Journal of Medicinal Plant Research

Volume 11 Number 47, 17 December, 2017

ISSN 1996-0875

Academic Journals

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Journal of Medicinal Plants Research

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academicJournals

Vol. 11(47), pp. 755-762, 17 December, 2017
DOI: 10.5897/JMPR2017.6487
Article Number: 986804166969
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Aqueous extract of *Persea americana* leaves ameliorates alloxan-induced hyperglycaemia and hyperlipidaemia in rats

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Received 3 September, 2017; Accepted 13 November, 2017

Decoctions of *Persea americana* Mill (Lauraceae) leaves are employed to manage diabetes mellitus among the Ibo communities of Eastern Nigeria. In this study, we studied the ameliorative effects of the aqueous extract of *P. americana* leaves (AEPAL) on alloxan-induced hyperglycaemia and hyperlipidaemia in Wistar rats. The oral median lethal dose (LD_{50}) of AEPAL was determined in rats. The effects of extract on blood glucose and lipids levels; and biochemical parameters were evaluated. The effects of AEPAL on relative organs weights, body weight changes as well as food and water consumption were monitored for 28 days in alloxan-induced hyperglycaemic rats and histopathological changes of the pancreas examined. The estimated oral LD_{50} of AEPAL was greater than 5000 mg kg^{-1}. AEPAL (125–500 mg kg^{-1}) significantly reduced the fasting blood glucose levels and hyperlipidaemia in the alloxan-induced hyperglycaemic rats. The extract significantly reversed the decreased body weight, increased food and water intake; and attenuated elevated levels of aspartate transaminase (AST), urea, total protein (TP), albumin (ALB), alanine transaminase (ALT) and alkaline phosphatase (ALP) in alloxan-induced hyperglycaemic rat. Histological examination of the pancreas showed regeneration of the β–cells of the Islet of Langerhans in the extract treated alloxan-induced hyperglycaemic rat. Our findings revealed that AEPAL contains biologically active components with potential hypoglycaemic activity, thus supporting its further development for the management of diabetic mellitus.

Key words: *Persea americana,* acute toxicity, antidiabetic, β–cells, metformin.

INTRODUCTION

Diabetic mellitus (DM), a group of metabolic disorders of carbohydrate, fat and protein resulting from defects in insulin's secretion, action or both (Triplitt et al*.,* 2008), is characterized by persistent elevated levels of blood glucose (hyperglycaemia), cholesterol, triglycerides and phospholipids as well as changes in lipoprotein composition (Tripathy et al., 2000). The development of DM involves several pathological processes including the

ones that destroy the β–cells of the pancreas resulting to insulin deficiency (Triplitt et al., 2008).

DM is classified into type 1 and type 2, with the type 1 accounting for 5-10% of the diabetic population (Whiting et al., 2011). The type 1 DM, which is diagnosed mainly in children (juvenile onset) and adolescents, is an autoimmune disorder where the body's own immune system attacks the β–cells of the Islets of Langerhans in the pancreas, destroying or damaging them sufficiently to reduce insulin production. Such diabetics are prone to ketoacidosis and often manifest the polytriad of diabetic symptoms including polyphagia, polyuria and polydipsia (Ukwe, 2006). The type 2 diabetes is characterized by relative insulin deficiency or insulin resistance (Triplitt et al., 2008).

The antidiabetic drugs currently in use do not provide cure nor prevent relapse and are often accompanied by serious adverse effects (Triplitt et al., 2008). The development of new pharmacological agents that can overcome these challenges is currently a major goal in diabetic research. The plant kingdom is the major area of interest in the search for safer and more efficacious drugs and lead compounds to treat this serious endocrine disorder.

For many years, preparations of *Persea americana* Mill (Lauraceae) have been used among the Ibo communities of Eastern Nigeria for the management of DM. In previous studies, the leaf extract of this plant demonstrated anti-ulcer (Oluwole et al., 2011), hypotensive (Adeboye et al., 1999) and hepatoprotective (Ekor et al., 2006) effects. The aim of this study was to evaluate the effects of the aqueous extract of *P. americana* leaves (AEPAL) in alloxan-induced hyperglycaemic Wistar rats to lend scientific support for the use of this plant in the Nigerian traditional medicine for the management of DM.

MATERIALS AND METHODS

Drugs and equipment

Alloxan monohydrate (Sigma-Aldrich Co. St. Louis,MO, USA), metformin (Merke Sante, France), chloroform, formalin (Sigma-Aldrich Co. St. Louis,MO, USA), ELITech reagent kits, (Vital Scientific, Netherlands), Glucometer and strips (Accu-check® Advantage, Roche Germany), hematocrit centrifuge (Denley BS400 centrifuge, England) and automated biochemical analyzer (Selectra XL, Vital Scientific, Netherlands) were used for the study.

Animals

Adult Wistar rats (150 to 200 g) of either sex obtained from Animal Facility Centre (AFC) of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria were used for this study. The animals were properly housed in transparent plastic cages padded with wood shavings, under standard conditions of temperature, relative humidity and light/dark cycles (12/12 h). They were fed with standard feeds and water *ad libitum* and were approved for use by the AFC ethical committee after reviewing the protocol (Ethical approval number: DAC/IW–OT/665/14). All the experiments were carried out in accordance with the National Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23) revised 1996. Efforts were made to minimize the number of rats used and their suffering.

Plant material

Fresh leaves of *P. americana* were collected from Zaria in Kaduna State, Nigeria in July, 2015. The leaves were identified and authenticated by Mallam U.S Gallah, a taxonomist from the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen (No. 992) was deposited at the Departmental Herbarium for future reference.

Preparation of the extract

The leaves were washed, shade-dried, pulverized into a coarse powder and stored in an air tight container until needed. To 500 g of powdered leaf material, 2 L of cold distilled water was added and stirred in a conical flask until evenly mixed. The mixture was left to stand for 24 h with occasional shaking and stirring. The mixture was filtered using muslin cloth followed by Whatman filter paper No 1. The solvent was removed under reduced pressure using a rotary evaporator and yielded 9.93% (w/w) of extract that was used for the study.

Acute toxicity study

The median lethal dose (LD_{50}) of the extract was determined orally in rats using methods described in detail by Lorke (1983) and modified by Amos et al. (2002).

Induction of hyperglycaemia

Hyperglycaemia was induced by a single intraperitoneal injection of freshly prepared alloxan monohydrate (150 mg kg^{-1}) in 16 h fasted rats with free access to water. Six hours after that, the rats were treated with 20% glucose solution orally to prevent fatal hypoglycaemia and the rats were fed with 5% glucose to prevent hypoglycaemia (Dhandapani et al., 2002). Seventy-two hours after alloxan administration, blood glucose concentrations were measured with the aid of digital glucometer and strips (Accu-Chek® Advantage, Roche Diagnostic, Germany). Rats with fasting blood glucose level ≥ 200 mg dL^{-1} were selected for the study and allowed to stabilize for another 3 days before commencement of the experiment.

Treatment

Rats were randomly selected and divided into six groups of six

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(n=6) of rats per group as follows: Group I received normal saline to serve as normal control, while group II received only alloxan monohydrate (150 mg kg^{-1}) to serve as hyperglycaemic controls. The alloxan-induced hyperglycaemic rats in groups III, IV and V received graded doses (125, 250 and 500 mg kg^{-1}) of the extract, while Group VI (alloxan-induced hyperglycaemic rats) received metformin 250 mg kg^{-1} to serve as positive control (Maithili et al., 2011). Freshly prepared normal saline, graded doses of the extract and metformin were orally administered daily using an oral cannula for a period of 28 days. The blood glucose levels were analyzed at regular intervals of 0, 7, 14, 21 and 28 days of the experimental period. The body weight of the rats were taken individually before the commencement of treatment and thereafter taken once at day 7, 14, 21 and 28 throughout the treatment period. The food and water consumptionin each group were measured daily as the difference between the quantity of feed and water supplied and the amount remaining after 24 h.

Biochemical analysis

The animals were sacrificed on the 29th day under anesthesia by cervical dislocation. Blood samples were collected by cardiac puncture into plain bottles, allowed to clot and centrifuged. The supernatant was collected for evaluation of biochemical parameters. Alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities, total protein (TP), albumin (Alb), serum urea and creatinine, total cholesterol(TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were analyzed with an automated chemistry analyzer (Cobas Mira, Roche), using commercial kits obtained from Randox Laboratories, UK.

Histology of the pancreas

The pancreas was carefully dissected out, washed in ice cold saline immediately and fixed in 10% formalin fixative solution for 48 h, processed routinely and embedded in paraffin wax. Histological sections were cut at 5–6 µm; and stained with haematoxylin and eosin as described by Strate et al. (2005). The slides were viewed at magnification of ×250 and photomicrographs were taken.

Statistical analysis

All data were presented as mean ± SEM and analyzed by one-way ANOVA followed by Dunnett's *post hoc* test using Graph Prism version 4.00 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Results were considered significant at $p < 0.05$.

RESULTS

The estimated oral LD_{50} of AEPAL was greater than 5000 mg kg^{-1} with no recorded signs of toxicity or mortality during the 24 h observation period.

Effect of the extract on alloxan-induced hyperlipidaemia and hyperglycaemia

The hyperglycaemic control showed a rise in blood glucose levels, which was significantly ($p < 0.05$) reduced in AEPAL and metformin treated groups from the 7th to 28th day of the treatment period (Figure 1).

In Figure 2, hyperglycemic control showed significant hyperlipidaemia compared to normal control. Treatment with AEPAL significantly reversed the hyperglycaemic-induced hyperlipidaemia in a dose related fashion. AEPAL (125 mg kg^{-1}) significantly ($p < 0.05$) increased HDL and decreased TG and LDL. Rats treated with 250 and 500 mg kg^{-1} AEPAL and 250 mg kg^{-1} metformin significantly ($p < 0.001$) reversed all the lipid profile of the alloxan-induced hyperglycaemic rats.

Effects of the extract on body weights variations

AEPAL (250 mg kg^{-1}) significantly ($p < 0.05$) reversed the body weight reduction caused by alloxan-induced hyperglycaemia on day 28 and standard drug (metformin 250 mg kg^{1}) on day 21. Rats treated with 500 mg kg^{-1} of the extract and metformin 250 mg kg^{-1} showed a significant ($p < 0.01$) weight gain on the last day of treatment (Figure 3).

Food and water intake in alloxan hyperglycaemia

The hyperglycaemic control rats showed an increase in food and water intake, which are characteristics of DM. The extract significantly ($p < 0.05$) reduced the feed consumption after week 1 in metformin (250 mg kg^{-1}) treated group and week 2 in 250 and 500 mg kg^{-1} treated groups (Figure 4). A significant ($p < 0.001$) reduction in water intake was observed in standard drug (metformin 250 mg kg^{-1}) group and in all AEPAL treated groups (Figure 4).

Biochemical parameters in alloxan hyperglycaemia

A significant reduction in AST, creatinine, urea, TP, ALB, ALT and ALP were observed in hypoglycemic rats treated with both metformin and graded doses of the extract (Table 1).

Histology

The pancreas was intact in the normal control group (Plate 1A). Pancreatic sections of hyperglycemic control rats showed severe necrotic changes of pancreatic islets, especially in the center of islets (Plate 1B). There were increased sizes of islets and hyperchromic nucleus from lowest to the highest dose of extract compared to hyperglycaemic control (Plate 1C to E). The metformin treated group had a lesser effect than the extract (Plate 1F).

DISCUSSION

From the data presented in this study, there are

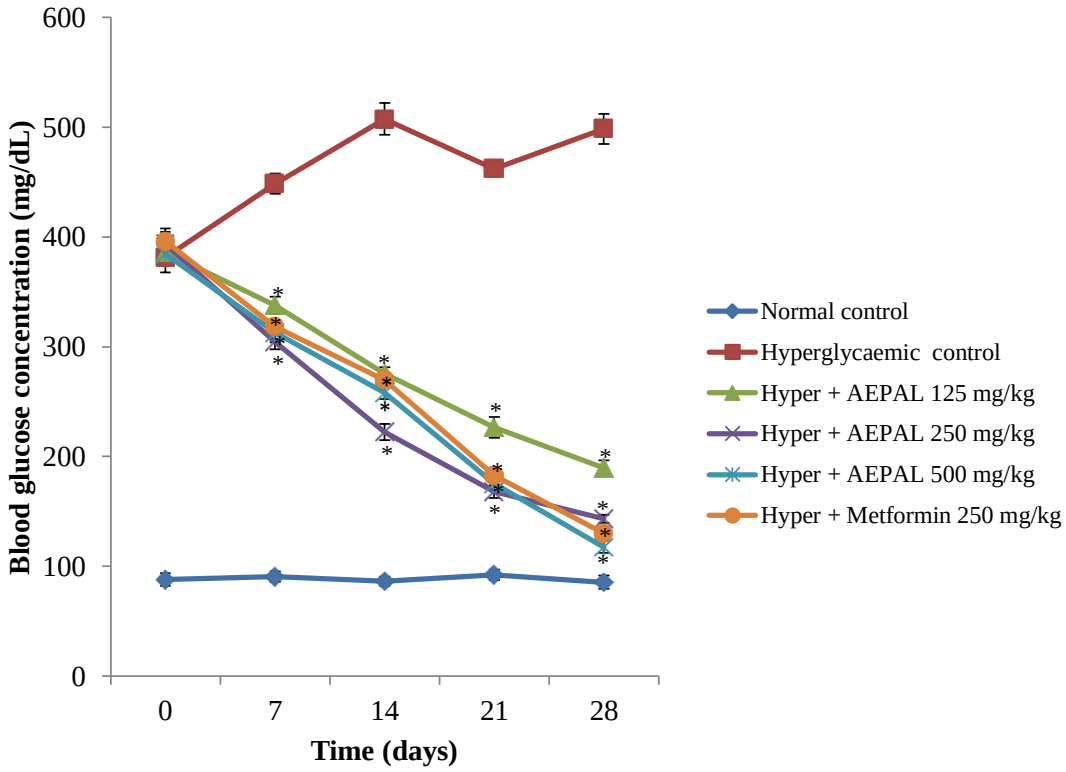


Figure 1. Effect of AEPAL on fasting blood glucose concentrations in alloxan-induced hyperglycemic Wistar rats. AEPAL, aqueous extract of *P. americana* leaf, n=6; values expressed as mean ± SEM.; data analyzed using one-way ANOVA followed by Turkey's post hoc test; *p < 0.05, statistically significant compared to hyperglycemic control.

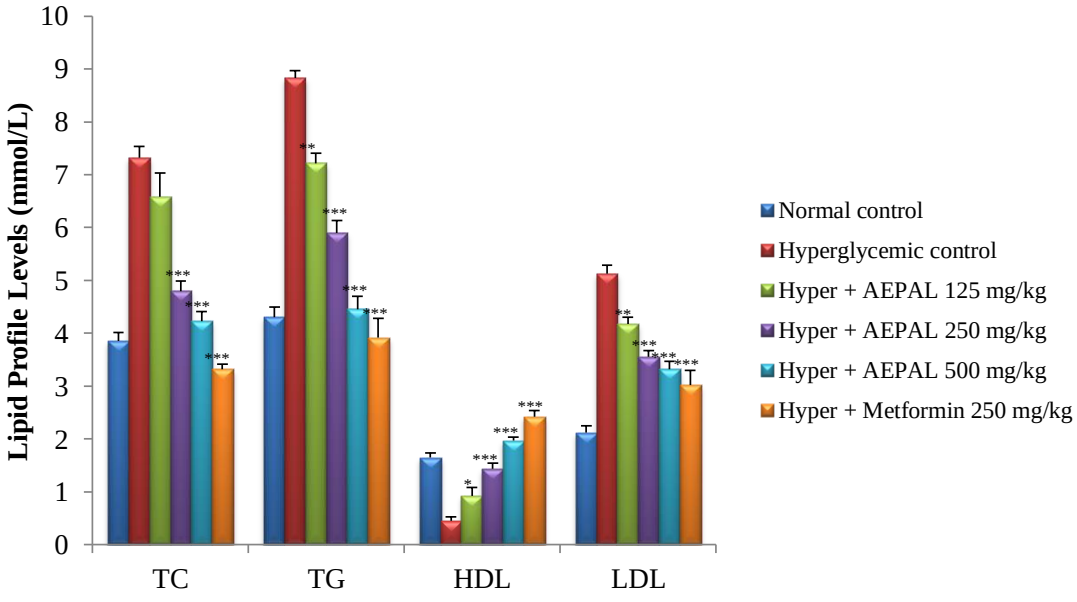


Figure 2. Effect of AEPAL on lipid profile levels in alloxan-induced hyperglycemic Wistar rats. AEPAL, aqueous extract of *P. americana* leaf; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein, n = 6; values expressed as mean ± SE. Data analyzed using one-way ANOVA followed by Turkey's post hoc test; *p < 0.05, **p < 0.01, ***p <0.001 statistically significant compared to hyperglycemic control.

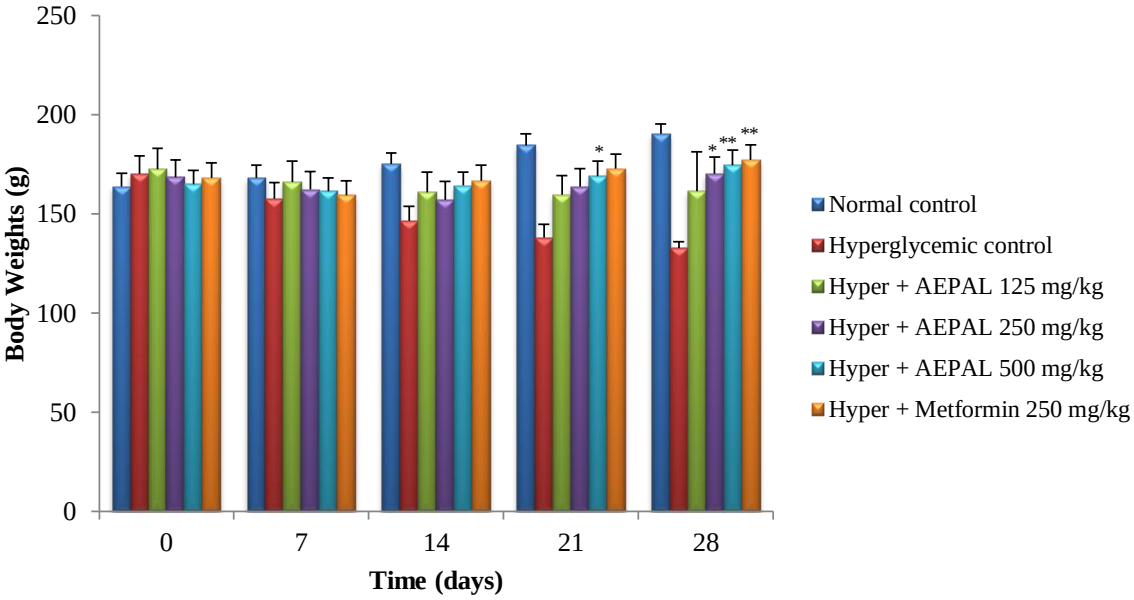


Figure 3. Effect of AEPAL on body weights of alloxan induced hyperglycemic rats. AEPAL – aqueous extract of *P. americana* leaf, n = 6; values expressed as mean ± SEM.; data analyzed using one-way ANOVA followed by Turkey's post hoc test; *p <0.05, **p <0.01, statistically significant compared to hyperglycemic control.

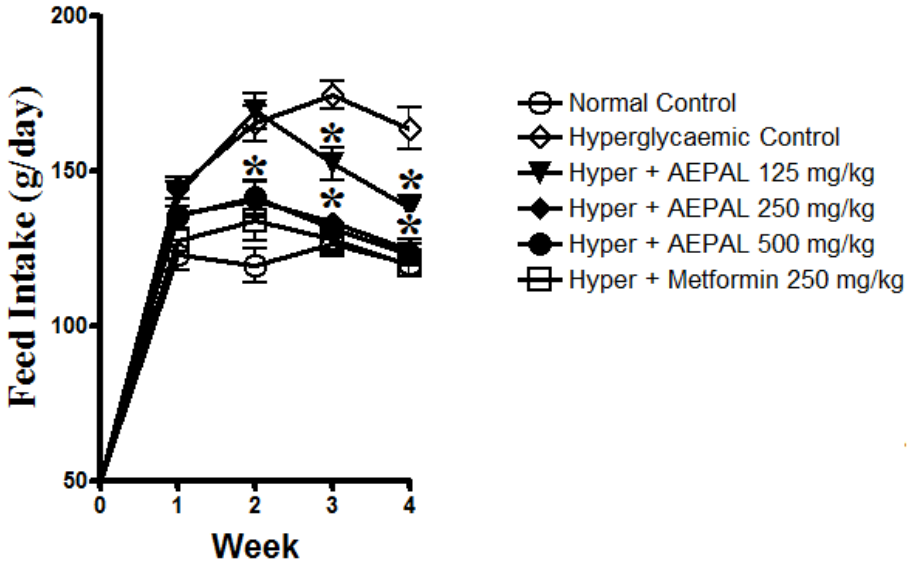
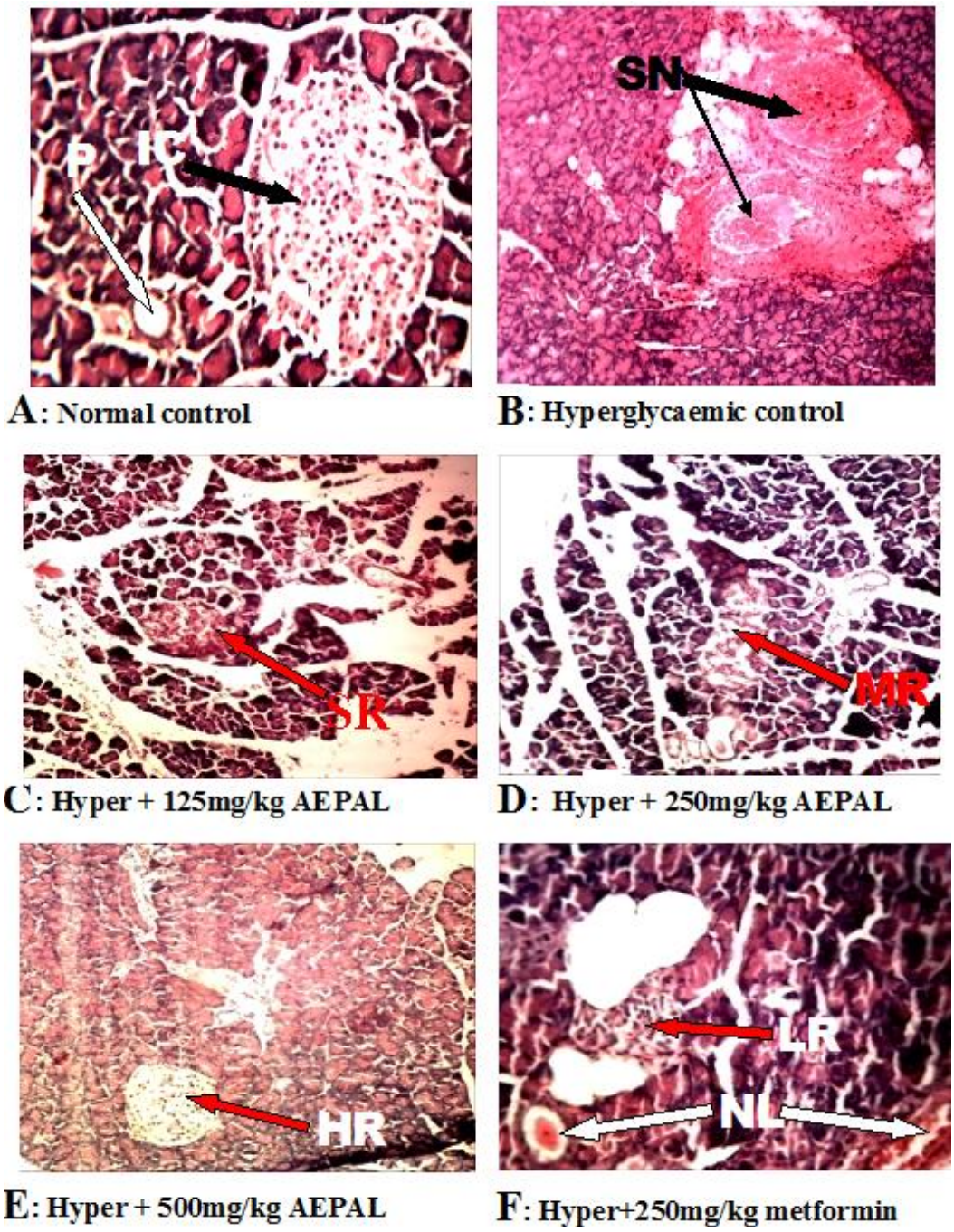


Figure 4. Effect of AEPAL on feed intake in alloxan-induced hyperglycemic Wistar rats. AEPAL, aqueous extract of *P. americana* leaf, n = 6; values expressed as mean ± SEM. Data were analyzed using one-way ANOVA followed by Turkey's post hoc test;*p<0.05, statistically significant compared to hyperglycemic control.

evidences that AEPAL contains biologically active constituents that are relevant in the management of diabetes mellitus. AEPAL significantly lowered blood glucose levels in alloxan-induced hyperglycaemic rats. Alloxan-induced hyperglycaemia is an acceptable model for Type I diabetes mellitus (Goldner and Gomori, 1944; Rohilla and Ali, 2012). Alloxan selectively destroys the insulin producing pancreatic beta cells (Szkudelski et al., 1998; Fasanmade and Alabi, 2008), resulting to various metabolic alterations including increased levels of blood



Plates 1. A, IC, Intact Islet cells; PL, normal pancreatic lobule. **B,** SN, Severe necrosis and atrophy of Islet cells; **C,** SR, slight regeneration of Islet cells; **D,** MR, moderate regeneration of Islet cells; **E,** HR, high regeneration and almost intact islet cells; **F,** LR, low regeneration of islet cells; NL, necrosis, lesion, distortion and atrophy still present.

glucose, cholesterol, alkaline phosphate and transaminases (Murugan et al., 2009). In this study, AEPAL (125 to 500 mg kg^{-1}) significantly decreased the blood glucose concentration, thus supporting the use of *P. americana* leaf preparations in folk medicine for the management of diabetes mellitus.

Hyperglycaemia, hyperlipidaemia and oxidative stress generally coexist in diabetic subjects (Mironova et al., 2000; Beckman et al., 2002). This study shows an increase in the concentration of total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C) and a decrease in HDL-C in hyperglycaemic control.

Table 1. Effect of AEPAL on biochemical parameters in alloxan-induced hyperglycaemic rats.

Treatment/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TP (g/L)	ALB (g/L)	Urea (mmol/L)	Creatinine (µmol/L)
Normal control	40.83±4.67	47.67± 5.11	62.83 ±7.94	65.00 ±6.55	37.17 ± 6.68	2.18 ±0.17	49.50 ±1.48
Hyperglycaemic control	78.67±5.46	90.50 ±11.42	102.17 ±8.28	28.00 ±3.90	19.17 ±4.31	5.05±0.20	68.50±2.57
Hyperglycaemic + AEPAL125	54.33±3.9**	71.67±5.65	93.50 ±5.97	32.33±3.46	23.83 ±2.97	4.62 ±0.28	60.50 ±1.34*
Hyperglycaemic + AEPAL250	57.50±3.30*	64.67±7.62	77.33 ±7.50	42.00 ±4.76	30.50±2.57	3.72±0.26**	45.67±1.20***
Hyperglycaemic + AEPAL500	43.67±6.49***	46.50±4.69**	61.50 ± 6.20**	57.00 ± 7.40**	46.50 ± 5.06**	2.95 ±0.17***	43.67±2.03***
Hyperglycaemic + Metformin 250	39.83 ±1.35***	52.83 ± 5.53**	58.68 ± 8.02**	65.33 ± 1.63***	38.67± 4.76	2.63 ± 0.17***	47.67±1.84***

AEPAL, aqueous extract of *P. americana* leaf, n = 6, values expressed as mean ± SEM., *p < 0.05, **p < 0.01, ***p < 0.001, statistically significant compared to hyperglycaemic control.

Diabetic-induced hyperlipidaemia is attributable to excess mobilization of fat from the adipose tissue due to underutilization of glucose (Krishnakumar et al., 2000). The lack of insulin stimulates lipolysis and enhanced release of free fatty acids from adipose tissue (Subbiah et al., 2006), which are converted to triglyceride (Suryawanshi et al., 2006). Lowering of serum lipid levels through dietary or drugs therapy may be associated with a decrease in the risk of vascular disease in diabetes (Claudia et al., 2006). In this study, graded doses of AEPAL significantly reduced serum levels of total cholesterol, triglyceride, low-density lipoprotein and increased serum levels of high-density lipoproteininalloxan-induced hyperglycaemic treated rats, suggesting that the plant extract may be useful in reducing the complications of hyperlipidaemia and hypercholesterolemia thatare often comorbidities in diabetics (Sharma, 2003).

The current study showed a progressive decrease in body weights of alloxan treated hyperglycaemic animals consistent with the reports of Andrade and Wiedenfeld (2001) and Eze et al. (2015). The reduction in body weights may be associated with degradation of structural proteins and increased muscle wasting (Cheng et al., 2013; Eze et al., 2015). AEPAL significantly reversed the body weight reduction, decreased food and water intake in alloxan-induced hyperglycaemic rats, suggesting that the AEPAL countered the basic polytriad symptoms of diabetes mellitus, polyphagia, and polydipsia and weight loss.

Furthermore, the study showed elevated AST, ALT and ALP in hyperglycemic control and treatment of hyperglycaemic animals with AEPAL and metformin produced a significant reduction in the levels of AST, ALT and ALP. The level of serum protein and albumin were reduced in hyperglycaemic control. An improvement in the total protein and albumin content was observed in AEPAL and metformin treated groups, which may be attributable to an increased in protein synthesis.

The blood urea and creatinine levels increased significantly in hyperglycaemic control group and reversed in AEPAL and metformin treated group. Increase in serum urea and creatinine concentrations, which are considered as markers of kidney dysfunction (Gross et al., 2005) may indicate diminished ability of the kidneys to filter these waste products from the blood and excrete them in the urine (Gross et al., 2005).

Alloxan caused degeneration of pancreatic β-cells in hyperglycaemic animals. AEPAL at tested doses increased sizes of islets and hyperchromic nucleus, relative granulated and normal beta cells compared to hyperglycaemic control.

Conclusion

The AEPAL may contain bioactive substances with potential hypoglycaemic and hyperlipidaemic properties, thus supporting further the development of these substances as antidiabetic agents. Studies are in progress in our laboratories to isolate and mechanistically characterize the biologically active components from this important medicinal plant that is already in common use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors are grateful to Joseph Oseh, John

Kono and Mallam Kabiru for their technical assistance.

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Vol. 11(47), pp. 763-769, 17 December, 2017
DOI: 10.5897/JMPR2017.6519
Article Number: F43165A66970
ISSN 1996-0875

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Journal of Medicinal Plants Research

Full Length Research Paper

Phytochemical screening and *In Vivo* antimalarial activities of crude extracts of *Lantana trifolia* root and *Premna oligotricha* leaves in plasmodium berghei infected mice

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Received 13 October, 2017; Accepted 9 November, 2017

Resistance of malaria parasites to several antimalarial drugs mandates the need for new compounds from affordable sources. Thus the current study was conducted to justify the traditional usage of Lantana trifolia roots and Premna oligotricha leaves to combat malaria. The powdered roots of Lantana trifolia and leaves of Premna oligotricha were macerated using 70% ethanol. Secondary metabolites present in the extracts were determined using the standard chemical method. The in vivo antiplasmodial effect of the crude extracts were evaluated using the four-day suppressive test in chloroquine (CQ) sensitive strain of Plasmodium berghei infected mice. Parameters such as parasitaemia, packed cell volume, body weight change and survival time of mice were then determined as using standard tests. The ethanol extracts showed a dose-dependent chemosuppression when compared to the negative control in this study. The chemosuppressive effect produced by all doses was very significant (P <0.001). Concentrations at 350, 500 mg/kg of leaf extract and 200 mg/kg from root extract have demonstrated prevention effect on weight loss of mice (P <0.001). All the test extracts employed in this study have no significant prevention effect on PCV loss of mice at all dose levels. The present study has demonstrated the efficacy of the extracts of Premna oligotricha leaf and Lantana trifolia root traditionally used in chemotherapy of malaria infection. These observations provide the basis for the traditional use of these plants in treatments of malaria and potential for development of novel therapeutics in the conventional medicine.

Key words: Antimalarial*, In vivo*, *Lantana trifolia,* parasitaemia, *Plasmodium berghei, Premna oligotricha*.

INTRODUCTION

Malaria is a life-threatening infectious disease caused by plasmodium parasite. It affects more than 200 million people worldwide with an estimated death of nearly 1 million people, mostly children. The disease is also

responsible for causing significant economic loss, especially in developing countries (WHO, 2015). In Ethiopia, for example, malaria adversely affects the health of the public and the country's economy as a result of significant expansion to previously non-affected areas with endemicity of about 75% (WHO, 2004; Adhanom et al., 2006; Gebreyohannes et al., 2017). Subsequently, it attracts significant attention from various stakeholders in designing a strategy to prevent and control the disease.

Treatment of malaria infection with conventionally used anti-malarial drugs are puzzled by the emergence of resistant parasites, which reduces the importance of these drugs in controlling malaria infection (Kilama, 2005; Birru et al., 2017). Various strategies are designed and undertaken to enhance the treatment outcomes of anti-malarial drugs, including combination of drugs and the discovery of new compounds from plants (Tchoumbougnang et al., 2004; Okafor et al., 2013). Most of the currently used antimalarial drugs including chloroquine, primaquine and artimisinin derivatives are derived from plants which have been traditionally used against malaria.

In addition, the society relies on traditional medicine practice; and plants having antimalarial properties is commonly used as an alternative therapy to combat malaria (Waako et al., 2007; Builders et al., 2011). These necessitates the importance of further studies on medicinal plants which are reported to have antimalarial activity. In Ethiopia, despite the presence of a huge number of plants which are claimed to have anti-malarial activity, only few studies have been conducted to evaluate the anti-malarial activity and safety margins of these plants (Tadesse and Wubneh, 2017).

The roots of *Lanata trifolia* are traditionally used for the treatment of various human ailments such as malaria, fever, dermatitis, wound, bleeding, asthma, cough, colds, diarrhea, and bronchitis (Atkin and Kadercit, 2004). The plant is also used in East Africa to treat fever, epilepsy and cerebral malaria (Mukungu et al., 2016; Nalubega et al., 2013). Consumption of *L. trifolia* fruit has also been reported in Konso, Ethiopia (Ocho, 2012). Previous study demonstrated that the plant has shown anti-inflammatory and anti-nociceptive activity (Silva et al., 2005; Johnson et al., 2017).

Based on ethnobotanical survey conducted in South Omo, the leaves of Premna oligotricha is used for the treatment of malaria (Waako, 2007). *P. oligotricha*, is distributed in Ethiopia, Kenya and Somalia. The plant is locally known as 'yeweba medhanit' or 'drug of malaria' by the society in South Omo, Southern Ethiopia. As a result of pleasant smelling, the plant is also used to fumigate and cleanse gourds in Marsabit districts of Ethiopia and Kenyas (Ketemma et al., 2013). Studies also revealed the presence of two antibacterial diterpenes, a clerodane and labdane (WHO, 2005; Habtemariam et al., 1990).

Although *L. trifolia* and *P. oligotricha* are traditionally used for the treatment of malaria in Ethiopia, there is no study which evaluates the anti-malarial activities and safety of these plants. Therefore, this study is designed to evaluate the in vivo antimalarial activities of crude extract of *L. trifolia* and *P. oligotricha* leave in Plasmodium berghei infected mice. The study also examined the safety profiles and phytochemical constituents of both plants.

MATERIALS AND METHODS

Collections and preparation of plant materials

The roots of *L. trifolia* were collected from the premises of Hawassa city, South Ethiopia. Whereas, the leaves of *P. oligotricha* were collected from the location 50 km from Bulle Hora on the highway to Yabello, Oromiya regional state, Ethiopia. After collection, the plant materials were confirmed by a taxonomist and a voucher specimen (AE-001/08) was deposited at the herbarium of Addis Ababa University (AAU), Ethiopia.

Extraction

Prior to extraction, the plant parts were washed with distilled water and dried under shade just after specimen collection. The powdered roots and leaves were macerated using 70% ethanol. Then, the extracts of both plants were filtered using Whatmann filter paper No. 1 and the ethanol was removed using rotary evaporator at average of 40 RPM under reduced pressure at a temperature of 40 to 45^{o}C. The crude extracts obtained were kept in a refrigerator at 4°C and fresh solutions using 3% tween 80 were prepared for each extract immediately before the test.

Laboratory animal

Swiss albino mice of 6 to 8 weeks of age, weighing 25 to 32g, were maintained in a standard room and acclimatized to the laboratory condition for 14 days in 12 h light and dark cycle. Animals were provided with a standard pellet diet and water on *ad libitum*. Mice were handled according to the international guidelines for the care and use of animals in the experiments (European Community Guidelines, 1986). The study was also approved by the insttuional review board (IRB/026/08) of Hawassa University, Ethiopia.

The parasite strain

The anti-malarial activity of *L. trifolia* roots and *P. oligotricha* leaves were tested on mice infected with chloroquine-sensitive *P. berghei* strain ANKA which was obtained from the Department of Biomedical Sciences, Addis Ababa University. Viable strains of *P. berghei* was maintained by a weekly passage of blood from an

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infected donor mouse (with a rising parasitemia of 30%) to non-infected mice. Blood from infected mouse was collected through cardiac puncture. Taking into account the level of parasitemia of infected mouse and the erythrocyte count of non-infected mouse, the blood was diluted to get 1×10^7 *P. berghei*- infected RBCs in 0.2 ml normal saline diluted blood. On the first day (D0), mice were injected intraperitoneally with the 0.2 ml of infected and diluted blood (Knight and Peters, 1980).

Grouping of animals

Then, infected mice were allocated randomly into 8 groups of 5 mice each. The first 6 groups received an oral dose of 200, 350 and 500mg/kg b. wt root and leave extracts of *L. trifolia* and *P. oligotricha*, respectively. The other two groups were served as positive and negative control groups, and received an equal volume of 3% of Tween 80 (vehicle) and chloroquine (25 mg/kg/day; orally) (Ethiopian Pharmaceutical Manufacturing, Ethiopia), respectively.

***In vivo* anti-malarial test**

Antiplasmodial activity of the test extracts was assessed following the standard four-day suppressive test protocol (Waako et al., 2005). The mice were divided randomly into five groups consisting of five mice each. 3 h after infection, 200, 350 and 500 mg/kg/day oral doses of *L.trifolia* and *P.oligotricha* crude extracts were given to the test groups. The control groups were treated with chloroquine (25 mg/kg/day, orally) and an equal volume of 3% Tween 80. Treatment of the test and control groups was conducted for four consecutive days (day 0 to day 3). On day 4, blood samples were collected from the tail of each mouse. Thin blood smears were made and stained with 10% Giemsa stain. The numbers of parasitized RBCs out of 100 RBCs in random fields were used to determine the parasitemia level. Then, average percentage parasitemia was determined using the following formula:

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBCs}}{\text{Total number of RBCs}} \times 100$$

In addition, the average percentage of parasitemia suppression was calculated using the following formula:

$$\% \text{ Suppression} = \frac{\text{Parasitemia in the negative control group- Pasitemia in test group}}{\text{Parasitemia in the negative control group}} \times 100$$

Body weight changes and packed cell volume (PCV)

The body weights and PCV at D0 and D4 of mice were taken to observe whether the test extracts prevented the weight loss and reduction in PCV that are commonly observed with increasing parasitaemia in *P. berghei* infected mice. The PCV was determined by the following equation:

$$\text{PCV} = \frac{\text{Total volume erythrocytes in a given volume of blood}}{\text{Total volume of blood}} \times 100$$

Determination of the percentage survival

The curative potential of the fractions was assessed by monitoring the mortality starting from the day of treatment in infected mice, daily. The number of animals survived from the time injecting blood containing the parasite up to 10th day was determined for each group. The comparison was made based on the percentage of animals that survived on the 10th day after treatment.

Test for acute oral toxicity studies

In order to assess the safety profile of crude extracts of *L. trifolia* and *P. oligotricha*, mice were divided randomly into two groups of six animals each (three male and three female). Mice were fasted for one night prior to administration of the extracts and provided with only water. Then, the extracts were given with an increasing doses of 2000, 3500, 5000 mg/kg using oral gavage following the standard guidelines (WHO, 2001). Animals were observed for any signs of toxicity for 14 days.

Observation was carried out for any signs of overt toxicity such as hair erection, salivation, lacrimation, diarrhea, tremor, convulsion, and mortality.

Phytochemical screening

Crude extracts of *L. trifolia* and *P. oligotricha* were evaluated for the presence of secondary metabolites such as flavonoids, alkaloids, triterpenoids, resine, tannins, saponins and steroids, according to the previously established protocols (Debella, 2002; Jones and Kinghorn, 2006).

Statistical analysis

Data were analyzed using Windows, statisitical package for social science (SPSS) Version 22.0. Comparison of percentage suppression, PCV, weight changes and percentage survival with the negative control group was made using One-way Analysis of Variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. P-values less than 0.05 was considered statistically significant.

RESULTS

Acute toxicity study

Oral acute toxicity studies on the extracts of *L. trifolia* roots and *P. oligotricha* leaves showed that the extracts were found to be safe up to a maximum dose of 5000 mg/kg. Administration of either of the extracts did not produce mortality, signs of morbidity and a significant behavioral and physical changes in graded doses given to experimental animals. Therefore, the results reveals that the medium lethal doses (LD_{50}) of the extracts are greater than 5000 mg/kg body weight.

Table 1. The effect of crude extracts of *P. oligotricha* leaf and *L. trifolia* root on survival time, percentage parasitemia and chemosuppression in *P. berghei* infected mice.

Treatment groups	% survival of animal on day 10	% parasitemia	% chemosuppression
P. oligotricha			
200 mg/kg leaf	80.40±0.50*	48.16±0.86*	26.21±2.05*
350 mg/kg leaf	80.60±0.50*	34.84±0.42*	46.66±0.70*
500 mg/kg leaf	80.60±0.50*	29.84±0.98*	54.29±1.40*
L. trifolia			
200 mg/kg root	80.40±0.40*	52.07±1.28*	20.26±2.82*
350 mg/kg root	80.60±0.24*	41.74±0.68*	37.12±1.12*
500 mg/kg root	90.00±0.63*	35.68±1.44*	46.93±2.13*
Choloroquine (25 mg/kg)	100.00±00*	00.00±0.00*	100.00±0.00*
Negative control	60.40±0.24	65.37±0.94	00.00±0.00

Results are expressed as Means ± SEM, n = 5. *P <0.05 versus negative control.

Table 2. The effect of crude extracts of *P. oligotricha* leaf and *L. trifolia* root on body weight of *P. berghei* infected mice.

Treatment groups	Weight (g) before treatment	Weight (g) after treatment	% change
P. oligotricha			
200 mg/kg	21.00±0.63	22.00±0.32	+4.54±1.64
350 mg/kg	28.20±0.58	26.40±0.60	-6.81±0.36*
500 mg/kg	24.60±0.24	26.60±1.29	+7.51±1.10*
L. trifolia			
200 mg/kg	27.80±0.80	28.00±0.80	+0.71±0.00*
350 mg/kg	22.20±0.49	21.20±0.80	-4.71±0.18
500 mg/kg	22.40±0.50	21.60±0.67	-3.70±0.07
Choloroquine (25 mg/kg)	20.80±0.37	19.40±0.24	-7.21±1.30
Negative control	20.40±0.67	18.40±0.40	-10.86±1.27

Results are expressed as means ± SEM, n = 5. *P <0.05 versus negative control.

Effect of the extracts on percentage parasitemia and survival of mice

The effects of treatment with crude extracts of *P. oligotricha* leaf and *L. trifolia* root on parasitemia suppression and survival of *P. berghei* infected mice are shown in Table 1. The study exhibited that crude extracts of both plants exhibited a significant (P<0.05) and dose-dependent chemosuppression compared with the negative control. The chemosuppression was maximum at higher doses for both extracts (54 and 46% for *P. oligotricha* and *L.trifolia,* respectively at 500 mg/kg dose) compared with the negative control.

In addition, 10th day assessment of infected and extract treated mice demonstrated that the extracts prevented mortality in 80 to 90% of mice. Accordingly, mice treated with all doses of extract lived longer than the negative control (Table 1).

Effect of the extracts on body weight loss

Doses of 350 and 500 mg/kg *P. oligotricha* leaf extract and 200 mg/kg *L. trifolia* root extract demonstrated a significant (P <0.05) weight gain in mice compared with the negative control group (Table 2). However, the 350 mg/kg dose of *P. oligotricha* leaf extract caused significant body weight loss (P <0.001) compared to the negative control group (Table 2).

Effect of the extracts on PCV

The extracts employed in this study failed to prevent PCV

Table 3. The effect of crude extracts of *P. oligotricha* leaf and *L. trifolia* root on PCV of *P. berghei* infected mice.

Treatment groups	PCV before treatment	PCV after treatment	% change
P. oligotricha			
200 mg/kg	57.32±2.53	53.02±3.04	-8.11±2.20
350 mg/kg	60.60±2.48	59.04±2.43	-2.64±1.40
500 mg/kg	62.38±1.44	56.04±2.11	-11.31±1.42
L. trifolia			
200 mg/kg	62.32±2.43	57.42±2.26	-8.53±1.18
350 mg/kg	60.42±1.91	56.20±2.28	-7.50±1.00
500 mg/kg	58.74±0.83	62.06±1.04	+5.34±0.24
Choloroquine (25 mg/kg)	60.52±1.44	63.72±1.08	+5.02±0.34*
Negative control	61.88±0.97	54.80±0.91	-12.9±0.15

Results are expressed as means ± SEM, n = 5. *P <0.05 versus negative control.

Table 4. Phytochemical constituents of crude ethanol extract of *P. oligotricha* leaf and *L.trifolia* root.

Secondary metabolite	*P. oligotricha* leaf	*L. trifolia* root
Phenol	+	-
Flavanoid	+	-
Tannin	+	-
Steroids	+	+
Terpenoids	+	+
Steroidal glycosides	+	+
Alkaloids	-	+
Quinines	-	-
Saponins	+	+
Resine	-	+
Glycosides	+	+

NB: (+) = presence, and (–) = absence.

loss at any dose (P >0.05), compared with the negative control group (Table 3).

Phytochemical screening

Phytochemical evaluation of the root and leaf extracts of *L. trifolia* and *P. oligotricha,* respectively was carried out following the standard protocols. Steroids, terpenoids, steroidal glycosides, saponins, and glycosides were found in the extracts of both plants (Table 4).

DISCUSSION

The antimalarial activity of a compound is commonly assessed using the *P. berghei* infected rodent model because of the similarity with human malaria infection, and the sensitivity of *P. berghei* to chloroquine. The model takes into account the contribution of the immune system in the elimination of infection (Waako et al., 2007).

The study demonstrated the antimalarial activity of crude ethanol extracts of *P. oligotricha* leaves and *L. trifolia* roots which are traditionally used in Ethiopia and elsewhere, against malaria infection. The four-day suppressive test was used to evaluate the impact of treatment with extracts on parasitemia suppression, survival time, PCV and body weight changes. Moreover, the acute toxicity profile and the phytochemical constituents of both plants were determined.

A compound which produces a minimum of 30% parasitemia suppression or greater percent survival compared to infected and non-treated mice is commonly considered to be an active antimalarial agent (David et al., 2004; Fentahun et al., 2017). The four-day

suppressive test showed that the crude extracts of *P. oligotricha* and *L. trifolia* demonstrated a significant and dose-dependent parasitemia suppression. The suppression caused by both plants was greater than 30% at 350 and 500 mg/kg doses.

The results indicates that the crude extracts of both plants are endowed with antimalarial activity with an optimal minimal dose of 350 mg/kg body weight. On the other hand, treatment with the crude extracts of both plants significantly enhance the percent survival of mice on the 10th day relative to the negative control group. The enhanced survival of mice in the extract treated group could be attributed to the suppressive effect of the extracts on parasitemia. The activity of the extracts, in suppressing parasitemia and enhancing survival time of infected mice, was in agreement with previous studies on other plant extracts such as *Echnopis kebericho* (Toma et al., 2015), *Croton Machrostachyus*, *Dodonaea angustifolia* (Mengiste et al., 2012) and *Nigella sativa* (Dikasso et al, 2006), at a relatively comparable doses.

Phytoconstituents are frequently reported for the therapeutic benefits of herbal preparations (Habtemariam et al., 1991; Ayoola et al., 2008). Secondary metabolites such as alkaloids, triterpenoids, quassinoids, xanthones, sesquiterpenes, flavonoids, quinines and phenolic compounds have shown significant antimalarial activities (Habtemariam et al., 1991; Nalubega et al., 2013). Therefore, the antimalarial activity of *P. oligotricha* and *L. trifolia* could be as a result of the individual or combined effects of the phytoconstituents present. Herbal remedies with immunomodulatory and antioxidant (Silva et al., 2005; Okokon et al., 2006) activities are shown to posses antimalarial activity. Moreover, inhibition of protein synthesis and prevention of invasion of new red blood cells (RBCs) by plasmodium parasites (Mukungu et al., 2016; Okokon et al., 2013) could also be considered as a possible mechanism of antimalarial activity.

Prevention of anemia and body weight loss in *P. berghei* infected mice is also a crucial criteria to consider as the best compound for antimalarial agent (Okokon et al., 2013). In this study, except chloroquine treated mice, the significant body weight gain was recorded only in 500 and 200 mg/kg doses of *P. oligotricha* and *L. trifolia*, respectively. On the contrary, treatment with all other doses demonstrated variable degrees of body weight loss. This might be due to the presence of appetite-suppressant metabolites in the extracts, which is supported by earlier studies on other plant extracts (Mukungu et al., 2016; Toma et al., 2015; Gebretsadik and Mekonnen, 2016; Verman et al., 2006).

Erythrocyte fragility, reduced PCV, and life-threatening anemia is commonly observed in *P. berghei* infected mice. The 500 mg/kg dose *L. trifolia* enhances the PCV of mice, which was comparable to the effects of chloroquine. However, *P. oligotricha* and the other doses of *L. trifolia* failed to prevent PCV reduction. The reduction of PCV might be associated with the presence of saponins, which are responsible to cause hemolysis of erythrocytes (Yang et al., 2005).

Mice treated with the ethanolic extract of *P. oligotricha* and *L. trifolia* were found to be safe at an increasing dose of up to 5 000 mg/kg b. wt. Any orally administered test substance with LD_{50} greater than 1000 mg/kg or higher than three times the minimum effective dose can be considered as nontoxic, and can be considered for further studies (Toma et al., 2015). Therefore, acute oral exposure to *P. oligotricha* and *L. trifolia*, at 5 g/kg can be considered non-toxic.

Conclusion

The crude ethanol extracts *P. oligotricha* and *L. trifolia* exhibited significant and dose-dependent antimalarial activity in *P. berghei* infected mice, which may justify the traditional uses of the plants against malaria. Further studies are required to isolate and identify the active compound(s) responsible for the antimalarial activity .

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are thankful to the Office of Vice President for Research and Technology Transfer of Hawassa University for financing this project.

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