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Full Length Research Paper

Evaluation of *Procavia capensis* hyraceum used in traditional medicine for antioxidant activity

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Hyraceum (HM) used in traditional medicine in Southern Africa is produced by the herbivore *Procavia capensis.* It is fossilized excreta derived from urine, faecal matter and plant material. In this study a qualitative phytochemical screening, determination of the *in vitro* antioxidant activity using the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide scavenging methods, and determination of the total phenolic content in the crude methanolic (95%) extract were done. Phytochemical screening detected the major phytochemical classes in the hyraceum extract as terpenoids, saponins, polyphenols, quinones, phlobatannins and coumarins with the minor components as flavonoids, alkaloids, tannins, simple phenols, anthocyanins, anthraquinones and amino acids. Total phenolics content was 37.339 mg gallic acid equivalents per gram dry weight (mgGAE/g DW). Effective concentration at 50% (EC_{50}) for HM and L-ascorbic (AA) in DPPH assay was 5.983 and 0.429 µg/ml respectively while in H_2O_2 scavenging assay EC_{50} was 5.059 and 1.666 µg/ml, respectively. The antioxidant activity of HM could have been due to the various phenolic and terpenoid antioxidants in the HM. The findings implied that HM was slightly stronger at scavenging H_2O_2 than at scavenging DPPH. Bioactive compounds in HM could potentially be exploited in further studies as potential antioxidants of therapeutic value.

Key words: Phytochemicals, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide.

INTRODUCTION

The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is the trigger of non-communicable environmental disease (Zeliger, 2015). The ROS and RNS damage biological molecules, *in vivo* via oxidative stress. Elevated levels of ROS, acting *via* molecular level toxic effects, are now thought to be responsible for a wide spectrum of diseases, including lipid peroxidation of cellular membranes which causes decrease in membrane fluidity, DNA attack, adduction,

enzyme inhibition, oxidative attack on the central nervous system and cell signaling; all of which have been linked to non-communicable diseases (NCDs) including neurodegenerative diseases (such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis), cancer, cardiovascular disease, diabetes, and others (Basu et al., 1999; Ames et al., 1993; Kovacic and Somanathan, 2012).

Reactive oxygen species (ROS) are continuously

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generated inside the human body while the generated ROS are detoxified by antioxidants present in cells. However, over production of ROS or inadequate antioxidant defences can lead to oxidative damage of various biomolecules including proteins, lipids, lipoproteins and DNA. Free radicals are the major cause of chronic and degenerative diseases such as coronary heart diseases, inflammatory stroke, diabetes and cancer (Scalbert et al., 2005).

An antioxidant is generally defined as any substance that effectively prevents or delays the adverse effects caused by free radicals and the amount of the antioxidant is less than that of the substance to be oxidized (Halliwell and Gutteridge, 1999). An antioxidant significantly delays or prevents oxidation of cell components (susceptible to oxidation) such as proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA). Due to their redox properties, antioxidants act as reducing agents, hydrogen donors, singlet oxygen quenchers and chelating metal (Tung et al., 2009; Lauro and Francis, 2000). Although several synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available, their toxicity has always been a concern and strong restrictions have been placed on their application in pharmaceuticals. There is therefore a need for more effective, less toxic and cost effective antioxidants. Recently, there has been an upsurge of interest in the therapeutic potential of plant-derived antioxidants in reducing free radical-induced tissue injury and the current trend is to substitute synthetic with naturally occurring antioxidants (Barlow, 1990). Several biologically active compounds of plant origin (phytochemicals) have been found to possess antioxidant, free radical scavenging activity and many are being applied therapeutically for free radical associated disorders (Lee et al., 2000).

The concoctions used in traditional medicine contain many secondary metabolites (SMs) of plant origin such as polyphenolic compounds and polysaccharides which interact synergistically (Eid et al., 2013, 2012; Mulyaningsih et al., 2010; Wink, 2015). The therapeutic benefit of plant-based traditional medicine is often attributed to the antioxidant properties and potential of the constituent phytochemicals (Hertog et al., 1993; Zhang et al., 2001).

In Lesotho, as in many other countries in the world, a system of traditional medicine based on the use of plants, birds, animals, their products and their combinations to treat a broad spectrum of communicable and non-communicable diseases is still being practiced (Padmanabhan and Sujana, 2008).

One of such products used in Lesotho from animals is the fossilized excreta of *Procavia capensis* (rock hyrax) called *hyraceum* or *"moroto oa pela"* in Sesotho. The Basotho use hyraceum to treat respiratory infections, urinary tract or bladder infections, measles and non-communicable diseases such as *diabetes mellitus.*

The hyraceum is also used in combination with other medicinal plant species to enhance its efficacy (Seleteng-Kose et al., 2015). The strongly aromatic hyraceum is also a well-known Khoikhoi medicine, often used as a post-natal medicine for mothers and babies and a remedy for hysteria and epilepsy (van Wyk, 2008). The fossil is formed from the faeces and urine of *P. capensis* as the major components which accrete to form dark brown, resin-like masses to which plant material, pollen grains and other digestive remains are trapped. Fossilization occurs with time in arid regions. *P. capensis* inhabits shelters in rocky outcrops in a variety of biomes and feeds on a variety of grasses, shrubs, tree leaves, fruits and berries including the bark of the tree (Olsen et al., 2008).

The only literature found on the biological activity of hyracei was that on its effect on the GABA-benziodiazepin receptor, which indicated that since the hyracei exhibited a high affinity for GABA-benziodiazepin they could be used to treat epilepsy, a non-communicable disease (Olsen et al., 2008). Other than that, no records were found on the scientific validation of traditional medicinal uses of hyraceum (van Wyk, 2008).

In view of complex nature of hyraceum, composed of animal metabolic waste and plant materials and its use to treat both communicable and non-communicable diseases, the present study was performed and involved the analyses of the crude methanolic (95%) extract of hyraceum for the content of phytochemical and antioxidant activity and the relationship between the total phenolic content and antioxidant activity.

MATERIALS AND METHODS

Hyraceum material

A sample of hyraceum was purchased at the main Maseru open air market. Maseru is the capital of Lesotho with the geographical coordinates of; Latitude: -29° 19' 0.01" S, Longitude: 27° 28' 59.99" E. Part of the sample was fragmented and dried in a fanned oven (Labcon) at 35°C to a constant weight and brittle. The dried hyraceum was ground to a fine powder using an electrical pulverizer (Kenwood) and assessed for antioxidant activity.

Preparation of extract

The crude methanolic (95%) extract of hyraceum was prepared according to the method of Adedapo et al. (2009) with slight modifications. Ground material was soaked with 95% methanol (v/v) in distilled water at 2 ml/g and shaken on an orbital shaker for 24 h. The extract was filtered through No. 1 Whatman filter paper using suction. The filtrate was concentrated using a Gallenkamp rotary evaporator and oven dried at 35°C overnight to constant weight and stored in glass Petri dish at 4°C until used.

Chemicals

All solvents and reagents were of analytical grade. L-ascorbic (AA), gallic acid (GA), Folin-Ciocalteau's phenol reagent and 1, 1-

Diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich, St Louis, MO, USA. Methanol (absolute) was a product of Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa); Hydrochloric acid, Glacial acetic acid and Hydrogen peroxide were products of UNILAB (Krugersdorp, South Africa). All spectrophotometric measurements were done on the Shimadzu 1201 UV-VIS Spectrophotometer (Shimadzu, Kyoto, Japan).

Qualitative phytochemical screening

The hyraceum extract was subjected to a qualitative screening for the presence of major phytochemical classes using standard phytochemical methods and the appropriate reagents and chemicals according to the modified methods of Trease and Evans (1984; Trease and Evans, 2002) by Nwaoguikpe et al. (2014), Soni and Sheetal (2013) and Uddin et al. (2014). The reaction mixture was visually assessed as in Lu et al. (2014), for precipitation, foam formation, colour change and colour intensity according to the following key: (+), Low presence; (++), Moderate presence; (+++), High presence and (-), Absence. The colour intensity of the solutions of hyraceum and (or) the appearance of solids or precipitates enabled a semi-quantitative evaluation for the presence of antioxidants in extract solutions (Chukwudi and Yusha'u, 2016).

Determination of total phenolic content (TPP)

Based on the results of the colorimetric phytochemical profile of hyraceum extract, which showed that most of the compounds present in the hyraceum were phenolic, only the total phenolic content was determined. The total phenolic content was determined by the methods of McDonald et al. (2001) and Sharma and Joshi (2011) using the Folin-Ciocalteu reagent and expressed in milligrams of gallic acid equivalents per gram dry weight (mgGAE/gram DW) of extract. A 1.0 mL volume of HM extract solution (5.0 g/L in ethanol) was mixed thoroughly with Folin-Ciocalteau reagent (1.0 mL) in a volumetric flask. After 3 min, 4.0 mL of sodium carbonate (Na_2CO_3) solution (0.7M) was added, and then the mixture was allowed to stand for 2 h at room temperature in the dark with intermittent shaking. The absorbance of the mixture was measured at 760 nm in a Shimadzu 1201 UV-VIS spectrophotometer. The absorbance of the solutions was measured at 760 nm. All determinations were done in triplicates. A standard calibration curve was obtained by mixing 1ml ethanolic solutions of gallic acid of various concentrations (0.025-0.200 mg/ml) with 1ml Folin-Ciocalteau's reagent (ten-fold dilution) and 4.0 ml sodium carbonate (0.7M) and treated in the same manner as the hyraceum extract. All tests were done in triplicate.

The total phenolic content of the extract was determined in milligrams of gallic acid equivalents per gram dry weight (mgGAE/g DW) of extract using the gallic acid standard curve, calculated from the following formula:

$$C = c \times V/m \qquad (1)$$

Where; C = Total phenolic content in mgGAE/g DW of hyraceum extract, c = Concentration of the gallic acid established from the gallic acid standard curve (mg/ml), V = Volume of the extract used in the assay (ml) and m = mass of the hyraceum extract used in the assay (g) (Genwali et al., 2013).

Antioxidant activities

The 2-diphenyl-2-picrylhydrazyl (DPPH) free radical and Hydrogen peroxide (H_2O_2) scavenging activity of hyraceum extract were used to determine antioxidant activity of the hyraceum.

determination of milligram gallic acid equivalents per gram of dry weight (mgGAE/g DW) of extract and the concentration of extract needed to inhibit oxidation by 50% (EC_{50}).

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of hyraceum extract

The free radical scavenging activity of the extract was evaluated using the *in vitro* DPPH free radical scavenging assay (Nwaoguikpe et al., 2014). Crude hyraceum extract solutions were prepared by dissolving 0.03 g powder of the extract in 10 ml of 50% methanol (v/v in water) to obtain a stock solution of 3000 µg/ml from which serial dilutions of concentrations (2000, 1500, 1000, 500, 200, 100 and 0 µg/ml) were obtained. An aliquot of 0.1 ml of each of the sample dilutions was placed into reaction tubes, followed by the addition of 1.0 ml of 0.1 mM DPPH solution dissolved in absolute methanol and 0.45 ml of 50 mM Tris-HCl buffer (pH 7.40). The contents were mixed and incubated at room temperature for 30 min.

The absorbance of the mixture was measured at 517 nm against the corresponding blank solution (50% v/v methanol in distilled water) in a Shimadzu 1201 UV-VIS spectrophotometer. The assay was performed in triplicates. L-ascorbic acid was used as positive control (Magama et al., 2013).

DPPH free radical scavenging was calculated as percentage inhibition of DPPH free radical based on the control reading, which contained DPPH and distilled water only using the formula:

DPPH scavenged (%) = $[(A_{cont}-A_{test})/A_{cont}] \times 100$, where A_{cont} and A_{test} are absorbance of control and test respectively.

The assay was performed in triplicates. The antioxidant activity of the hyraceum extract was expressed as EC_{50}; the EC_{50} value being defined as the concentration (in µg/ml) of the extract that was required to scavenge 50% of the initial concentration of the DPPH free radical and was calculated from the equation of the linear regression curve of the graph of concentration versus percent scavenging of DPPH free radical using the means of the three determinations (Moyo et al., 2013; Do et al., 2014; Elfalleh et al., 2018).

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the hyraceum was estimated by replacement titration (Nwaoguikpe et al., 2014) using 100 µl of the solutions (3000, 2000, 1000, 500 and 0 µg/ml). All tests were done in triplicate. The percentage of hydrogen peroxide scavenged was calculated as follows (Vinodhini and Lokeswar, 2014; Abayomi et al., 2014):

Hydrogen peroxide scavenged (%)=$[(V_{cont}-V_{test})/V_{cont}] \times 100$, (2)

Where V_{cont} and V_{test} was the volume of sodium thiosulphate used to titrate control sample in the presence of hydrogen peroxide (without extract) and volume of sodium thiosulphate solution used in the presence of the extract, respectively.

The EC_{50} value in the hydrogen peroxide scavenging assay was defined as the concentration (in µg/ml) of the extract that reduced the initial hydrogen peroxide concentration by 50% (Moyo et al., 2013) and was calculated from the equation of the linear regression curve of the graph of concentration versus percent inhibition of H_2O_2 using the mean values obtained from three determinations (Do et al., 2014). L-ascorbic was used as a standard.

Table 1. Qualitative phytochemical screening of the hyraceum crude extract.

Antioxidant	Name of test	Colour for positive test	Results
Flavonoids	Shinoda	pink	+
Alkaloids	Wagner	Blue-black	+
Tannins	Ferric chloride	Blue-black/Green	+
Terpenoids	Salkowski	Reddish-brown	+++
Saponins	Foam test	Foam formation	+++
Simple phenols	Ferric chloride	Green	+
Polyphenols	Ferric chloride	Blue	+++
Anthocyanins	NaOH	Blue-green	++
Quinones	HCl	Green	+++
Phlobatannins	HCl	Red precipitate	+++
Anthraquinones	HCl+chloroform+ ammonia	Rose-pink/violet	+
Coumarins	NaOH+chlroform	Yellow	+++
Sterols	Salkowski	Red	-
Cardiac glycosides and Cardenolides	Keller-Kiliani's	Brown-red ring	-
Reducing sugars	Benedict's	Red precipitate	-
Proteins	Biuret	Violet	-
Amino acids	Ninhydrin	purple	+
Fatty acids	Diethyl ether	Transparent stain	-

+, Low presence; ++, Moderate presence; +++, High presence; -, Absence.

Data analysis

Data was expressed as means ± standard deviations of three replicate determinations using Microsoft excel 2013. Differences between controls and treatment groups were determined using Student's t-test. P-values of less than 0.05 were considered statistically significant using the IBMSPSS statistics, version 20 software. Regression equations and graphs were used for the

RESULTS

Qualitative phytochemical profile of the hyraceum extract

The yield of the hyraceum crude methanol extract from the whole mass of hyraceum was 6.17%. In Table 1 is presented the qualitative biochemical profile of hyraceum extract obtained from the various tests. Colour intensities of the hyraceum solutions (ranging from absence of antioxidant (-) to high presence (+++) for the most intense colour reaction, gave a semi-quantitative indication for the presence of different classes and relative the amounts of phytochemicals in the crude extract (Lu et al., 2014; Chukwudi and Yusha'u, 2016). Most of the classes of compounds identified in the hyraceum were phenolic in nature (flavonoids, alkaloids, tannins, terpenoids, saponins, phenols, polyphenols, anthocyanins, quinones, phlobatannins, anthraquinones and coumarins) and amino acids. No sterols, cardiac glycosides, reducing sugars, fatty acids and proteins were detected in the hyraceum extract. The highest colour intensities (+++) indicating a high presence were exhibited by terpenoids, saponins, polyphenols, quinones, phlobatannins and coumarins followed by anthocyanins (++). The lowest colour intensities, indicating a low presence (+), were given by flavonoids, alkaloids, tannins, phenols, anthraquinones, and amino acids.

Quantitative determination of total phenolics content (TPP)

In Figure 1 is presented the gallic acid calibration curve for determination of total phenolics of the hyraceum extract. The regression equation obtained was $y = 0.053x - 0.033$, $R^2 = 0.971$; where y was the mean absorbance of the sample at 760 nm and x, the concentration established from the gallic acid calibration curve. The total phenolic content of the hyraceum extract was found to be 37.339 mg GAE/g dry weight (37.339 mg GAE/g DW).

DPPH free radical scavenging assay

The results of the DPPH assay are presented in Table 2. A dose-dependent decrease in absorbance was observed which also reflected a dose-dependent increase in the percentage scavenging of the DPPH free radical for both the hyraceum and L-ascorbic acid. The highest concentration (3000 μg/ml) of the hyraceum crude extract and the reference compound L-ascorbic acid gave the lowest absorbance values at 517 nm while
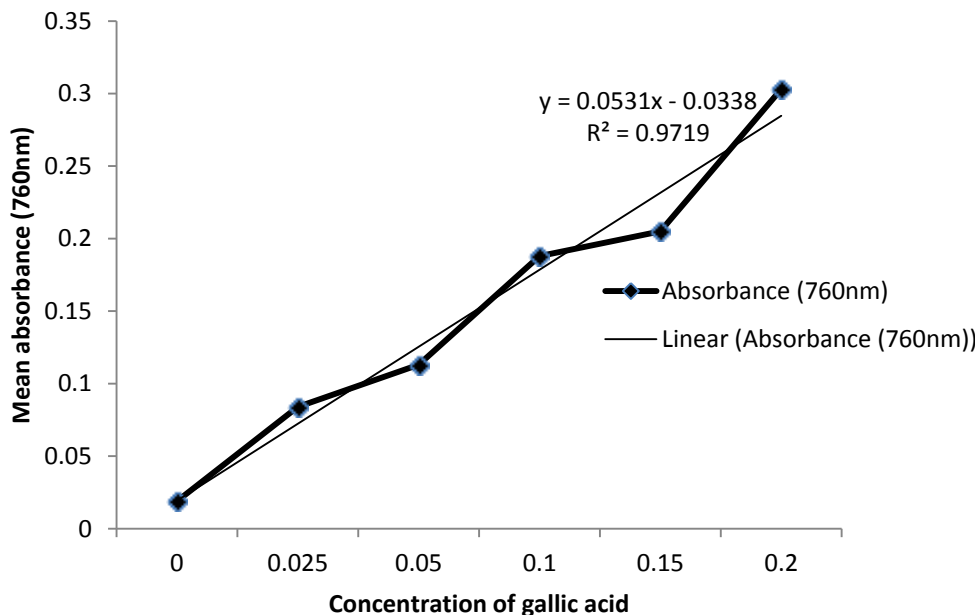
Figure 1. Gallic acid calibration curve for determination of total phenolic content of HM.

Table 2. Absorbance and percentage (%) inhibition of the DPPH free radical by the hyraceum (HM) and L-ascorbic acid (AA) at different concentrations (µg/ml).

Concentration (µg/ml)	Parameter	Absorbance (517nm)									
		L-Ascorbic acid (AA)#					Hyraceum (Hyr)				
		1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
0*	Absorbance	0.364	0.364	0.359	0.362	0.003	0.308	0.307	0.307	0.307	0.001
	% inhib.	0	0	0	0.00	0.000	0	0	0	0.00	0.00
100	Absorbance	0.023	0.024	0.024	0.024	0.001	0.285	0.286	0.288	0.286	0.002
	% inhib.	93.68	93.41	93.41	93.50	0.159	7.468	6.84	6.189	6.83	0.64
200	Absorbance	0.014	0.013	0.015	0.014	0.001	0.276	0.274	0.274	0.275	0.001
	% inhib.	96.15	96.43	95.88	96.15	0.275	10.39	10.75	10.75	10.63	0.21
500	Absorbance	0.015	0.013	0.014	0.014	0.001	0.245	0.238	0.24	0.241	0.004
	% inhib.	95.88	96.43	96.15	96.15	0.275	20.455	22.48	21.82	21.59	1.03
1000	Absorbance	0.011	0.011	0.013	0.012	0.001	0.202	0.195	0.198	0.198	0.004
	% inhib.	96.98	96.98	96.43	96.79	0.317	34.416	36.48	35.51	35.47	1.03
1500	Absorbance	0.014	0.013	0.01	0.012	0.002	0.167	0.173	0.176	0.172	0.005
	% inhib.	96.15	96.43	97.25	96.61	0.572	45.779	43.65	42.67	44.03	1.59
2000	Absorbance	0.015	0.01	0.01	0.012	0.003	0.14	0.151	0.147	0.146	0.006
	% inhib.	95.88	97.25	97.25	96.79	0.793	54.545	50.81	52.12	52.49	1.89
3000	Absorbance	0.011	0.01	0.01	0.010	0.001	0.137	0.139	0.137	0.138	0.001
	% inhib.	96.98	97.25	97.25	97.16	0.159	55.519	54.72	55.38	55.21	0.42

*, Negative control; #, Positive control. % inhib., percentage inhibition

the lowest concentration (0 µg/ml), being the negative control, gave the highest absorbance as shown.

As shown in Table 2, the highest percentage (%) inhibition of the DPPH free radical by the hyraceum

Figure 2. Linear regression graph of percent (%) inhibition of the DPPH free radical by hyraceum (HM) and L-ascorbic acid (AA) against concentration (µg/ml).
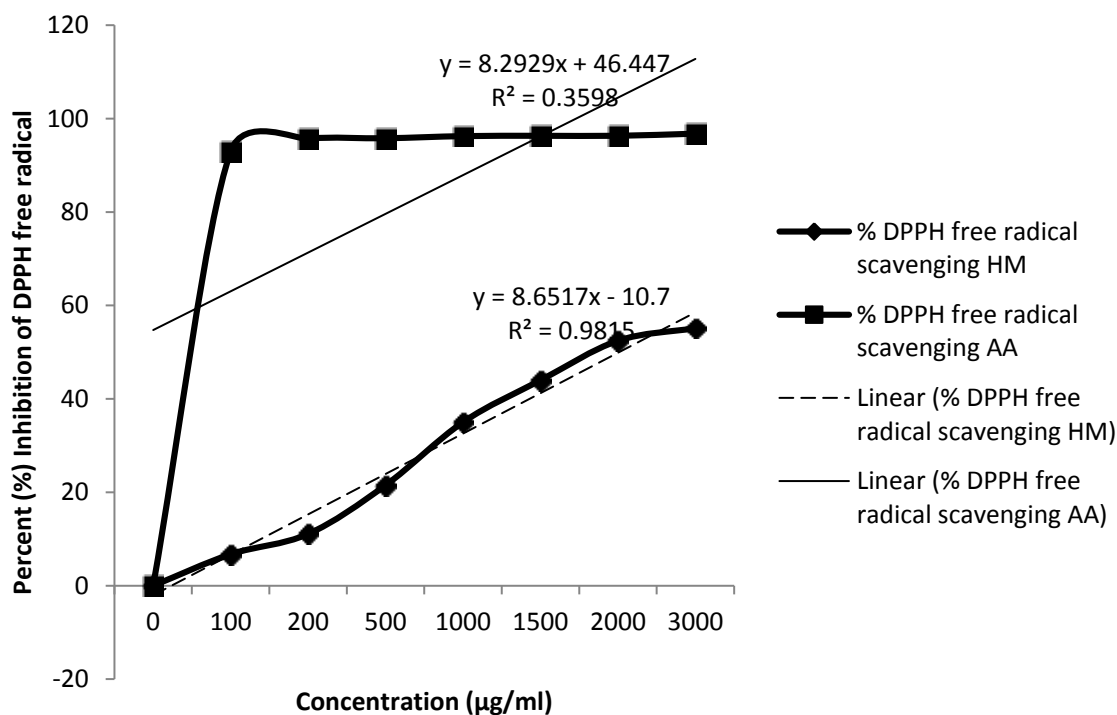
extract (55.21±0.42%) was observed at the maximum concentration of 3000 µg/ml used in the study, while at the same concentration, L-ascorbic acid scavenged 97.16 ±0.16% of the DPPH free radical. The lowest value for the DPPH free radical scavenging activity by the hyraceum extract (6.83±0.64%) was observed at100 µg/ml while at that same concentration L-ascorbic acid scavenged 93.50±0.16% of the free radical. The EC_{50} for hyraceum (HM) extract was 5.983 µg/ml calculated from the linear regression equation $y = 8.651x-10.7$, $R^2=0.981$ while the EC_{50} for L-ascorbic acid (AA) was 0.4293 µg/ml, calculated from the equation $y = 8.292x + 46.44$, $R^2 = 0.359$ shown in Figure 2.

The crude hyraceum extract showed significant DPPH free radical scavenging activity, of 55.21±0.42% at the maximum concentration of 3000 µg/ml. At the highest concentration used in the study, there was a significant ($p<0.05$) difference in DPPH free radical scavenging activity between the hyraceum extract and L-ascorbic acid.It was observed that antioxidant values of the hyraceum extract were lower than those of L-ascorbic acid and with a significant difference from those of the standard L-ascorbic acid ($p\leq0.05$).

Hydrogen peroxide scavenging assay for the hyraceum

In Table 3 is presented the results of the hydrogen

peroxide scavenging assay. From 0 to 500 µg/ml the hydrogen peroxide scavenging effect of the hyraceum was insignificant, being similar to that of the negative control (0%), while at 5 µg/ml L-ascorbic acid scavenged 77.78±4.81% of the hydrogen peroxide. L-ascorbic acid in the concentration range from 500 to 3000 µg/ml was very potent; the hydrogen peroxide scavenging reaction being instantaneous following the addition of one drop of thiosulphate and was therefore recorded as being greater than 77.78±4.81%.

The EC_{50} for hydrogen peroxide scavenging by L-ascorbic acid (AA) was calculated from the equation $y = 16.17x+23.06$, $R^2=0.571$ while that for the hyraceum (HM) was calculated from the regression equation $y = 16.05x -31.2$, $R^2=0.844$ (Figure 3). The EC_{50} obtained for AA (1.666 µg/ml) was lower than that for HM (5.059 µg/ml) indicating a higher antioxidant activity of the AA in this assay.

**DISCUSSION**

The qualitative phytochemical screening of HM revealed the presence of different classes of phytochemicals in the crude methanolic (95%) extract of the hyraceum. There was a high presence (+++) of terpenoids, saponins, polyphenols, quinones, phlobatannins and coumarins (Table 1) in the solution of the hyraceum based on the intensity of the colour in the colorimetric tests, which

Table 3. Percent (%) hydrogen peroxide (H_2O_2) scavenged by the hyraceum (HM) and L-ascorbic acid (AA) at different concentrations (μg/ml) using the back titration method.

Concentration (μg/ml)	Parameter	Volume of sodium thiosulphate (mL)									
		Hyraceum (Hyr)					L-Ascorbic acid (AA)#				
		1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
0*	Volume	0.6	0.4	0.4	0.47	0.09	0.6	0.4	0.4	0.47	0.12
	% inhib.	0	0	0	0	0	0	0	0	0	0
5	Volume	0.6	0.4	0.4	0.47	0.09	0.1	0.1	0.1	0.1	0.00
	% inhib.	0	0	0	0	0	83.33	75	75	77.78	4.81
500	Volume	0.4	0.5	0.6	0.5	0.08	<0.1	<0.1	<0.1	<0.1	<0.1
	% inhib.	33.33	-25	-50	-13.89	34.92	>77.78	>77.78	>77.78	>77.78	ND
1000	Volume	0.3	0.6	0.3	0.4	0.14	<0.1	<0.1	<0.1	<0.1	<0.1
	% inhib.	50	-50	25	8.33	42.49	>77.78	>77.78	>77.78	>77.78	ND
2000	Volume	0.3	0.2	0.2	0.23	0.05	<0.1	<0.1	<0.1	<0.1	<0.1
	% inhib.	50	50	50	50.00	0.00	>77.78	>77.78	>77.78	>77.78	ND
3000	Volume	0.1	0.15	0.1	0.12	0.02	<0.1	<0.1	<0.1	<0.1	<0.1
	% inhib.	83.33	62.5	75	73.61	8.56	>77.78	>77.78	>77.78	>77.78	ND

*, Negative control; #, Positive control; <0.1: Volumes could not be determined-very high potency, instant scavenging of H_2O_2 due to high bioactivity of L- ascorbic acid at these concentrations, hence volumes were recorded as < 0.1 mL since only a drop of thiosulphate was used; ND, Not determined. % inhib., percentage inhibition.
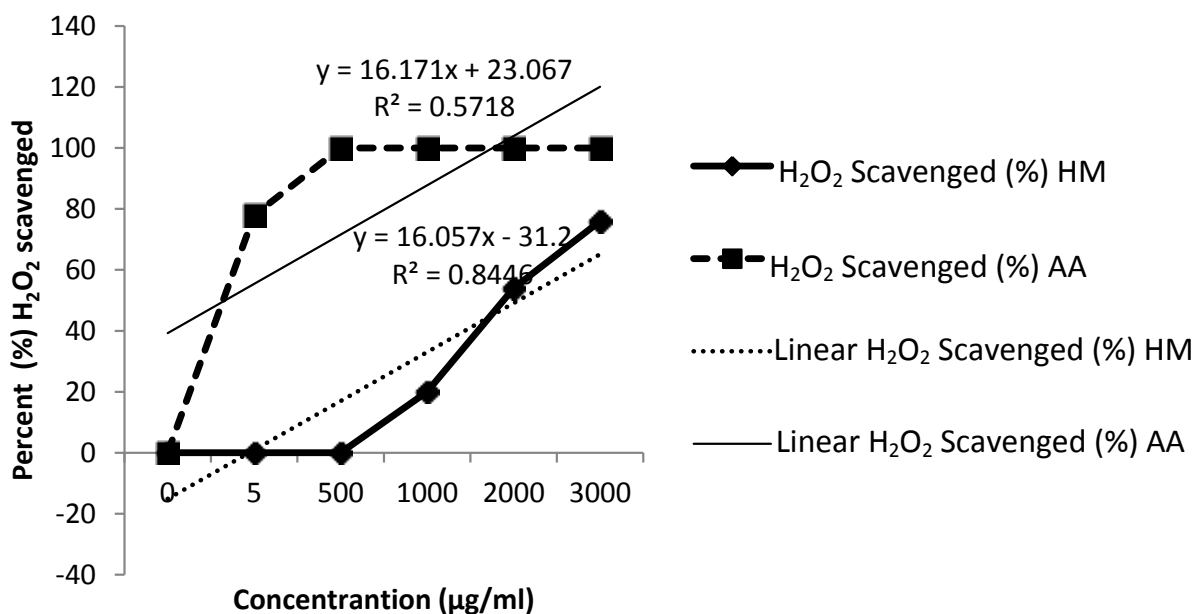
Figure 3. Linear regression graph of percent (%) hydrogen peroxide (H_2O_2) scavenged by hyraceum (HM) and L-ascorbic acid (AA) against concentration (μg/ml).

indicated that these phytochemicals were most abundant. Other phytochemicals that were detected at smaller quantities were anthocyanins with a moderate presence (++), while flavonoids, alkaloids, tannins, simple phenols, anthraquinones and amino acids exhibited a low presence (+). Sterols, cardiac glycosides and cardenolides, reducing sugars, proteins and fatty acids were not detected and hence assumed to be absent or below detection limit of the methods used. With the exception of terpenoids, all the phytochemicals detected

in the hyraceum extract were phenolic in nature even though they vary in structure. Terpenoids are highly conjugated molecules also with antioxidant properties just like phenolic compounds. According to Bang et al. (2015) phytochemicals such as carotenoids, terpenoids, ascorbates, reducing carbohydrates and tocopherols contribute to the antioxidant activity of natural products. The antioxidant activity of the crude methanolic (95%) extract of HM could therefore be attributed to the antioxidants detected in the hyraceum solution (Table 1). Phenolic compounds have antioxidant activity because they can donate hydrogen atoms to neutralize free radicals and the phenolic compounds form will be stabilized by resonance. Tannins act as antioxidants because of their ability to stabilize lipids and inhibition of lipoxygenase. Hydrolysable tannins have anti-ischemic activity. Alkaloids, particularly indole, are efficient at inhibiting the chain reaction of free radicals. All types of phenolic compounds are reported to exhibit antioxidant activity but to different degrees (Ahmad et al., 2014) and flavonoids are reported to exhibit antitumour, anti-inflammatory. The antioxidant activity of phenolic compounds is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, singlet oxygen quenchers and also may have a metallic chelating potential (Rice-Evans et al., 1996). Synergism among the antioxidant compounds in the crude extract makes the antioxidant activity is not only dependent on the concentration but also on the structure and interaction between the antioxidant compounds (Djeridane et al., 2006).

The phytochemicals identified in the hyraceum listed in Table 1 were also identified in the beaver castoreum, another complex product of animal metabolic waste and plant materials which exudes from the castor sacs of the mature North American beaver (*Castor canadensis*) and the European beaver (*Castor fiber*) (Müller-Schwarze, 2003).
Pyrolysis gas chromatography mass spectrometry analysis of hyraceum identified nitrogen-containing aromatic compounds, notably benzamide (Carr et al., 2010). The solvent-extractable lipids of hyraceum comprised homologous suites of long-chain n-alkanes (C_{24}–C_{34}) and n-alkanols (C_{16}–C_{26}) characteristic of higher plant leaf waxes, along with an abundance of animal-derived sterols, higher plant sterols and terpenoids as well as benzamide (Carr et al., 2010). As early as 1879, Green and Parker (1879) analyzed hyraceum and found that about 56% of it was soluble in water, the remaining insoluble material was organic and composed of woody fibre, sand and other inorganic substances as mixtures of various salts such as soda and lime (having the highest proportion among the salts). The organic matter contained traces of urea together with uric, hippuric, and benzoic acids due to the fact that hyraceum is partly derived from urine and faecal matter (Green and Parker, 1879). No study was found on the phytochemical composition of the hyraceum with which to compare the results.

Only the total phenolics content of hyraceum crude extract was determined in this study. The determined value of total phenolics (Figure 1) of the hyraceum extract of 37.339 mg GAE/g dry sample of the hyraceum was much higher than the highest value of 1.699 mg GAE/g observed with cow dung, another complex product of animal metabolic waste and plant materials by Jirankalgikar et al. (2014) most probably due to the fossilized nature of the hyraceum.

The hyraceum extract was tested for antioxidant activity using the DPPH free radical and the hydrogen peroxide (H_2O_2) scavenging assays.

As shown in Table 2, at 3000 µg/ml the highest percentage inhibition of the DPPH free radical and the EC_{50} for the hyraceum (HM) extract were 55.21±0.42% and 5.983 µg/ml respectively and for L-ascorbic acid (AA) were 97.16±0.16% and 0.4293 µg/ml respectively. The EC_{50} is the concentration of extract that causes a 50% decrease in the initial concentration of the DPPH free radical (Do et al., 2014). A lower IC_{50} represents higher antioxidant activity (Do et al., 2014; Proestos et al., 2013). Jarald et al. (2008) observed an EC_{50} of 5.10 µg/ml in studies with cow urine while Jirankalgikar et al. (2014) observed an EC_{50} range of between 12.810 and 41.554 mg/ml with cow dung samples collected at different times of the day in a six day period of study. In the same study by Jirankalgikar et al. (2014), the EC_{50} of L-ascorbic acid was 20.13 µg/ml. The EC_{50} of the hyraceum extract of 5.983 µg/ml obtained in the current study is similar to that obtained by Jarald et al. (2008) of 5.10 µg/ml in studies with cow urine but lower than the values of between 12.810 and 41.554 mg/ml obtained in studies with cow dung by Jirankalgikar et al. (2014).

In this study, hydrogen peroxide scavenging activity was estimated by the replacement titration method. As shown in Table 3, at 3000 µg/ml the highest percentage scavenging of H_2O_2 and the EC_{50} for the hyraceum (HM) extract were 73.61±8.56% and 5.059 µg/ml, respectively and for L-ascorbic acid (AA) were greater than 77.78±4.81% and 1.666 µg/ml, respectively. No literature on hydrogen peroxide scavenging activities by mammalian metabolic waste products was found for comparison with the results of this study. However, in a study by Vinodhini and Lokeswari (2014) the methanolic leaf extract of *Toona ciliate* scavenged a maximum of 55.69 ± 1.04% of hydrogen peroxide.

Conclusion

The colorimetric tests in the present study detected the major phytochemical components of the hyraceum extract as terpenoids, saponins, polyphenols, quinones, phlobatannins and coumarins and the minor phytochemicals as flavonoids, alkaloids, tannins, simple

phenols, anthocyanins, anthraquinones and amino acids. Sterols, cardiac glycosides and cardenolides, reducing sugars, proteins and fatty acids were not detected. The total phenolic content of the hyraceum extract was 37.339 mg GAE/gram dry weight. The hyraceum extract was more effective at scavenging H_2O_2 (73.61±8.56%) than the DPPH free radical (55.21±0.42%). Bioactive compounds in HM could potentially be exploited in further studies as potential antioxidants of therapeutic value in preference to synthetic antioxidants as they have negative side-effects in the body.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Towards accomplishing the roll back malaria initiative: Phytochemical screening and antimalarial activity of ethanolic leaf extract of *Ricinus communis* L. (Euphorbiaceae)

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Malaria is a major debilitating disease caused by *Plasmodium* species and spread by female *Anopheles* mosquitoes. This research was conducted to determine the efficacy of ethanolic leaf extracts of *Ricinus communis* L. against *Plasmodium berghei* (NK65) infection in mice. Phytochemical components of the extract were analyzed and elucidated in order to reveal the constituents with antimalarial potentials. The safety of the extract in the experimental mice was ascertained by determining the median lethal dose (LD_{50}). Result of the phytochemical screening revealed the presence of compounds notable for antimalarial effects such as alkaloids, flavonoids and anthraquinones. From the findings, it was established that a dosage of 141.42 mg/kg of the extract represents the acute lethal dose (LD_{50}) in mice. Hence, three separate doses of the extract (10, 20 and 40 mg/kg) were prepared for the curative test. All the three doses portrayed a remarkable antimalarial activity as compared to the standard reference drug (chloroquine, 5 mg/kg). The extract dosage of 20 mg/kg showed the highest average suppression of 81.6% among the treatments. No significant differences were however observed among the treated groups (P>0.05). On the other hand, a highly significant difference was observed between the treated and control groups (P≤0.001). The leaf extracts of *R. communis* thus possess antimalarial properties and is therefore recommended as a new candidate for antimalarial drug development.

Key words: *Ricinus communis,* chloroquine, phytochemical compound, antimalaria, dose, *Plasmodium berghei*.

INTRODUCTION

Malaria is an infectious debilitating disease which is continuously associated with considerable morbidity, mortality as well as significant social and economic impact around the globe (Balogun et al., 2009). It is the most common protozoan parasite disease in the tropical and subtropical regions of the world with more than 40%

of the world's population at risk (Snow et al., 2005). It was estimated that by the year 2015, there were 214 million cases of malaria worldwide resulting in about 438,000 deaths, with about 90% of all cases of mortality due to malaria occurring in Africa (WHO, 2016).

Nigeria has the highest prevalence of malarial cases in Africa as transmission of the disease occurs all year round in the southern part of the country while in the northern part, the disease is more seasonal occurring mostly during the rainy season (WHO, 2008). Pregnant women, their unborn foetus and children below the age of 5 years are more vulnerable to malaria which serves as the major cause of maternal and infant anemia (Hartman et al., 2010).

Malaria in human is transmitted by the bite of a female *Anopheles* mosquito infected with *Plasmodium* species. Species that generally cause malaria in humans are *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, Plasmodium ovale* and *Plasmodium knowlesi* (Collins, 2012). It is traditionally believed that *P. falciparum* accounts for the majority of deaths due to malaria (Sarkar et al., 2009). Recent evidence also suggests that *P. vivax* is associated with potentially life-threatening conditions (Baird, 2013).

It was earlier estimated that about 80% of the population of third world countries are dependent on medicinal plants for their primary health care needs due to poverty and lack of access to modern medicine (WHO, 1997). Despite recent developments in modern health care development, traditional medicine is still a norm in many parts of the world. Medicinal plants have been used since time immemorial in all cultures to treat ailments (Hoareau et al., 1999). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. Medicinal plants are plants that have one or more of their parts containing important phytochemicals that can be used for therapeutic purposes or as additives in pharmaceutical products (Bentley and Trimen, 2007). These phytochemicals appear to be of great benefits to humans and their consumption has less side effects as compared to pharmaceutical synthetic drugs (Barisi and Omodele, 2014). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Rabe and Vanstoden, 2000). A major challenge in the efforts to curtail malaria is the drug resistance (Randrianarivelojosia et al., 2003). Drug resistance is responsible for the spread of malaria to new areas and resurgence in areas where it had been eradicated (Ridley, 1997). As a result of this increasing problem, there is every need for the quest to develop novel antimalarial drugs to combat the spreading drug of resistant parasites (Abdulelah et al., 2010).

Ricinus communis, commonly known as Castor oil plant is a tropical flowering plant belonging to the family Euphorbiaceae. It is ubiquitous in nature (de Assis Junior et al., 2011). Inhabitants of rural communities often exploit different parts of this plant for the treatment of various ailments (Jena and Gupta, 2012). In the northern part of Nigeria, rural dwellers occasionally utilize the bark and leaves of this plant for local treatment of malaria. To the best of our knowledge there is no any published research that investigates the potentials of this plant in malaria therapy. This study therefore is designed to investigate the *in vivo* antimalarial potentials of *R. communis* leaves extract against chloroquine sensitive strain of *Plasmodium berghei.*

MATERIALS AND METHODS

Plant collection

The leaves of the plant were collected within the months of June and July, 2017 from Dutsin-ma area, Katsina State (Lat. 12°27'18" N and Long. 7°29'29" E) by the researchers and identified by a taxonomist at the Department of Biology, Umaru Musa Yar'adua University, Katsina State, Nigeria. Further confirmation was done by the departmental herbarium officer and voucher specimen was deposited for future reference, before embarking on the research.

Preparation of the plant extract

Fresh leaves of *R. communis* obtained were washed and air dried at room temperature. The dried leaves were later blended into powder and stored in a clean air tight plastic container to avert moisture absorption and any possible contamination. The plant extracts were prepared via cold extraction method as described by Barisi and Omodele (2014).

Phytochemical screening

A preliminary phytochemical screening of the leaf extract of *R. communis* was carried out to detect the presence or absence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, tannins and terpenoids using Dragendoff's reagent, sulphuric acid-chloroform test, Keller-Killani's test, ammonium test, frothing test, ferric chloride test, and Salkowski's test, respectively. This was done with adherence to standard procedures as described by Trease and Evans (1989) and Sofowara (1993).

Experimental animals

Swiss albino mice (both male and female) with considerable weights were obtained from the animal breeding unit of the Department of Pharmacognosy and Drug Development, Ahmadu Bello University Zaria, Kaduna State. The mice were housed in metal cages and maintained under standard laboratory conditions with free access to standard pelleted feed and water *ad-libitum*.

Ethical consideration

The research protocol was conduct by strictly adhering to the principle of Laboratory Animal Care (NIH publication #85-23, revised in 1985). The research protocols were also approved by the Postgraduate Research Committee of the Department of Biology and the Umaru Musa Yar'adua University Board of Research. Further permission and approval was obtained from the Research Ethics Review Committee of Katsina State Ministry of Health via

clearance certificate No. MOH/ADM/SUB/1152/1/149 dated 13th July, 2017. This was done to ensure conformity with the ethical provisions expected of this kind of research.

Determination of median lethal dose (LD$_{50}$)

The LD$_{50}$ of the extract (Table 2) was estimated using Swiss albino mice by oral administration route as previously described (Lorke, 1983).

Parasite inoculation

A chloroquine-sensitive strain of *P. berghei* (NK-65) was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Oyo State, Nigeria. Inoculum preparation was made from blood of donor mouse infected with *P. berghei*. The desired blood volume was drawn from the donor mouse by slaughtering and dilution in normal saline solution. The final suspension produced contains about

1×10^6 infected red blood cells in every 0.2 ml of the suspension (Abdulelah et al., 2011). Each mouse was intraperitoneally inoculated on the first day or day 0 (D$_0$) with 0.2 ml of infected blood containing about 1×10^6 *P. berghei* parasitized red blood cells (Ishih et al., 2004).

In vivo antimalarial assay

In vivo curative antimalarial assay was carried out in order to evaluate the possible antimalarial activity of the ethanolic leaf extract of the plant (*R. communis*) at 10, 20 and 40 mg/kg doses as compared to control groups treated with 0.5 ml of distilled water and reference drug group treated with standard drugs (chloroquine 5 mg/kg). The percentage parasitaemia was determined by counting the number of parasitized red blood cells in random fields of microscope. This experiment was conducted in duplicate. The average percentage suppression of parasitaemia was calculated in comparison with the control as described previously (Abdulelah et al., 2011).

Average percentage suppression is given by:

$$\frac{Average\ \%\ parasitemiaincontrolgroups\ -Average\ \%\ parasitemiaintreatedgroups}{Average\ \%\ parasitemiaincontrolgroups} \times 100$$

Curative antimalarial activity

Thirty Swiss albino mice were inoculated intraperitoneally with 0.2 ml suspension containing 1×10^6 *P. berghei* each on day zero (D$_0$). The mice were then divided into five groups with each group containing six mice. After 72 h, different doses (10, 20 and 40 mg/kg/day) of the extract were orally administered to the experimental groups. The reference drug group was treated with chloroquine (5 mg/kg) and the control group received 0.5 ml of distilled water. The treatment was continued once daily for five days (Etetim et al., 2008). On the sixth day, thin blood film was prepared from the blood obtained from the tail of each mouse, stained with Giemsa stain and examined under microscope to determine the percentage of parasitaemia and average percentage of suppression following treatment.

Statistical analysis

All the data obtained were carefully cleaned, filed and entered into GraphPad InStat 3 statistical software for further analysis. The results were expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was used to determine the mean differences between the different treatments. Differences at 95% level of confidence ($P \leq 0.05$) were considered significant. Tukey-Kramer multiple comparisons test was also used to compare the variations between the treated groups to ascertain the level of significance of the various treatments on the mice.

RESULTS

Phytochemical screening

Qualitative phytochemical investigation of ethanolic extract of *R. communis* revealed that the leaves contain secondary metabolites such as alkaloids,

anthraquinones, flavonoids, saponins and tannins shown in Table 1.

Median lethal dose

After the two-phase test for the median lethal dose (Table 2), a value of 141.42 mg/kg was obtained as the dose that will kill half the test subjects (mice). Going by this value, doses of 10, 20 and 40 mg/kg of the extract was chosen for the bioassay. Asthenia, lethargy and ataxia were observed as the symptoms of toxicity of the extract. At the highest dose of 1000 mg/kg, these symptoms continued till death of the mice.

Curative antimalarial activity of *R. communis* ethanolic leaf extract

The results as presented in Table 3, indicates that the ethanolic extract of *R. communis* leaves exhibited a remarkable reduction of parasitaemia in mice infected with *P. berghei*. Analysis of variance (ANOVA) indicated an extremely significant difference between the control, treated and standard drug groups (P < 0.001). The post-test (Tukey-Kramer multiple comparisons test) showed that there were no significant differences among the treated groups administered with the doses of 10, 20 and 40 mg/kg (P > 0.05) of the extract. These findings however showed high significant differences between the treated groups (administered with doses 10, 20 and 40 mg/kg) and the control group (P < 0.001). Furthermore, there were no significant differences between the groups

Table 1. The tests/reagents and the presence or absence of secondary metabolites.

Chemical constituents	Tests/Reagents	Results
Alkaloids	Dragendoff's reagent	+
Anthraquinones	Sulphuric acid-chloroform layer test	+
Cardiac glycosides	Keller-Killani's test	−
Flavonoids	Ammonium test	+
Saponins	Frothing	+
Tannins	Ferric chloride test	+
Terpenoids	Salkowski's test	−

+: Present; −: Absent.

Table 2. The acute oral toxicity of the ethanolic extract of *Ricinuscommunis* leaves administered orally to mice.

Dose (mg/kg)	Mortality	Toxic symptoms
10	0/3	None
*50	0/1	None
100	1/3	Lethargy, asthenia
*100	0/1	Lethargy
*200	1/1	Lethargy, asthenia
*400	1/1	Lethargy, asthenia, ataxia
1000	3/3	Lethargy, asthenia, ataxia

*Refers to the second phase of LD_{50}.

Table 3. The curative antimalarial activity of ethanolic extract of *Ricinus communis* in mice.

Drug or extract	Dosage (mg/kg/day)	Average parasitemia	Average % suppression	P-value
	10	9.4 ± 1.8	74.2	P < 0.001
Ricinus communis	20	6.7 ± 1.5	81.6	P < 0.001
	40	6.8 ± 0.9	81.3	P < 0.001
Chloroquine (standard)	5	3.9 ± 0.3	89.3	-
Distilled water (control)	0.5 ml	36.4 ± 3.5	-	-

Data are expressed as Mean ± SEM for six mice per group.

treated with different doses of the extract and those treated with the standard or reference drug group (P >0.05). This implies that the doses are comparatively effective in the reduction of parasitaemia.

DISCUSSION

The phytochemical screening of the ethanolic extract of *R. communis* revealed that the leaves contain alkaloids, anthraquinones, flavonoids, saponins and tannins. Alkaloids and flavonoids are usually implicated in the possession of antimalarial activity. Similar results to the potentials of these phytochemicals were obtained in previous studies (Miliken, 1997; Abdulelah et al., 2010). Flavonoids are forms of phenolic compounds which are known to exhibit significant anti-parasitic activities against different strains of *Plasmodium, Trypanosoma* and *Leishmania* species (Kim et al., 2004; Monbrison et al., 2006; Tasdemir et al., 2006). The antimalarial activity of this extract could be due to single or synergistic action of these chemical compounds. Nonetheless, it is important to identify and ascertain the active principle which could be isolated and purified to improve the potency of theextract.

In the curative test, the plant extract showed a dose-dependent antimalarial activity which was found to be statistically significant when compared with the control.

The lack of significant differences among the doses when compared with the standard drug (chloroquine) confirms their competitive curative antimalarial efficacy against *P. berghei* infection. The result of this study agrees with the findings of a previous similar research conducted in Malaysia (Abdulelah et al., 2010). Thus, the doses are fairly equally effective in the reduction of parasitaemia with regards to their high percentage suppression.

The decline in activity at higher doses of the extract could be as a result of reduced or no effect of thecomponents present in the extract at higher doses. This agrees with the findings from the research conducted by Rao et al. (2001). Plants that exhibit antimalarial activities are known to do this either by initiating elevation of red blood cells oxidation or by preventing protein synthesis depending on the phytochemical constituents within them (Etkin, 1997). Phytochemicals such as saponins and phenols (flavonoid is also a phenolic compound) are reported to be good antioxidants in a study by Barisi and Omodele (2014). These compounds could be responsible for the activity displayed by *R. communis* extract since antioxidant property is another mechanism by which antimalarial effect can be exerted. Previous works by Abdulelah et al. (2011) suggests that anti-plasmodial activities could be related to antioxidant effects of some phytochemicals. Saponins are known to aid in the fight against parasitic infections by boosting the immune system while other phytochemicals having good antioxidant properties that exhibit capabilities of protecting or elevating resistance of red blood cells to oxidative damage (Barisi and Omodele, 2014). On another note, it has been suggested by Bapna et al. (2014), that the smell of some medicinal plants may repel mosquitos thereby reducing the incidence of malaria infection. Based on the findings from this research, the ethanolic extract of *R. communis* proved to be very effective for malaria therapy.

Conclusion

The results of the present study revealed the potent antimalarial properties possessed by the ethanolic leaf extract of *R. communis* due to its abilities to significantly suppress *P. berghei* infection in the evaluation test conducted. The antimalarial potentials exhibited by the leaf extracts of *R. communis* could be attributed to the active anti-plasmodial components within the extracts which are mainly alkaloids, flavonoids, anthraquinones and saponins. The use of the *R. communis* plant extract as a potential candidate for future anti-malaria drug development is therefore recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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