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Full Length Research Paper

In vitro evaluation of fruit extracts of *Annona stenophylla diels* and *Flacourtia indica* for incorporation into formulations for management of cancer

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There are many reports which show a positive relationship between free radical damage and diseases such as cancer. Free radicals are formed when humans are exposed to ionizing radiation or during oxidation process in human beings can damage tissues and may potentially lead to development of cancers. Since radiation may be used in the management of cancer, there is need for the development of a radioprotective herbal medicine that can be used to increase the therapeutic index of radiotherapy and chemotherapy treatments by offering protection to non cancerous cells. Since most fruits contain polyphenols, they are radical scavengers which can effectively prevent cancer development due to increased reactive oxygen species. The aim of this research was to evaluate how fruit extracts of *Annona stenophylla diels* and *Flacourtia indica* may help in the prevention of cancer using UV light induced red blood cell (RBC) haemolysis and1, 1-diphenyl-2-picrylhydrazyl (DPPH) assays. Since cancer pathogenesis may involve lipid peroxidation, UV induced haemolysis and DPPH assays were used to examine the role of the fruit extracts in radioprotection and free radical quenching in order to prevent peroxidation. Phenolic compounds were extracted using aqueous methanol and the two assays were carried out using concentrations ranging from 20 to 200 µg/ml. The results were analysed using T test. The fruit extracts from the two plants showed a marked antioxidant and radioprotection efficacy against lipid peroxidation in the presence of UV light. Both assays showed a significant absorbance inhibition at ($p \leq 0.05$) implying that the fruit extracts were effective radical scavengers and radioprotectors. The results obtained suggest that the fruit extracts were good plasma membrane stabilizers and good radical quenchers. The membrane of red blood cells was stabilized by preventing UV induced peroxidation, a process in which free radicals are formed. These findings suggests that fruit extracts obtained from *A. stenophylla diels* and *F.indica* plants can be incorporated in pharmaceuticals formulations as nutraceuticals for prevention cancer and improvement of radiotherapy and chemotherapy therapeutic index.

Key words: DPPH, radioprotective, cancer, UV visble, *A. stenophylla diels, F.indica*, reactive oxygen species.

INTRODUCTION

Role of radiation and free radicals in cancer development

Radiation exposure to human beings may trigger mutation which may lead to development of cancer. Normal people who are exposed to radiation include professionals handling radioactive materials and patients undergoing radiotherapy or radio-diagnosis. Ionizing radiation generates free radicals in healthy cells and may induce mutations which lead to the development of cancer.

Most degenerative diseases including cancer are caused by DNA damage due to oxidative stress. The radicals $O_2^{\bullet -}$ and OH• that are produced during oxidative stress are chief culprits responsible for oxidative DNA damage (Fulgentius et al., 2013). Several studies have determined 8-hydroxy-2- deoxyguanosine (8-OHdG) as the target for oxidative attack on DNA. 8-hydroxy-2- deoxyguanosine (8-OHdG) was initially identified as a marker of carcinogenesis by Floyd (1990). In other studies it was found that sugar-phosphate backbone of DNA is also attacked by these free radicals (Prasad and Amit, 2017).

Need for pharmaceutical radioprotection

X rays were discovered by Roentgen in 1895 and Becquerel then discovered radioactivity in 1896. These two discoveries were considered to be a turning point in human health care since X rays were able to get inside the human body. The harmful effects of radiation were reported within months after the discovery of X rays; however, the extent of damage could not be ascertained. Studies of workers exposed to radiation were later carried out on workers such as physicians and research scientists handling radioactive material. The studies gave a clear picture of the harmful effects of radiation and the evidence was further strengthened by survivors of 1945 Japanese atomic bomb (Smith et al., 2017; Ganesh, 2007). It is now known that radiation has harmful effects to living organisms and it is reasonable to do research on pharmacological agents of plant origin to safeguard human beings against radiation exposure. Lead shielding can be used by workers handling radiation, however it may not protect against radiation exposure due to accidents, background radiation and terror attacks; therefore, the use of pharmacological medicines of plant origin either alone or to complement lead shielding to protect humans against ionizing radiation is still recommended. Free radical damage forms the basis of some chemotherapy drugs in cancer treatment such as anthracyclines (Mut-Salud et al., 2016). The side effects of chemotherapy which include hair loss and immune-suppression are well documented. These side effects which result from the barrage of free radicals that indiscriminately attack both malignant and health cells can be reduced by radioprotectors (Mut-Salud et al., 2016).

Chemical radioprotection and radical protection

The need to safeguard humans against radiation from atomic weapons was first attempted in 1949 after World War II by Patt and co-workers. Patt and co-workers wanted to use a free radical scavenger amino acid cystein which proved to be efficacious in preclinical studies. However, due to the high toxicity of this thiol compound it could not be used and this necessitated search for safe alternative agents; thereafter, it was thought that agents of plant origin can be used as nontoxic radioprotectors (Shukla and Gupta, 2010).

In view of the above, most investigators have now diverted their attention towards natural products of plant origin in the last decades. Plants have played an important role in drug discovery and development. This is interestingly true in areas of infectious diseases and cancer, where more than 75 and 60% of the drugs respectively, were derived from plants (Smith et al., 2017). A chemical protector is considered to be good if it can protect against harmful effects of ionising radiation and background irradiation. A desirable radioprotector should be cheap and should possess the following pharmacological properties; non toxic over a wide dose range, should be administered orally, quickly absorbed in the gut and act through several mechanisms. This implies that the best radioprotector should consist of a mixture of herbal extracts to increase its capacity to quench the effects of radiation through multiple mechanisms.

Basically radioprotection process involves radical scavenging; therefore, radio-protectors can also act as radical quenchers and can play a significant role in the prevention of cancer due to oxidative stress. The advantage of using agents of plant origin is that plants are used in traditional health systems that are widely accepted by human beings; however, their use as radioprotectors requires scientific investigation and validation (Mut-Salud et al., 2016).

Radio-protective potential of plants and herbs

Plants can be useful radio-protectors, notably those that have antioxidant, anti-inflammatory, antimicrobial and anti-stress properties. Radio-protection capacity is due to phenolic compounds that are produced by plants. These phenolic compounds are typically found in leaves, roots, stem, rhizomes, fruits and seeds. Phenolic compounds are normally produced in response to specific physical or physiological noises (Adeboye et al., 2014) which include exposure to UV light or infection by parasites and extreme temperatures (Soto et al., 2015). Phenol compounds are synthesized by three pathways which are the pentose, shikimate and phenylpropanoid pathways (Adeboye et al., 2014). Phenolic compounds are basically produced by nearly all plants. Phenol implies that the complex secondary metabolite contains one or several

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phenyl ring moieties in its structure (Lattanzio, 2013). Each of the phenyl moieties in these complex structures contains one or more hydroxyl groups (Min et al., 2015). Phenol compounds prevent effects of free radicals and radio-induced radicals by accepting the radical or donating hydrogen, thus terminating the possible propagation step which generates more radicals. Short term in vitro tests for free radical and antioxidant status and lipid peroxidation of the pharmacological agent may provide some leads with regard to radioprotective and free radical scavenging potential of the agent. The best approach to select a desirable plant candidate with radio-protective effects is to look for a plant with anti-inflammatory, antioxidant, antimicrobial, immuno-modulatory or free radical scavenging properties. Lipid peroxidation tests can be done by irradiating red blood cells with UV light, whereas the scavenging capacity can be done by determining the effects of plant extracts on DHHP absorbance.

The mechanisms of cancer prevention

Cancer prevention mechanism targets the physiological processes necessary for tumour development. Plant extracts can block insults that cause damage to cellular DNA thus preventing cancer initiation, which is achieved by quenching free radicals. Radical quenchers can prevent the formation of carcinogens from precursor substances (Gopalakrishnan and Kong, 2008).

Cancer initiation blockers also stimulate molecular pathways including Nrf2 (nuclear factor-erythroid 2p45 (NFE2)- related factor 2) to promote the synthesis of protective phase II enzymes such as UDP-glucuronyltransferases, acetyltransferases, glutathione-*S*-transferases (GSTs), NAD(P)H: sulphotransferases and quinone oxidoreductase (Gopalakrishnan and Kong, 2008). It has also been reported that dietary antioxidants perform their protective effects both as cancer initiation blockers and by inducing *de novo* expression of genes that code defensive genes (Gopalakrishnan and Kong, 2008).

These genes that are stimulated by xenobiotics in response to stress contain a common *cis*-element genes, called antioxidant response elements (ARE). The expression of these genes is modulated by transcription factors that include NRF (NF-E2 related factor). These transcription factors bind to specific ARE sequences to promote expression of genes (Mut-Salud et al., 2016).

The cancer promotion suppressors are agents that can activate specific cellular pathways to express signals that induce apoptosis in abnormal cells. They can also terminate malignant cell growth by repairing damaged DNA material (Mut-Salud et al., 2016). Mitogen-activated protein kinases (MAPKs) are intracellular signalling pathways responsible for regulation of cellular proliferation (Yu and Kensler, 2005: Kwon et al., 2007). Dietary antioxidants targeting different moieties in MAPKs can silence or activate the signalling system, thereby preventing abnormal cell proliferation (Mut-Salud et al., 2016). Excessive stimulation of NF-κB leads to increased resistance to cellular apoptosis and neoplastic cellular transformation. Several phytochemicals have been found to target the NF-κB genes to suppress the expression of these genes thus preventing development of cancer (Martin-Moreno et al., 2008).

Classification of radio-protectors

Primary radio-protectors

These are compounds that donate an electron to radiation induced free radical present in the system (for example, lipid radical) forming a new radical more stable than the initial one. Primary antioxidants include compounds such as flavonoids, tocopherol and ascorbic acid.

Secondary radio-protectors

Secondary radio-protectors remove ROS initiators by quenching chain-initiating catalysts. This can be accomplished by deactivation of high-energy species like 02-, absorption of UV light, chelations of metal catalysing free radical reactions, or by inhibition of peroxidases, such as xanthine oxidase or lipoxygenases (Shukla and Gupta, 2010).

Radio-protective formulations

The prevalence of cancer has remained high despite people taking nutritional supplements containing antioxidants such as ascorbic acid. Biological oxidation and radiation exposure are the sources of reactive oxygen species that exerts damaging effects to essential biomolecules such as DNA, proteins and lipids. Damage to the DNA may cause mutations which can lead to the development of cancer. There is converging evidence that herbs play a major role in degenerative diseases such as cancer, with several reports in agreement on the fact that these herbs should be taken in combination rather than as single entities. Therefore, a good radio-protective pharmaceutical formulation should be a mixture of herbs in order to provide a wide range of poly-phenols. This will enable the herbs to be effective in long term, and will improve the quality of life by postponing the onset and preventing degenerative diseases through several mechanisms. This implies that radioprotective formulations should consist of a mixture of herbs for them to be effective (Mut-Salud et al., 2016).

Plant selection

The plants are native to Zimbabwe and have never been tested for cyto-protective properties. Large quantities of phenols are usually found in fruits which are normally coloured, hence the choice of these two plants (Soto et al., 2015).

Uses of *F. indica* in traditional medicine

The pulp is sweet but with an acidic tang, and it can be eaten raw or used to make jelly or jam. The fruit can be fermented to make wine, while the leaves infusion or roots extracts are used for treatment of snakebite. The bark is an effective herbal medicine for arthritis. The leaf is an astringent and utilized as a tonic, an expectorant and for asthma, gynaecological disorders, as an antihelmintic and in treatment for pneumonia. The decoction of the root is used to relieve body pains. In Madagascar, the bark oil is utilized as anti-rheumatic liniment. The ash of burnt root is used for kidney disorders (Orwa et al., 2009), while the leaves are chewed by mouth to treat diarrhoea (Maroyi, 2011).

Uses of *A. stenophylla diels* in traditional medicine

The pulp of ripe fruit is sweet with a pleasant smell and taste. Ripe fruits are soaked in water, squeezed and filtered for juice. The orange-yellow is 25 – 45 mm long, containing a soft edible pulp full of numerous black, shiny seeds. The roots are used for treatment of diabetes mellitus II (Taderera et al., 2015). *A. stenophylla diels has* been reported to treat gonorrhoea, syphilis and abdominal pains. Infusions, which are made with other plants, are taken by mouth. The roots provide a strong medicine for treating tooth pain and the infusion is cooled down before using it to rinse the mouth, and it is spat out. Roots paste applied on the boils and extract is drunk as treatment for chest pains and STI remedy; mixed with roots of *Securidaca longipedunculata Fresen* and sprinkled around homestead as snake repellent (Maroyi, 2011).

Chemicals and equipment

Chemicals

DPPH 2,20-diphenylpicrylhydrazyl, hexane, aqueous methanol, gallic acid Folin Ciocalteu's reagent, sodium carbonate (20%), Quercetin, aluminium chloride DPPH, sodium chloride , disodium hydrogen phosphate, sodium dihydrogen phosphate, distilled water,stock RBC suspension, ascorbic acid.

Equipment

Centrifuge machine (Universal centrifuge: Model PLC-034H), UV visible spectrophotometer (MRC SPECTRO Uv-63pc), PH meter, UV lamp, rotor vapour, thermostat, incubator, stop watch, Buchner funnel, refrigerator, mortar and pestle.

METHODOLOGY

Collection of fruits

F.Indica fruits were obtained from Mvuma area that is located 200 km south of Harare whilst the *A.stenophylla diels* was obtain from Mhondoro that is located 90 km south west of Harare. The voucher specimens can be found at the national herbarium of Zimbabwe.

Phenolic compounds extraction

The extractions were done using hexane and aqueous methanol solvents, as in method described by (Markham, 1982). 100g of dried *A. Stenophylla diels* and *F.indica* fruits was ground using mortar and pestle. The dried fruit powder was mixed with 50 ml of 85% aqueous methanol for 48 h. The mixture was then filtered using a Buchner funnel to remove the slurry. The crude extract was obtained after removing methanol on rotary evaporator. The crude aqueous extract was purified by extracting it with hexane for four times to ensure that the lipids are removed. The solvents were then removed using a rotary evaporator to obtain a solid extract. The solid extract obtained was weighed and stored at -4°C in a refrigerator.

Determination of total phenol content

Concentrations ranging from 20 to 100 µg/ml of gallic acid were prepared following procedure described by Kamtekar et al. (2014) and Roya and Fatemeh (2013). 1ml of gallic acid aliquots with concentration ranging from 20 to 100 µg/ml were added to a test tube. To the same test tube 5ml of water and 0.5 ml of Folin Ciocalteu's reagent was added and the mixture was shaken. 1.5 ml of Sodium carbonate (20%) was added after five minutes and distilled water was then added to make the total volume 10 ml. The mixture was incubated for 2 h at room temperature to allow an intense blue colour to develop. Absorbance was then measured at 750 nm using UV visible spectrophotometer. The blank was performed in which the reagent is replaced with water. The results obtained were used to plot calibration curve of standard gallic acid. The phenol content of extracts was determined using method described by Bhalodia et al. (2011) and Patel et al. (2010).

$$T = c\ V/m$$

Where; T is total phenolic content in mg/g dry extract of gallic acid equivalent C concentration of graph obtained from calibration curve in mg/ml, V is volume of extract in ml, m mass of the extract in grams.

Determination of flavanoid

Different concentrations of quercetin were prepared following procedure described by Kamtekar et al. (2014**)**. Aliquots of Quercetin with concentration ranging from 10 to 50 µg/ml were added to a test tube. 1 ml of quercetin was added to test tube and then 1 ml of 2% aluminium chloride in methanol was added. The mixture was incubated for 10 min and the absorbance of the mixture was then measured at 430 nm using UV visible spectrophotometer. Results obtained were used to plot a calibration

Table 1. Showing free radical scavenging of plant fruit extracts **as % inhibition of absorbance.**

Concentration in (µg/mL)	**Annona Stenophyla Diels**	***Flacourtia* indica**	**Ascorbic acid**
20	53.25 ± 0.39	39.85 ±1.15	49.81 ± 1.15
40	61.30 ± 2.30	48.28 ± 0.77	56.70 ± 0.58
60	71.26 ± 0.38	58.24 ± 1.34	59.77 ± 0.77
80	74.71 ± 2.27	68.97 ±1.34	67.05 ± 1.16
100	81.22 ± 0.59	83.52 ± 0.76	71.26 ±1.34
IC50 (µg/mL)	19	45	49

IC50 is the minimum concentration to cause 50% inhibition and determine potency of the extracts.

curve which was then used to estimate the total flavanoid content of plant extracts.

Assessment of antioxidant activity of plant extracts

The capacity of plant extracts to scavenge free radical was assessed according to standard method used by Prakash et al. (2017). Plant extract samples were diluted to obtain concentrations ranging from 20 to 100 µg/ml. 50 ml of sample extract containing various concentrations were prepared. 50 ml of samples were then mixed with 5 ml of a 4 mg/100 ml methanol solution of DPPH. Ascorbic acid was used as a positive standard. The negative control was prepared by replacing extract with an equal volume of methanol. The mixture was shaken and then incubated for 30 min in dark fume hood at room temperature. After incubation in darkness, the absorbance was measured at 517 nm and methanol was used as a blank. Concentration of extracts that caused 50% inhibition (IC50) was estimated by plotting a graph of % inhibition against concentration, using a nonlinear regression algorithm. IC50 can also be calculated using the equation below.

$$IC50 = 50 - Y\ Intercept/\text{slope}$$

DPPH scavenging capacity was calculated as follows:

DPPH radical scavenging activity percentage = (Ab Blank - Ab sample / Ab Blank) x 100

Radioprotective efficacy

Stock solution

The stock 0.9% saline solution buffered at pH 7.4 was prepared by dissolving sodium chloride (90 g), disodium hydrogen phosphate (13.65 g) and sodium dihydrogen phosphate (2.34 g) in 1 L of distilled water.

Treatment of sheep Blood

The sheep blood was washed with buffered 0.9 saline solutions for three times. Each blood wash was followed by centrifugation for ten minutes at 3000 rpm. After washing a 40% v/v suspension was prepared using 0.9% isotonic buffered saline solution. The suspension was then used as stock RBC suspension.

Procedure for testing radioprotective efficacy

Radioprotective testing was done according to method described by Patel et al. (2012) with some modifications. The experimental sample was prepared by mixing 2 ml of extract, 5 ml of 0.9 % saline stock solution and 5 ml of RBC suspension. The negative control sample was prepared by replacing of fruit extract in experimental sample with 2 ml of 0.9% saline solution. The preparation of standard (positive control) was similar to that of test except that it had 2 ml of ascorbic acid instead of herbal extract. The concentration of herbal extracts and ascorbic acid ranged from 80-200 µg/ml. All the prepared mixtures were exposed immediately to UV (wavelength 365 nm) light for 30 min at room temperature. The mixtures were then centrifuged for 10 min at 3000 rpm. The absorbance of the supernatant was measured using UV visible spectrophotometer at 540 nm. Radioprotective efficacy of extracts was then determined by the extent of radiation induced haemolysis. Radioprotective efficacy was calculated as percentage inhibition of haemolysis using the following equation (Gunathilake et al., 2018).

% Inhibition of haemolysis = 100 x [A 1 –A 2 /A 1]

Where: A 1 = Absorbance of isotonic buffered solution alone and RBC cell suspension after exposure to UV light; A 2 = Absorbance of isotonic buffered solution alone and RBC cell suspension test solution containing (Herbal extracts or ascorbic acid) exposed to UV light.

RESULTS AND DISCUSSION

DPPH assay

DPPH is a free radical compound with maximum absorption band ranging from 515 - 528 nm; therefore, it is a useful reagent for use in evaluation of antioxidant effects of phenolic compounds (Sánchez-Moreno, 2002). DPPH assay involves the reduction of DPPH by antioxidants to a yellow coloured compound called diphenylpicrylhydrazine. The reduction extent depends on hydrogen donating capacity of antioxidants. The hydrogen donating capacity of *Annona Stenophyla Diels* and *F.Indica* fruit extracts of concentrations ranging from 20 to 100 µg/ml were studied. The results obtained exhibited dose dependent DPPH radical scavenging activity as presented in Table 1. All the extracts and the positive control significantly decreased haemolysis ($P \leq 0.05$). At low concentrations, *F.Indica* extract was less potent than ascorbic acid (standard) ($P \leq 0.05$). There was no significant difference between % inhibition of

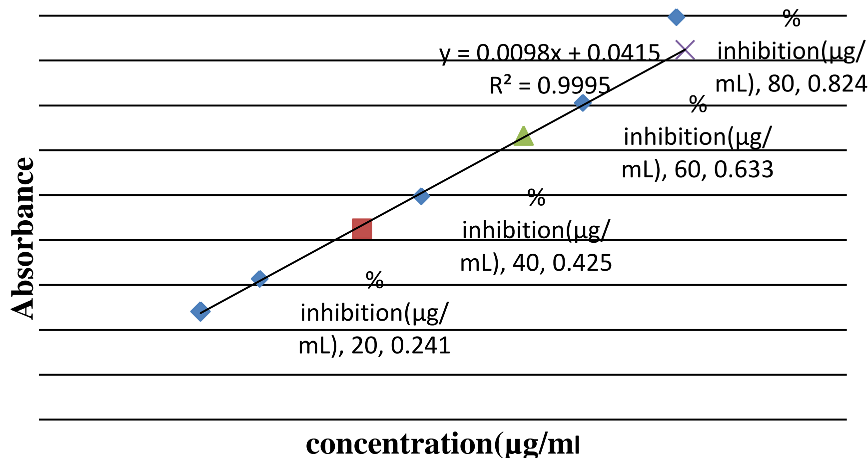

Figure 1. Standard gallic acid curve for determination of total phenol content.

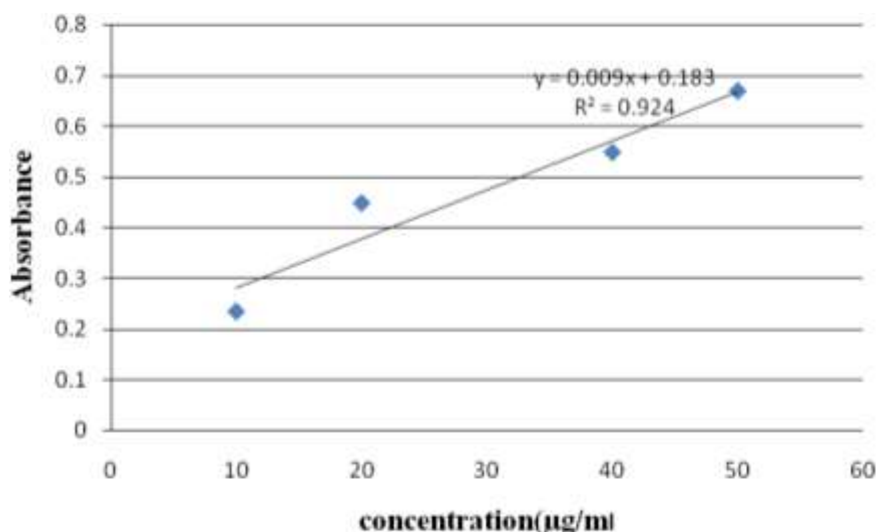

Figure 2. Standard Quercetine curve for determination of total flavonoids content.

Annona Stenophyla Diels and ascorbic acid (positive standard) ($P \leq 0.05$). This implies that ascorbic acid and *Annona Stenophyla Diels* have equal antioxidant potency. However % IC_{50} which is also an indicator for potency shows that the extracts were more potent than ascorbic acid (standard) as depicted in Table 1. The results obtained in this study suggest that the fruit extracts are good radioprotectors and radical quenchers; therefore, eating these fruits can protect or postpone development of degenerative diseases such as cancer (Figures 1 to 8).

RBC haemolysis

Red blood cells are the most abundant cells in the human body, and they have their unique physiological and morphological characteristics (Hamidi and Tajerzadeh, 2003). Their membranes are rich in polyunsaturated fatty acids and envelopes haemoglobin carrying high oxygen concentrations; hence red blood cells are extremely prone to oxidative damage (Arbos et al., 2008). Oxidative damage of red blood cell membrane was implicated in haemolysis by (Arbos et al., 2008). In view of this, it is reasonable to use red blood cells in the study of oxidative stress and radio-protective effects of fruit plant extracts by exposing them to UV light, a free radical initiator. The antioxidant effect of plant fruit extracts was confirmed in sheep erythrocytes exposed to concentrations ranging from 100 to 200 µg/ml. All extracts showed concentration dependent inhibition of haemolysis as shown in Table 2. The extracts of both plant fruits and the positive control

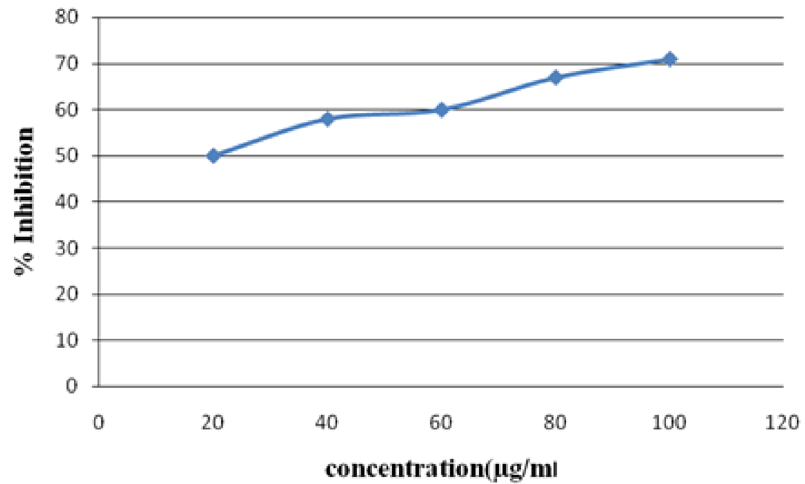

Figure 3. Showing % inhibition against concentration of Ascorbic acid.

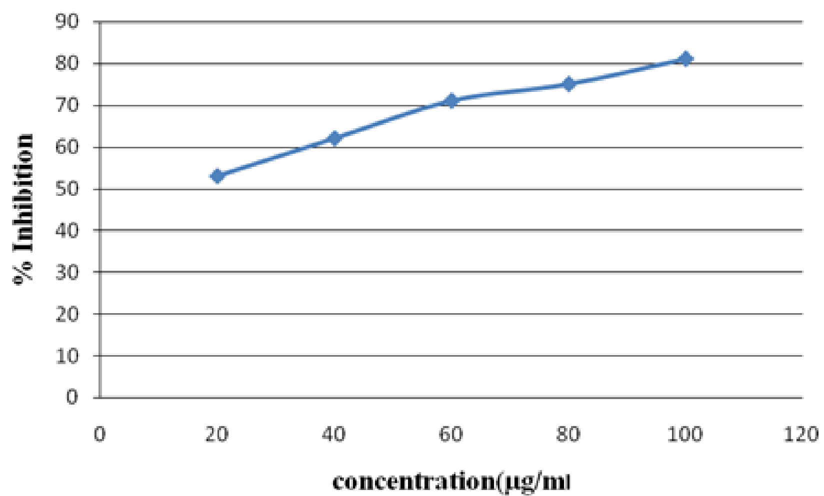

Figure 4. Showing % inhibition against concentration of *Annona Stenophyla Diels.*

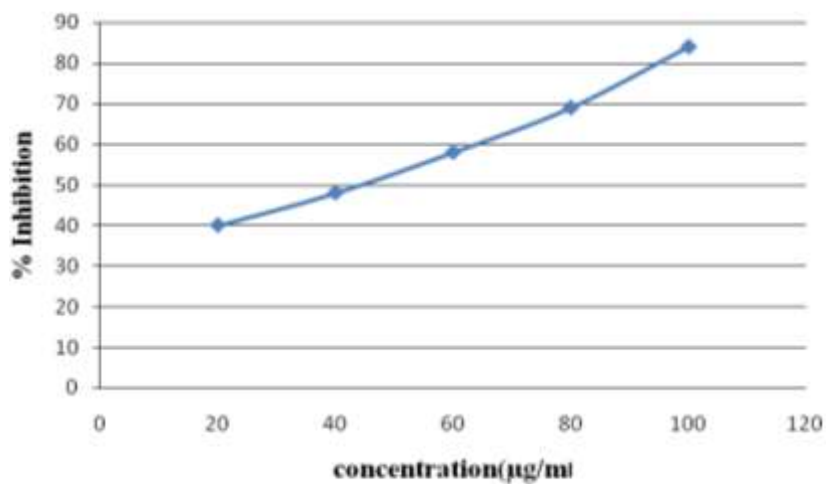

Figure 5. Showing % inhibition against concentration of *F.Indica.*

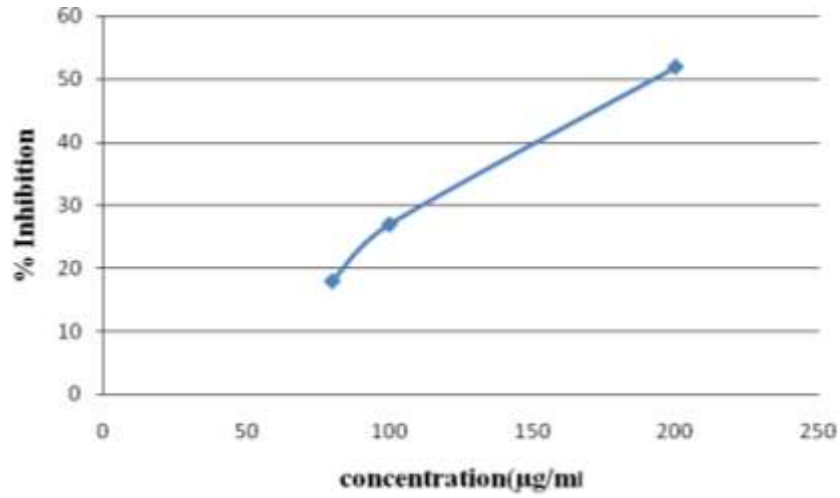

Figure 6. Showing % haemolysis inhibition against concentration of ascorbic acid.

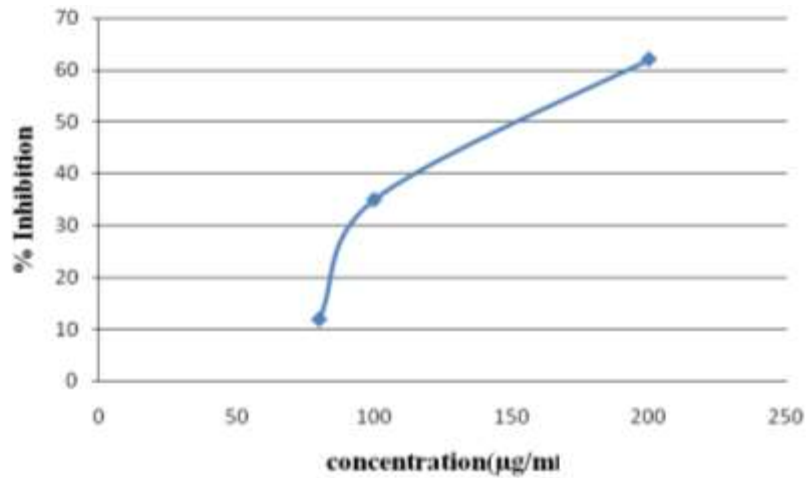

Figure 7. Showing % haemolysis inhibition against concentration of *F.Indica.*

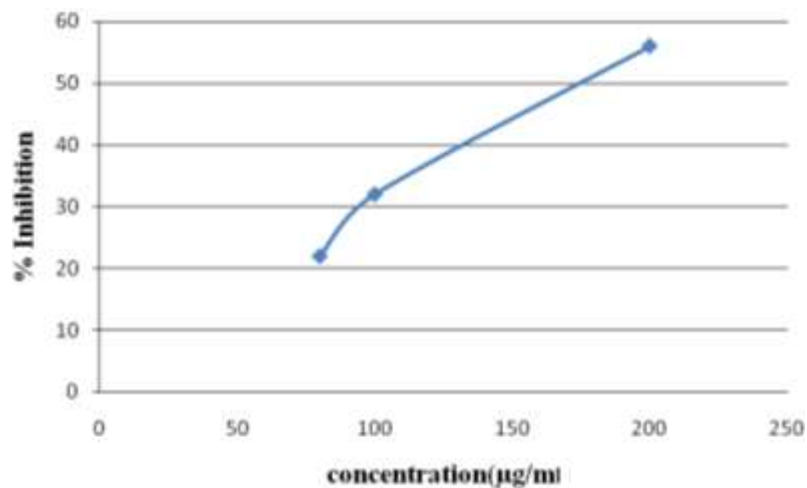

Figure 8. Showing % haemolysis inhibition against concentration of *Annona Stenophyla Diels.*

Table 2. Showing % radio-protective effects of plant extracts and IC50 (μg/mL).

Concentration in (μg/mL)	*Annona Stenophyla Diels*	Indica	Ascobic acid
80	21.63 ±0.60	11.54± 0.36	17.42 ±0.49
100	31.96 ± 0.49	35.08 ± 1.48	27.12 ± 0.73
200	57.54 ±0.73	61.98 ±0.73	51.82 ± 0.49
IC50 (μg/mL)	149	157	152

IC50 is the minimum concentration to cause 50% inhibition and determine potency of the extracts.

significantly decreased haemolysis (P ≤ 0.05). At higher concentrations *F.Indica* extract was more potent than ascorbic acid, the positive standard (P ≤ 0.05). There was no significant difference between % haemolysis inhibition of *A. stenophyla diels* and ascorbic acid (positive standard) (P ≤ 0.05). This implies that ascorbic acid and *A. Stenophyla Diels* have equal radio-protective potents. These results obtained show that if plant extracts can protect red blood cells from UV induced membrane peroxidation mediated haemolysis, they have the capacity to protect all other cells from UV induced mutations leading to cancer. UV light is a radical initiator; the plasma membrane forms peroxyl radicals upon UV light exposure. These radicals then undergo self-perpetuating reactions, forming more radicals leading to damage of important biomolecules such as proteins and DNA.

Phenol and flavanoid content

Studies have uncovered that free radicals such as hydroxyl, peroxide and superoxides play an important role in the pathogenesis of cancer, and plants are the main sources of antioxidants. Hence, plant derived supplements can be useful in maintenance of good health and combating degenerative diseases including cancer (Demiray et al., 2009). Results obtained in this study indicate that the phenol content of *Annona Stenophyla Diels* and *F.Indica* was 306 and 384 mg/gGAE (gallic acid equivalent) respectively, and the flavanoid content was 157 and 170 mg/gQE (quercetin equivalent) respectively.

Conclusion

The main aim of this research was to investigate the cytoprotective properties of plant fruit extracts using DPPH assay and UV induced haemolysis assay. The results obtain shows that the plant extracts inhibited DPPH absorption and can be used to protect cells from damaging reactive oxygen species. The plant extracts also suppressed UV light induced haemolysis. These findings suggest that fruit extracts can be used as armoury for protection against radiation exposure which induces peroxidation of lipids, forming radicals which can potentially damage DNA leading to the development of cancer. The results obtained further suggest that the fruits of *A. Stenophyla Diels* and *F.Indica* can be used in the formulation of nutraceuticals for prevention of cancer. These fruit extracts are alternative radio-protectors of plant origin which may provide radioprotection to other normal tissues during radiotherapy since radiation genotoxicity is largely free radical mediated and chemotherapy treatments whose mechanism involves free radical attack. The study was carried out in vitro, hence there is need to carry out in vivo studies to enable the inclusion of fruit extracts in herbal cancer prevention formulations. Further research should also focus on the potential use of fruit extracts to reduce the side effects of radiotherapy and its role in recovery of patients after a chemotherapy session. Further research should also focus on studies which include use of in-vitro cell lines as well as in-vivo studies.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

The study was approved by Harare Institute of Technology.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Chemical fingerprint of *Bacopa monnieri* L. and *Rosmarinus officinalis* L. and their neuroprotective activity against Alzheimer's disease in rat model's putative modulation *via* cholinergic and monoaminergic pathways

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Alzheimer's disease is characterized by progressive degeneration of cortical and hippocampal neurons. This study aims to characterize the metabolic profiles of the hydro-ethanolic extracts of *Bacopa monnieri* L. (BM) and *Rosmarinus officinalis* L. (RO) cultivated in Egypt *via* UPLC–ESI/MS analyses and reveal their possible mechanism of the prophylactic effect(s) on neuro-degeneration in rat model of Alzheimer's disease (AD). Here, UPLC–ESI/MS analyses were employed for the characterization of hydro-ethanolic extracts. Forty-two male albino rats were intra-peritoneally injected with Aluminum chloride at a dose of 4.2 mg/kg to induce AD. The extracts of BM and RO were separately orally administered at doses of 300 and 450 mg/kg, and Donazil® was orally administered at dose 2.5 mg/kg. Serum levels of malondialdehyde (MDA), and total antioxidant capacity (TAC) were measured using ELISA. Further, Amyloid β-protein, acetylcholinesterase (AChE), τ-protein and serotonin levels were measured in brain tissue using ELISA. The UPLC–ESI/MS analyses revealed the presence of fifteen and seventeen active metabolites in BM and RO extracts respectively which may account for their effects on neuro-degeneration. Serum level of MDA, amyloid β-protein, AChE and τ-protein were significantly decreased in herbal treated groups when compared to AD group (P value < 0.0001). On the other hand, TAC and serotonin levels were significantly elevated in groups treated with BM and RO compared to AD group (P value < 0.0001). Consequently, BM and RO extracts were found to have a potential neuroprotective effect in AD rat model due to their variety of active metabolites.

Key words: Alzheimer's, serotonin, anti-AChE, antioxidant, *Bacopa monnieri, Rosmarinus officinalis.*

INTRODUCTION

Aluminum (Al) is the most abundant metal on earth, it can enter the body through diet, drinking water, aluminum containing drugs and so enters the brain; deposited in the cortex, hippocampus and cerebellum which are crucial for

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memory and cognition. Al was reported as a main risk factor for the cause and development of neuro-degenerative diseases as: Alzheimer’s disease (AD), amyotrophic lateral sclerosis and Parkinson’s disease (PD) (Thenmozhi et al., 2016).

Aluminum induced neurotoxicity was previously reported by many authors in which administration of aluminum chloride hexahydrate 25 mg/kg/day for one month orally (Zaky et al. 2013) and daily treatment with $AlCl_3$ at dose 100 mg/kg orally for 42 days was observed (Lin et al., 2015).

Alzheimer’s disease is characterized by progressive degeneration of cortical and hippocampal neurons. This results in deterioration of the persons’ memory and cognitive ability (Ghoneim et al., 2015). AD is the most common cause of dementia among elderly population (Zhang et al., 2016) and has a progressive and devastating nature that poses a huge financial and social burden on families and caregivers of the elderly (Sica, 2015; Goren et al., 2016). Precise worldwide prevalence of AD is difficult to estimate; however, prevalence of AD is expected to increase in the coming decades as a consequence of aging of the world population (Goren et al., 2016; Brookmeyer et al., 2007; Carter, 2008).

Although not well understood, it has been postulated that loss of cholinergic function at the central nervous system as well as accumulation of reactive oxygen species are the key components implicated in AD pathophysiology (Bartus, 2000; Murphy and Steenbergen, 2008). Accumulation of amyloid β (Aβ) protein and appearance of neurofibrillary tangles of tau (τ) protein are the most prominent pathological hallmarks of AD (Iqbal and Grundke-Iqbal, 2010). To date, there is no effective treatment for AD that is why finding preventive measure to reduce the disease incidence is of crucial significance (Sica, 2015).

Bacopa monnieri L. (BM), commonly known as “Brahmi” named after Brahma, the creator god of the Hindu pantheon of deities, is an herb that is used in traditional Indian medicine for its antioxidant and anti-inflammatory as well as memory-enhancing effect. This effect is believed to date back more than 3000 years in India (Rathee et al., 2008; Chaudhari et al., 2017).

Rosmarinus officinalis L. (RO) is used since antiquity to enhance the memory. The ancient Egyptians had a famous tradition of laying rosemary across the coffin or upon a tombstone during the embalming process and it was considered sacred to ancient Egyptians, Romans and Greeks (Burlando, 2010). It was traditionally burned for the Greek students prior to their exams to boost their mental performances and was considered a loyalty symbol between lovers due to this trait (Rathee et al., 2008). Rosemary is an herb reportedly known for its antioxidant and anti-inflammatory effects (Aruoma et al., 1996; Lipton et al., 2016; Posadas et al., 2009).

Herbal extracts pose a potential hope for prevention and treatment of many age-linked diseases such as atherosclerosis (Kabiri et al., 2012), hypertension (Reinhart et al., 2008), type 2 *Diabetes mellitus* (Li et al., 2004) and osteoarthritis (Aborehab et al., 2017). Focusing on nootropic herbal extracts, it is well established that *Ginkgo biloba* (Birks and Grimley, 2007), *Piper nigrum* (Subedee et al., 2015), *Hericium erinaceus* (Zhang et al., 2016), *Withania somnifera* (Bhattacharya et al., 2001) and *Panax ginseng* (Petkov et al., 1993) extracts improve learning and memory deficits and relieve the neuropsychological symptoms associated with animal models of AD. Recently, BM showed cholinergic effects in mice similar to current treatments of AD (Le et al., 2013). Also, attention has been drawn to use RO in treatment of AD as its metabolite, carnosic acid (CA), was found to enhance memory and learning of AD mice model (Lipton et al., 2016).

The objective of this study is to characterize the metabolic profiles of the hydro-ethanolic extracts of BM and RO cultivated in Egypt and to investigate their neuroprotective effects compared to standard medication Donazil® as a selective acetylcholinesterase inhibitor as well its potential mechanism of its neuroprotective actions in a rat model of AD.

METHODOLOGY

Plant material

The aerial parts of “Brahmi” *B. monnieri* L. (Plantaginaceae) and “Rosemary” *R. officinalis* L. (Lamiaceae) were provided from El Orman Botanical Garden, Giza, Egypt and kindly identified by Dr. Mohamed El-Gebaly (National Research Institute, Dokki, Giza, Egypt). Voucher specimens of both collected samples were kept at Pharmacognosy Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA) with codes (MSA-2017-8 and MSA-2017-9, respectively). The Fresh aerial parts (500 g) were air dried at room temperature, then ground into fine powder. The extracts were obtained using a Soxhlet extraction over a period of 8 h using 70% ethanol, filtered, and the solvent was evaporated under reduced pressure in a rotatory evaporator at a temperature as low as 45°C. The dried extracts were kept in a desiccator for further chemical and biological investigations.

UPLC-Electro spray ionization-mass spectroscopy (UPLC–ESI-MS) apparatus

40 µg of BM and RO hydro-ethanolic extracts were separately dissolved in 1 mL HPLC grade methanol, filtered using a 0.2 µm membrane disc filter and degassed by sonication prior to injection. UPLC–ESI-MS analyses were performed on an Agilent® 1100 Series using ACQUITY UPLC - BEH C18 column, (1.7 µm - 2.1 × 50 mm, i.d.), with an integrated pre-column. 10 µL of each extract was eluted using gradient mobile phase composed of two eluents: eluent A is nano-pure H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid with the flow rate of 0.2 mL/min for 35 min. A XEVO TQD triple quadruple instrument, Waters® Corporation, Milford, MA01757, U.S.A mass spectrometer connected to a PDA detector with standard flow cell (10 mm path length, 14 µL volume, 40 bar maximum pressure) was used for mass spectrometric analysis. ESI interface was employed in both negative and positive ion modes using N_2 as a drying and nebulizing gas. At 250°C capillary temperature, the spray and capillary voltages were 4.48 kV and 39.6 V respectively and the full scan mode was in mass range of *m/z* 100–2000. The peaks and

spectra were interpreted using the Masslynx 4.1® software and tentatively assigned by comparing their mass spectrums with the reported data.

Chemicals and drugs

Aluminum chloride ($AlCl_3$) was purchased from Sigma-Aldrich Chemicals Co, Egypt, dissolved in saline 0.9% and injected intraperitonealy (I.P) at dose 4.2 mg/kg/day for 28 days according to methodology of Bitra et al. (2014) and Nayak and Chatterjee (2001). Ethanol (analytical grade) was purchased from El Gomhoreya Co., Egypt. Donepezil hydrochloride, is the main active ingredient in Donazil®; it acts as a selective acetylcholinesterase inhibitor and hence, enhancing the cholinergic activity in the brain which is insufficient with AD (Seltzer, 2005). Donazil was purchased from Eva Pharma, Egypt, and was dissolved in Carboxy Methyl Cellulose (CMC) (0.25%) and administered orally.

Animals

Forty-two male albino rats, weighting 200 ± 20 g at the start of the experiments were used. Prior to the initiation of the studies, the animals were randomized and assigned to treatment groups. Four rats were housed per cage (size 26 × 41 cm) and placed in the experimental room for acclimatization 24 h before the test. The animals were fed with standard laboratory diet and with tap water ad libitum, and kept in an air-conditioned animal room at 23 ±1°C with a 12 h light/dark cycle. Animal care and handling was performed in conformity with approved protocols of MSA University, Faculty of Pharmacy, Research Ethics Committee and Egyptian Community guidelines for animal care.

Experimental groups

The dose of BM extract was determined according to methodology of Sathiyanarayanan et al. (2010). The LD_{50} of RO extract was determined previously (Anadon et al., 2008) which is 2000 mg/kg of body weight. Rats were randomly allocated into seven groups of six animals each. Rats were orally given the hydro-ethanolic extracts of BM and *RO* for a period of 2 weeks prior to injection of $AlCl_3$.

Group 1: Control group injected (I.P) by 0.9% saline.
Group 2 ($AlCl_3$): Rats injected $AlCl_3$ at dose 4.2 mg/kg/day (I.P) for 28 days.
Group 3 (BM 300): Rats received BM extract 300 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 4 (BM 450): Rats received BM extract 450 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 5 (RO 300): Rats received RO extract 300 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 6 (RO 450): Rats received RO extract 450 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 7 (Donazil® 500): Rats received Donazil 2.5 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.

Blood samples and biochemical analysis

Preparation of blood samples

At the end of the study, rats were fasted overnight, anesthetized with thiopental sodium (50 mg/kg) (Vogler, 2006) and blood samples were collected in the morning (5 ml per rat). Blood samples were centrifuged at 3000 rpm for 15 min after 30 min of collection and stored at –80°C until analyzed for the analysis of Malondialdehyde (MDA), and Total anti-oxidant capacity (TAC).

Preparation of brain samples

Animals were euthanized by cervical dislocation, and then the brain was rapidly removed from each rat. Part of each brain was fixed in formalin-saline for 48 h for histopathological study. Another part of the brain was homogenized, using glass homogenizer (Universal Lab. Aid MPW-309, mechanika precyzyjna, Poland), with 5 ml phosphate buffer saline (PBS) then centrifuged using cooling ultra-centrifuge. The homogenate was divided into four aliquots for measuring Amyloid β (Aβ) - peptide, acetylcholinesterase (AChE), tau (τ) protein and serotonin.

Biochemical analysis

Analysis of serum was carried out for measuring MDA, and TAC levels using corresponding colorimetric Cell Biolabs, Inc, USA and Zen-Bio, ABTS antioxidant assay kit, Inc., USA respectively. Aβ-peptide, tissue AChE, τ-protein and serotonin were measured using corresponding rat enzyme immunoassay kits Wuhan EIAAB Science Co, Ltd, China, Kamiya Biomedical Company, USA, Elabscience, USA, and Lifespan Biosciences Inc. USA, respectively.

Histopathological examination

As mentioned earlier, brain tissue was fixed in 10% formalin and then routinely processed and embedded in paraffin. Five microns' sections were cut and stained with hematoxylin and Eosin (H&E) and Congo Red.

Statistical analyses

All data were expressed as mean ± SD and analyzed using Prism program version 6. For all parameters, comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. All *P* values reported are two-tailed and $P < 0.05$ was considered significance.

Ethics approval

Animal care and handling was performed in conformity with approved protocols of MSA University and Egyptian Community guidelines for animal care.

RESULTS AND DISCUSSION

Characterization of major metabolites in BM and RO extracts *via* UPLC-ESI-MS

Chromatographic fingerprints of the hydro-ethanolic extracts of BM and RO were obtained using UPLC-ESI-MS as depicted in Figures 1 and 2 respectively. The identities, elemental compositions, relative percentages and observed molecular and product ions for individual components in both positive and negative modes are presented in Tables 1 and 2 respectively. With the optimized LC and MS conditions, a total of 15 and 17

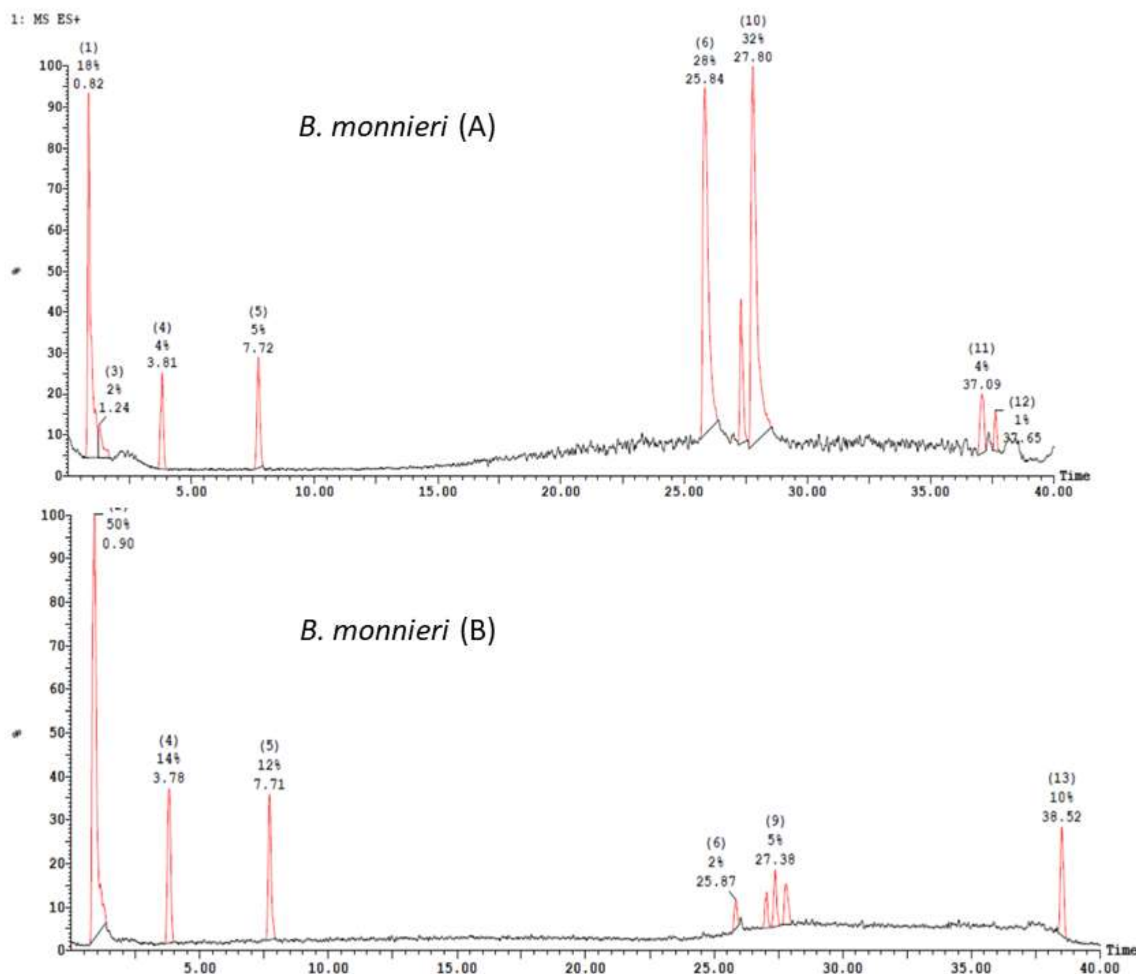

Figure 1. The LC–ESI/MS Chromatograms of the hydro-ethanolic extracts of *B. monnieri* (A): positive mode and (B): negative mode.

metabolites were tentatively characterized in BM and RO extracts respectively on the basis of their elemental compositions and MS fragmentation patterns compared to the data previously reported in literature.

The major metabolites of BM are dammarane-type of steroidal saponins that are categorized into jujubogenin saponin glycosides and pseudojujubogenin saponin glycosides. Pseudojujubogenin is the isomer of jujubogenin having different position of the prenyl side chain (Nuengchamnong et al., 2016), in addition to, cucurbitacins and sterol glycosides. The UPLC-ESI-MS analyses revealed the presence of 5 jujubogenin saponin glycosides, namely; bacoside A1, bacopasaponin F, bacopaside N1, deoxy-jujubogenin-Ara-Glc and deoxy-jujubogenin-2-Glc; and 7 pseudojujubogenin saponin glycosides, namely; bacopaside (I, II and III), oxy-bacopaside I, bacopasaponin (C and D) and pseudojujubogenin-Glc-Glu-Ara. Cucurbitacins (bacobitacin B and C) and bacosterol-Glc were also identified in the hydro-ethanolic extract of BM.

The UPLC-ESI-MS analyses of RO hydro-ethanolic extracts led to the characterization of rosemary landmarks represented in 6 phenolic diterpenes, namely; carnosol, carnosic acid, rosmanol, epirosmanol, rosmadial and methyl carnosate, 3 phenolic acids namely; Gallic acid, caffeic acid and rosmarinic acid. Flavonoids of flavone and flavanone types were also detected such as apigenin, luteolin, cirsimaritin, hesperidin and their glycosides, in addition to the dihydrochalcone (phloridzin) and the lignan (medioresinol).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on serum MDA and TAC levels

Mean serum level of MDA was significantly increased in $AlCl_3$ induced Alzheimer's group compared to the control group (*P* value was < 0.001). Mean serum level of MDA was significantly reduced in BM 300, BM 450, RO 300,

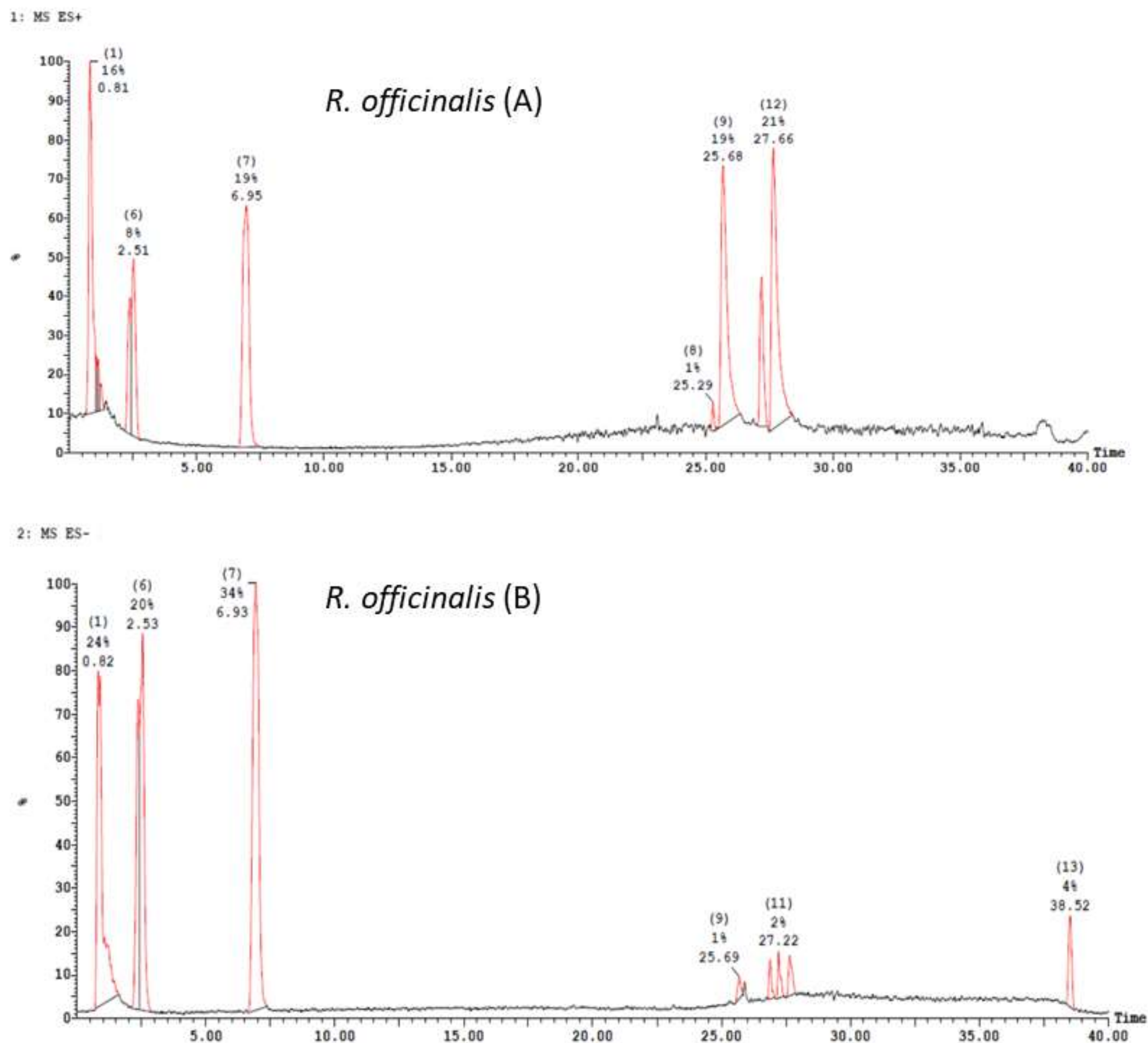

Figure 2. The LC–ESI/MS Chromatograms of the hydro-ethanolic extracts of *R. officinalis* (A): positive mode and (B): negative mode.

RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (*P* value < 0.001). RO 450 treatment reduced MDA level compared to BM 450 and Donazil groups (*P* value was < 0.001).

Similarly, mean serum level of TAC was significantly decreased in $AlCl_3$ induced Alzheimer's group compared to the control group (*P* value was < 0.0001). The mean serum level of TAC was significantly raised in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.001). TAC was increased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figures 3 and 4; Table 3).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue amyloid beta protein and τ-protein levels

Mean tissue level of amyloid β peptide was significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value was < 0.0001). On the other hand, mean tissue level of amyloid β peptide was significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Amyloid β peptide was decreased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figure 5 and Table 4).

Table 1. Peak assignment of metabolites in the hydro-ethanolic extract of *Bacopa monnieri* using LC–ESI/MS in the positive and negative modes.

Peak No.	Positive Ionization		Negative Ionization		Elemental composition	Tentative compound assignment	Relative (%)	References
	$[M+H]^+$ *(m/z)*	Product ion fragments *(m/z)*	$[M-H]^-$ *(m/z)*	Product ion fragments *(m/z)*				
Jujubogenin saponin glycosides								
1	736.69	455	n.d.	n.d.	$C_{40}H_{64}O_{12}$	Bacoside A1	2.65	Nuengchamnong et al. (2016)
2	n.d.	n.d.	1059.38	765, 633	$C_{52}H_{84}O_{22}$	Bacopasaponin F	2.13	
3	797.47	779 , 635, 455	795.39	633	$C_{42}H_{68}O_{14}$	Bacopaside N1	1.47	
4	749.43	437	747.32	435	$C_{41}H_{64}O_{12}$	Deoxy-Jujubogenin-Ara-Glc	4.39	
5	779.62	437	n.d.	n.d.	$C_{46}H_{66}O_{10}$	Deoxy- Jujubogenin -2 Glc	2.81	
Pseudojujubogenin saponin glycosides								
6	979.1	767, 605, 473	977.46	845, 241	$C_{46}H_{74}O_{20}S$	Bacopaside I	32	Sookying et al. (2017)
7	n.d.	n.d.	927.48	795, 633	$C_{47}H_{76}O_{18}$	Bacopaside II	5.11	Nuengchamnong et al. (2016)
8	847.41	765, 515, 393	n.d.	n.d.	$C_{41}H_{66}O_{16}S$	Bacopaside III	14.18	
9	n.d.	n.d.	993.51	861, 505, 389	$C_{46}H_{74}O_{21}S$	Oxy-Bacopaside I	4	
10	n.d.	n.d.	897.03	765, 603	$C_{46}H_{74}O_{17}$	Bacopasaponin C	3.5	
11	767.5	605, 473	n.d.	n.d.	$C_{41}H_{66}O_{13}$	Bacopasaponin D	7.71	
12	n.d.	n.d.	941.53	809, 647	$C_{47}H_{74}O_{19}$	Pseudojujubogenin-Glc-Glu-Ara	28.06	
Cucurbitacins								
13	599.37	479, 443	n.d.	n.d.	$C_{34}H_{46}O_9$	Bacobitacin B	6.23	Bhandari et al. (2006)
14	1113.47	981, 835	n.d.	n.d.	$C_{54}H_{80}O_{24}$	Bacobitacin C	17.5	
Sterol glycosides								
15	575.89	414	n.d.	n.d.	$C_{35}H_{60}O_6$	Bacosterol-Glc prevent	9.72	Bhandari et al. (2006)

*n.d.: Not detected, Glc: glucose, Glu: glucouronide, Ara: arabinose.

Similarly, mean tissue level of tau-protein was significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value < 0.001). Also, the mean tissue levels of τ -protein was significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.001). τ -protein was decreased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figure 8 and Table 5).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue acetylcholinesterase (AChE) levels

Mean tissue levels of AChE were significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value < 0.0001). AChE were significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Also, AChE was reduced in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.0001 (Figure 6).

Table 2. Peak assignment of metabolites in the hydro-ethanolic extract of *Rosmarinus officinalis* using LC–ESI/MS in the positive and negative modes.

Peak No.	Positive Ionization		Negative Ionization		Elemental composition	Tentative compound assignment	Relative (%)	References
	$[M+H]^+$ (m/z)	Product ion fragments (m/z)	$[M-H]^-$ (m/z)	Product ion fragments (m/z)				
Phenolic diterpenes								
1	331.34	287	n.d.	n.d.	$C_{20}H_{25}O_4$	Carnosol	8.32	Hossain et al. (2010)
2	n.d.	n.d.	331.24	287, 244	$C_{20}H_{27}O_4$	Carnosic acid	19.47	
3	347.19	285	345.25	283	$C_{20}H_{26}O_5$	Rosmanol	1.87	Achour et al. (2018)
4	347.18	285	345.16	283	$C_{20}H_{26}O_5$	Epirosmanol	0.85	Borras-Linares et al. (2014)
5	n.d.	n.d.	343.37	315, 300	$C_{20}H_{23}O_5$	Rosmadial	16.28	Hossain et al. (2010)
6	347.2	303, 288	n.d.	n.d.	$C_{21}H_{29}O_4$	Methyl carnosate	0.72	
Phenolic acids								
7	171.01	127	169.03	125	$C_7H_5O_5$	Gallic acid	0.93	Hossain et al. (2010)
8	n.d.	n.d.	179.12	161, 135	$C_9H_7O_4$	Caffeic acid	0.95	
9	n.d.	n.d.	359.07	197, 161	$C_{18}H_{16}O_8$	Rosmarinic acid	19.39	Borras-Linares et al. (2014)
Flavonoids: Flavones								
10	n.d.	n.d.	269.1	269	$C_{15}H_{10}O_5$	Apigenin	3.75	Achour et al. (2018)
11	n.d.	n.d.	576.95	269	$C_{27}H_{29}O_{14}$	Apigenin-7-O-rutinoside	5.36	Hossain et al. (2010)
12	n.d.	n.d.	285.05	263, 191	$C_{15}H_{10}O_6$	Luteolin	24.36	Achour et al. (2018)
13	n.d.	n.d.	478.91	315	$C_{22}H_{22}O_{12}$	Nepetrin	1.5	Borras-Linares et al. (2014)
14	n.d.	n.d.	313.09	298, 283	$C_{17}H_{13}O_6$	Cirsimaritin	12.19	
Flavonoids: Flavanone								
15	611.07	463, 303	n.d.	n.d.	$C_{28}H_{34}O_{15}$	Hesperidin	20.24	Achour et al (2018)
Dihydrochalcone								
16	437.15	275, 169	435.13	273, 167	$C_{21}H_{23}O_{10}$	Phloridzin	0.73	Hossain et al. (2010)
Lignan								
17	389.23	209, 165	n.d.	n.d.	$C_{21}H_{24}O_7$	Medioresinol	1.28	Mena et al. (2016)

*n.d.: Not detected.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue serotonin level

Mean tissue level of serotonin was significantly decreased in $AlCl_3$ induced Alzheimer's group compared to control group (P value < 0.0001). Also, mean tissue levels of serotonin was significantly elevated in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Serotonin was increased in RO 450 group compared to Donazil and BM 450 groups at P

Table 3. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on serum MDA and TAC levels.

Groups	Serum MDA (umol/ml)	Serum TAC (umol/ml)
Control	12.4 ± 1.40	100 ± 7.43
ALZ	38 ± 4.56 [a]	41.9 ± 3.80 [a]
BM 300	26.5 ± 0.87 [ab]	53 ± 3.80 [ab]
BM 450	20.9 ± 1.24 [abc]	68 ± 3.82 [abc]
RO 300	32.5 ± 3.66 [ab]	54.4 ± 4.39 [ab]
RO 450	17.7 ± 1.32 [ab]	84.2 ± 5.52 [ab]
Donazil	21.4 ± 1.28 [abc]	76.7 ± 4.94 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a **=** Significant from control at P < 0.001, b **=** Significant from ALZ at P < 0.001, c = Significant from RO 450 at P < 0.001.

Table 4. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue amyloid beta protein and acetylcholinestrase levels.

Groups	Tissue amyloid beta peptide (Pg/gm tissue)	Tissue acetylcholinestrase (ng/gm tissue)
Control	9.3 ± 0.92	0.81 ± 0.08
ALZ	30.9 ± 4.01[a]	3.14 ± 0.26[a]
BM 300	21.8 ± 1.37[ab]	2.2 ± 0.08 [ab]
BM 450	17.3 ± 1.09[abc]	1.79 ± 0.08 [abc]
RO 300	21.8 ± 1.74[ab]	2.34 ± 0.15 [ab]
RO 450	13.2 ± 0.95[ab]	1.21 ± 0.11 [ab]
Donazil	18.9 ± 0.9[abc]	1.67 ± 0.11 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a **=** Significant from control at *P* < 0.0001, b **=** Significant from ALZ at P < 0.0001, c = Significant from RO 450 at P < 0.01.

value < 0.0001 (Figure 7).

Histopathological changes associated with herbal treatment

Brain sections from the control group (C) showed normal histological appearance in both the neurons and the blood vessels. The ALZ group showed marked neuronal degenerative changes and marked amyloid deposits on the blood vessels. All other groups showed different histological changes in both neurons and blood vessels illustrated in Figures 9 and 10.

The present study showed that *Bacopa monnieri* (BM) and *Rosmarinus officinalis* (RO) have a neuroprotective effect in AD rat model. This effect is possibly mediated *via* anti-AChE and antioxidant, and monoaminergic pathways modulation. Both extracts are potential candidates in the management of AD.

BM significantly increased serum TAC levels on the other hand significantly reduced MDA, a well-known oxidative stress biomarker, in $AlCl_3$ induced AD rat model. This clearly indicates the enhancement of the antioxidant activity, which is a key component in AD pathogenesis (Bartus, 2000). This result comes in agreement with several studies that have shown that BM exerts antioxidant activity both *in vivo* and *in vitro* (Russo et al., 2003; Bhattacharya et al., 2000; Chaudhari et al., 2017). Jyoti et al. (2007) reported that BM inhibited the reduction of superoxide dismutase (SOD) activity in $AlCl_3$ induced neurotoxicity of rat brain. It also showed that BM inhibited thiobarbituric acid reactive substance (TBARS), an index of lipid peroxidation (LPO) and its accumulation associated with $AlCl_3$ neurotoxicity of rat brain (Jyoti et al., 2007; Jyoti and Sharma, 2006). Our results further elaborate and confirm these reports of the antioxidant effect of BM as a neuroprotective agent in AD that is comparable to current medications used (Figures 3 and 4). In addition, it was shown that BM reduced Aβ and τ - protein levels in AD rat model. It was also observed that

Table 5. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue serotonin in Alzheimer's rats.

Groups	Tissue serotonin (ng/gm tissue)	Tissue Tau-protein (Pg/gm tissue)
Control	47.7 ± 6.27	8.2 ± 1.02
ALZ	6.07 ± 0.77 [a]	30.7 ± 2.91 [a]
BM 300	23.6 ± 2.31 [ab]	23.5 ± 0.97 [ab]
BM 450	21.9 ± 3.24 [abc]	17.3 ± 1.17 [abc]
RO 300	12.8 ± 1.22 [ab]	22.8 ± 1.78 [ab]
RO 450	37.5 ± 2.93 [ab]	14.7 ± 1.14 [ab]
Donazil	14.9 ± 0.73 [abc]	17.3 ± 1.42 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.0001$.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on serum MDA of ALZ model in rats

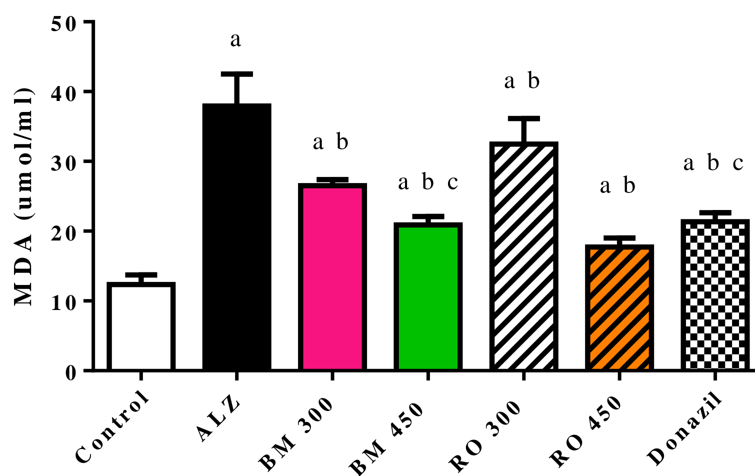

Figure 3. Serum level of MDA (µmol/ml) in the experimental groups. BM and RO extract reduced serum level of MDA in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.001$, b = Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.001$.

BM at tested doses reduced Aβ and τ -protein levels in AD rat model brain when examined histologically using H&E and Congo Red staining compared to control. Neurons exhibited unremarkable degenerative changes while blood vessels showed moderate and unremarkable amyloid thickening (Figures 9 and 10).

We also demonstrated that BM extract inhibited significantly acetylcholinesterase (AChE) activity in AD rat model, a result that further support its use as a neuroprotective agent in AD. It is well documented that cholinergic dysfunction is implicated in AD pathogenesis although the mechanism is not well understood (Bartus, 2000). Our results support the hypothesis that the observed neuroprotective effect of BM extract can be attributed to inhibition of AChE consequently preserving ACh longer at the synapses and compensating for the

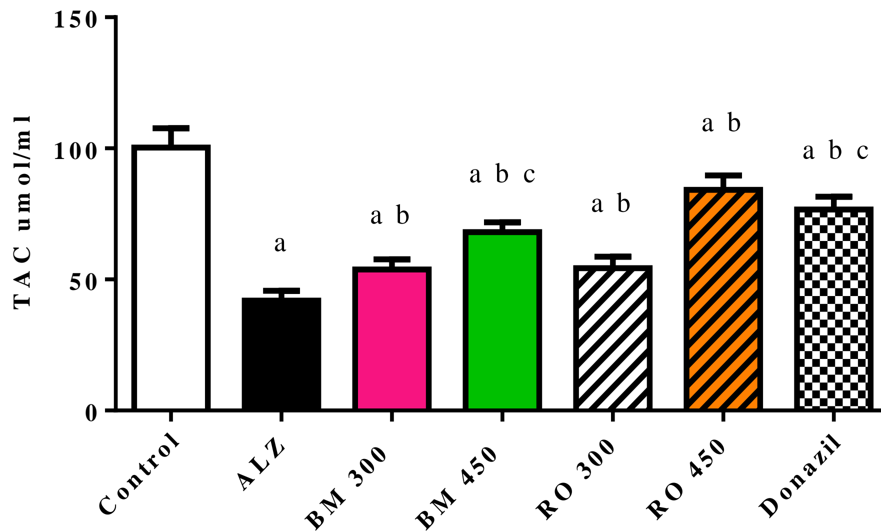

Figure 4. Serum level of TAC (µmol/ml) in the experimental groups. BM and RO extract raised serum level of TAC in Alzheimer's rats at the end of 2 month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.01$.

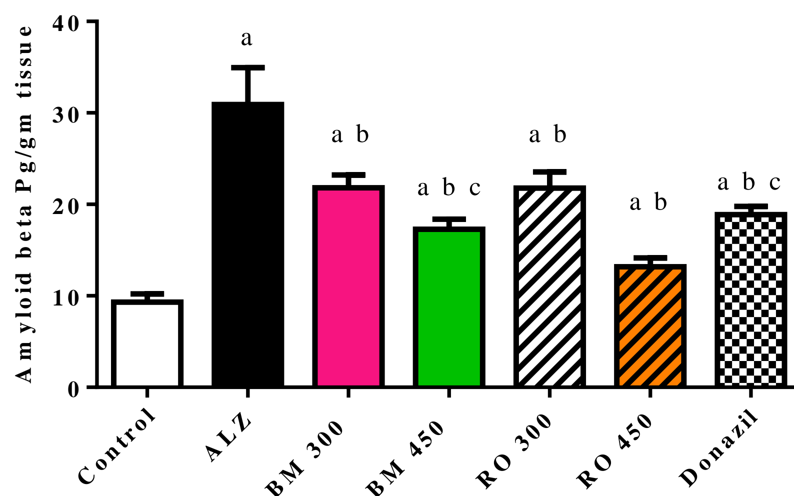

Figure 5. Tissue level of amyloid beta peptide (Pg/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of amyloid beta in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.01$.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue acetylcholinestrase of ALZ model in rats

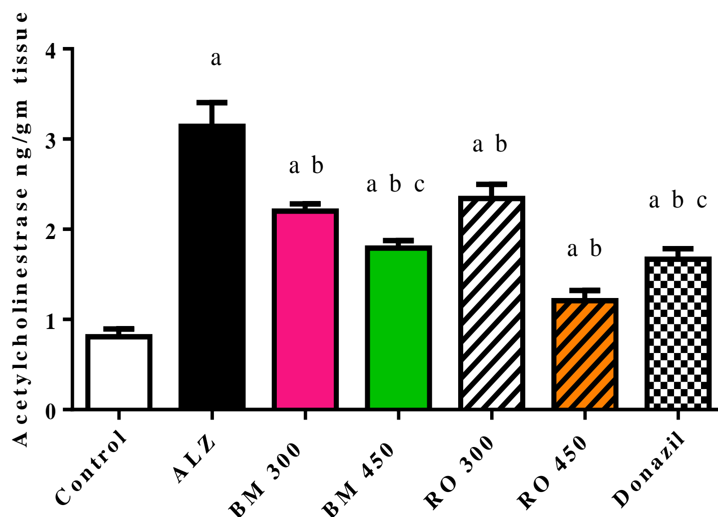

Figure 6. Tissue level of acetyl cholinestrase (ng/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of amyloid beta in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a **=** Significant from control at $P < 0.0001$, b **=** Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.0001$.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue serotonin of ALZ model in rats

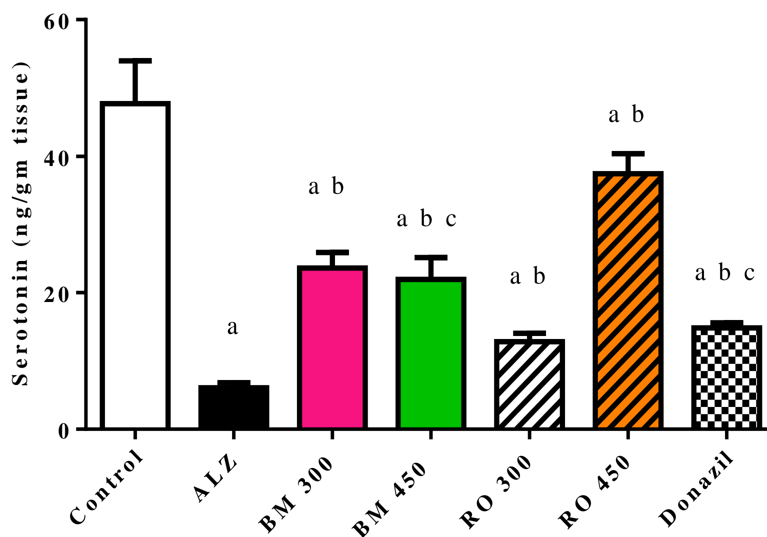

Figure 7. Tissue level of serotonin (ng/gm tissue) in the experimental groups. BM and RO extract raised tissue level of serotonin in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = B. monnieri extract 300 mg/kg; BM 450 = B. monnieri 450 mg/kg; RO 300 = R. officinalis extract 300 mg/kg; RO 450 = R. officinalis extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.0001$.

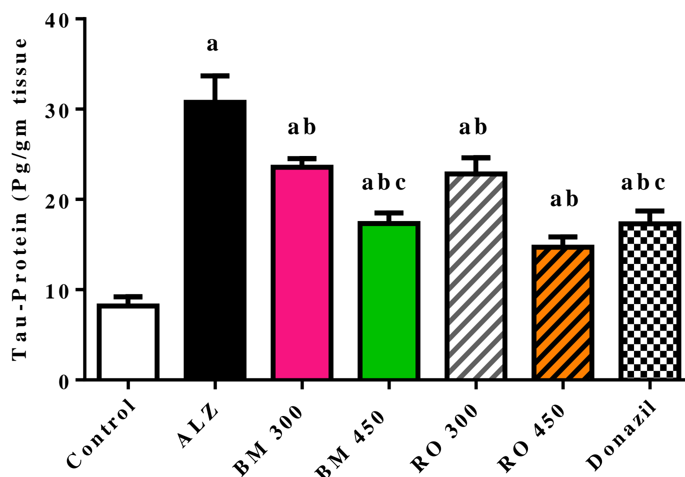

Figure 8. Tissue level of Tau-Protein (Pg/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of tau-protein in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a **=** Significant from control at $P < 0.001$, b **=** Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.01$.

lost cholinergic function. To our knowledge, this is the first demonstration of BM effect on AChE.

The mechanism underlying the protection of Brahmi against Aβ25–35-mediated neurotoxicity demonstrated that Aβ 25–35 induced neurotoxicity causes the elevation of intracellular AChE activity and so the elevation of AChE activity was diminished by co-treatment of cortical cells with Brahmi extract. Also, AChE was proved to be neurotoxic both *in vitro* and *in vivo* models. This observation suggests the neuroprotection of Brahmi through its inhibitory effect on amyloid peptide-activated intracellular AChE activity (Limpeanchoba et al., 2008).

The presence of the nootropic metabolite; Bacopaside I (Pseudojujubogenin- 3-O-[α-l-arabinofuranosyl-(1→2)]-6-O- sulfonyl-α-D-glucopyranosyl-(1→3)-α-l-arabinopyranoside) is believed to account for the neuroprotective effect of BM extract as it reverses the depressive-like symptoms caused by reserpine, which is mediated through antioxidant and noradrenergic activations. It also stimulates PI3/Akt signaling in organotypic hippocampal slice cultures (Yin et al., 2016). In accordance, a study conducted by Le et al. (2015) concluded that bacopaside I played a role in neuroprotective effects in both *in vitro* and *in vivo*, where in the *in vitro* experiment, the hippocampal slice cultures (OHSCs) were incubated with triterpenoid saponins from BM, where bacopaside I exhibited potent neuroprotective effects against OGD-induced neuronal cell damage (Le et al., 2015). However, the role of each dammarane steroidal saponin of BM in the neuroprotection is still uncovered.

The presence of Bacoside metabolite is thought to have anti-oxidant and free radical scavenging as it inhibits lipid peroxidation and elevates the anti-oxidant enzymes in prefrontal cortex, hippocampus, and striatum which also possess a significant iron chelating property; also, iron and other divalent metals interact with Aβ protein and modulate several effects that are thought to be the pathogenic effects of that protein (Chaudhari et al., 2017).

Similar to BM, *R. officinalis* (RO) extract also exhibited antioxidant effects in our $AlCl_3$ induced AD rat model. This effect was demonstrated by its significant reduction of the oxidative stress biomarker MDA and enhancement of TAC (Figures 3 and 4). This result complement previous reports that Carnosic acid (CA), a metabolite found in rosemary and sage, had antioxidant effects in both *in vitro* and transgenic mice (Lipton et al., 2016). Also, Rasoolijazi et al. (2013) reported on its role in memory and learning scores improvement. CA decreased

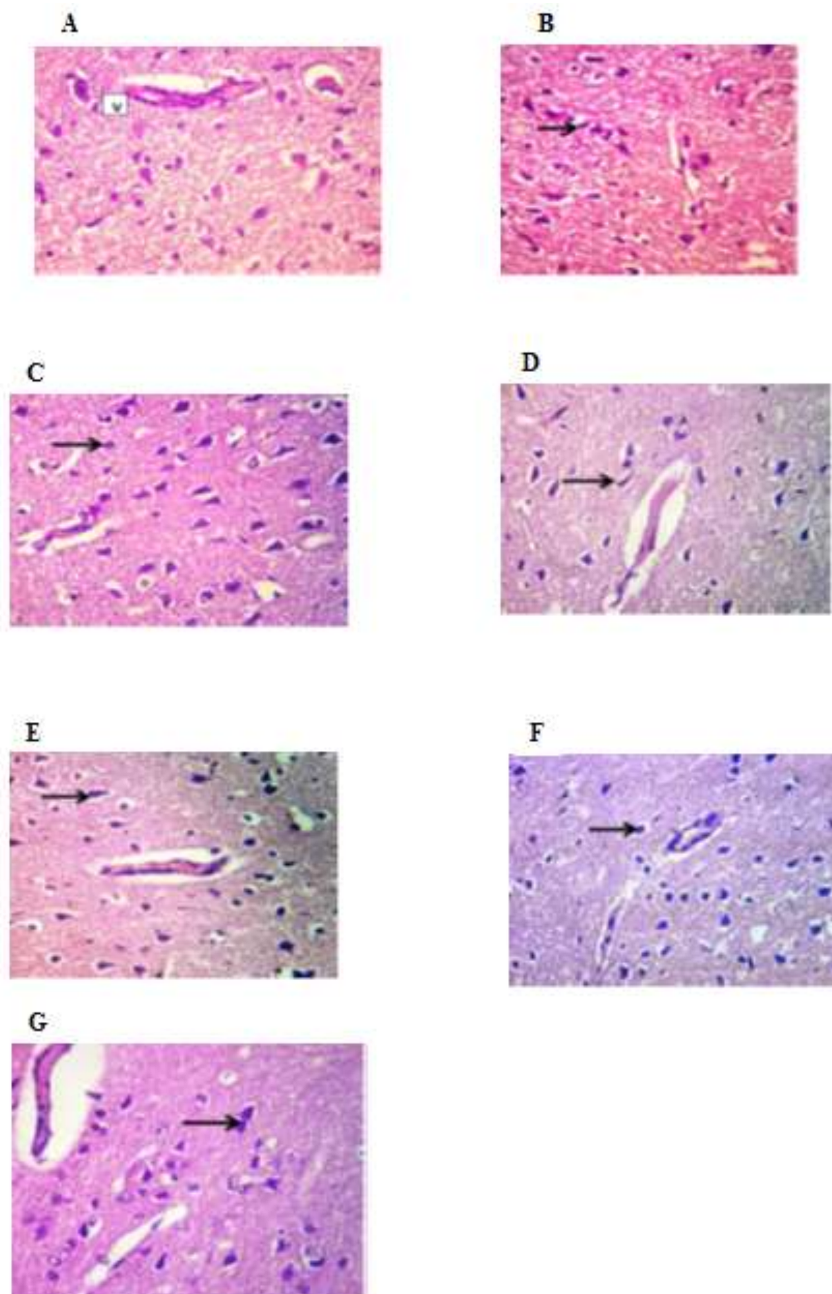

Figure 9. Histopathological sections of brain tissue illustrating herbal treatment effect in all experimental groups. **A:** negative control, illustrates unremarkable changes in both neurons and vessels (V). **B:** ALZ group exhibiting neuronal marked degenerative changes (thin arrow). **C:** RO 300 group showed minimal neuronal degenerative changes (thin arrow). **D:** RO 450 group showed unremarkable neuronal degenerative changes (thin arrow). **E:** BM 300 group demonstrating un remarkable neuronal degenerative changes (thin arrow). **F:** BM 450 group demonstrating un remarkable neuronal degenerative changes (thin arrow). **G:** Donazil group showing unremarkable degenerative changes of neurons (thin arrows). H&E staining. Magnification 200, arrow pointing at neurons, V blood vessels.

52% of the infarct volume from brains under ischemia/reperfusion *in vivo*, and also protected the PC12 cells from hypoxic injury *via* reducing oxidative stress biomarkers and enhancing cell viability *in vitro*

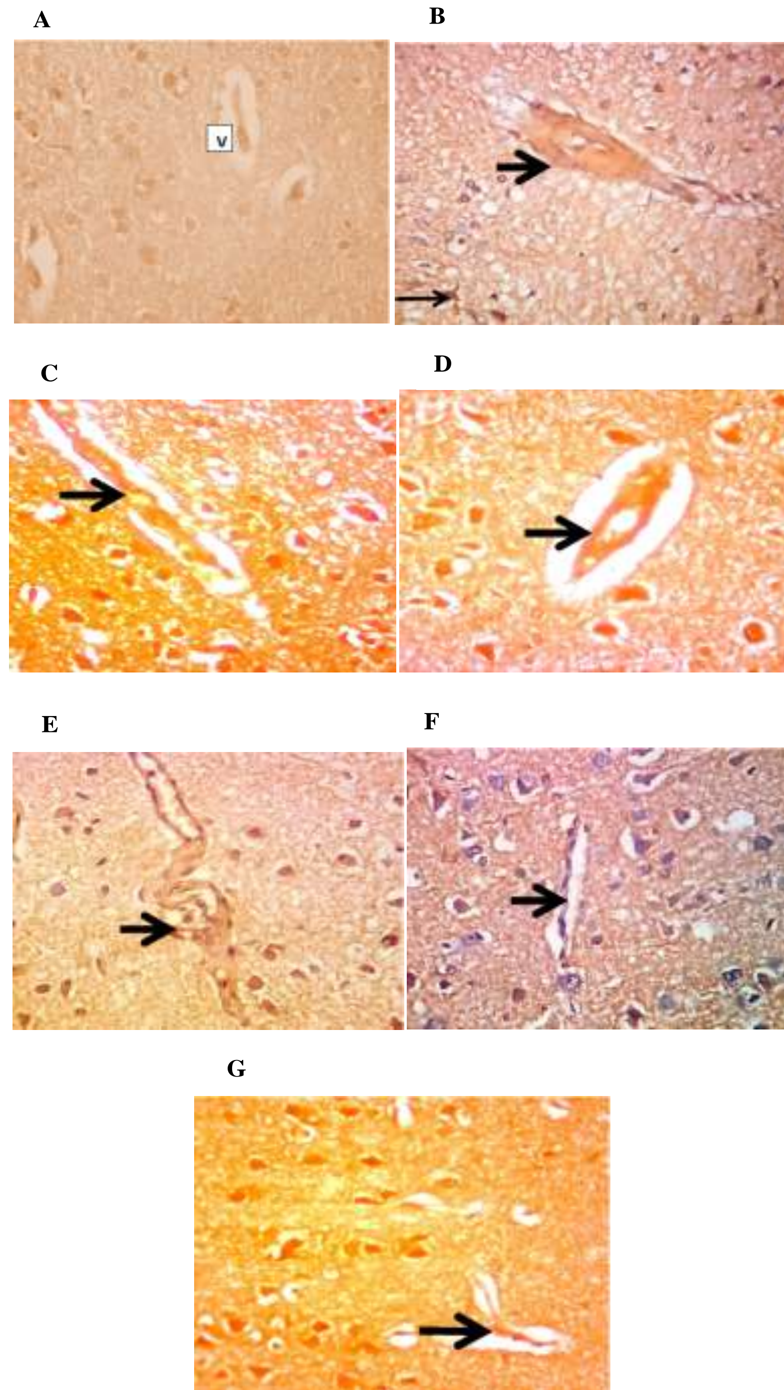

Figure 10. Histopathological sections of brain tissue illustrating herbal treatment effect in all experimental groups. **A:** negative control illustrates unremarkable changes in both neurons and vessels (V). **B:** ALZ group exhibited marked thickening of the vessel wall by the amyloid deposits on blood vessels (thick arrow). **C:** RO 300 group showed mild thickening of the vessel wall by the amyloid deposits (thick arrow). **D:** RO 450 group showed moderate thickening of the vessel wall by the amyloid deposits (thick arrow). **E:** BM 300 group exhibiting moderate thickening of the vessel wall by the amyloid deposits (thick arrow). **F:** BM 450 group exhibiting unremarkable thickening of the vessel wall by the amyloid deposits (thick arrow). G: Donazil group showing unremarkable thickening of the vessel wall by the amyloid deposits (thick arrow). Congo Red staining. Magnification 200, arrow pointing at neurons, V blood vessels.

(Hou et al., 2012).

Not only that CA has a nootropic effect, but also, the administration of Rosmarinic acid (RA) averted cognitive impairment induced by chronic ethanol (Hasanein et al., 2017). Actually, the mechanism of action of RO pertaining to neuroprotection could also be attributed to the synergistic effects of phenolic and polyphenolic metabolites that possess well-known antioxidant and anticholinesterase activities.

Our experiments also showed that RO at selected doses reduced Aβ and τ -protein levels in AD rat model brain, a result that comes in agreement with a previous report of the effect of CA on U373MG human astrocytoma cells probably *via* activation of α-secretase (Yoshida et al., 2014). This may also explain our histopathological examination of RO treated rat brains that revealed mild to unremarkable degenerative changes as well as mild to moderate amyloid thickening of vascular walls (Figures 9 and 10).

Since there seems to be an agreement that AD pathogenesis results primarily from defective brain cholinergic function (Bartus, 2000; Iqbal and Grundke-Iqbal, 2010; Murphy and Steenbergen, 2008), we investigated the possibility that RO neuroprotective effects in our AD rat model may be due to enhancement of cholinergic function. It was found that, similar to BM, RO significantly inhibited AChE. This also agrees with previous report that RO improved long-term memory and inhibited the AChE activity of rat brain (Ozarowski et al., 2013). These results may also explain the observed effectiveness of rosemary aromatherapy in human (Jimbo et al., 2009).

It is well-known that nitrocatechol derivatives exhibit anti-aggregation properties against Aβ protein. It is also documented that Rosmarinic acid had two cathecol moieties, which consequently induces morphological and signature changes in the secondary structure of tau-protein once it is interacted with, thus, preventing aggregation and β-sheets assembly and also reducing fibril progression (Cornejo et al., 2015). Both RO and BM effects on all afore-mentioned biochemical markers and histopathological results were comparable to Donazil®™, a standard selective inhibitor of brain cholinesterase commonly used in AD management (Bitra et al., 2014; Nayak and Chatterjee, 2001).

Serotonin (5-hydroxytryptamien, 5-HT) has been linked to emotional and motivational aspects of human behavior and memory (Meneses and Liy-Salmeron, 2012). Recently, it has been documented that the serotonin as a neurotransmitter is involved in the pathophysiology of AD (Ramirez, 2013; Maccioni et al., 2018). Hence, the 5-HT_6 receptor is a promising target for cognitive disorders AD (Amat-Foraster et al., 2017) where we aimed to evaluate tissue serotonin in both our AD model and with herbal therapy (Figure 7).

Indeed, our results have shown the diminution of tissue serotonin as well as its partial restoration with our herbal treatment.

Both extracts exhibited significant elevation of tissue serotonin levels with a prominent effect for RO 450 mg/kg compared to the controls. This result is the first demonstration that RO and BM extracts may exert their neuroprotective effects in AD rat model *via* serotoninergic system. It was previously reported that RO extract exhibited antidepressant-like activity in mice *via* the monoaminergic system (Machado et al., 2009). BM, on the other hand, elevated serotonin levels rats subjected to stress (Sheikh et al., 2007). Our results is in line with other reports of BM and RO influencing the monoaminergic system (Rajan et al., 2015; Machado et al., 2009), however, we are the first to report this effect in AD rat model.

Since both BM and RO have similar antioxidant and monoaminergic effects, combining both RO and BM can have synergistic effects in the treatment of AD. Ramachandra et al. (2014) reported its neuroprotective effectiveness in contrast to its individual use in embryonic cell line (Ramachandran et al., 2014). The effect in animal model still needs to be investigated.

This study showed that *B. monnieri* (BM) and *R. officinalis* (RO) have a neuroprotective effect in AD rat model at both histological and biochemical levels which is due to their interesting variety of bioactive metabolites. This effect is possibly mediated *via* anti-AChE, monoaminergic and antioxidant pathways modulation. Further pharmacological and clinical studies are needed to confirm these results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of the *in vivo* antiplasmodial activity of ethanol leaf extract and fractions of *Jatropha gossypifolia* in *Plasmodium berghei* infected mice

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The study evaluates the *in vivo* antiplasmodial activity of ethanol leaf extract and fractions of *Jatropha gossypifolia* in *Plasmodium berghei* infected mice. Phytochemical, physicochemical analyses, median lethal dose (LD_{50}), effects on biochemical parameter were evaluated and the schizonticidal effects during early and established infections were investigated. The extract (50-200 mg/kg) was screened for antimalarial prophylactic and curative activities against 50 (20-30 g) *P. berghei* infected mice using standard method. The prophylactic and curative activity tests were repeated for the fractions of the extract. The phytochemical analysis done on *J. gossypifolia* leaves showed presence of tannins, saponins, phlobatannins, alkaloids, flavonoids, terpenoids and cardiac glycosides. It contained total ash (6.3%); acid insoluble ash value (3.8%), water soluble ash value (2.5%), ethanol extractive value (4.5%) and moisture content (7%). LD_{50} was 4472.14 mg/kg. There was significant increase in alanine transaminase (ALT), alanine phosphatase (ALP) and aspartate aminotransferase (AST) at 500 mg/kg on the 28th day, indicating liver injury at high dose and prolonged administration. The crude extract of *J. gossypifolia* (50-200 mg/kg) exhibited moderate prophylactic and significant ($P < 0.05$) curative activities in both day 4 and 7 tests with a mean survival time comparable to the standard drug, quinine 100 mg/kg. The fractions of the leaf extract of *J. gossypifolia* exhibited moderate to good prophylactic and curative activities, with ethyl acetate fraction eliciting the best activity in both test models. The leaf extract and fractions of *J. gossypifolia* administration are safe and possess good antiplasmodial activity, which confirmed its folkloric antimalarial medicinal use.

Key words: Malaria, antimalarial, *Plasmodium berghei, Jatropha gossypifolia*, toxicity, biochemical parameters.

INTRODUCTION

Malaria is a mosquito borne infectious disease of humans and other animals caused by parasitic protozoan belonging to the genus *Plasmodium*. The disease is transmitted by the biting of an infected female *Anopheles* mosquito, and the symptoms usually begin ten to fifteen days after being bitten. Malaria is typically diagnosed by

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the microscopic examination of blood using blood films, or with antigen-based rapid diagnostic tests. Methods that use the polymerase chain reaction to detect the parasite's DNA have been developed, but are not widely used in areas where malaria is common due to their cost and complexity (Caraballo, 2014; Nadjm and Behrens 2012).

Malaria can be associated with several serious complications among which include development of respiratory distress, encephalopathy, splenomegaly, hepatomegaly, hypoglycemia, and hemoglobinuria spontaneous bleeding and coagulopathy (Taylor et al., 2012; Adebayo and Krettli, 2011; Ferri, 2009; Korenromp et al., 2005; Beare et al., 2011).

Malaria in pregnant women is an important cause of stillbirths, infant mortality, abortion and low birth weight, particularly in *Plasmodium falciparum* infection, but also with *Plasmodium vivax* (Hartman et al., 2010). Malaria is widespread in the tropical and subtropical regions (Caraballo, 2014). Malaria is commonly associated with poverty and has a major negative effect on economic development (Gollin and Zimmermann, 2007). The World Health Organization reports that there were 198 million cases of malaria worldwide in 2013. This resulted in an estimated 584,000 to 855,000 deaths, the majority (90%) of which occurred in Africa (Murray et al., 2012; Filler et al., 2003).

Many antimalarial agents have been developed and used in the malaria treatment, with significant cases of drug failures and the attendant side effects associated with these agents. Majority of the rural dwellers depend on traditional medicine as a source of primary health care including malaria. *Jatropha gossypifolia* has many folkloric ethnomedicinal uses, including malaria treatment. It is called Bellyache bush, Cotton leaf and Physic nut. *J. gossypifolia* is native to tropical America, but is now cultivated widely in tropical countries throughout the world (Wikipedia, 2019; Csurhes, 1999).

Throughout tropical Africa, different parts of *J. gossypifolia* are used for a range of ethnomedicinal purposes. The oil-rich seeds and seed oil are used as a drastic purgative and emetic and to expel internal parasites. The leaves and bark have the same purgative effect. The oil is applied internally as abortifacient, and externally as rubefacient to treat rheumatic conditions and a variety of skin infections, although its use on the skin may also cause an irritative rash.

In Senegal the seed oil is also applied against leprosy and rabies. The sap has a widespread reputation for healing wounds, as a haemostatic and for curing skin problems. It is applied externally to treat infected wounds, ulcers, cuts, abrasions, ringworm, eczema, dermatomycosis, scabies and venereal diseases. The sap has a styptic effect and is used against pains and bee and wasp stings. Dried and pulverized root bark is made into poultices and is taken internally to expel worms and to treat oedema. A decoction of the leaves is taken to treat colic, stomach-ache and fever, including malaria (Abbiw, 1990; Csurhes, 1999).

Despite the grand variety of popular uses and the data from *Jatropha* species, *J. gossypifolia* has been scarcely studied regarding biological activities; with the exception of the antimalarial activity, some biological activities have been studied and listed as follows: antihypertensive action (Abreu et al., 2003); anti-inflammatory and analgesic action (Bhagat et al., 2011); wound healing action (Vale et al., 2006); haemostatic action; anti-cholinesterase action; antioxidant action (Kharat et al., 2011); contraceptive action; tocolytic action; antineoplasic action (Kupcan et al., 1970); neuropharmacological action (Apu et al., 2012).

Predicting the emergence and spread of resistance to current antimalarials and newly introduced compounds is necessary for planning malaria control and instituting strategies that might delay the emergence of resistance (Hasting et al., 2000). Throughout tropical Africa, different parts of are used for a range of medicinal purposes, but the antimalarial activity has been largely uninvestigated; making antimalarial evaluation of this potential biostore to become apt. The aim of this study is to evaluate the antimalarial activity of *J. gossypifolia* leaf extracts and fractions in mice infected with *Plasmodium berghei* using prophylactic and curative models, and also evaluate their toxicity profile.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *J. gossypifolia* were collected in April 2015 in Agulu, Anambra State, Nigeria. The plant was identified by a technologist in the Pharmacognosy and Traditional Medicine Department of Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Nigeria.

Reagents and chemicals

Ethanol (JHD, China), n-hexane (JHD, China), ethyl acetate(JHD, China), water (NBC, Nig), ammonia solution (Shakti Chemicals, India), aluminium chloride (Neel Chemicals, India), chloroform, glacial acetic acid (JHD, China), ferric chloride (Ecosia Chemical, Hong Kong).

Equipment

Microscope (Finlab, Nig), syringes and needles (1, 2 and 10 ml capacity), electronic weighing balance (Ohaus Corp, USA), water bath (Serological, England), beaker (Pyrex; 10, 50, 100 and 1000 ml), spatula, measuring cylinder, refrigerator (Thermocool, England), cotton wool (Pyrex).
Organism: *Plasmodium berghei*

Preparation of the plant material

The leaves were put in water to remove dust and unwanted particle. They were spread on a clean flat tray and allowed to air dry at room temperature for two weeks. The dried leaves were pulverized with

an analytical milling machine. 605.72 g of the dried pulverized leaves was macerated in 2500 ml of ethanol for 72 h. The mixture was sieved using porcelain cloth and filtered with a filter paper. The filtrate was dried *in vacuo* at 40°C. The extract was stored in a refrigerator for use.

Preliminary phytochemical analysis

Qualitative phytochemical analysis of the crude powder of the leaves collected was determined according to standard procedure to identify the constituents present in the plant extracts (Sofowora, 1993; Trease and Evans, 1989).

Experimental animals and housing

Albino mice (158) of both sexes with an average weight in the range of 20-30 kg were selected for the experiment. They were obtained from the Zoology Department of University of Nigeria Nsukka, Enugu State. They were fed with growers mash, with free access to water. The mice were allowed to acclimatize for 7 days.

Collection and Identification of *P. berghei*

Three infected mice obtained from University of Nigeria Nsukka. Blood was collected from the tail of the infected donor mice and placed on a clean glass slide. The glass slide was placed horizontally on the working bench. The slide and spreader were held at a suitable angle (45°), the spreader was pulled back to touch the drop of blood on the slide and spread it along. The film was fixed with methanol and lowered into an already prepared Giemsa stain (1 ml of Giemsa +19 ml of buffer) and allowed to stain for 45 min. The slide was lifted off the bench and air dried. The parasitaemia was examined microscopically under oil immersion and parasitized cells were counted.

Acute toxicity study

The acute toxicity study of *J. gossypifolia* was carried out according to the method employed by Lorke (1983) method but modified, using a total of 17 mice (Bruce et al., 2016).

Anti-plasmodial activity of *J. gossypifolia*

In-vivo evaluation of the anti-plasmodial activity of *J. gossypifolia* was studied in two models using the method described by Ezenwa et al. (2017) and Adebayo et al. (2012):

(a) Prophylaxis test and
(b) Curative test

Prophylaxis test procedure

In this procedure, a total of 25 mice were used. They were grouped into 5 groups of 5 mice per group. The animals received treatment for 3 days as follows:

Group 1 received 5 ml/kg 5% tween 80 ‘per os’, by mouth (P.O)
Group 2 received 22.5 mg/kg of sulfadoxine/pyrimethamine P.O
Group 3 received 50 mg/kg of extract
Group 4 received 100 mg/kg of extract
Group 5 received 200 mg/kg of extract

On the Day 4, all the animals were infected with *P. berghei* by a single 0.2 ml intraperitoneal administration of the diluted infected blood, which contains approximately 1×10^7 infected red blood cells. The animals were left for 72 h. Then a thin film blood smear was made from the tail vein stained in Giemsa stain and viewed under the microscope to examine the presence of parasitemia. Then the mean % parasitemia inhibition was calculated using the formula below:

Mean % Parasitemia inhibition = 100-(a/b x 100)

Where a = mean % parasitemia in treatment group, b = mean % parasitemia in control group

Curative test procedure

In this model a total of 25 mice were used. They were grouped into 5 groups of 5 mice per group. All the animals were infected with *P. berghei* by a single intraperitoneal administration of 0.2 ml of diluted infected blood, which contains approximately $1\text{x}10^7$ infected red blood cells. The animals were left for 72 h for the infection to be established. A thin blood film was made from the tail vein of each animal to confirm parasitemia and was termed the basal parasitemia. Then the animals received treatment as follows:

Group 1 received 5 ml/kg of 5% tween 80 P.O
Group 2 received 100 mg/kg of quinine P.O
Group 3 received 50 mg/kg of extract P.O
Group 4 received 100 mg/kg of extract P.O
Group 5 received 200 mg/kg of extract P.O

On Day 4 post treatment, the parasitemia was examined as above. Then the animals still continued treatment, with the parasitemia re-examined on day 7 post treatment. Then the mean % parasitemia inhibition was calculated using the relation.

Mean % parasitemia inhibition = 100-(a/b × 100)

Where a = mean % parasitemia on treatment day, b = mean % parasitemia on Day 0.

Prophylaxis test procedure for fractions

In this procedure, a total of 40 mice were used. They were grouped into 8 groups of 5 mice per group. The animals received treatment for 3 days as follows:

Group 1 received 250 mg/kg of N-hexane fraction of extract P.O
Group 2 received 500 mg/kg of N-hexane fraction of extract P.O
Group 3 received 250 mg/kg of Ethyl acetate fraction of extract P.O
Group 4 received 500 mg/kg of Ethyl acetate fraction of extract P.O
Group 5 received 250 mg/kg of aqueous fraction of extract P.O
Group 6 received 500 mg/kg of aqueous fraction of extract P.O
Group 7 received 22.5 mg/kg of sulfadoxine/pyrimethamine P.O
Group 8 received 5 ml/kg 5% tween 80 P.O

On the Day 4, all the animals were infected with *P. berghei* by a single 0.2 ml intraperitoneal administration of the diluted infected blood, which contains approximately 1×10^7 infected red blood cells. The animals were left for 72 h. Then a thin film blood smear was made from the tail vein stained in Giemsa stain and viewed under the microscope to examine for the presence of parasitemia. Then the mean % parasitemia inhibition was calculated using the relation.

Mean % Parasitemia inhibition = 100-(a/b × 100)

Where a = mean % parasitemia in treatment group, b = mean % parasitemia in control group.

Curative test procedure for fractions

In this model a total of 40 mice were used. They were grouped into 8 groups of 5 mice per group. All the animals were infected with *P. berghei* by single intraperitoneal administration of 0.2 ml of diluted infected blood such that it contains approximately 1×10^7 infected red blood cells. The animals were left for 72 h for the infection to be established. A thin blood film was made from the tail vein of each animal to confirm parasitemia and was termed the basal parasitemia. Then the animals received treatment as follows:

Group 1 received 250 mg/kg of N-hexane fraction of extract P.O
Group 2 received 500 mg/kg of N-hexane fraction of extract P.O
Group 3 received 250 mg/kg of Ethyl acetate fraction of extract P.O
Group 4 received 500 mg/kg of Ethyl acetate fraction of extract P.O
Group 5 received 250 mg/kg of aqueous fraction of extract P.O
Group 6 received 500 mg/kg of aqueous fraction of extract P.O
Group 7 received 100 mg/kg of quinine P.O
Group 8 received 5ml/kg 5% tween 80 P.O

On Day 4 post treatment, the parasitemia was examined as above. Then the animals still continued treatment the parasitemia was also examined on Day 7 post treatment. Then the mean % parasitemia inhibition was calculated using the relation.

Mean % parasitemia inhibition = 100-(a/b × 100) [Where a = mean % parasitemia on treatment day, b = mean % parasitemia on Day 0]

Physicochemical analysis

Physicochemical parameters on the plant materials were determined using the procedures described by Ezugwu et al. (2011).

Biochemical evaluation results

The effects of the administration of the leaf extract of *J. gossypifolia* on the biochemical parameters were evaluated using the method described by Ezenwa et al. (2017).

Statistical analysis

The data was analyzed using one-way analysis of variance (ANOVA). Data was tabulated as Mean ± SEM (Standard error of mean) $P < 0.05$ was considered significant.

RESULTS

Phytochemical analysis

The results of phytochemical analysis are presented on Table 1. Tannins, saponins, phlobatannins, flavonoids, alkaloids, terpenoids and cardiac glycosides are present.

Acute toxicity

The results of the median lethal dose test are presented on Table 2. The amount of the leaf extract of *J. gossypifolia* required to kill 50% of the test population LD_{50} is 4472.14 mg/kg.

Prophylaxis and curative tests

The results of the prophylaxis and curative tests of the extract are presented in Tables 3 and 4. Chemosuppression achieved in this study was dose dependent with chemosuppression rate of 85.42% at the highest dose 200 mg/kg when compared with the 91.70% of 22.5 mg/kg of sulfadoxine/ pyrimethamine (positive control) in Table 3. The curative effect of the 200 mg/kg crude extract which gives the percentage parasitaemia clearance of 88.23% (day 4) and 96.07% (Day 7) compared with the 100 mg/kg quinine (positive control) which also gives the percentage parasitaemia clearance of 88.37% (Day 4) and 93.02% (Day 7) in Table 4.

Prophylactic and curative tests results for the fractions

The results of the prophylaxis and curative tests of the fractions are presented in Tables 5 and 6. Chemosuppression achieved in this study was dose dependent with chemosuppression rate of 79.97% at the highest dose 500 mg/kg and 69.99% at the dose of 250 mg/kg, when compared with the 90.03% of 22.5 mg/kg of sulfadoxine/pyrimethamine (positive control) in Table 5. The curative effect of the 500 mg/kg ethyl acetate fraction which gives the percentage parasitaemia clearance of 66.69% (Day 4) and 87.15% (Day 7) and 250 mg/kg of ethyl acetate fraction which also gives the percentage parasitaemia clearance of 58.52% (Day 4) and 75.64% (Day 7), compared with the 100 mg/kg quinine (positive control) which gives the percentage parasitaemia clearance of 85.37% (Day 4) and 90.27% (Day 7) in Table 6.

Statistical comparison of results

The statistical comparisons of the prophylactic and curative test results for the crude extract are presented on Tables 7 and 8 respectively. In the prophylactic test result, the result of positive control compared those of 100 and 200 mg/kg extract treatments. There was no significant statistical difference in these treatments (Table 7). In the curative test result, the result of positive control compared those of 50, 100 and 200 mg/kg extract treatments. There was no significant statistical difference in these treatments at Days 4 and 7, except for the curative treatment group (Table 8).

Comparison of prophylaxis and curative test results for fractions

The comparison of the prophylactic test results of the fractions with the naïve group showed a significant difference in all the cases of comparison except for 250

Table 1. Phytochemical analysis.

Tests	Results
Tannin	+
Saponin	+
Phlobatannins	+
Flavonoids	+
Steroids	_
Alkaloids	+
Terpenoids	+
Cardiac glycosides	+

+ = Present-- =Absent.

Table 2. Acute toxicity result.

Phase	Dose (mg/kg)	No. of death	Inference
1	10	0/3	Active
	100	0/3	Active
	1000	0/3	Active
2	2000	0/2	Active
	3000	0/2	Active
	4000	0/2	Calm
	5000	½	Dead

$LD_{50}= \sqrt{axb}$ Where a = the highest dose that did not kill, b= the lowest does that killed $\sqrt{4000x5000}$ $\sqrt{20000000} = 4472.14$ mg/kg $LD_{50}= 4472.14$ mg/kg .

Table 3. Prophylaxis test result of crude extract.

Group	Treatment	Mean parasitemia	Percentage prophylaxis
1	50 mg/kg extract	2.3 ± 0.2550	52.10
2	100 mg/kg extract	1.3 ± 0.3391	72.92
3	200 mg/kg extract	0.7 ± 0.1225	85.42
4	22.5 mg/kg of Sulfadoxine and Pyrimethamine	0.4 ± 0.1871	91.70
5	0.5 ml 5% Tween 80	4.8 ± 0.0.4062	0.00

mg/kg aqueous fraction of extract (Table 9). The comparison of the curative test results of the fractions with the basal showed a significant difference with the positive control, 250 and 500 mg/kg ethyl acetate fraction at Day 4; at Day 7 there was a significant difference with the positive control, 250 and 500 mg/kg ethyl acetate fraction, and moderately significant difference with 500 mg/kg n-hexane fraction (Table 10).

Physicochemical analysis

The result of the physicochemical analysis is presented on Table 11, showing a total ash value of 6.3% and a moisture content of 7.0%.

Biochemical evaluation

The result of the biochemical evaluations is presented on Tables 12 to 14. The result showed elevated effect of extract on AST at 500 mg/kg treatment at Days 14 and 28 in Table 12 and the elevated effect of extract on ALT and ALP at 125 mg/kg treatment at Days 14 and 28 in Tables 13 and 14.

DISCUSSION

Traditional medicinal plants have proved to be rich sources of new drugs coupled with the fact that antimalarial drugs in use presently were either obtained

Table 4. Curative test result of crude extract.

Group	Treatment	Basal Parasitemia	Day 4 Parasitemia	Day 4 % Curative	Day 7 Parasitemia	Day 7 % Curative
1	50 mg/kg extract	6.0 ± 0.65	1.5 ± 0.47	75.00	0.8 ± 0.25	86.67
2	100 mg/kg extract	0.7 ± 0.88	0.8 ± 0.20	85.96	0.5 ± 0.15	91.22
3	200 mg/kg extract	5.1 ± 0.57	0.6 ± 0.29	88.23	0.2 ± 0.12	96.07
4	100 mg/kg quinine	4.3 ± 0.86	0.5 ± 0.22	88.37	0.3 ± 0.12	93.02
5	0.5 ml 5 % Tween 80	4.4 ± 0.50	6.2 ± 0.77	-40	11.1 ± 2.15	-177.40

Table 5. Prophylactic test result for the fractions.

Group	Treatment	Mean parasitemia	% prophylaxis
1	250 mg/kg N-hexane fraction	8.33 ± 0.333	37.51
2	500 mg/kg N-hexane fraction	6.67 ± 1.202	49.96
3	250 mg/kg Ethyl acetate fraction	4.00 ± 0.574	69.99
4	500 mg/kg Ethyl acetate fraction	2.67 ± 0.667	79.97
5	250 mg/kg aqueous fraction	11.67 ± 0.333	12.45
6	500 mg/kg aqueous fraction	9.00 ± 1.528	32.48
7	22.5 mg/kg Sulfadoxine and Pyrimethamine	1.33 ± 0.333	90.03
8	0.5 ml 5% Tween 80	13.33	0.00

Table 6. Curative test result for the fractions.

Group	Treatment	Basal parasitemia	Day 4 parasitemia	Day 4 percentage curative	Day 7 parasitemia	Day 7 percentage curative
1	250 mg/kg N-hexane fraction	10.67 ± 0.882	8.33 ± 1.202	21.93	7.00 ± 1.000	34.39
2	500 mg/kg N-hexane fraction	11.00 ± 1.155	8.67 ± 0.882	21.18	6.00 ± 1.000	45.45
3	250 mg/kg Ethyl acetate fraction	13.67 ± 0.333	5.67 ± 0.667	58.52	3.33 ± 0.333	75.64
4	500 mg/kg Ethyl acetate fraction	13.00 ± 1.155	4.33 ± 0.882	66.69	1.67 ± 0.333	87.15
5	250 mg/kg aqueous fraction	11.00 ± 1.530	10.00 ± 1.530	9.09	8.00 ± 1.000	27.27
6	500 mg/kg aqueous fraction	13.67 ± 0.333	10.00 ± 1.528	28.85	7.68 ± 1.202	43.82
7	100 mg/kg Quinine	13.67 ± 0.333	2.00 ± 0.577	85.37	1.33 ± 0.333	90.27
8	0.5ml 5% Tween 80	12.67±1.667	16.00±0.577	-26.28	18.67 ±0.882	.47.36

directly from plants or developed using chemical structures of plant-derived compound as templates. The feasibility of discovering new potent antimalarials from traditional medicinal plants is very promising. Therefore, this study demonstrates the *in vivo* antiplasmodial activity of the ethanol leaf extract and fractions of *J. gossypifolia* in *P. berghei* infected mice.

The preliminary phytochemical analysis done on *J. gossypifolia* leaves showed that it contains different secondary metabolites such as tannin, saponin, phlobatannins, flavonoids, terpenoids and cardiac glycosides. This correlates with an earlier study done by Murugalakshmi et al. (2014) which reveals the presence of alkaloids, tannins, flavonoids, phenolic compounds, steroidal sapogenins (saponins), unsaturated sterols, triterpenoids and essential oils. The anti-plasmodial activity observed in many plants is assumed to result from single or combined action of the metabolites which could be the same for the present study. These constituents have been found in other natural plant products which possess antiplasmodial activity. The alkaloid constituent of *J. gossypifolia* explains its relevance in treatment of malaria (Vijayta et al., 2015).

The different classes of alkaloids were believed to block protein synthesis in *P. falciparum*. Triterpenoid and steroid saponins have been found to be detrimental to several infectious protozoans such as *P. falciparum*. Tannins are complex phenol polymers which have

Table 7. Statistical comparison of the prophylaxis results.

Compared variables for prophylaxis	Significance	P value
22.5 mg/kg of sulfadoxine/pyrimethamine vs. 50 mg/kg extract	**	P < 0.01
22.5 mg/kg of sulfadoxine/pyrimethamine vs. 100 mg/kg extract	NS	P > 0.05
22.5 mg/kg of sulfadoxine/pyrimethamine vs. 200 mg/kg extract	NS	P > 0.05
22.5 mg/kg of sulfadoxine/pyrimethamine vs. Tween 80	**	P < 0.01
50 mg/kg Crude Extract vs. 100 mg/kg Crude Extract	*	P > 0.05
100 mg/kg Crude Extract vs. 200 mg/kg Crude Extract	NS	P > 0.05

NS= not significant; *= moderately significant; **= significant.

Table 8. Statistical comparison of the curative results.

Compared variables for curative	Significance	P value
100 mg/kg quinine vs. 50 mg/kg Crude Extract Day 4	NS	P > 0.05
100 mg/kg quinine vs. 100 mg Crude Extract Day 4	NS	P > 0.05
100 mg/kg quinine vs. 200 mg/kg Crude Extract Day 4	NS	P > 0.05
100 mg/kg quinine vs. 0.5ml Tween80 Day 4	**	P < 0.01
100 m/kg Quinine vs. 50 mg/kg Crude Extract Day 7	NS	P > 0.05
100 mg/kg quinine vs. 100 mg/kg Crude Extract Day 7	NS	P > 0.05
100 mg/kg quinine vs. 200 mg/kg Crude Extract Day 7	NS	P > 0.05
100 mg/kg quinine vs. 0.5 ml Tween80 Day 7	**	P < 0.01
50 mg/kg Crude Extract vs. 100 mg/kg Crude Extract Day 4	NS	P > 0.05
50 mg/kg Crude Extract vs. 200 mg/kg Crude Extract Day 4	NS	P > 0.05
100 mg/kg Crude Extract vs. 200 mg/kg Crude Extract Day 4	NS	P > 0.05
50 mg/kg Crude Extract vs. 100 mg/kg/kg Crude Extract Day 7	NS	P > 0.05
50 mg/kg Crude Extract vs. 200 mg/kg Crude Extract Day 7	NS	P > 0.05
100 mg/kg Crude Extract vs. 200 mg/kg Extract Day 7	NS	P > 0.05
50 mg/kg Crude Extract Day 4 vs. 50 mg/kg Extract Day 7	NS	P > 0.05
100 mg/kg Crude Extract Day 4 vs. 100 mg/kg Extract Day 7	NS	P > 0.05
200 mg/kg Crude Extract Day 4 vs. 200 mg/kg Extract Day 7	NS	P > 0.05

NS= not significant; *= moderately significant; **= significant.

Table 9. Comparison of prophylaxis test results for fractions.

Compared variables for prophylaxis	Significance	P value
0.5 ml 5% Tween 80 vs. 250 mg/kg N-hexane fraction of extract	**	P < 0.01
0.5 ml 5% Tween 80 vs. 500 mg/kg N-hexane fraction of extract	**	P < 0.01
0.5 ml 5% Tween 80 vs. 250 mg/kg Ethyl acetate fraction of extract	**	P < 0.01
0.5 ml 5% Tween 80 vs. 500 mg/kg Ethyl acetate fraction of extract	**	P < 0.01
0.5 ml 5% Tween 80 vs. 250 mg/kg aqueous fraction of extract	NS	P > 0.05
0.5 ml 5% Tween 80 vs. 500 mg/kg aqueous fraction of extract	**	P < 0.01
0.5 ml 5% Tween 80 vs. 22.5 mg/kg Sulfadoxine and pyrimethamine	**	P < 0.01

NS= not significant; *=moderately significant; **= significant.

antioxidant activity which may also contribute to the antimalarial activity due to inhibition of haemo-polymerization. Specifically, the parasitaemia suppression effect of the extract may be attributed to the presence of alkaloids (Murugalakshmi et al., 2014).

The acute toxicity result showed that the extract has good safety margin with LD_{50} of 4472.14 mg/kg, slightly toxic at doses higher than the LD_{50}. *J. gossypifolia* leaf

Table 10. Comparison of curative test results for fractions.

Group	Treatment	Compared variables for curative	Significance	P-value	Compared variables for curative	Significance	P-value
1	250 mg/kg n-hexane fraction	Basal vs. Day 4	NS	P > 0.05	Basal vs. Day 7	NS	P > 0.05
2	500 mg/kg N-hexane fraction	Basal vs. Day 4	NS	P > 0.05	Basal vs. Day 7	*	P < 0.05
3	250 mg/kg Ethyl acetate fraction	Basal vs. Day 4	**	P < 0.01	Basal vs. Day 7	**	P < 0.01
4	500 mg/kg Ethyl acetate fraction	Basal vs. Day 4	**	P < 0.01	Basal vs. Day 7	**	P < 0.01
5	250 mg/kg aqueous fraction	Basal vs. Day 4	NS	P > 0.05	Basal vs. Day 7	NS	P > 0.05
6	500 mg/kg aqueous fraction	Basal vs. Day 4	NS	P > 0.05	Basal vs. Day 7	*	P < 0.05
7	100 mg/kg quinine	Basal vs. Day 4	**	P < 0.01	Basal vs. Day 7	**	P < 0.01
8	0.5 ml 5% Tween 80	Basal vs. Day 4	NS	P > 0.05	Basal vs. Day 7	*	P < 0.05

NS= not significant; *=moderately significant; **= significant.

extract showed good therapeutic index and is safe for use at lower doses. Four day chemo-suppresive test showed that the crude extract achieved good suppression of the *Plasmodium* parasite in mice infected with *P. berghei*. Chemosuppression achieved in this study was dose dependent with chemosuppression rate of 85.42% at the highest dose 200 mg/kg when compared with the 91.70% of 22.5 mg/kg of sulfadoxine/ pyrimethamine (positive control). The chemosuppression rate of 85.42% at the highest dose 200 mg/kg recorded is quite good, when compared with the percentage suppression achieved by the ethanolic extract of *Alstonia boonei* which reported its chemosuppression rate of 54.68% at the highest dose of 800 mg/kg (Idowu et al., 2015). This reveals that the extract has a considerable high antiplasmodial effect. This may be due to the synergistic effect of some active constituents of the plants. Significant reduction of parasitaemia (P < 0.05) was observed in all groups of mice treated with the *J. gossypifolia* leaf extract when compared to the negative control.

The curative test revealed that the extract has a considerable high antiplasmodial effect. The curative effect was also dose dependent, but with a very small change with increase in dosage. The curative effect of the 200 mg/kg crude extract which gives the percentage parasitaemia clearance of 88.23% (Day 4) and 96.07% (Day 7) compared with the 100 mg/kg quinine (positive control) which also gives the percentage parasitaemia clearance of 88.37% (Day 4) and 93.02% (Day 7). This may also be due to the synergistic effect of some active constituents of the plants. Significant reduction of parasitaemia (P < 0.05) was observed in all groups of mice treated with the *J. gossypifolia* leaf extract when compared to the negative control. This result compared with the curative effect reported for the combination of powdered seed of *Picralima nitida*, stem bark of *Alstonia boonei* and leaves of *Gongronema latifolium* extract at 400 mg/kg body weight with the percentage parasitaemia clearance of 83.84%; and was in the same range with that of *A. boonei* leaves extract used alone, which with the percentage parasitaemia clearance of 81.36% (Iyiola et al., 2011). The antimalarial activity of the extracts could be attributed to the presence of some phytochemicals like alkaloids, tannins, saponins, flavonoids and terpenes present in the study. Specifically, the parasitaemia curative effect of the extract may be attributed to the presence of alkaloids.

The prophylactic and curative test results for the fractions of the extract *J. gossypifolia* are presented on Tables 5 and 6 respectively. The four day chemosuppresive test showed that the ethyl acetate fraction achieved good suppression of the *Plasmodium* parasite in mice infected with *P. berghei*. Chemosuppression achieved in this study was dose dependent with chemo-suppression rate of 79.97% at the highest dose 500 mg/kg and 69.99% at the dose of 250 mg/kg, when compared with the 90.03% of22.5 mg/kg of sulfadoxine/pyrimethamine (positive control). However, the ethyl acetate fraction exhibited greater percentage inhibition compared with other fractions. The curative test also revealed that the ethyl acetate fraction has a considerable high antiplasmodial effect.

The curative effect was also dose dependent, but with a very small change with increase in dosage. The curative effect of the 500 mg/kg ethyl

Table 11. Physicochemical analysis result.

Parameter	Values (%)
Total ash	4.3
Acid insoluble ash	3.8
Water insoluble ash	2.5
Moisture content	7.0
Ethanol extractive value	4.5

Table 12. Effect of extract on aspartate transaminase (AST).

Group	Treatment dose	Mean absorbance		
		Basal	14 Days	28 Days
1	125 mg/kg	35.573 ± 0.2133	36.01 ± 0.2829	36.48 ± 0.3124
2	250 mg/kg	34.316 ± 0.5340	37.12 ± 1.064	41.19 ± 2.801
3	500 mg/kg	38.09 ± 0.5180	44.55 ± 0.988**	49.56 ± 1.017**
4	0.5 ml of 15 % Tween 80	35.573 ± 0.2133	35.86 ± 0.1976	36.73 ± 0.7622

Values are mean ± SEM; n = 5; **means level of significant difference.

Table 13. Effect of extract on alanine transaminase (ALT).

Group	Treatment dose	Mean absorbance		
		Basal	14 Days	28 Days
1	125 mg/kg	72.52 ± 0.240	72.84 ± 0.4288	72.90 ± 0.563
2	250 mg/kg	63.01 ± 5.136	64.71 ± 5.078	66.16 ± 5.278
3	500 mg/kg	57.13 ± 0.671	60.45 ± 0.445*	63.48 ± 0.743**
4	0.5 ml of 15% Tween 80	62.46 ± 5.175	62.91 ± 5.197	63.003 ± 5.162

Values are mean ± SEM; n = 5; * and ** mean levels of significant difference.

Table 14. Effect of extract on alanine phosphatase (ALP).

Group	Treatment dose	Mean absorbance		
		Basal	14 Days	28 Days
1	125 mg/kg	70.863 ± 5.511	73.16 ± 5.131	74.38± 5.034
2	250 mg/kg	61.98 ± 0.979	63.996 ± 0.127	64.71 ± 0.600*
3	500 mg/kg	54.31 ± 1.091	57.295 ± 0.472*	58.75 ± 0.315**
4	0.5 ml of 15 % Tween 80	61.32 ± 0.575	61.34 ± 0.623	61.62 ± 0.875

Values are mean ± SEM; n = 5; * and **mean level of significant difference.

acetate fraction which gave the percentage parasitaemia clearance of 66.69% (Day 4) and 87.15% (Day 7) and 250 mg/kg of ethyl acetate fraction which also gave the percentage parasitaemia clearance of 58.52% (Day 4) and 75.64% (Day 7), compared with the 100 mg/kg quinine (positive control) which gave the percentage parasitaemia clearance of 85.37% (Day 4) and 90.27% (Day 7). The antiplasmodial property of the plant extracts may be attributed to presence of some phytochemicals which might have conferred some protective / antioxidative effect against oxidative stress induced in the host parasitized red blood cells (RBCs) by the malaria parasite (Nethengwe et al., 2012). When the positive control was compared with the test, there was no significant difference $P > 0.05$ in the rate at which they suppressed the parasitic load. Therefore, when the

antiplasmodial activity of Day 4 and 7 were compared there was also no significant difference P > 0.05.

The result of the physicochemical analysis showed moisture content value (7%), total ash value (6.3%), acid insoluble ash value (3.8%), water soluble ash value (2.5%), and ethanol extractive value (4.5%). Low moisture content is desirable for preservation and long shelf life of the plant part. The moisture content of the *J. gossypifolia* leaves which is 7% which is not more than 10% (British Pharmacopoeia, 2011). Total ash value indicates the absence of contamination, substitution and adulteration of the plant part. The total ash value which is 4.3% is desirable for leaves, which is not more than 5% (British Pharmacopoeia, 2011). Water soluble ash value is useful in ascertaining the quality and purity of the crude drug is 2.5% is desirable for leaves, which is not less than 2.0% (British Pharmacopoeia, 2011). Lastly, the ethanol extractive value of *J. gossypifolia* leaves is 4.5% (British Pharmacopoeia, 2011).

The result of the biochemical parameter screening, when compared to the basal values presented on Tables 12, 13 and 14 showed a significant increase in alanine transaminase (ALT), alanine phosphatase (ALP) and aspartate aminotransferase (AST) levels with increase in the dose of the extract. There was significant difference in the value of the experimental animals when compared with the basal control, providing sufficient evidence that the impact was due to the extract. This has also been reported in earlier findings on other traditional medicinal plants such as *Chrozophora senegalenesis* (Ali et al., 2011) and ethanolic extract of *Magnifera indica* (Ogbe et al., 2012). There was significant difference in the value of the experimental animals when compared with the two controls, providing sufficient evidence that the impact was due to the extract. The level of the ALP at 400 mg/kg showed no difference in all the experimental animals, suggesting that the leakage in AST and ALT was from the liver and not bile duct. Considering the observation in the three parameters, it can be adduced that the extract had an impact on the liver, thereby allowing a leakage of AST and ALT into the blood stream (Idowu et al., 2015).

Conclusion

The results of this study have shown that the ethanol leaf extract and fractions of *J. gossypifolia* possess antiplasmodial property as seen in its ability to suppress malaria in *P. berghei* infection in the two models evaluated. The leaves also contains the normal range of values for moisture content values, ash values and extractive values as specified above, which is an important aspect of standardization so as to establish the correct identity of the crude drug before inclusion in the Pharmacopoeia. This justifies the traditional use of this plant as malaria remedy. It also provides scientific basis for the continuous use of this plant in the treatment of malaria in parts of Nigeria. This study could form basis for further research on this plant, which has exhibited antiplasmodial activity, for possible development of antimalarial drug or preparation.

Ethical statement

All authors hereby declare that the principles of laboratory animal care “(NIH publication No 85-23, revised 1985), were adopted. The research work did not involve human subjects, thus consent to participate is not applicable here.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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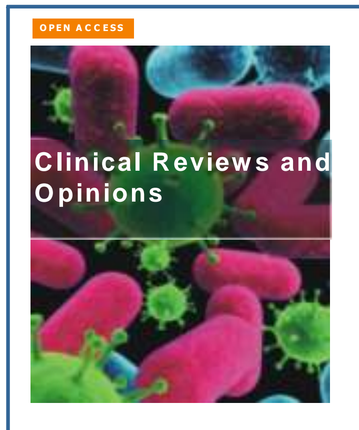

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