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Antioxidant Activities of Methanol and Dichloromethane Blend Extracts of *Caesalpinia volkensii* Harms. and *Carissa edulis* (Forssk.) in Vitro

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Abstract:- Oxidative stress is the primary cause of many human ailments, including aging. Synthetic antioxidants are unaffordable and are associated with severe effects. This necessitates the need for alternative antioxidant agents. This investigation aimed to determine the antioxidant activities and qualitative phytochemical composition of DCM and MeOH blend extracts of root barks of *Carissa edulis* and leaves of *Caesalpinia volkensii*. The antioxidant assays included ferric reducing antioxidant power (FRAP), H_2O_2 radical scavenging and DPPH radical scavenging activities, as well as total flavonoid and total phenolic content tests. The extracts revealed potent FRAP and DPPH and H_2O_2 radical scavenging activities, including a considerable amount of total flavonoids and phenolics contents. The *C. edulis* extract noted better antioxidant activities than *C. volkensii* extract. The antioxidant effects of the two studied extracts were concentration-dependent. In addition, the *C. edulis* extract had a considerably higher amount of total phenolic and total flavonoid contents relative to *C. volkensii* extract. Phenolics, terpenoids, alkaloids, cardiac glycosides, saponins, steroids, and flavonoids were detected in the qualitative phytochemical analysis, except for alkaloids and steroids in *C. volkensii* extract and cardiac glycosides in *C. edulis* extract. In conclusion, the two extracts have potent antioxidant activities and are endowed with phytochemicals associated with antioxidant activities. The two extracts, therefore, may be used as alternative antioxidant agents.

Keywords:- *Antioxidant; oxidative stress; phytochemicals; flavonoids; phonolics*

I. INTRODUCTION

Reactive oxygen species (ROS) refers to extremely reactive molecules with oxygen atoms and unpaired electrons. These molecules can damage biomolecules, including proteins, lipids, and deoxyribonucleic acid, thereby causing many pathological conditions in the body [1]. Free nitrogen radicals, singlet oxygen, hydroxyl radicals and superoxide anion radicals are some examples of ROS [2]. Free radicals are produced due to intense physical exercise, improper diet, long-term stress conditions and exposure to ultraviolet radiation [1]. In a normal cell, an appropriate balance between oxidants and antioxidants exists [3]. When the oxidants levels increase and antioxidants decrease, this balance shifts, causing oxidative stress that causes physiological disorders such as Parkinsons disease, Alzheimers disease, atherosclerosis, diabetes mellitus, premature aging and cancer, among others [4]. The harmful action caused by ROS can be naturally controlled by enzymatic antioxidants such as glutathione, superoxide dismutase and catalase, among others [5]. Free radicals can be inhibited, scavenged, or chelated by antioxidants. Antioxidants convert free radicals into safe molecules by donating an electron or an active hydrogen atom [6].

Synthetic antioxidants like butylated hydroxytoluene, propylgallate and tertiary butyl-hydroquinone are known to ameliorate oxidative stress. However, the use of these antioxidants is associated with cancer and liver toxicity [7]. For this reason, there has been a lot of interest in searching for alternative agents that are efficacious, and non-toxic. Lately, the utilization of natural antioxidants in the treatment of ailments and disorders has drawn more attention [8]. Many medicinal plants have long been used to relieve oxidative stress [9]. Nevertheless, there is insufficient scientific evidence to validate these claims [10].

Medicinal plants possess phytochemicals (secondary metabolites) that exert antioxidant activities. These secondary metabolites such as saponins, alkaloids, phenolic acids, carotenoids, tocopherols, flavonoids and cinnamic acids have been documented to possess potent antioxidant effects [11]. They ameliorate oxidative stress through scavenging or mopping up free radicals [12]. The root barks of *Carissa edulis* and leaves of *Caesalpinia volkensii* are used traditionally by Kenya communities to manage oxidative stress. However, no empirical scientific data have been documented to ascertain these claims. This investigation aimed to assess *in vitro* antioxidant effects, including total phenolic and total flavonoid contents of *C. edulis* and *C. volkensii*, as well as qualitative phytochemical composition.

II. MATERIALS AND METHODS

A. *Medicinal sample collection and preparation*

Fresh root barks of *C. edulis* and leaves of *C. volkensii* were collected with the assistance of a traditional medical herbalist from Mbeere North, Embu County, Kenya. The medicinal samples were availed to a recognized taxonomist for botanical identification and voucher specimens (SJK 001 and SJK 002 for *C. edulis* and *C. volkensii*, respectively) deposited in the herbarium of Kenyatta University. Samples were then sorted out properly, cleaned, chopped, air-dried and milled into a fine homogenous powder.

B. *Extraction*

500 grams of each finely powdered medicinal sample was soaked in two liters of MeOH and DCM in the ratio of 1:1 followed by regular shaking for two hours and then let to stand for 48 hours. The mixture was passed through Whatman filter paper number 1, and then the filtrate concentrated using a rotary evaporator under reduced pressure (40 °C). A semisolid extract was then dried using an oven at 38 °C.

C. *Determination of FRAP*

A method described by [13] was used to assess the FRAP of the two studied extracts. The extracts as well as the standard used concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. One milliliter of the extract was put into 2.5 ml of phosphate buffer (pH 6.6, 0.2 M). The resultant mixture was added to 2.5 ml of potassium ferricyanide, incubated for 20 minutes at 50 °C and consequently centrifuged at 3000 rpm (revolutions per minute) for 10 minutes after adding 2.5 ml of 10% trichloroacetic acid. 2.5 ml of the supernatant was aspirated, and then blended with 2.5 ml of freshly made $FeCl_3$ solution and distilled water (2.5 ml). The assay used distilled water as the blank. A spectrophotometer was used to determine the optical densities of the samples at a wavelength of 700 nm.

D. *Determination of DPPH radical scavenging activity*

A protocol used by [14] was adopted to assess the DPPH scavenging effects of the two extracts. 50 ml of MeOH was used to dissolve 2.66 mg of DPPH to prepare a 0.135 mM DPPH radical solution. 1 ml of each extract or ascorbic acid concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml was mixed with 1 ml of the DPPH solution. The resultant mixture was vortexed and then allowed to stand for half an hour in a dark room. MeOH served as the blank. The sample absorbances were determined at 517 nm by the use of a spectrophotometer. The samples' percentage DPPH radical scavenging activities were calculated as follows:

$$\text{DPPH scavenging activity (\%)} = \frac{Ac - As}{Ac} \times 100$$

Where,
As = Sample absorbance
Ac = Blank absorbance (control)

E. *Determination of hydrogen peroxide radical scavenging activity*

A procedure described by [15] was used to assess the two extracts' *in vitro* H_2O_2 radical scavenging abilities. 4.53ml of H_2O_2 was diluted with 1 L of phosphate buffer (0.1 M; pH 7.4) to form 40 Mm of H_2O_2 solution. Concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/ml were prepared for the two extracts and ascorbic acid. Then, 1 ml of the sample and 2 ml of the H_2O_2 solution were mixed for 10 minutes. The phosphate buffer solution served as the blank. The sample absorbances were detected at 560 nm by the use of a spectrophotometer. The H_2O_2 radical scavenging (%) effect was computed as follows:

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{Ac - As}{Ac} \times 100$$

Where,
As = Sample absorbance
Ac = Blank absorbance (control)

F. *Evaluation of total flavonoid contents*

A method described by [16] was adopted to assess for total flavonoid content. 0.5 ml of the extract (1 mg/ml) was blended with 10% $AlCl_3$ (0.1 ml), 1 M potassium acetate (0.1 ml), MeOH (1.5 ml), and distilled water (2.8 ml) to make a volume of 5 ml. It was then left standing for 30 min. Distilled water was utilized as the blank and quercetin as the standard. The samples' absorbances were measured at 415 nm using a spectrophotometer. Concentrations of 50, 100, 150, 200 and 250 mg/ml of quercetin were used to draw a standard curve. Afterwards, the total flavonoid concentration was calculated using the standard calibration curve equation, and the findings were presented as milligrams of quercetin equivalence per gram of the extract.

G. *Evaluation of total phenolic contents*

A protocol by [17] was used to assess total phenolic contents. A volume of 2.5 ml of Folin-Ciocalteau's reagent (diluted 1/10) and two milliliters of sodium bicarbonate (7.5%, w/v) were added to each sample (0.5 ml) prior to incubation at 45°C for 15 minutes. Using a spectrophotometer, the sample absorbances were detected at 765 nm. Gallic acid (standard) values of 50, 100, 150, 200, and 250 mg/ml were utilized to draw a standard curve. The gallic acid equivalence was computed using a standard curve equation and results were expressed as milligrams of gallic acid equivalence per gram of the extract.

H. *Qualitative phytochemical analysis*

The DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* were subjected to several assays to determine their qualitative phytochemical analysis.

➢ *Alkaloids test*

Five milliliters of each extract was first acidified with 1 M HCl. After heating the acidic medium, Dragendroff's reagent was added. Alkaloids were present when reddish-brown or orange precipitate formed [18].

➢ *Flavonoids (Sodium hydroxide test)*

Two milliliters of each extract was blended with two milliliters of diluted NaOH. Positive outcomes were indicated by an intense/golden-yellow precipitate [19].

➢ *Terpenoids (Salkowski test)*
After mixing 0.5 g of each extract with chloroform (2 ml) and 1 ml of petroleum ether, 3 ml of concentrated H_2SO_4 was gradually added to form a layer. Terpenoids were identified by a reddish-brown colored interface [20].

➢ *Saponins (Froth test)*
Two milliliters of each extract was blended with a few drops of $NaHCO_3$ solution, vigorously shaken, and let to stand for 15 to 20 minutes. The formation of foam exceeding 1 cm was indicative of saponins [21].

➢ *Steroids test*
Two milliliters of chloroform was used to dilute a mass of 0.5 g of the extract. The test tube's sides were carefully filled with 3 ml of concentrated H_2SO_4 to form a layer. The steroidal ring formed a reddish-brown coloration at the interface [15].

➢ *Phenolic test*
One milliliter of $FeCl_3$ solution was put into 2 ml of the extract. Phenolics were present as evidenced by the formation of a blue to green coloration [22].

➢ *Cardiac glycosides (Keller-Kilian test)*
A volume of two milliliters of glacial acetic acid and 2 drops of a 10% $FeCL_3$ solution were used to dissolve 0.5 g of the extract. Consequently, one milliliter of concentrated H_2SO_4 was added slowly. Cardiac glycosides were indicated by a violet, brown, or greenish ring at the interphase [23].

I. *Statistical data analysis*
Raw data was tabulated in the Microsoft Excel Spreadsheet, cleaned and exported to GraphPad Prims version 9 statistical software for analysis. The mean±standard deviations (SD) were used to express descriptive statistics. One-way ANOVA (analysis of variance) was utilized to perform inferential statistical analysis between distinct treatment groups. Tukey's multiple comparisons were computed to ascertain the group differences when one-way ANOVA revealed a significant variation. An independent t-test was computed to compare the total flavonoid content and total phenolic content of the two extracts. The level of significance was set at p less than 0.05. Tables and graphs were used to present the study outcomes.

III. RESULTS

A. *Ferric reducing antioxidant power*
The DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml revealed FRAP (Figure 1). The two extracts at 0.2, 0.8 and 1 mg/ml noted a considerable variation ($p>0.05$) in FRAP. However, the FRAP of *C. edulis* extract concentrations of 0.4 and 0.6 mg/ml were statistically greater than those ($p<0.05$) recorded in *C. volkensii* extract. The FRAP of ascorbic acid was substantially higher relative to those of the two extracts ($p<0.05$) at the same concentrations (Figure 1).

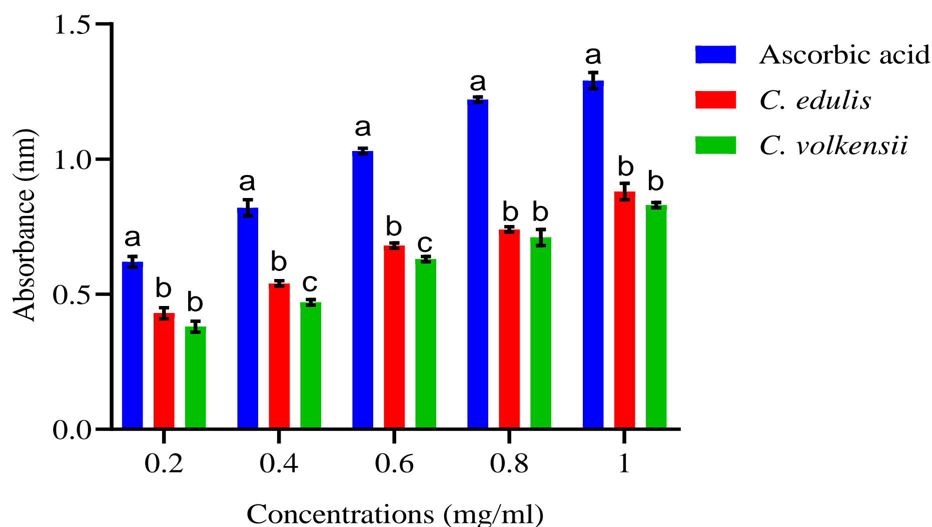

Fig. 1: FRAP of *C. edulis* and *C. volkensii* extracts. Bars with distinct letters differ statistically ($p<0.05$) at the same concentration using one-way ANOVA and Tukey's multiple comparisons.

B. *DPPH radical scavenging activity*
The DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* noted potent *in vitro* DPPH scavenging effects (Figure 2). At the corresponding concentrations, the effect of *C. edulis* extract had a considerably higher percentage of DPPH radical scavenging activity than the effect of *C. volkensii* extract ($p<0.05$). The effect of the standard had a statistically higher percentage of DPPH radical scavenging activity relative the effect ($p<0.05$) of the two extracts at all the tested concentrations (Figure 2). The ascorbic acid as well as *C. edulis* and *C. volkensii* extracts had half-maximal inhibitory concentration (IC_{50}) of 2.16, 3.15 and 3.52, respectively.

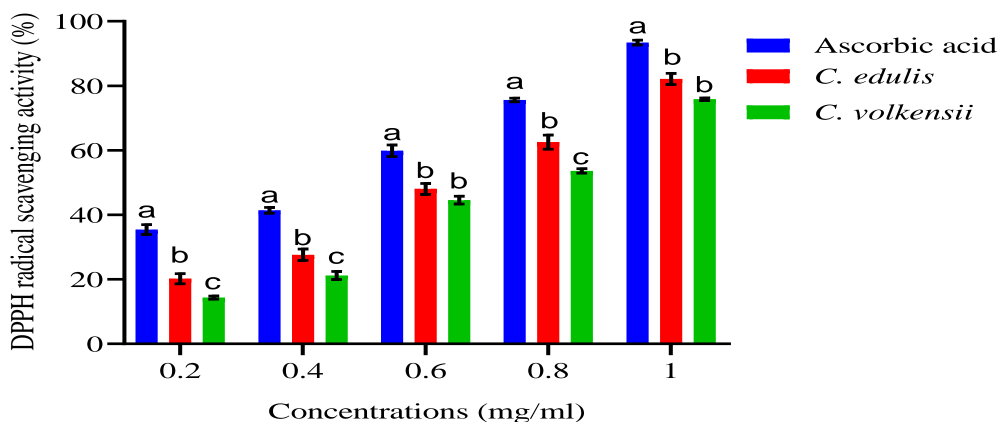

Fig. 2: DPPH radical scavenging effects of *C. edulis* and *C. volkensii* extracts. Bars with distinct letters differ statistically ($p<0.05$) at the same concentration using one-way ANOVA and Tukey's multiple comparisons.

C. Hydrogen peroxide radical scavenging activity

The DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* showed *in vitro* H_2O_2 radical scavenging effects at all the concentrations tested (Figure 3). At the concentrations of 0.2, 0.6, 0.8, and 1 mg/ml, the percentage of H_2O_2 radical scavenging activity of the *C. edulis* extract was significantly higher than that of the *C. volkensii* extract ($p<0.05$). Ascorbic acid exhibited a significantly higher percentage of H2O2 radical scavenging activity when compared to the two extract concentrations of 0.2 and 0.4 mg/ml ($p<0.05$). Nevertheless, the percentage of H_2O_2 scavenging activity of the standard and *C. edulis* extract showed no significant differences at the concentrations of 0.6, 0.8, and 1 mg/ml ($p>0.05$; Figure 3). The IC_{50} of *C. edulis* and *C. volkensii* extracts as well as ascorbic acid were 1.45, 2.08 and 0.19, respectively.

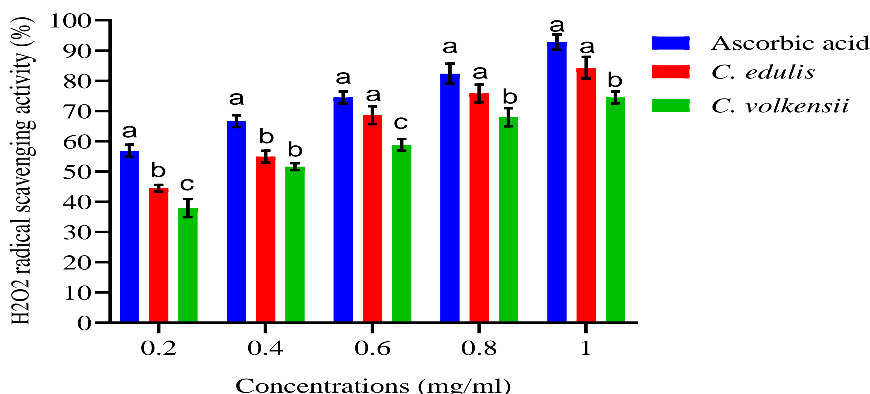

Fig. 3: Hydrogen peroxide radical scavenging effects of C. edulis and C. volkensii extracts. Bars with distinct letters differ statistically ($p<0.05$) at the same concentration using ANOVA and Tukey's multiple comparisons.

D. Total phenolic and total flavonoid contents

The *C. volkensii* extract had significantly lower total flavonoid and total phenolic contents than those of *C. edulis* extract ($p<0.05$; Figure 4).

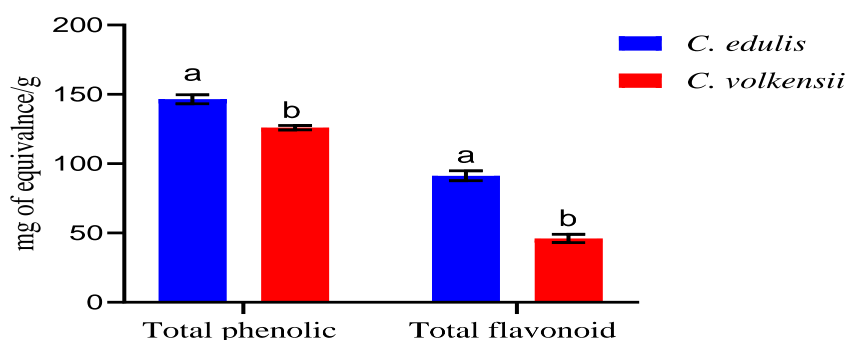

Fig. 4: Total flavonoid content and total phenolic content of *C. edulis* and *C. volkensii* extracts. Bars with distinct lowercase letters differ significantly using ($p<0.05$) an independent t-test.

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E. Qualitative phytochemical screening

The results demonstrated that *C. edulis* extract had saponins, terpenoids, alkaloids, steroids, flavonoids, cardiac glycosides and phenolics. On the other hand, *C. volkensii* extract had saponins, terpenoids, cardiac glycosides, flavonoids, and phenolics. However, cardiac glycosides were absent in *C. edulis* extract, while steroids and alkaloids were absent in *C. volkensii* extract (Table 1).

Table 1: Qualitative phytochemical composition of *C. edulis* and *C. volkensii* DCM and MeOH blend extracts

Phytochemicals	***C. edulis***	***C. volkensii***
Saponins	+	+
Alkaloids	+	-
Flavonoids	+	+
Terpenoids	+	+
Cardiac glycosides	-	+
Steroids	+	-
Phenolics	+	+

+ = present; - = absent

IV. DISCUSSION

Oxidative stress causes numerous ailments like diabetes mellitus, arthritis, cancer, Alzheimer's, liver cirrhosis, including aging. This occurs as a result of an imbalance between antioxidants and oxidants produced in the body [24]. Synthetic antioxidant agents are used to manage oxidative stress. Nevertheless, these agents have been documented to possess severe effects [7], necessitating the need for alternative antioxidant agents. This study revealed that DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* had potent FRAP, H_2O_2 radical scavenging and DPPH radical scavenging activities, as well as a considerable quantity of total flavonoid and total phenolic contents, suggesting antioxidant activity. The two extracts also showed the presence of major phytocompounds that are linked with antioxidant potential.

FRAP analysis assesses the ability of antioxidants to donate electrons. It is an efficient and quick way to evaluate a substance's antioxidant impact [25]. A reducing agent contains atoms that can donate one or more of their electrons to react with free radicals and convert them into more stable products. This, therefore, terminates or blocks the radical chain reactions [26]. The substances that have FRAP react with potassium ferricyanide (Fe^{3+}) to generate potassium ferrocyanide (Fe^{2+}), which then reacts with $FeCl_3$ to generate a ferrous complex. This complex is Perl's Prussian blue in color. The maximum absorption of the ferrous complex is 700 nm. The FRAP of a compound is thus explained by the ability to donate an electron [27].

This study's findings noted that the two extracts had significant FRAP, an indication of antioxidant activity. The phytochemicals present in the two extracts were therefore attributed to the reduction of Fe^{3+} to Fe^{2+}. The FRAP of the two extracts suggested that the two extracts have reducers that serve as electron donors and can terminate radical chain reactions, hence minimizing tissue oxidative damage [28]. The standard (ascorbic acid) proved to have better ferric-reducing ability than the two extracts. Moreover, *C. edulis* extract had a considerably higher FRAP than *C. volkensii* extract. This could be explained by the fact that *C. edulis* had additional alkaloids that have been documented to possess antioxidant effects [29]. The increased absorbance at 700 nm indicated an increase in the extract's ferric-reducing abilities. The study also showed that the FRAP of the two extracts was concentration-dependent. Studies by [30-32] have reported similar findings on the FRAP.

DPPH generates a free radical and is used in determining radical scavenging activities [33]. The DPPH test is a popular choice for in vitro antioxidant screening due to its ease of use, convenience, and simplicity in evaluating antioxidant activity. The color of DPPH changes when an antioxidant substance donates an electron, and the electron is accepted by DPPH. The change of color from purple to yellow is an indication of the presence of a radical scavenger [34].

The results of this investigation show that the two extracts have the potential to scavenge DPPH radicals. The percentage of DPPH radical scavenging activity in *C. edulis* extract was significantly higher than that of *C. volkensii* extract. The findings also showed that ascorbic acid noted a considerably higher percentage of DPPH radical scavenging activity than those of the two extracts. Numerous studies have shown a correlation between higher percentages of DPPH radical scavenging activities of medicinal plant extracts and high total phenolic and total flavonoid contents [35]. The high levels of total flavonoid content and total phenolic content that were detected in the two extracts may be attributed to a greater percentage of DPPH radical scavenging activities. As a result, the two examined extracts may be regarded as proton donors and may be utilized as substitute medicinal agents for the treatment of oxidative stress.

The two extracts' abilities to scavenge DPPH radicals followed a dose-dependent pattern. The percentage of DPPH radical scavenging activity was highest in the highest concentrations of the extracts. The findings on DPPH radical scavenging activity were consistent with those reported by [36-38].

The term "IC_{50}" is used to describe the quantity of extract required to scavenge 50% of the free radicals. The scavenging effects of the extracts are inversely correlated with IC_{50}. This is because the smaller the IC_{50}, the greater the scavenging potential [39]. According to the results of this study, *C. edulis* extract noted a considerably higher percentage of DPPH scavenging activity than that of *C.*

volkensii extract. These results, therefore, suggest that *C. edulis* extract had a better DPPH radical scavenging effect than *C. volkensii* extract.

Hydrogen peroxide is produced *in vivo* from a reaction of enzymes such as superoxide dismutase. They can rapidly cross the cell membrane and cause toxic effects by reacting with Cu^{2+} and Fe^{2+} ions, forming highly reactive hydroxyl radicals through Fenton reaction [40]. Polyphenols (flavonoids and phenolic acids) can protect body cells from the cytotoxicity effect that is caused by H_2O_2. The polyphenols have hydroxyl groups that have a remarkable potential to scavenge hydrogen peroxide [41]. Therefore, by inhibiting H_2O_2, the production of these reactive radicals will be prevented and hence the body systems are protected. The concentration of H_2O_2 is decreased by compounds that have scavenging effects. These compounds accelerate the conversion of H_2O_2 to water and oxygen [40].

This study demonstrated that the two studied extracts scavenged H_2O_2 in a concentration-dependent response. *Carissa edulis* extract showed a better H_2O_2 scavenging ability than *C. volkensii*. The differences in the H_2O_2 radical scavenging abilities could be associated with the moieties of their active compounds which determine their ability to donate electrons or active hydrogen atoms. The percentage of H_2O_2 radical scavenging effects of *C. edulis* and *C. volkensii* can be associated with phenolics and flavonoids. These polyphenols can donate electrons to H_2O_2 hence neutralizing it to form water and oxygen. This study's findings on H_2O_2 radical scavenging activity corroborate with other similar studies by [42, 43].

The antioxidant properties of medicinal plants are attributed to phytocompounds like flavonoids, alkaloids, phenolics, and even terpenoids [44]. The results obtained from qualitative phytocompounds analysis of *C. edulis* and *C. volkensii* extracts reported the presence of steroids, phenols, alkaloids, terpenoids and flavonoids, except for alkaloids in *C. volkensii* extract. The presence of these phytocompounds in the two extracts could be linked to their good antioxidant potential. The major phytochemicals that have been reported to possess antioxidant properties include flavonoids, phenolic saponins and alkaloids [45, 46]. Their antioxidant actions have been linked to their metal chelating and redox properties [47].

V. CONCLUSIONS

It was concluded that the DCM and MeOH blend extracts of *C. edulis* and *C. volkensii* had potent *in vitro* FRAP, DPPH radical scavenging and H_2O_2 radical scavenging activities, as well as considerable quantities of total flavonoid content and total phenolic content. The qualitative phytochemical analysis of the two extracts also revealed phytochemicals associated with antioxidant effects such as flavonoids, terpenoids, alkaloids and phenolics. The two extracts, therefore, may be used as novel alternative antioxidant agents against oxidative stress.

DECLARATION

A. *Author Contributions:*

- Scolar Jepkorir Kibiwott: Carried out experiments, analyzed data and reported data, as well as developed the manuscript.
- Mathew Piero Ngugi: Conceived the study idea, developed the study design, interpreted data, and supervised the development of the manuscript.
- Mary M. Ng'ang'a: Contributed reagents, provided technical support, interpreted data and proofread the manuscript.

B. *Funding*

This study did not secure external funding.

C. *Conflicts of Interest*

The authors affirm that they have no competing interests.

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