PHARMACOGNOSTIC STANDARDIZATION, PHYSICOCHEMICAL ANALYSIS AND PHARMACOLOGY OF *EUPHORBIA WALLICHII* RHIZOME.

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Summery

Euphorbia wallichii is a potent medicinal herb of Himalaya. The current was designed with the aim to screen *Euphorbia wallichii* rhizome for its therapeutic potential and to validate its traditional uses on scientific basis. Rhizome of *Euphorbia wallichii* was assisted for mineral composition through AAS revealed various macro and micronutrients in different concentration. Nutraceutical profile showed content of carbohydrates, crude lipids, protein, fiber, ash and moisture. In-vivo acute toxicity was assessed using swiss albino mice showed critical dose for morbidity and mortality as 1500mg/kg b.w and 2000mg/kg.bw respectively. Rhizome extract of *E. wallichii* (REEW) significantly shown dose dependent antipyrexia in brewer yeast induced hypertherimc mice. Alloxan monohydrate induced diabetic rabbits showed significant reduction in serum glucose level (SGL) treated with REEW compared to saline treated animals. LD_{50s} of 24.17 µg/ml and 34.03 µg/ml were observed for Brine shrimps cytotoxicity and *Aedes aegypti* larvae respectively while Lemna phytotoxicity was observed with FI50 347.42 µg/ml.

Key Words: Phytochemistry, Antidiabetics, Antipyretics, Cytotoxicity, Phytotoxicity, *Euphorbia wallichii*

BACKGROUND

Herbal medicines are promising choice over modern synthetic drugs. They show minimum or no side effects and are considered to be safe [1]. Plants have been used by man to cure diseases and heal injuries since time immemorial. The universal role of plants in the treatment of disease is exemplified by their employment in all major systems of medicine irrespective of the underlying philosophical premise [2]. Plants have been used throughout the world in folk medicine and as local cures for common ailments. Folk medicine gave rise to traditional systems of medicine in various diseases. In recent past, extensive consideration had been gained by the utilization of ecofriendly and biofriendly plant based products for healing and preclusion of various human diseases. Considering the high degree of side effects of synthetic medicines, the global population is searching for natural remedies, which are relatively safe and effective [3]. People of developing countries cannot meet the expense to spend millions of dollars to import costly allopathic medicines and hence now going to encourage the use of traditional medicine as a major and integral component of public health care program [4]. About 70 percent population of Pakistan lives in rural areas and mostly depends on traditional system of Medicine [5,6]. Phytotherapy was the only major system used for sustaining health till the discovery of synthetic organic molecules in 19th century [7]. According to World Health Organization (WHO) about 80% of the global population relies on traditional medicinal systems for their primary health needs [8,9] and about 25 percent of the global human population completely depend upon the traditional system of medicines for curing various ailments [10]. Despite of remarkable attainment in the field of pharmaceutics, synthetic medicines possess ill effects, due to which the significance of the “alternative treatment by natural products” has tremendously increased [11].

Phytomedicines are moving from fringe to a major stream, used by greater number of people who seek remedies for health with no or low side effects which is caused by synthetic medicines. In recent era, people of both developing and developed countries have paid considerable attention to ecofriendly and biofriendly plant based products for curing and prevention of various human diseases [3]. However a key hindrance, which has stalled the acceptance of the herbal medicines in the developed countries, is the lack of proper documentation and strict quality control [12]. Medicinal herbs are the indispensible source of therapeutic preparations, both curative and preventive. With the revival in the demand and consumption of medicinal herbs, World Health Organization renowned the need for their standardization and quality control [13]. In recent era, the increasing demand for plant based products has led to a quantum jump in volume of herbal materials traded within the countries and across the border. Secondary phytochemicals (plant metabolites) with unknown pharmacological potentials has been intensively investigated to be recognized as source of medicinal agents [14]. *Euphorbia wallichii* is herb belongs to family euphorbiaceae having perennial rhizome from which multiple stems arise. The plant has wide range ethnobotanical profile. The whole plant is tonic, stimulant, laxative, carminative and also used for heart and skin diseases [6]. Plant juice is used against ringworm while dried leaves and seeds are given to children in constipation [15]. Underground parts of *E. wallichii* have been used in Tibetan traditional medicines for curing skin disorders, exanthema, edema, furuncle and cutaneous anthrax [16]. The whole plant is poisonous, highly laxative and causes severe diarrhea [17] and stem latex is used for healing of external wounds [18]. Rhizome latex is yellow colored which is highly poisonous, also used externally to cure skin infections and warts [19].

MATERIALS AND METHODS

For the preparation of REEW, 100 gm of dried powder of *E. wallichii* rhizome was extracted with hydroethanol (30%:70% v/v) trice and the filtrate was concentrated using rotary evaporator at 50±3^{o}C.

Phytochemical test

Detective tests for various phytochemicals like Proteins, carbohydrates, oils, alkaloids, terpenoids, anthraquinones derivatives, phytosterols, saponins, glycosides, flavonoids, phenols tannins and anthocyanins was carried out following [20,21].

Nutraceutical analysis

Crude fibers, total lipids, crude protein and ash were investigated according to AOAC [22]. For determination of moisture content, four gram of powdered samples was taken in pre weighed Petri-dish, covered with lid, heated in oven upto 110^{o}C for 5 hours, till constant mass was acquired, cooled and final mass obtained. Percent carbohydrates value was determined by using given formula viz; [100 − (fats% + proteins% + moisture% + ash%)], where total energy values (kcal/100 g) was determined using equation;

Kcal/100 g = [4 (carbohydrate %) + 9 (crude lipids%) + 4 (protein %)].

Elemental analysis

To 0.5 gm of REEW, 10 ml conc. Nitric Acid was added and allowed to overnight. 4ml of perchloric acid was added, kept for 25 minutes, heated on hot plate till the appearance of white fumes, cooled the sample, diluted with distilled water and filtered. The samples were analyzed for quantitative detection of both macro and micro nutrients through AAS [22].

Pharmacology

Acute toxicity assay of REEW were determined against Swiss albino mice of either sex. Animals were acclimatized to laboratory conditions for 10 days prior to experimentation. The animals were fasted overnight and allowed free excess to water for experimentation. The experiment was performed in randomized complete block design where animals were divided into five groups each having six mice. Each group was assigned the treatments at random as [23].;

Group I: Received normal saline at 10ml/kg of body weight, p.o (Control).

Group II: Received 500mg/kg of REEW p.o.

Group III: Received 1000mg/kg of REEW p.o.

Group IV: Received 1500mg/kg of REEW p.o.

Group V: Received 2000mg/kg of REEW p.o.

Antipyretic assay of REEW extracts was determined against Swiss albino mice of either sex (27±3.7 g b.w). Initial rectal temperature of all animals was recorded using digital thermometer. Hyperthermia was induced in overnight fasted animals (access to drinking water only) though subcutaneous administration of 10 mL/kg b.w of 20% aqueous suspension of Brewer's yeast and rectal temperature was recorded after 24 h and animals were divided into five groups of six mice each. Group A (-ve control) received 10 mL/kg b.w saline *i.p*, Group B (Standard) received 100 mg/kg b.w of paracetamol *i.p*, Groups C, D and E (Test) received 100, 200 and 300 mg/kg b.w *i.p.* of REEW respectively. Rectal temperature of animals of all groups was recorded after 2, 4 and 6 hours of drugs administration and percent antipyrexia was calculated as;

$$\text{Antipyrexia (\%)} = \frac{T1 - T2}{T2} \times 100$$

The antidiabetic activity of REEW was evaluated in alloxan induced diabetic rabbits. Hyperglycemia was induced by administration of 150mg/kg b.w of alloxan (i.v) in all experimental animals and after seven days serum glucose level (SGL) was tested using glucometer. Animals with SGL more than 200mg/dl were selected for experimentation. Hyperglycemic rabbits were divided into five groups (n=6). Group A (-ve control) received 10 mL/kg b.w saline, Group B (Standard) received 0.5 mg/kg b.w of Metformin, Groups C, D and E (Test) received 100, 200 and 300 mg/kg b.w. of REEW respectively. SGL was monitored at 0.5, 2, 4, 8, 12 and 24 hours of drugs administration using glucometer while collecting blood from marginal ear vein.

Phytotoxic bioassay was carried out using *Lemna minor* toxicity assay, growth medium for *Lemna* was prepared following [24,25]. The medium was autoclaved for 15 minutes at 121^{o} C. 10 ml of each five different concentrations (10, 50, 100, 500 and 1000µg/ml) of REEW (in growth medium) was put into petri dishes having 10 individual *Lemna minor* plants with rosette of 3 fronds each. Petri plates with 2% DMSO in growth media served as control. After 72 hours number of fronds per petri dish were counted and Percent growth regulation was calculated as;

$$\text{\% Growth Regulation} = 100 - \frac{\text{Number of fronds in test samlpe}}{\text{Number of fronds in possitive control}} \times 100$$

Cytotoxic efficacy of REEW was evaluated using brine shrimps lethality assay (BSLA). 10ml of each concentration (10, 50, 100, 500 and 1000 µg/ml) of REEW (in brine solution) was transferred to glass vials having 10 brine shrimps larvae. 10 ml of brine solution act as negative

control and whole experiment was triplicated. The number of survivors was recorded in each vial after 24 hours and the % mortality was determined as [24];

$$\% \text{ Mortality} = 100 - \frac{Number\ of\ larvae\ alive\ in\ test\ sample}{Number\ of\ larvae\ alive\ in\ negative\ control} \times 100$$

Larvicidal potential of subjects was carried out against fourth instar larvae of *Aedes aegypti*. Larvae were obtained from Department of Zoology, University of Peshawar, Pakistan. Larvae were fed with powdered dog biscuits and yeast with the ratio of 1:3 and kept at 30 ± 2º C. The larvae at their fourth instars stages were used for larvicidal bioassay. The experiment was performed through completely randomized design with three replicates each with 10 larvae in 49 ml of water with larvae food and 1 ml of different concentrations (10, 50, 100. 150, 200 and 250 µg/ml) of REEW [26,27]. After 24 hours of exposure, the numbers of survivors were counted and % mortality rate was determined using formula:

$$\% \text{ Mortality} = 100 - \frac{Number\ of\ larvae\ alive\ in\ test\ sample}{Number\ of\ larvae\ alive\ in\ control} \times 100$$

RESULTS AND DISCUSSIONS

Elemental composition

The elemental composition of *E. wallichii* rhizome is summarized in figure 1. The K concentration was 215. 63 mg/l, Na concentration was 24.68 mg/l, Ca, 418.40 mg/l, Magnesium 61.63mg/l, Fe 5.21mg/l, Manganese (Mn) 7.71mg/l, Copper (Cu) 1.17mg/l, Zink (Zn) 1.09mg/l, Cobalt (Co) 0.91mg/l and Nickel (Ni) concentration was 1.61mg/l. It is an established fact that phytonutrients just like secondary metabolites, playing significant role in combating various

illnesses [28]. Potassium (K) is an essential macromineral, act as a cofactor for multiple enzymes associated protein and carbohydrates metabolism. It is essential for the normal functioning of nerves, maintain body osmoregulation, blood pressure and actively participate in constipation as well as controlling cardiac rhythm [29, 30]. Na is helpful in the transmission of nerves impulses also helps in regulation of body osmotic balance [31]. Calcium assist blood clotting, regulate blood pressure and heart rhythms and maintain proper nerve and muscle functions [32, 33]. Magnesium deficiency is closely associated with Diabetes mellitus as Mg is vital for releasing insulin essential for the metabolism of fats and carbohydrate [29, 34]. Iron (Fe) is an essential part of many enzymes as well as that of myoglobin and of hemoglobin. Fe helps in digestion and helps to maintain a healthy immune system, also makes tendons and ligaments [29]. Deficiency of Mn causes hypercholesterolemia, poor blood clotting, skin damage, higher skin cancer rates, fertility problems, anemia, poor bone formation and birth defects. Mn is essential for the development of immune system, bone growth, normal digestion and cell reproduction. It also helps in glucose metabolism [35]. Mn is an essential micronutrient has good antioxidant potency, hence combat oxidative stresses caused by ROS in the body, also required for normal functioning of CNS [36]. Copper (Cu) acts to regulate cardiovascular and immune functions and act as a cofactor of many enzymes. Its deficiency cause cardiovascular disfunctioning promote anemia and causes infirmity of bones [29]. Plants with promising Cu and Zn contents were found to possess anticancer property as both elements are required in growth and proliferation of normal cells [37]. Zinc (Zn) act as cofactor for insulin and hence play vital role in carbohydrate, promotes sexual maturity [30, 34]. Deficiency of Zn leads to reproductive failure, delay healing, causes loss of appetite, impairs immune functions and coronary artery disease [38, 39]. Chronic Zn deficiency causes dwarfism and hypogonadism [35, 37]. Cobalt is an essential microelement

required to the body in trace. Co is an integral part of cobalamin, vitamin B12 and help in the production of RBCs as well in the formation of myelin nerve coverings. It is stored in pancreas, liver, spleen, kidney, RBCs and in blood plasma. The normal cobalt level in body ranged between 80-300 mcg. Its deficiency is mainly associated with pernicious anemia and nerve damage [40, 41]. Nickel is essential micro mineral with significant impact on the processes of hemoglobin synthesis, stimulation of erythropoietin production and haematopoiesis. Deficiency of nickel is strongly associated to anemic condition [40, 42].

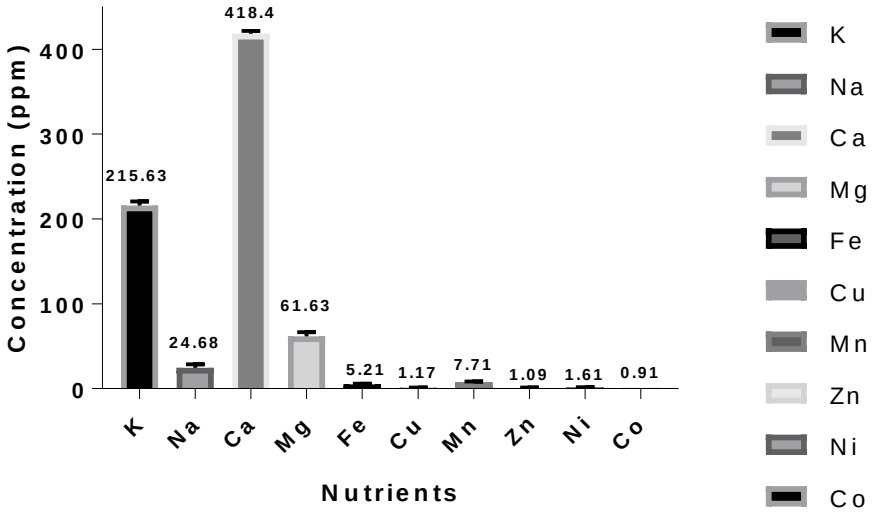

Figure 1. Mineral content of *E. wallichii* rhizome. The bars represent Mean± SE at p<0.05 of a triplicate experiment.

Proximate Analysis

The evaluation of proximate composition of medicinal herbs can be helpful in understanding the nutritional worth of these plants for both man and animals [43]. Proximate analysis of rhizome of *E. wallichii* revealed 7.21% ash, 55.82% carbohydrate, 6.52% fiber content, 16.43% protein, 3.63% fats and 10.39% moisture. The gross root energy for rhizome is 272.38 K. cal/100g. Several other workers carried out proximate analysis of different medicinal plants include *Boerhavia diffusa, Ricinus communis, Chrozophora tinctoria, Peganum harmala, Fagonia cretica* and *Tribulus terrestris* and reported diversity in carbohydrates, protein, fats, moisture and fibers contents in different parts of the plant [44, 45].

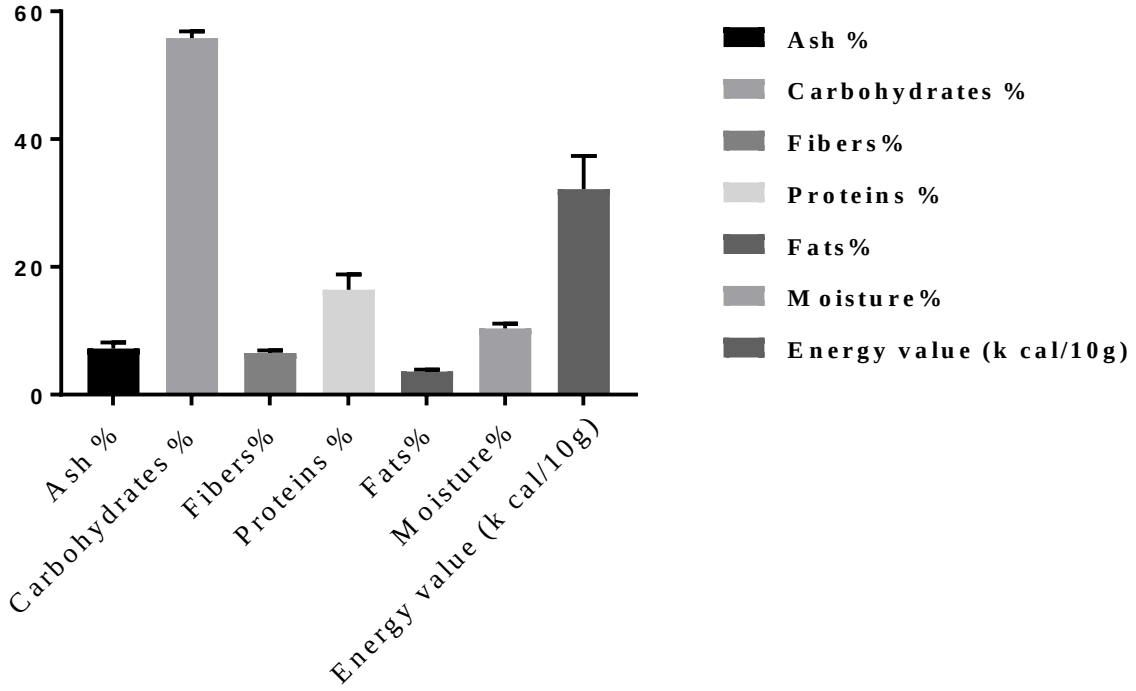

Figure 2. Nutraceutical composition of *E. wallichii* rhizome. The bars represent Mean± SE at p<0.05 of a triplicate experiment.

Preliminary Phytochemical evaluation

The phytochemical evaluation of REEW showed the presence of alkaloids, glycosides, reducing sugars, phenols, Proteins, Tannins, saponins and Phytosterols while Flavonoids showed negative detective tests (Table 1).

Table 1. Preliminary phytochemical profile of REEW.

Phytometabolites	**Result**
Reducing sugars	+ +
Alkaloids	+ +
Flavonoids	-
Tannins	+
Phytosterols and Triterpenes	+
Saponins	+
Glycosides	+
Proteins	+
Phenols	+ +

(+ +) high concentration, (+) Presence, (-) Absence

Acute toxicity

The evaluation of medicinal plants or their preparations for their toxicity is conducted in order to enhance the level of confidence in their safety to humans [46]. In the present study acute toxicity assay of REEW was examined in swiss albino mice. Rhizome extract at the highest experimental dose (2000 mg/kg.bw) induced mortality to all the tested animals, while at 1500 mg/kg b.w the experimental animals exhibit morbidity. Animals feed with 1000mg/kg.bw extract were observed with no apparent toxic symptoms (Table 2). Evaluation of toxic potential of a drug is of commence importance when considering for public health protection, as exposure of human to phytochemicals can be hazardous and the results may be adverse [47]. By applying *in vivo* toxicity assays, the expressions of tested animals in the form of distress, allergic reactions, pain and change in normal physical behavior is recorded. However, acute toxicity does not assess the vital processes of CNS, respiratory system and cardiovascular system [48].

Table 2. Acute toxicity assay of REEW, the response of experimental animals (n=6) is shown as a mean effect.

Doses /Kg.bw	**Response**
Saline10ml	Alive (normal)
REEW 500 mg	Alive (normal)
REEW 1000 mg	Alive (normal)
REEW 1500 mg	Alive (morbid)
REEW 2000 mg	dead

Anti-diabetic efficacy

REEW exhibited significant dose and time dependent antidiabetic activity against alloxan induced hyperglycemic rabbits at experimental doses of 100, 200 and 300mg/kg b. w. The rate of reduction in SGL was significantly increased up to 4 hours of treatment administration and in coming hours incline in SGL was observed (Figure 3). Metformin produced significantly hypoglycemia i.e. 26.82 % at 2 hours of administration. REEW at dose of 300mg/kg b. w reduced SGL up to 19.44% at 12 hours of administration. Insulin is secreted by beta cells of pancreas, responsible for proper utilization of blood glucose level responsible for normal body functions. Alloxan monohydrate has cytotoxic effect on beta cells and hence cause IDDM or NIDDM. The pronounced antidiabetic activity of REEW may be attributed to the adequate amount of Zinc (evident from elemental profile) which act as cofactor for insulin and hence play vital role in glucose metabolism [30, 34], as well have the sulfur containing phytochemicals (Alkaloids, flavonoids etc) which have the tendency for the inhibition of chemical species competing with insulin for their SH-group [49], hence confirming pancreatotrophic action of REEW for hypoglycemia.

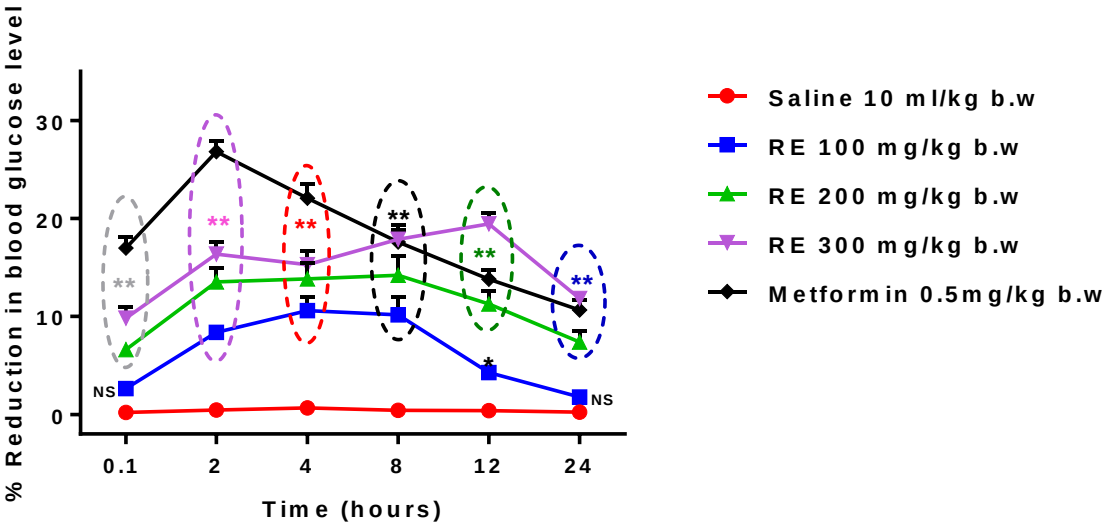

Figure 3. Comparative hypoglycemic effect of various doses REEW with saline (control) and metformin (standard) at different time intervals. Reduction in SGL (%) is represent by mean ± SEM at $p < 0.05$ of n=6 each. OWANOVA revealed significant SGL reduction compare to saline. Significant ** represent highly significant effect compare to saline ($p < 0.01$, $p < 0.05$), * represent significant effect ($p < 0.05$) where NS represent statistically non-significant differences among the treatments.

Antipyretic potency

Sub-cutaneous injection of 10 mL/kg b.w of 20% aqueous suspension of Brewer's yeast significantly induced hyperthermia (Figure 4). REEW exhibited dose and time dependent antipyretic effect in yeast induced mice. REEW at all the experimental doses (100, 200 and 300 mg/kg b.w) produced significant alleviation in rectal temperatures in all the experimental animals at 2 to 5 hours of drug administration except REEW at 100 mg/kg b.w which produced nonsignificant hypothermic effect at 2 hours of administration. Brewal yeast inducing hyperthermic (pathogenic fever) modal presents a cost effective and reliable way for evaluating novel antipyretics. Yeast prompts the production of interferon-α (IFN-α), interleukin-1β (IL-1β) and IL-6, tumor necrosis factor-α (TNF-α) and prostaglandins like PGE2 responsible to modulate the thermoregulatory center of CNS at elevated temperature [50-52]. REEW presented antipyretic effected most probably reducing prostaglandins level, enhancing antipyretic message within brain [53].

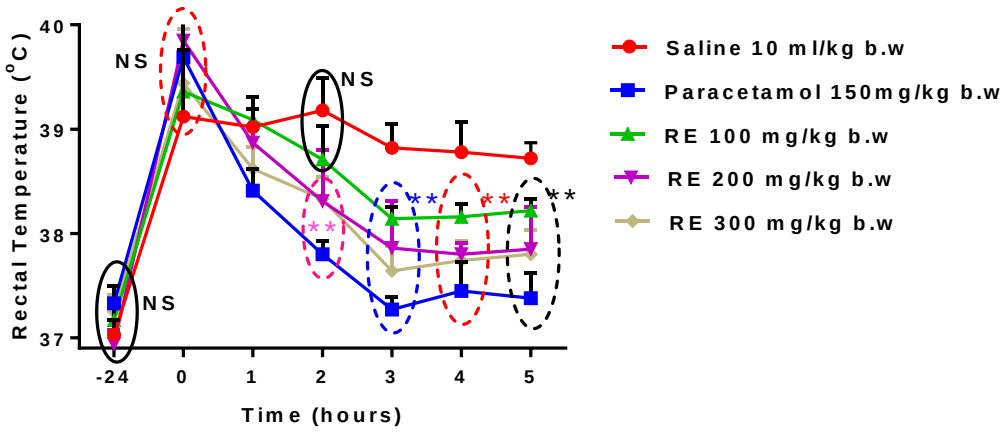

Figure 4. Antipyretic effect of various doses (100, 200, 300mg/kg b.w) of REEW. Saline at 10ml/kg b.w is control condition where paracetamol at 150mg/kg b.w act as reference drug. Reduction in rectal temperature is represent as mean ± SEM at $p < 0.05$ of n=6. OWANOVA revealed significant rectal temperature reduction compare to saline condition. ** represent highly significant effect ($p < 0.01$, $p < 0.05$), * represent significant effect ($p < 0.05$) where NS represent statistically non-significant () compare to saline.

Phytotoxic bioassay

REEW showed significant dose dependent *Lemna minor* toxicity. REEW exhibited 11.11%, 28.89%, 55.56%, 75.56% and 93.33% *Lemna* frond proliferation inhibition at 50 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml and 5000 µg/ml doses respectively having FI_{50}, FI_{70} and FI_{90} values of 347.42 µg/ml, 873.49 µg/ml, 3313.26 µg/ml respectively. Plants containing secondary metabolites as allelochemicals are responsible for their phytotoxic potential. Polyphenols, alkaloids and other organic acids are mostly related to the phytotoxic nature of a plant. The phytochemical architect reveals the presence of alkaloids, polyphenols, glycosides and

phytosterols which may be responsible for inhibiting the *Lemna* growth [54]. Members of genus euphorbia like *Euphorbia aellenii*, *Euphorbia prostrata, Euphorbia dracunculoides, Euphorbia helioscopia* and *Euphorbia hirta* have also been reported with tremendous phytotoxicity [55-57].

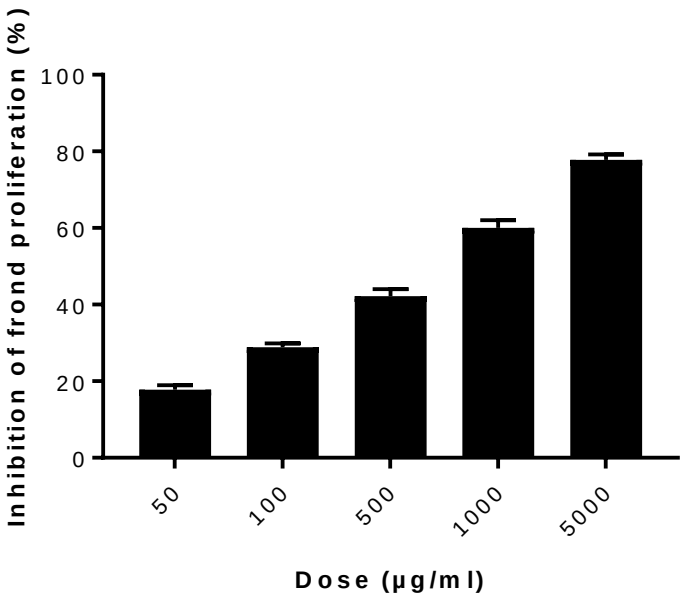

Figure 5. Frond proliferation inhibition (%) of *Lemna minor* under different concentration of REEW. Histograms represent Mean±SEM at $p<0.05$ of a triplicate data.

Brine shrimps cytotoxicity

The Cytotoxic potential of REEW was evaluated against *Artemia salina* larvae using BSLA (Brine shrimps lethality assay) model. The mortality was observed to be dose dependent and strongly positively correlated to the dose concentrations. The lethal concentrations of rhizome extract for 50%, 70% and 90% mortality (LD_{50}, LD_{70} and LD_{90}) were 24.17 µg/ml, 53.66 µg/ml and 169.94 µg/ml respectively. The percent mortality at 10, 50, 100 and 500 µg/ml

dose were 35.86%, 53.68%, 87.53% and 100% respectively. The high lethality index of *Artemia salina* larvae in REEW indicates the presence of antitumor metabolites in *E. wallichii* rhizome. According to [58] phytochemicals are active cytotoxic if they has an LC_{50} value of less than 1000 µg/mL. Member of genus Euphorbia (*E. hirta*) has significant cytotoxic potential as having the LC_{50} is less than 1000 µg/mL [59]. Brine shrimps toxicity is positively correlated with 9KB (human nasopharyngeal carcinoma) cytotoxicity [60, 61]. Plants with adequate content of saponins have promising cytotoxic effect as they works by stopping cellular mutations, a major cause of cancer development [62]. Recently other phytocomponents have been observed with effective cytotoxic property. Among these flavonoids, polyphenols, alkaloids, coumarines and anthraquinones are the most effective [63]. The promising cytotoxic effects of REEW is due to the presence of active metabolites like saponine, alkaloids, flavonoids and other phenolics, confirmed through chemical tests.

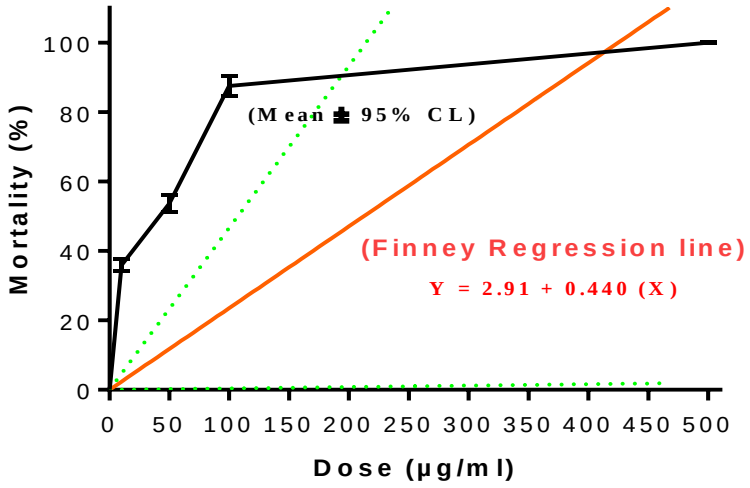

Figure 6. Cytotoxicity of various doses (10, 50, 100 and 500 µg/ml) of REEW against *Artemia salina* larvae. The mortality rate (%) is a mean ± 95% CL of mean of triplicate

experiment. The lethal doses (LD50, LD70 and LD90) were determined using Finney probit analysis and the regression line is fit for dose mortality relation.

Larvicidal potential

The larvicidal potential of REEW was assessed against the fourth instar larvae of *Aedes aegypti*. A dose dependent toxicity was observed where the toxicity was ranged from highly significant to moderately significant at various experimental doses. The percent mortality of *Aedes aegypti* larvae was recorded as 39.28, 44.72, 50.00, 53.68 and 70.39% at 10, 20, 50, 80 and 100µg/ml respectively. Probit analysis revealed that lethal concentrations of REEW for 50%, 70% and 90% (LC_{50}, LC_{70} and LC_{90}) larval mortality were 34.03, 234.91 and 3838.43µg/ml respectively. Larvicidal potential of other members of Euphorbia were reported with significant larvicidal potential where the degree of potency is strongly positive correlated to the experimental dose [64]. The latex of *Pergularia daemia* has significant effect upon the first, second and third instar larvae of *A. aegypti* while it's forth instar larvae showed unnoticeable mortality [65].

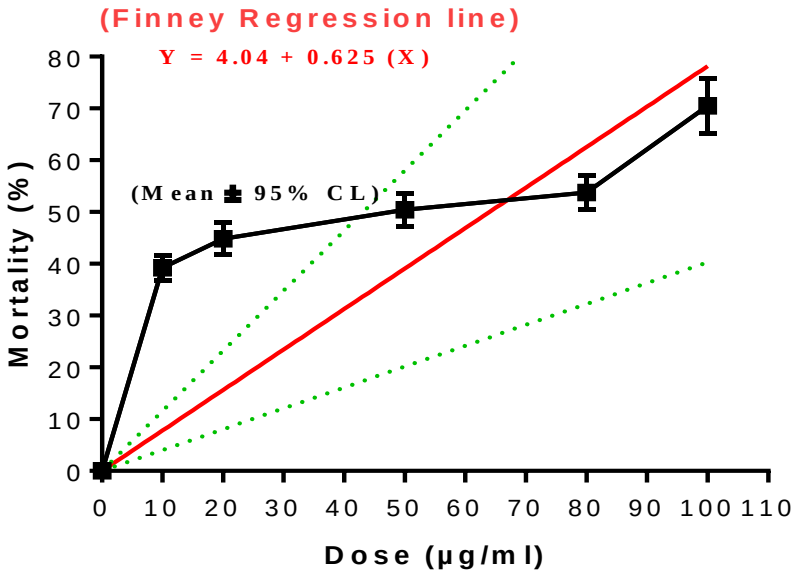

Figure 6. Larvicidal effect of various doses (10, 20, 50, 80 and 100µg/ml) of REEW against fourth instar larvae of *Aedes aegypti*. The mortality rate (%) is a mean ± 95% CL of mean of triplicate experiment. The lethal doses (LD50, LD70 and LD90) were determined using Finney Probit analysis and the regression line is fit for dose mortality relation.

Conflict of interest

The authors declare that they have no conflict of interest.

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