrations, but they become toxic to the body at high concentrations. When excess free transition metals are present, including copper and iron, these oxidants generate more reactive species attacking proteins, lipids and deoxyribonucleic acid (DNA) (Valko et al., 2007). Organisms contain non-enzymatic and enzymatic antioxidant systems eliminating ROS. These antioxidant systems maintain an antioxidant balance, sustain the cell life and stabilise tissues. Oxidative stress is a mediator and a consequence of various diseases, mainly driven by oxidative stress cascade (Jothy et al., 2012). Oxidative stress cascade is initiated by free radicals including ROS and reactive nitrogen species (RNS), when

they gain stability by pairing with the biological molecules including lipids in healthy and diseased human cells through a process called 'lipid peroxidation'. The formation of free radicals in the body shortens the life span of cells and resembles signs of ageing (Valko et al., 2007). Thus, ROS and RNS interact with different lipids, especially unsaturated fatty acids and cholesterol (Ayala et al., 2014). Possible mechanisms of metals-induced oxidative stress associated with human diseases, are well documented (Jomova and Valko, 2011).

Iron is essential for existence, due to its redox properties. An iron overload can result in toxicity, through producing ROS and RNS from its redox properties. Tight systemic and cellular controls are required to maintain physiological levels of iron in the body. This regulation seems to be mainly located in the liver (Mitchell and Mendes, 2013). This is because liver is the major organ in the body, responsible for approximately 500 body functions, including immune defence, digestion, carbohydrate and lipid metabolism, providing the body with the energy it needs. The liver processes about entirely what humans consume, breathe or absorb through the skin (Franciscus, 2012). Treatment with medicinal plant extracts and thiobarbituric acid (TBA) experiments elucidate and validate extracts reducing lipid peroxidation (Veeru et al., 2009). Medicinal plants are rich in secondary metabolites, essential to the human body as they may serve as antioxidants, contributing to reducing ROS and RNS under physiological and pathophysiological conditions respectively (Ahmad et al., 2013). Oxidative stress remains important in various chronic diseases of lifestyles (communicable and non-communicable). The treatment of these diseases involves antioxidants (Cichoz-Lach and Michalak, 2014).

B. variegata Linn of the family Leguminosae is native to Asia and is an invasive plant species in South Africa. This plant species spreads by seed dispersal and is found in five provinces in South Africa: Limpopo, Gauteng, Mpumalanga, kwaZulu Natal and Eastern Cape. *B. variegata* is an evergreen tree with purple flowers and round leaves. Its legume fruits are dark brown when they are dry and green when they are still fresh. Flowers appear during August to October. Due to adaptation of the plant to new environment and climatic change, some species of *B. variegata* in the Limpopo province of South Africa, flower during May to June (Bansal et al., 2014).

Each part of *B. variegata* comprises its own medicinal purposes (Ahmed et al., 2012). *In vitro* and *in vivo* studies revealed, water and organic solvent extracts of various parts of *B. variegata* Linn, especially flowers, leaves, stem-barks and roots, possess antioxidant activity and other medicinal benefits (Aljobouri et al., 2015). Flowers and leaves of *B. variegata* are traditionally used as a vegetable, implying this plant's nutritional values, calling for commercialisation in the food process industries to explore its medicinal and nutritional properties, respectively. This plant also possesses several hidden medicinal properties, remaining to be scientifically evaluated (Bansal et al., 2014). Properties of seed extracts of *B. variegata* are partly investigated, but not yet for the prevention and treatment of lipid peroxidation, one of the markers of oxidative stress.

Milk thistle (also known as *Silybum marianum*), belongs to the Asteraceae family. This plant is one of the well-researched plants in the treatment of liver diseases. *S. marianum* has green leaves producing milk like substances in their veins as its name milk thistle and brown black seeds. Milk thistle is native to the Mediterranean and invasive to other continents including Africa. Seeds of milk thistle contain 70-80% silymarin flavonolignans and 20-30% of chemically unknown fraction, comprising mostly polymeric and oxidised polyphenolic compounds. Silymarin is a complex mixture of four flavonolignan isomers (Dixit et al., 2007). Seeds of this plant have antioxidant activity through various mechanisms (Ghosh et al., 2010). An increase in protein synthesis in the damaged liver provides evidence of milk thistle influencing liver regeneration. Milk thistle tablets and capsules are still considered an effective method treating liver diseases (Althagafy et al., 2013). In this study, 80% acetone and absolute methanol sequential extracts of defatted seeds of *B. variegata* were used to preliminary screen for their antioxidant and hepatoprotective potential against FeCl₃-induced lipid peroxidation in the chicken liver homogenate *in vitro*, in comparison with ascorbic acid, and absolute acetone and methanol extracts of non-defatted seeds of milk thistle.

Materials and Methods

Chemicals and reagents

Methanol, chloroform, trichloroacetic acid (TCA) and n-hexane were purchased from Merck (Pty) Limited, South Africa. Toluene, FeCl₃, chloroform, methanol, ascorbic acid and potassium dihydrogen orthophosphate (KH₂PO₄) were purchased from BDH Chemicals Limited, England. Ethyl acetate and acetone were purchased from Rochelle Chemicals and Lab Equipment (South Africa). All the chemicals and reagents were of analytical grade.

Collection and processing of seeds of *B. variegata*

Seeds of *B. variegata* were collected in January 2014 from Polokwane central, Limpopo province in South Africa. Voucher specimen (UNIN121167) was deposited at the Larry-Leach Herbarium, University of Limpopo and plant materials were identified by Dr B. Egan. Seeds were washed with water and dried in the shade. Dried seeds (5 g) were weighed, using the standard laboratory weighing scale (Mettler AJ100, Switzerland) and ground to fine powder. The powdered seeds were stored in zip seal plastic bags, covered with foil to protect it from direct light and kept in a cupboard at room temperature (RT) until use.

Extraction of *B. variegata* and milk thistle seeds

Extracts of powdered seeds of *B. variegata* were prepared by the successive extraction method using three organic solvents, specifically n-hexane, acetone and methanol as described by the World Health Organization (WHO) protocol CG-06 with minor modifications (Patel and Verma, 2013). Powder of the seeds was defatted by n-hexane according to the method described by the International Organization for Standardization (ISO, 1998). A solution of 80% acetone and absolute methanol were used successively to prepare the experimental seed extracts of *B. variegata*. A powdered seed (20 g) was weighed in the glass beaker, using the analytical weighing balance (DIGI model DS-708, Japan). n-Hexane (200 mL) was added into the glass beaker with 20 g of seed powder, mixed and placed in the Labcon incubator shaker (Rochelle Chemicals and Lab Equipment, South Africa), set at 111 revolutions per minute (rpm) at RT for 3 hours (hrs). The mixture was filtered through the Macherey Nagel filter papers MN 615.Ø 100 mm (Germany). The remaining residue was incubated in the shaker overnight with freshly added 200 mL of n-hexane under the same conditions. The solution was filtered and the remaining residue was washed three times with 100 mL of 80% acetone [acetone: water, 4:1, volume/volume (v/v)] to remove hexane. Extraction using acetone was performed with the same procedure used for n-hexane. Briefly, 200 mL of 80% acetone was mixed with the washed residue, shook for 3 hrs at RT and filtered. The residue was extracted overnight with another 200 mL of 80% acetone and filtered. The remaining residue was washed three times with absolute methanol to remove acetone. BVMe was prepared using 200 mL of absolute methanol at the same conditions used for the BVAc. Filtrates of each experimental extraction solvent were combined in a pre-weighed labelled glass bottle and fan dried at RT.

Absolute acetone and methanol extracts of non-defatted seeds of milk thistle were successively prepared from capsules, purchased from local pharmacies in South Africa. A powdered seed of milk thistle (20 g) was non-defatted because it is completely solubilised in n-hexane. MSMe and MSAc extracts were prepared with a similar procedure applied for the BVMe and BVAc extracts. The resulted mass of extracts from both plant seeds were calculated using the gravimetric method (Singh and Rugged, 2012).

The yields of BVAc and BVMe extracts were 702 mg (3.5%) and 32 mg (0.16%), respectively. These two dried extracts of *B. variegata* were dissolved in 100% dimethyl sulfoxide (DMSO) yielding concentrations of 351 mg/mL BVAc and 80 mg/mL BVMe and kept in 5.7 (2 mg) µL and 25 µL (2 mg) aliquots. The yields of acetone and methanol extracts of non-defatted seeds of milk thistle, MSAc and MSMe were 5880 mg (29.4%) and 7.2 mg (0.036%). These extracts were dissolved in 100% DMSO yielding concentrations of 0.606 mg/µL (oil) MSAc and 0.0036 mg/µL MSMe, respectively. These two extracts were kept in 3.31 µL (2 mg) and 1 mL (2 mg) aliquots. All extracts, BVAc, BVMe, MSAc and MSMe were stored in aliquots at -20°C to avoid contamination and repetitive freeze-thaw (Jung et al., 2006).

Qualitative phytochemical and antioxidant screening

The presence of phenolic and flavonoid compounds in the acetone and methanol extracts of defatted seeds of *B. variegata* and non-defatted seeds of milk thistle was tested respectively according to the methods described by Chandrashekar et al. (2013). Phytochemicals of seed extracts were also analysed using the thin layer chromatography (TLC) plate, according to the method described by Chandrashekar et al. (2013). The TLC method was used for coupling it with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) to check for antioxidant activity of the plant extracts.

Phenols test

Several methods are used to measure the presence of phenols in plant extracts. In this study, qualitative FeCl₃ method was used. Briefly, 5% FeCl₃ solution was added drop-wise into 500 µL of 2 mg/mL plant extract solution. In the presence of phenols, the colour changes to dark green/blue. In the absence of phenolic, the mixture solution remains yellow (Chandrashekar et al., 2013).

Flavonoids test

Several methods are used, testing for the presence of flavonoids in plant extracts. In this study, the qualitative aluminium chloride (AlCl₃) method was used. Briefly, 1% AlCl₃ solution was added drop-wise into a 500 µL of 2 mg/mL plant extract solution and mixed. The developed yellow colour was recorded as the presence of flavonoids, and non-development with the absence of flavonoids (Hossain et al., 2011).

Thin layer chromatography analysis

Thin layer chromatography was used to develop the compounds in the crude plant extracts as described by Eloff *et al.* (2011). Various TLC systems were prepared to achieve polar, intermediate and non-polar mobile phases. Ethyl acetate-methanol-water (EMW, 96:3:1, v/v/v) and chloroform-methanol (CM, 1:1, v/v) are polar mobile phases, acetone-methanol-water (AMW, 6:2:1, v/v/v) is an intermediate mobile phase and hexane-ethyl acetate-ethanol (HEE, 5:4:1, v/v/v) is a non-

polar mobile phase, used to develop secondary metabolites of the 80% acetone extract of *B. variegata* (BVAc) and absolute acetone extract of milk thistle, according to their polarity. Milk thistle acetone extract (MSAc) served as a positive control.

TLC plates (Silica gel 60 F₂₅₄ on aluminium sheet, Merck) were cut 10 cm high and 4.5 cm wide, and extracts were spotted at 1 cm from the bottom. All extracts were diluted in the solvent used during their extractions. The TLC plates could absorb the mobile phase to develop compounds in the spotted extracts. The mobile phase was left to move until it reaches ±1 cm from the top of the TLC plate. Plates were viewed under the UV-light to visualise bands invisible under the visible light. The plates were sprayed with vanillin reagent and backed at 110°C for 5 minutes in the incubator (Prolab PL100I, Labex, South Africa). Plates were scanned for record-keeping.

Qualitative antioxidant analysis

The DPPH radical scavenging assay based on the quantitative measurement of radical scavenging activity was performed using the TLC method as described by (Rajan et al., 2011). The qualitative DPPH method on the TLC plate may also specify what compound has the antioxidant activity, based on the principle of free radical scavenging activity. Phytochemical compounds are firstly separated by the mobile phase, followed by reaction with the DPPH. In most studies, the DPPH scavenging activity was measured in the microtiter 96-well plates. The spectrophotometer quantification of the DPPH scavenging activity does not identify the compound with antioxidant activity separately (Nithianantham et al., 2013). A qualitative TLC-DPPH method was prepared simultaneously with the TLC method described, with minor differences. Instead of spraying the TLC plates with vanillin reagent, freshly prepared 0.2% DPPH in methanol was used to detect the compounds with antioxidant activity (Anthony and Saleh, 2012).

Determining lipid peroxidation levels

Thiobarbituric acid (TBA) assay was applied to determine the levels of lipid peroxidation as described by Ahmad et al. (2013), with minor modifications. TBA reacts with reactive species giving multiple bimolecular breakdown products, undergone free radical attack to form TBARS. The TBA assay is not only specific for malondialdehyde (MDA), one of many products from damaged lipids. Non-specificity results from the acid-heating step of the TBA assay causing formation of the MDA-TBA like derivatives (Ayala et al., 2014).

Proteins were precipitated and removed by centrifugation prior to adding the TBA solution and heating steps. Chicken liver samples were supplied by Mike's chicken abattoir (Ladana, Polokwane in South Africa), frozen in liquid nitrogen and kept at -80°C until required. Chicken liver homogenate (w/v) (10%) was prepared according to the method described by Ahmad et al. (2013), with minor modifications. One-gram (1 g) wet chicken liver was homogenised in 10 mL ice-cold potassium chloride (KCl) buffer (pH 7.4) in the hand glass homogeniser (Leighton Buzzard, England) placed in ice.

Table 1: Preparations of FeCl₃ to induce lipid peroxidation in chicken liver homogenate

Final concentration of FeCl ₃ (mM)	100 mM FeCl ₃ stock solution (µL)	0.136% KH ₂ PO ₄ solution (µL)	Liver homogenate (µL)
0	0	400	800
2	25	375	800
4	50	350	800
8	100	300	800
17	200	200	800
33	400	0	800

Table 1 indicates the preparation of controls and experimental samples in 2 mL Eppendorf tubes using a FeCl₃ final concentrations range of 0-33 mM. Briefly, after adding and mixing the blanks and samples with and without FeCl₃ as shown in Table 1, at zero minute of incubation, 300 µL from each Eppendorf tube was transfer to new tubes in ice and 600 µL of ice-cold 15% TCA was added in all the tubes to stop the reaction. Tubes were kept in ice for 10 minutes to precipitate

proteins. To separate the protein precipitates from the supernatant, tubes were centrifuged at 3000 rpm for 15 minutes at 4°C using the Beckman coulter bench-top microcentrifuge (USA). The clear supernatants were transferred to new tubes and 900 µL of 0.6% TBA was added. The mixture was incubated in the shaking water bath (Lasec, Germany) at 95°C for 30 minutes. A pink colour developed, an indicator of the MDA-TBA adduct. After cooling at RT, 250 µL from each tube were transferred into clear 96-well plates in triplicate. The absorbance was read at 450 nm using the Glomax multi-detection system with four optical wavelength fitted standard filters for various assays (450 nm, 560 nm, 600 nm and 750 nm, Promega; E8032). The remaining samples in the control and experimental tubes were incubated at 37°C. After every 30 minutes of incubation for 90 minutes, 300 µL from each Eppendorf tube was transferred to a new tube and followed the similar procedure of the samples collected at zero minute of incubation. The MDA levels were calculated as mM/mg liver, using the molar extinction coefficient of 1.56×10^5 MDA/M/cm.

TBARS inhibition assay

Table 2: Preparations of TBARS (lipid peroxidation) inhibition

Final extract concentration (µg/mL)	Liver homogenate (µL)	0.136% KH ₂ PO ₄ (µL)	1800µg/mL ascorbic acid or seed extract stock (µL)	48 mM FeCl ₃ stock (µL)	Final FeCl ₃ concentration (mM)
blank	200	100	0	0	0
0	200	50	0	50	8
50	200	48.437	1.562	50	8
100	200	46.875	3.125	50	8
150	200	43.75	6.25	50	8
200	200	37.5	12.5	50	8
250	200	25	25	50	8
300	200	0	50	50	8

Table 2 indicates control and experimental samples used to elucidate inhibition of FeCl₃-induced lipid peroxidation in chicken liver homogenate in 2 mL Eppendorf tube. Using the TBARS assay procedure mentioned above, various concentrations of each plant extract (BVAc, BVMe, MSAc and MSMe) and ascorbic acid were dissolved in 1% DMSO at a range of 0-300 µg/mL and added into the experimental and control samples, respectively. Each Eppendorf tube contained 200 µL of liver homogenate, a defined volume of buffer, 50 µL of 48 mM FeCl₃ and a certain concentration of plant extract as shown in Table 2. Blank was prepared using 1% DMSO. Lipid peroxidation in chicken liver homogenate was induced by 8 mM FeCl₃. Ascorbic acid, and MSAc and MSMe extracts were used as antioxidant and hepatoprotective controls.

The mixtures were incubated at 37°C for 60 minutes. After incubation, 500 µL of ice-cold 15% TCA was added to all the tubes, precipitating the proteins in ice for 10 minutes. The tubes were centrifuged at 3000 rpm for 15 minutes at 4°C using the Beckman coulter micro-centrifuge. The clear supernatants were transferred to new tubes and 750 µL of 0.6% TBA was added. The mixtures were incubated in a shaking water bath at 95°C for 30 minutes. After cooling at RT, 250 µL from each tube was transferred into a clear 96-well plate in triplicate. The absorbance was read at 450 nm using the Glomax multi-detection system (Promega, USA). The % TBARS inhibition was calculated using the following equation (Bibhabasu *et al.*, 2008):

$$\% \text{ inhibition} = \frac{\text{AbsC} - (\text{AbsS} - \text{AbsB})}{\text{AbsC}} \times 100$$

Results

Qualitative phytochemicals screening

Table 3: Phenols and flavonoids of seed extracts

Bioactive compounds	BVAc	BVMe	MSAc	MSMe
Phenols	++	+	+++	++
Flavonoids	++	-	+++	++

B. variegata acetone extract (BVAc), *B. variegata* methanol extract (BVMe), Milk thistle acetone extract (MSAc), Milk thistle methanol extract (MSMe). (-) absent; (+) less present; (++) moderately present; and (+++) highly present.

Table 3 indicates the two phytochemicals, namely phenols and flavonoids of acetone and methanol extracts of defatted seeds of *B. variegata* and non-defatted seeds of milk thistle, respectively. The results indicated the presence of phenolic compounds in the four extracts and the presence of flavonoids in BVAc, MSMe and MSAc, respectively.

Thin layer chromatography analysis

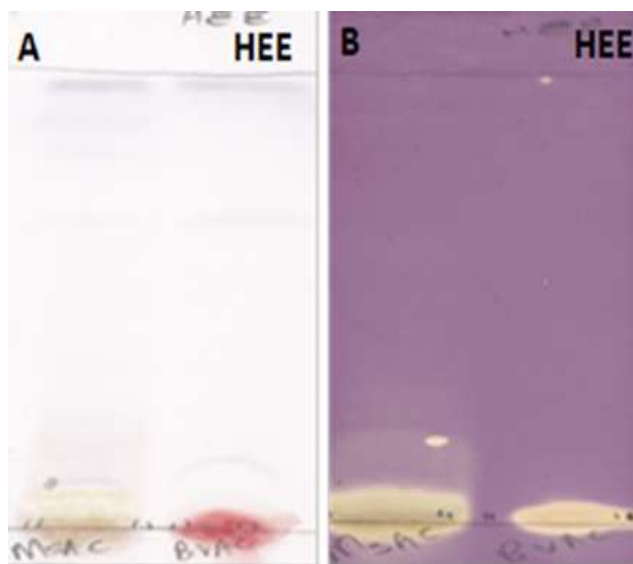


Figure 1: Chromatograms of acetone extracts of non-defatted seeds of milk thistle (MSAc) and defatted seeds of *B. variegata* (BVAc) (A) TLC plate developed in hexane-ethyl acetate-ethanol (HEE, 5:4:1, v/v/v) and sprayed with vanillin reagent, (B). TLC plate developed in HEE and was sprayed with DPPH.

The non-polar mobile phase HEE demonstrated slightly developing compounds in MSAc and BVAc extracts, after spraying the plate with vanillin reagent. MSAc indicated a complex yellow compound, while BVAc indicated a complex dark red compound. Both complex compounds did not migrate longer on the TLC. Other compounds in these two seed extracts were well developed (Figure 1A). Both extracts indicated antioxidant activity by changing the purple colour of DPPH into yellow (Figure 1B). Notably, MSMe and BVMe were not analysed on the TLC, resulting low extraction yields from the starting seed materials. These results revealed antioxidant compounds in acetone extracts of non-defatted seeds of milk thistle (MSAc) and defatted seeds of *B. variegata* (BVAc).

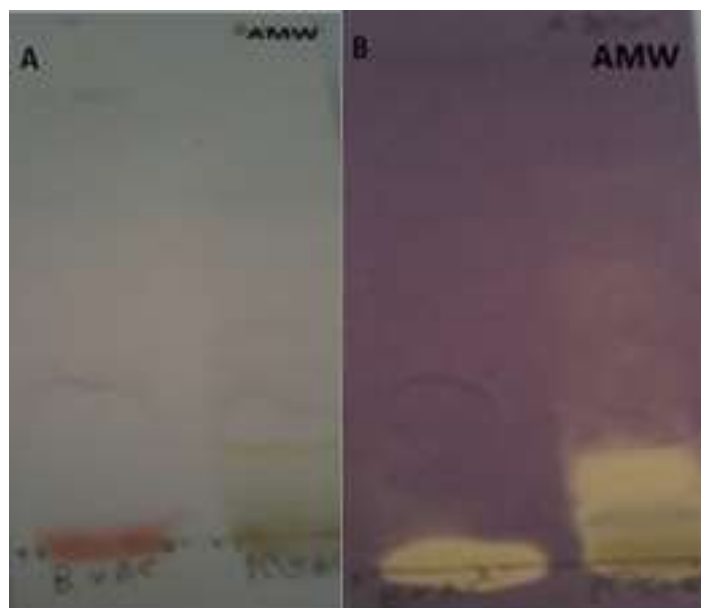


Figure 2: Chromatograms of acetone extracts of defatted seeds of *B. variegata* (BVAc) and non-defatted seeds of milk thistle (MSAc). (A) TLC plate developed in acetone-methanol-water (AMW, 6:2:1, v/v/v) and sprayed with vanillin reagent. (B) TLC plate developed in AMW and sprayed with DPPH.

The intermediate mobile phase (AMW) developed several compounds with various colours in both BVAc and MSAc (Figure 2A). It also revealed that the nature of some compounds with antioxidant activity in MSAc, may be different from that in BVAc. Some of the active compounds in MSAc are intermediate, while those in BVAc are neither non-polar nor intermediate (Figure 2B). These results demonstrate that a BVAc compound with antioxidant activity may be a complex as compared with that detected in Figure 1.

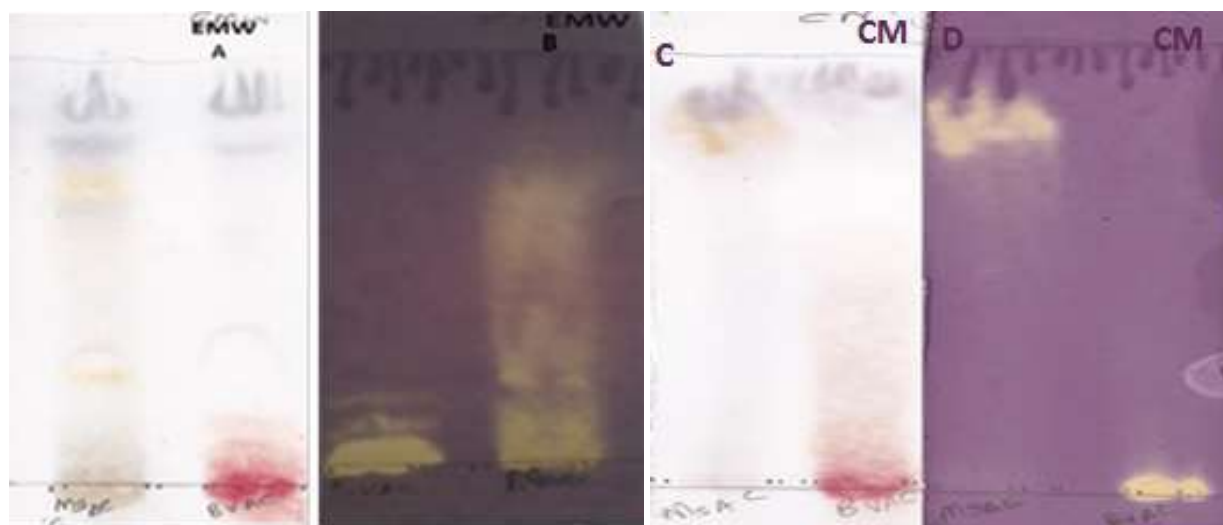


Figure 3: Chromatograms of acetone extracts of non-defatted seeds of milk thistle (MSAc) and defatted seeds of *B. variegata* (BVAc). (A) TLC plate developed in ethyl acetate-methanol-water (EMW, 96:3:1, v/v/v) and sprayed with vanillin, (B) TLC plate developed in EMW and sprayed with DPPH, (C) TLC plate developed in chloroform-methanol (CM, 1:1, v/v) and sprayed with vanillin (D) TLC plate developed in CM and sprayed with DPPH.

Polar mobile phase (EMW) developed different compounds of black, blue, yellow and brown colours in MSAc and BVAc. Both extracts indicated similar compounds; the last band at the top is dark blue and brown in colours. BVAc indicated a red thick band with antioxidant activity at the bottom of the chromatogram. Most MSAc bands indicated antioxidant activity (Figures 3A and B).

Polar mobile phase CM was also used, resulting in MSAc compounds with antioxidant activity moving to the top of the TLC plate, suggesting that this extract contains more polar antioxidant compounds. The BVAc compounds with antioxidant activity were not separated by CM, suggesting this extract contains antioxidant compounds with dissimilar polarity than MSAc (Figures 3C and D). These results demonstrate that an MSAc compound with antioxidant activity may be a complex made of more polar, moderate neutral and less non-polar compounds, compared with the chromatograms of Figures 3A and B.

BVAc compound with antioxidant activity may be a complex made of polar (Figures 3A and B) and moderate neutral compounds compared with the chromatograms of Figures 2A and B. Following several optimisations of the mobile phases, it is acknowledged that the TLC plates remain under-developed in the four mobile phases. The TLC-DPPH results allowed the study to elucidate antioxidant compounds in the extracts of *B. variegata* seeds in comparison with those of milk thistle.

FeCl₃-induced lipid peroxidation in chicken liver homogenate

Time and concentration-dependent of FeCl₃-induced lipid peroxidation in chicken liver homogenate was measured by using the TBARS method (Figure 4). At zero minute, the measured absorbance was below 0.01. The reading of samples taken at zero minute slightly increased as FeCl₃ concentration increases from 0 to 33 mM. After 30 minutes of incubation at 37°C, the absorbance readings were taken and they were above 0 minute as expected. At 30 minutes of incubation, concentration of 33 mM reached the absorbance reading of 0.032, indicating incubation time influences the induction of lipid peroxidation by FeCl₃. From 0 to 8 mM, a change in absorbance readings in all series was high. From the concentration of 8 to 17 mM, there was a gradual increase in both absorbance readings and TBARS levels. All the concentrations increase, followed the same trends, being time-dependent as observed after 60 and 90 minutes respectively, of incubation. The shape of the graph indicated, when the FeCl₃ concentrations increase, an increase in the absorbance with incubation time occurred (Figure 4A). The MDA/mg of liver weight indicated the higher the incubation time and concentration of FeCl₃ lead to increase in the levels of TBARS, defined as MDA/mg of liver. FeCl₃ at final concentration of 8 mM resulted in a linear graph, even after 60 minutes of incubation compared to other concentrations used (Figure 4B).

Percentage lipid peroxidation (TBARS) inhibition

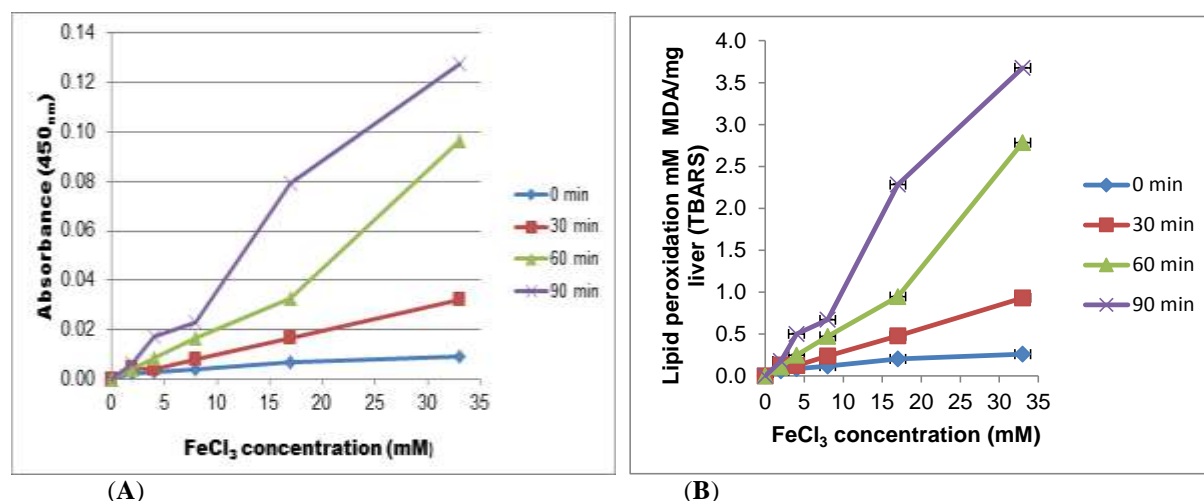


Figure 4: Time and concentration-dependent of FeCl₃-induced lipid peroxidation in chicken liver homogenates, measured in triplicate. (A) Absorbance at 450 nm, (B) Lipid peroxidation levels.

Lipid peroxidation inhibition potential of ascorbic acid, BVAc, BVMe, MSAc and MSMe against 8mM FeCl₃-induced lipid peroxidation in chicken liver homogenate was conducted using the TBARS assay (Figure 5). From the lipid peroxidation induction assay (Figure 4), 8mM FeCl₃ and 60 minutes incubation time were chosen in the lipid peroxidation inhibition experiment with the seed extracts and ascorbic acid. The blank, control and experimental samples were incubated at the same conditions (Table 2).

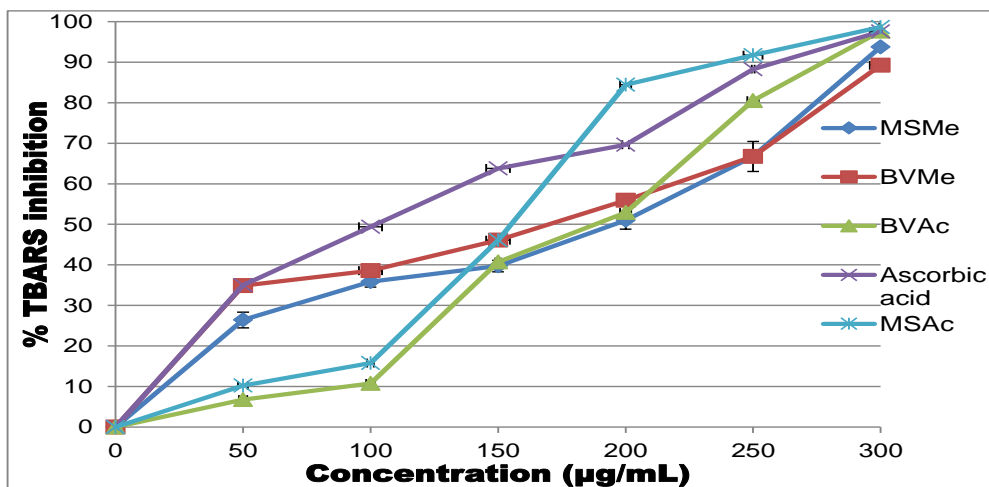


Figure 5: % TBARS inhibition with ascorbic acid, MSAc, MSMc, BVAc and BVMe. **BVAc**, *B. variegata* acetone extract, **BVMe**, *B. variegata* methanol extract, **MSAc**, milk thistle acetone extract, **MSMe**, milk thistle methanol extract.

All the extracts indicated activity by inhibiting FeCl₃-induced lipid peroxidation in the liver homogenate (Figure 5). Ascorbic acid indicated the highest activity at the initial concentration. At higher concentration, MSAc indicated the highest inhibition of TBARS (lipid peroxidation). Both MSAc and BVAc extracts at the concentrations range from 50 to 100 µg/mL inhibited less than 20% of TBARS. With the same concentration ranges, BVMe, MSMc and ascorbic acid were above 20% of TBARS inhibition.

Ascorbic acid at 100 µg/mL concentration indicated 49% of TBARS inhibition. This indication supports effectiveness of ascorbic acid at a lower concentration than those of the seed extracts. At 150 µg/mL concentration, both BVAc and MSMc indicated the same TBARS inhibition of 40%; a drastic increase in BVAc and a slight increase in MSMc. This might be a cause of MSMc inhibition already being above 20% at 100 µg/mL concentration, while BVAc's was less than 20%.

Both BVMe and MSAc also indicated the same inhibition of 46% at 100 µg/mL concentration. This was a high increase in MSAc from 15.8% to 46%, while the increase was slight in BVMe, indicating the TBARS inhibition of 40% at similar concentration. This revealed that BVMe inhibition increased with 6%. Ascorbic acid maintained the highest TBARS inhibition when compared with all the seed extracts; it achieved 63.8% at 100 µg/mL. From concentration of 200 to 300 µg/mL, MSAc and ascorbic acid (positive controls) indicated the highest TBARS inhibition abilities. MSAc indicated an increase from 46% to 84%; the highest change in inhibition ability. From 84% it started to increase slowly to 98.7% and inhibition was reached. Ascorbic acid was overlapped by MSAc, but still higher than MSMc, BVAc and BVMe extracts. Ascorbic acid at the highest concentration of 300 µg/mL indicated 97.5% TBARS inhibition.

BVAc increased rapidly from 200 g/mL with TBARS inhibition of 52.9% to 250 g/mL with 80.5%. In addition, BVAc overlapped ascorbic acid at 200 µg/mL concentrations. At 300 µg/mL concentration, BVAc indicated 97.6% TBARS inhibition, 0.1% higher than that of ascorbic acid. Methanol extracts of both plants (BVMe and MSMc) indicated similar patterns of TBARS inhibition at 250 µg/mL. Highest concentrations of MSMc and BVMe indicated TBARS inhibition of 93.8% and 89%, respectively (Figure 5). All extracts inhibited TBARS production in a concentration dependent manner, compared with ascorbic acid.

Discussion

The antioxidant and hepatoprotective potential of the acetone and methanol extracts of defatted seed of *B. variegata* comparing to non-defatted seed of milk thistle were investigated with the *in vitro* model. This study has limitations, including lack of affordability to use the modern qualitative methods such as liquid chromatography-mass spectrophotometry (LC-MS) amongst others, and screening of phytochemicals.

Preliminary screening of the phytochemicals revealed the presence of phenolics and flavonoids in the extracts of both *B. variegata* and milk thistle (Table 3). Separation of secondary metabolites on the TLC chromatograms was not well developed (Figures 1 to 3). Various mobile phase systems were used, but none of them could successfully develop the antioxidant compounds and other unknown compounds in the plant extracts.

Further studies are required to optimise the mobile phases (non-polar, intermediate and polar) to successfully develop compounds of the acetone defatted seed extract of *B. variegata*. Various compounds with numerous colours were observed in the TLC plates sprayed with vanillin. The collective colours in acetone extract of *B. variegata* were blue,

yellow and red. Some of these compounds were also present in the acetone extract of milk thistle, used as a positive control in this study, due to its known antioxidant and hepatoprotective effects (Anthony and Saleh, 2012).

The TLC-DPPH system analysed the antioxidant potential of *B. variegata* seed extracts compared with those of milk thistle. The *B. variegata* acetone extract indicated antioxidant activity, even when the compounds were not well developed, compelling it impossible identifying a specific compound with antioxidant activity. One developed band of both BVAc and MSAc on the same position indicated antioxidant activity. This indicates, milk thistle and *B. variegata* extracts might contain some similar phytochemicals (Olaleye et al., 2010).

To determine whether the plant extracts possess antioxidant activity *in vitro*, lipid peroxidation was induced by FeCl₃. Inducing lipid peroxidation by FeCl₃ uses the same mechanism as CCl₄ generating biological active phospholipids, including N-modified phospholipids (Davies and Guo, 2014). FeCl₃ was used as it contains iron, with a function in many reactions in the body. Iron ions can bind to many molecular moieties including proteins; excess iron in the body mediates molecular damage (Poddar, et al., 2004). FeCl₃ does not only damage the liver, but it also damages the arteries conveying blood (Li et al., 2013). The increase in the concentration of FeCl₃ leads to more production of MDA. According to Ayala et al. (2014), MDA concentration measures the damage inside the liver. In this study, MDA-TBA adduct was measured at 450 nm, due to the spectrophotometer having fixed filters of 450 nm, 560 nm, 600 nm and 700 nm (Figure 4).

TBARS was used again, measuring inhibition of lipid peroxidation in plant extracts and ascorbic acid. Various concentrations were used to monitor hepatoprotective effect of extracts. Ascorbic acid confirmed the antioxidant activity causing it to be used as positive control in this study (Mohsen et al., 2014). The presence of antioxidant compounds in milk thistle was again confirmed by the activity of the acetone extract in the inhibition of lipid peroxidation, after it was confirmed by DPPH on the TLC plates (Ghosh et al., 2010). Other plants such as *chrysanthemum fontanesii* leaf extracts have antioxidant activity, proven to have ascorbic acid as one of the vitamins (Amrani et al., 2014). Only few plants are found with antioxidant activity from the seed extracts, like *B. variegata* and milk thistle. Most of the seeds of various plants are not consumed by animals. This is an advantage as there will be more seeds available for medical purposes and a disadvantage that seeds are needed for dispersal of the plant. Seeds extract of milk thistle was confirmed active in rats (El-Gazayerly et al., 2014).

From the preliminary results of phytochemical screening, it was found that both plant extracts contain phenolic and flavonoids compounds. The presence of polyphenols is known, providing the plant antioxidant activity (El-Gazayerly et al., 2014). Flavonoids are also known with a potential activity against oxidative stress. As observed in this study, milk thistle extracts have the higher content of phenols and flavonoids compounds, possessing higher percentage of lipid peroxidation (TBARS) inhibition of up to 98.7% as compared with up to 97.6% of *B. variegata* extracts (Figure 5). Silymarin is a flavonoid complex compound of milk thistle, found to have an antioxidant and hepatoprotective on carbon tetrachloride-induced hepatotoxicity in rats (El-Gazayerly et al., 2014). It remains necessary to test for similar compounds that may be common in seed extracts of milk thistle and *B. variegata* or whether there are unknown compounds, requiring identification (Althagafy et al., 2013).

The methods and solvents used for extraction have some effects on the activity of plant part extract. Defatting seeds of *B. variegata* removes lipids (Arain et al., 2012) permitting inactivate antioxidant activity in their presence. Lipids may also distract the reaction and mechanism of extract reacting with FeCl₃ in the liver. Variations of solvents extract different compounds depending on their polarities. Whether plant materials were boiled or soaked can also affect the activity of extract. Extracts in this study were prepared at room temperature to avoid denaturing active compounds of seeds. It was difficult to measure the Retention factor (R_f) values of the studied extracts of both plants because the TLC plates were not well developed. TLC profiling and qualitative analysis of sequential extracts of defatted seeds of *B. variegata* provide preliminary evidence of the presence of phytochemicals with antioxidant and hepatoprotective potential against FeCl₃-induced lipid peroxidation in chicken liver homogenate.

Conclusion

Ferric chloride was confirmed to induce lipid peroxidation in chicken liver homogenate at time- and concentration-dependent manner. With phenols and flavonoids present in 80% acetone defatted seeds extract of *B. variegata* proves antioxidant activity, also observed on the TLC-DPPH systems. This observation may support hepatoprotective ability of seed extracts of *B. variegata* against FeCl₃ induced-lipid peroxidation. Both acetone and methanol extracts of this plant collected after defatting seeds with n-hexane indicated hepatoprotective activity against lipid peroxidation by TBARS inhibition at a concentration-dependent manner. These findings were comparable to those of ascorbic acid and non-defatted seed extracts of milk thistle, respectively. The study concludes that treatment of FeCl₃-induced lipid peroxidation with defatted seed extracts of *B. variegata* was effective. Further studies are recommended to test for the cytotoxicity and other metabolic effects of defatted seed extracts of *B. variegata* using the *in vitro* and *in vivo* models. Purification and identification of acetone defatted seed extract of *B. variegata* are also recommended for future studies. These recommendations can assist preventing and treating various diseases mediated by oxidative stress in humans and animals using this seed extract of *B. variegata*.

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Conflicts of Interest: The authors declare that this research presents no conflicts of interest.

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