

Evaluation of Anti-obesity and Antioxidant Activities of Ethanolic Extract of *Achyranthes aspera* Leaves.

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ABSTRACT: Leaves of *Achyranthes aspera* are traditionally used in different therapeutic applications in countries like Nepal, India, and some part of Africa. The leaves of *A.aspera* was investigated for total phenolic content (TPC) and total flavonoid content (TFC). The total phenolic content was determined using the Folin-Ciocalteu method, while the total flavonoid content was determined using quercetin method. MSG induced obese rat were used for the anti obesity test; Body weight, biochemical, and locomotive activities were analyzed. The TPC in the leaves extract of *A.aspera* was calculated as 275.21mg/g and TFC was 140.4mg/g gallic acid equivalent GAE/g of dry weight. The extractive supplementation with dose of 200mg/kg and 400mg/kg were capable of lowering the body weight, triglyceride, total cholesterol and significantly increasing the locomotion of the rat. Also, the investigation shows that the ethanolic extract of leaves *achyranthes aspera* posses antioxidant activities and it was done using the Nitric oxide, hydroxyl radical technique and phosphomolybdenum method. The reaction showed that it is a concentration-dependent reaction and the results obtained here were expressed in terms of IC₅₀ values using gallic acid as standard. The leaves of *A. aspera* justified its potential as an anti-obesity agent and antioxidant, 5 groups of animals (3 rats per group) showed a significant decrease in body weight and biochemical parameters, and the increase in exercise activity and concentration-dependent results of antioxidant effect. It can be said that *Achyranthes aspera* has both lipolytic and antioxidant properties due to its chemical constituents (flavonoids, tannins, terpenoids and alkaloids, etc.).

KEYWORDS: Anti-obesity; antioxidant; *Achyranthes aspera*; rats; Body weight; Biochemical.

1. INTRODUCTION

Obesity is a complex chronic medical condition distinguished by excess body fat that emerges as a result of a long-term energy imbalance, i.e., excessive caloric intake and limited energy output. This surplus energy is stored in the form of adipose tissue. Body mass index (BMI), is a measurement of weight-and-height-based, identifies persons as overweight if their BMI is between 25 and 30 kg/m², and obese if their BMI is higher than 30 kg/m² [1].

A sedentary lifestyle negatively impacts health by increasing the risk of developing fatal and non-fatal diseases such as hypertension, heart disease, type 2 diabetes, gallbladder disease and various cancers, as well as non-fatal diseases such as back pain, arthritis, infertility and poor psychosocial behavior. Nearly one-third of the world's population is now overweight or obese, according to data showing that "the global prevalence of overweight and obesity has tripled since 1975 [2]. The identification of new anti-obesity drugs could be aided by naturally occurring secondary plant compounds.

1.1. Obesity and Human Health

Obesity is defined as an energy imbalance between energy intake and energy expenditure over time. Weight gain, body fat accumulation, and adiposity expansion are all promoted in a pathologic way when energy intake (calories) is increased and physical activity is decreased [3]. Obesity is associated with increased risk of major depression, emotional disturbance, early death, disability, menstrual irregularities, infertility, miscarriage, and poor pregnancy outcomes. According to the research, there is also conclusive evidence that obesity is associated with higher morbidity and mortality. Obesity is associated with a number of chronic

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diseases, such as: hypertension, dyslipidemia (high total cholesterol or high triglyceride levels), type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea, and respiratory disease [4].

1.2. Etiology of obesity

The cause of excess fat is not known. Numerous factors, including environmental, hereditary, nutritional, physiological, emotional, social, cultural, and others, have been associated with the development and progression of the disease [5]. The pathophysiology of obesity seems clear: a persistent excess of food intake relative to energy expenditure. However, due to the complexity of the neuroendocrine and metabolic systems that regulate energy intake, storage, and utilization, it has been difficult to quantify all of the major components.

Achyranthes aspera, (Family: Amaranthaceae), commonly known as Prickly chaff flower in English, and is an erect, perennial herb, found on road sides, field boundaries and waste places as a weed throughout India up to an altitude of 2100 m. It is widely used in traditional system of medicine as purgative, laxative, liver complaints, rheumatism, scabies and other skin diseases. The plant is also well known for its spermicidal, hypoglycemic, hepatoprotective, anti-inflammatory, analgesic, antipyretic and antiarthritic activity [6, 7]. In traditional medicine (Ayurvedic Pharmacopeia) it has been mentioned to treat medroga [6]. But no systematic studies have been reported in its ethanolic extract for antiobesity activity. Therefore, the present study has been designed to evaluate the anti-obesity potential of ethanolic extract of leaves of *Achyranthes aspera* in rats fed with high fat diet thereby providing scientific evidence to this traditional healer

2. RESULTS

2.1 Acute oral toxicity test

In acute toxicity study, no signs or symptoms of toxicity or mortality were observed during the observation period at doses of 200 mg/kg and 400 mg/kg. Therefore, 200 mg/kg was selected as the starting dose for the obesity evaluation and 400 mg/kg was selected as the higher dose.

2.2 Phytochemical analysis

Table 1. Qualitative Analysis of Ethanolic Leaf Extract of *Achyranthes Aspera*

S/No	Name of test	Results	Conclusion
01	Test for alkaloids		
	Mayer's Test	+	Present
	Dragendroff's Test	+	Present
02	Test for tannins		
	FeCl ₃ Test	+	Present
	Gelatin Test	+	Present
03	Test for Flavonoids		
	Alkaline reagent test	+	Present
	Lead acetate Test	+	Present
04	Test for Terpenoids		
	Salkowski Test	+	Present
05	Test for Saponins		
	Foam Test	+	Present
06	Test for Reducing Sugars		
	Fehling Test:	+	Present

Primary phytochemical screening of extracts determined the presence of alkaloids, glycosides, tannins, saponins, carbohydrates, and flavonoids as major constituents in EEAA.

2.3. Total phenolic content in Ethanolic extract of leaves *Achyranthes aspera*

The crude ethanolic extract of *Achyranthes aspera* leaves was used to determine the total phenolic content (TPC). It was calculated as gallic acid equivalent (GAE) in the extract and the value was 275.21 mg per g dry weight.

2.4. Total Flavonoids content in Ethanolic extract of leaves *Achyranthes aspera*

The crude ethanolic extract of *Achyranthes aspera* leaves was used to determine the total flavonoid content (TFC). It was calculated as gallic acid equivalent (GAE) in the extract and the value was 140.4 mg per g dry weight.

Table 2. Results of Quantitative Analysis of Ethanolic Leaf Extract of *Achyranthes Aspera*

Flavonoids (mg/g)	Phenols (mg/g)
140.4	275.21

2.5. Anti-obesity activity

A digital weighing balance was employed here to measure the weight of the rats for 28 days and it was recorded after every 7 days after the administration of the drug, the drug produce a significant decrease in the body weight of the rat which indicated that it posses the anti obesity activity.

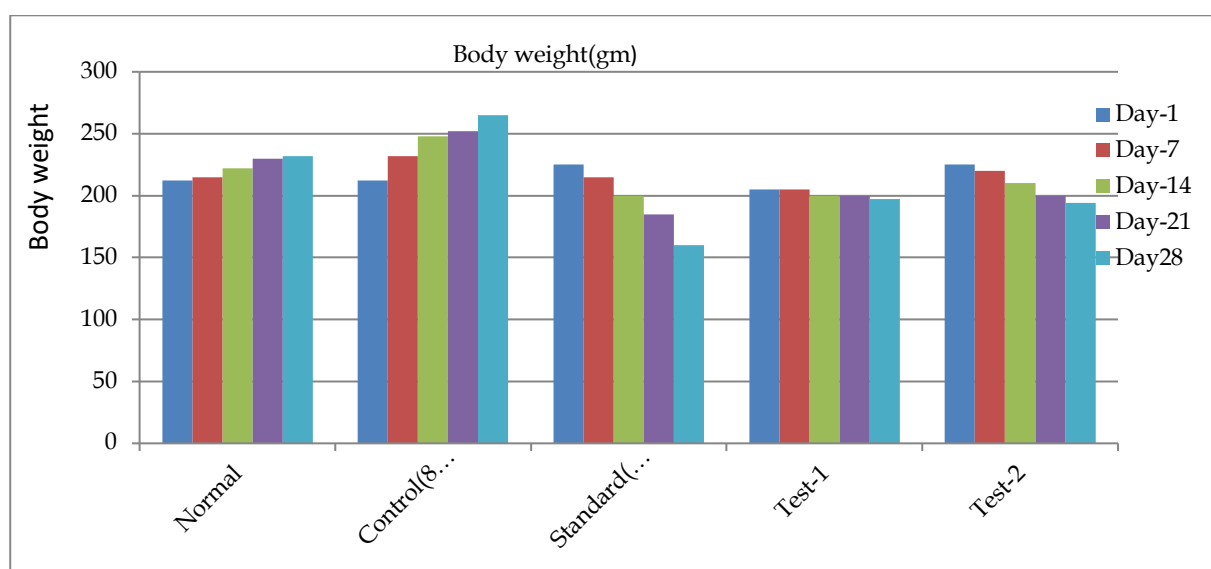


Figure 1. Effect of Ethanolic Leaf Extract of *Achyranthes Aspera* on Body Weight in MSG Induced Obesity Rats

Values are expressed as mean \pm SEM, n=3; *
 P< 0.005 vs control
 P< 0.005 vs HFD

2.6. Locomotive activity

Each week for 28 days, locomotor activity was measured using a digital actophotometer with an observation period of 5 minutes after drug administration. Compared to the normal groups, the MSG control rats had significantly lower locomotor activity. Compared to the normal group, administration of extracts (200mg/kg, 400mg/kg) resulted in significantly higher locomotor activity. The standard shows high locomotor activity, indicating that the drug has anti-obesity activity.

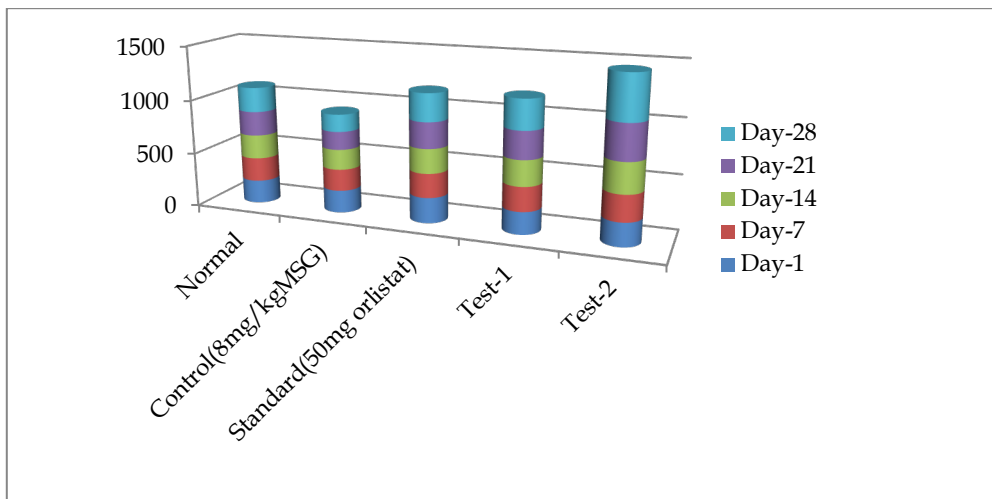
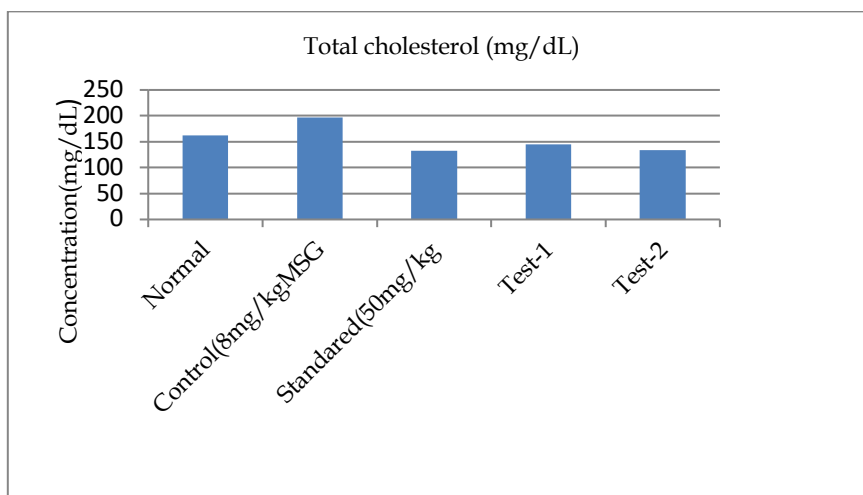


Figure 2. Effect of Ethanolic Leaf Extract of *Achyranthes Aspera* on locomotive activity in MSG Induced Obesity Rats.

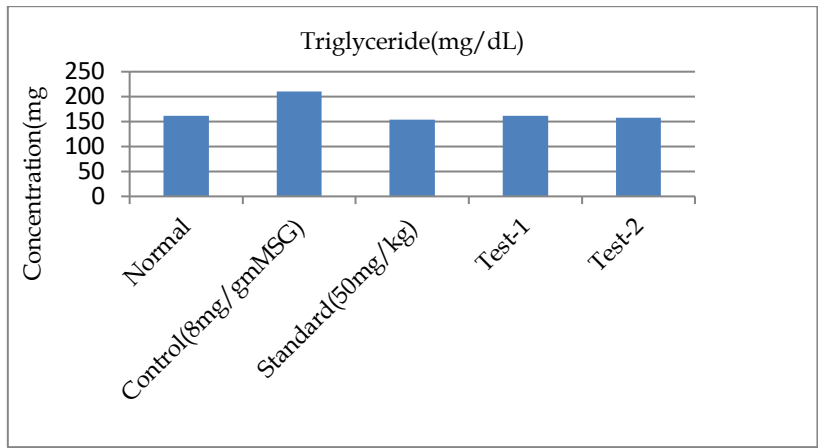
Values are expressed as mean \pm SEM, n=3*
 P< 0.005 vs control
 P<0.005 vs HFD.

2.7. Biochemical parameter

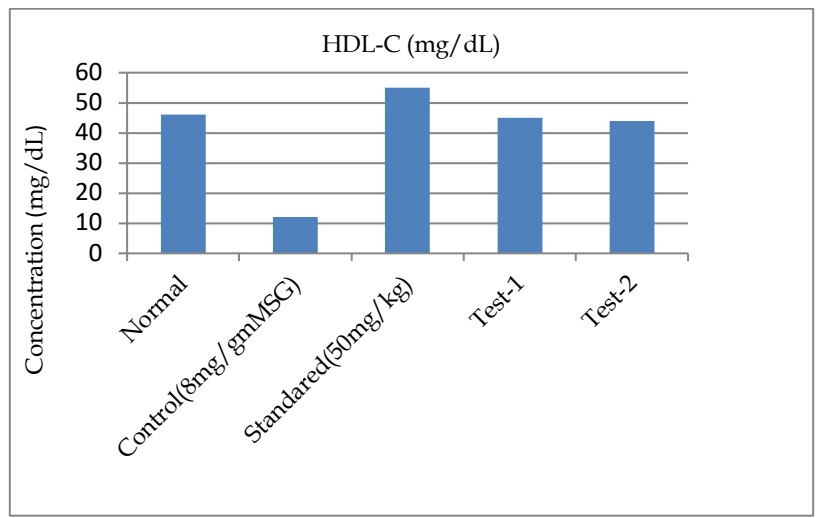
A biochemistry analyzer is used to calculate levels of LDL, HDL, VLDL, total cholesterol, and triglycerides in conjunction to blood sugar levels. As the estimation was finished, the findings of each group were tabulated, revealing a significant drop in blood glucose, total cholesterol, LDL-C, VLDL-C, and triglyceride, as well as a significant increase in HDL-C, when compared to the normal control group. The ethanolic extract of *achyranthes aspera* was found to lower blood glucose, total cholesterol, LDL-C, and VLDL-Triglycerides.



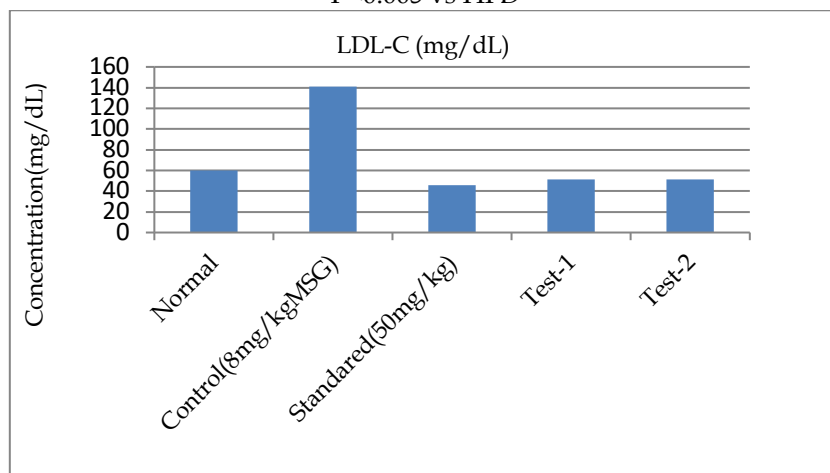
Values are expressed as mean \pm SEM, n=3*
 P< 0.005 vs control
 P< 0.005 vs HFD



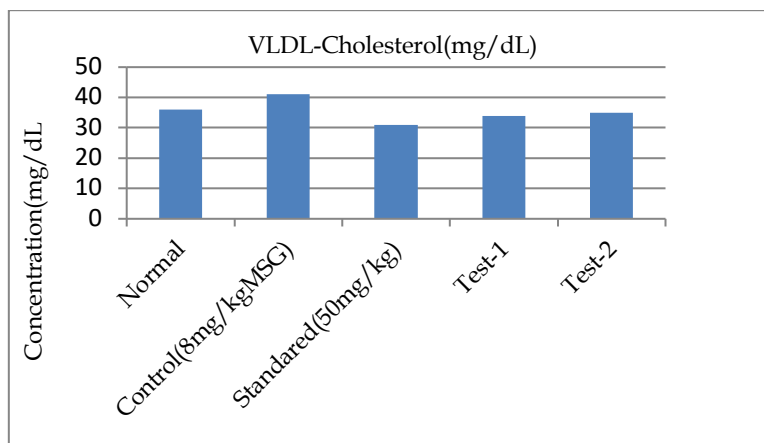
Values are expressed as mean \pm SEM, n=3*
P< 0.05 vs control
p <0.05 vs HFD



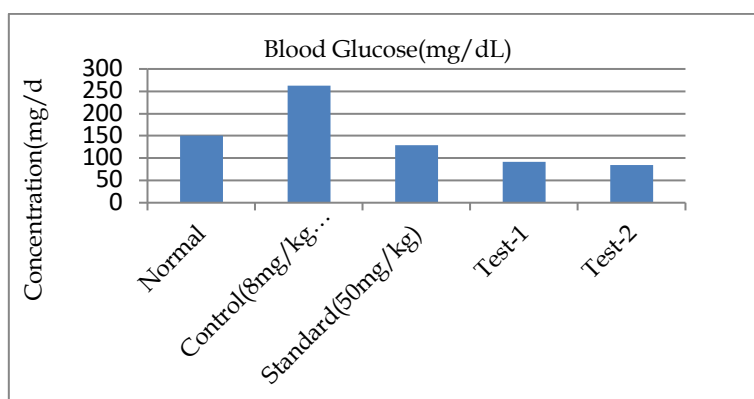
Values are expressed as mean \pm SEM, n=3*
P< 0.005 vs control
P<0.005 vs HFD



Values are expressed as mean \pm SEM, n=3*
P< 0.005 vs control
P<0.005 vs HFD



Values are expressed as mean \pm SEM, n=3*
 P< 0.005 vs control
 P<0.005 vs HFD



Values are expressed as mean \pm SEM, n=3
 P< 0.005 vs control
 P<0.005 vs HFD

Figure 3. Effect of Ethanolic Leaf Extract of *Achyranthes Aspera* on Biochemical parameters on MSG Induced Obesity Rats.

2.8. In vitro antioxidant activity

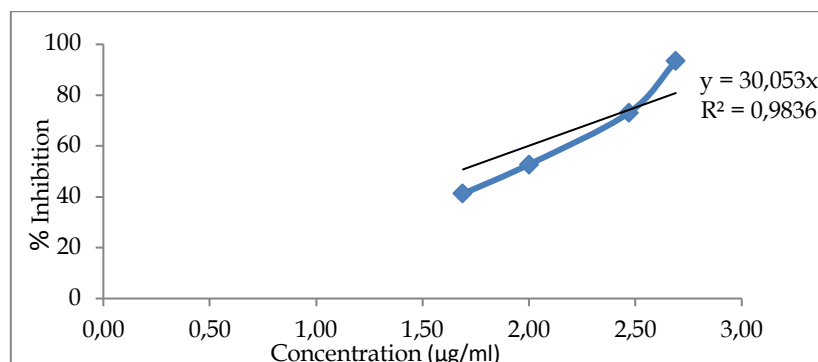


Figure 4. Effect of ethanolic extract of *Achyranthes Aspera* on nitric oxide scavenging assay

Values are expressed as mean \pm SEM, n=3
 P< 0.005 vs control

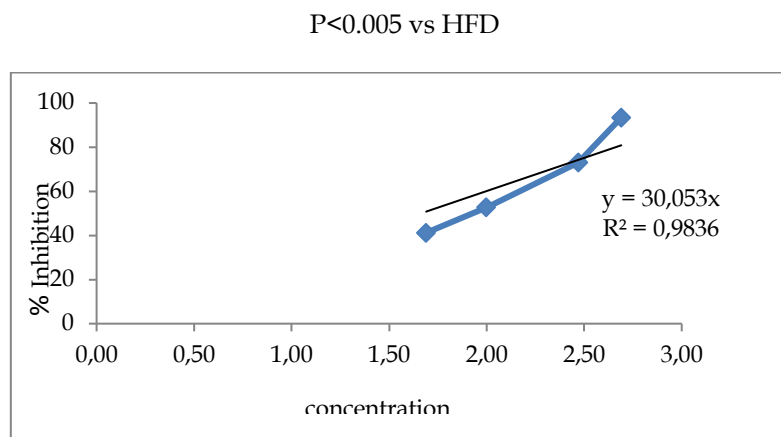


Figure 5. Effect of ethanolic extract of *Achyranthes Aspera* on hydroxyl radical scavenging assay

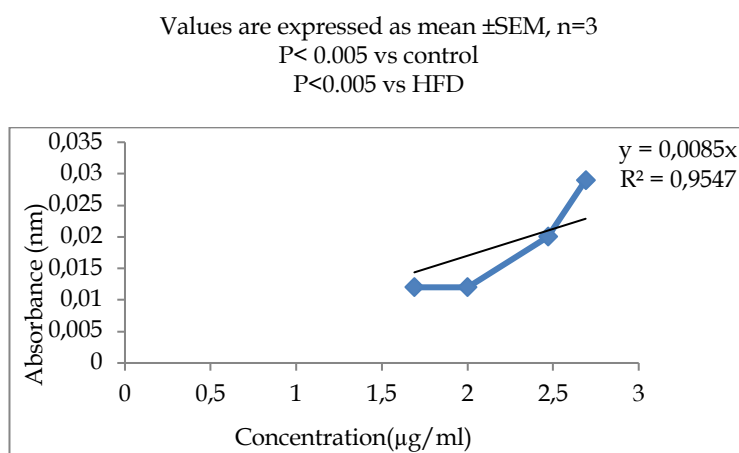


Figure 6. Effect of ethanolic extract of *Achyranthes Aspera* on phosphomolybdenum assay

Values are expressed as mean \pm SEM, n=3
P< 0.005 vs control
P<0.005 vs HFD

3. DISCUSSION

Achyranthes aspera is a long-lived, erect, occasionally sprawling herb that belongs to the Amaranthaceae family. It has been studied for its anti-obesity, antioxidant, and other related effects. Although the lethal dose of this test extract was not determined in the experiment, we did not observe any deaths in experimental animals at the dose used for the study [6]. The result of the present work clearly shows that the ethanolic leaf extract of *Achyranthes aspera* has both anti-obesity and antioxidant properties.

Obesity is caused by excess caloric intake [7] and this can be improved by inhibiting pancreatic lipase activity and by inhibiting or delaying lipid absorption [8]. Inhibition of α -amylase activity and inhibition of carbohydrate absorption also play an important role in the prevention and treatment of diabetes [30]. α -amylase, one of the digestive enzyme secreted from the pancreas and salivary glands, is involved in an important biological process such as digestion of carbohydrates. Many crude drugs inhibit α -amylase activity [9]. Natural α -amylase inhibitors have been demonstrated to be beneficial in reducing postprandial hyperglycemia by slowing down the digestion of carbohydrates and, consequently the absorption of glucose. Reducing postprandial hyperglycemia prevents, glucose uptake into adipose tissue to inhibit synthesis and accumulation of triacylglycerol [10]. On the other hand, it is well known that dietary lipid is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase. The two main products formed by the hydrolysis of triglycerides by pancreatic lipase are fatty acid and 2-monoacylglycerol. Based on these facts, inhibition of these digestive enzymes is beneficial in treatment of obesity.

All animals' body weights were tracked from the first day to the 28th day. In comparison to the normal group in the early stages of drug treatment, the MSG control, standard, and test groups had higher body weights, according to the results. Following the conclusion of the pharmacological treatments, Rats in the normal and test groups had significantly lost some weight, as seen fig 2.5.1. Every week for 28 days, locomotive activity was measured using digital actophotometer with 5-minute observation. When compared to normal groups, MSG control rats have considerably lower locomotive activity. When compared to the normal group, extract administration (200mg/kg, 400mg/kg) resulted in considerably higher locomotive activity. Standard reveals a lot of locomotive action. Which shows the drug poses antiobesity activity as shown in Table 2.6.1.

Orlistat is the only FDA-approved pancreatic lipase inhibitor on the market today for the treatment of obesity [11]. It works by decreasing lipase activity and boosting fat excretion in the feces. However, prolonged use of orlistat has been associated to side effects such steatorrhea (Oily, smelly stools, which often float.), bloating (A buildup of gas in the stomach and intestines), oily spotting, faecal urgency etc [12]. As a result, the search for a safe and effective pancreatic lipase inhibitor continues. From the investigation we can say that the ethanolic extract of *Achyranthes aspera* possess antiobesity activity.

Phytochemical compounds such as flavonoids, alkaloids, triterpenoids, saponins which are the major phytochemical groups with biological activity are responsible for the antiobesity activity as both the seeds, and root possess a major constituent which is saponin.

Fig 2.8.1 and 2.8.2 summarize the results of the antioxidant, using several antioxidant methodology, the results of the antioxidant activity were expressed in terms of IC₅₀ values. The computed IC₅₀ values for *Achyranthes aspera* and gallic acid using Nitric oxide and hydroxyl radical technique are 46.02ug/ml and 71.94ug/ml and for gallic acid it is 15.99ug/ml and 38.81ug/ml. A lower IC₅₀ values indicate a more effective protective function [13].

Fig. 2.8.3 summarizes the results of the phosphomolybdate antioxidation experiment: There is a concentration-dependent increase in absorbance. When *Achyranthes aspera* leaf extract reacts with the hydrogen donor in the antioxidant principle, it reduces the radicals to the appropriate hydrazine, according to the results. The radical scavenging effect of *Achyranthes aspera* is also a concentration-dependent reaction [14].

The results of the study show that the ethanolic extracts of *Achyranthes aspera* leaves contain a variety of phytochemical compounds that can effectively prevent cell damage by free radicals through their radical scavenging activity, and thus can be used as a potent source of natural antioxidant compounds. Moreover, the phenolic compounds can be characterized by reversed-phase HPLC analysis. This report also indicates the low antibacterial and hemolytic activity of *A. aspera*. From all these results, we can conclude that *A. aspera* can be used as a source of safe and natural antioxidant compounds [15].

The phytochemicals contained in the *Achyranthes aspera* leaves may be responsible for antiobesity and antioxidant effect. The findings implies that *Achyranthes aspera* leaves extract have strong antiobesity and antioxidant effect in concentration-dependent manner and that they could be a valuable energy expenditure.

4. CONCLUSION

In conclusion, *Achyranthes aspera* leaves can prevent obesity by reducing the excessive accumulation of body fat and altering the lipid profile in serum. In rat obesity models provoked by MSG, the ethanolic extract showed efficacy against obesity by decreasing body mass, food intake, and serum levels of TRG, LDL, VLDL, GLU, and CHO, and increasing HDL levels at moderate and high doses compared to the standard. All phytochemical components such as alkaloids, triterpenoids, saponins, and flavonoids are soluble in the ethanolic extract, suggesting that it possesses antioxidant and anti-obesity properties.

5. MATERIALS AND METHODS

5.1.2 Plant material collection and authentication

The *Achyranthes aspera* leaves were obtained from the Aditya educational institution's campus in Aditya nagar, on the ADB road in the east Godavari district of Andhra Pradesh. RAGHURAM Taxonomist, Maharani College, Peddapuram, performed the plant authentication.

5.1.2 Preparation of ethanolic extract

The leaves were dried and grinded into a fine powder, and the powdered materials were weighed before being macerated in ethanol and subjected to hot percolation for 3 hours. The extract was then filtered and concentrated through distillation, and the extract was kept in desiccators for further usage and storage.

5.1.3 Drug and Instrument

In addition to orlistat injection and monosodium glutamate, other equipment such as biochemical kits, centrifuge machines, centrifuge tube micropipettes, digital balances, test tubes, and biochemistry analyzers were also employed.

5.1.4 Experimental animal

For this investigation, 15 healthy wistar albino rats of either sex, aged 3-4 months and weighing 180-230gm, were employed. Individually housed in polypropylene cages the animals were cared for according to industry standards. (12 hours of light and 12 hours of darkness, 23±5°C and 40-60% humidity). They were given water and fed a typical rat pallet diet.

5.1.5 Total Phenolic Content

The total phenolic content of *achyranthes aspera* leaves was determined using the Folin–Ciocalteu method [16]. Briefly, 1 mL of *A. Asper* (100–500 µg/mL) solution was mixed with 2.5 mL of 10% (w/v) Folin–Ciocalteu reagent. After 5 min, 2.0 mL of Na₂CO₃ (75%) was subsequently added to the mixture and incubated at 50 °C for 10 min with intermittent agitation. Afterwards, the sample was cooled and the absorbance was measured utilizing a UV Spectrophotometer (Shimazu, UV-1800) at 765 nm against a blank without extract. The outcome data were expressed as mg/g of gallic acid equivalents in milligrams per gram (mg GAE/g) of dry extract.

5.1.6 Total Flavonoid Contents

The total flavonoid contents of *achyranthes aspera* leaves was determined as per the Dowd method [17]. An aliquot of 1 mL of *A. Aspera* solution (25–200 µg/mL) or quercetin (25–200 µg/mL) were mixed with 0.2 mL of 10% (w/v) AlCl₃ solution in methanol, 0.2 mL (1 M) potassium acetate and 5.6 mL distilled water. The mixture was incubated for 30 min at room temperature followed with the measurement of absorbance at 415 nm against the blank. The outcome data were expressed as mg/g of quercetin equivalents in milligrams per gram (mg QE/g) of dry extract.

5.1.7 Acute oral toxicity test

The acute oral toxicity study for EEAA was carried out according to OECD guidelines 423.[18] albino rat were fasted overnight. The EEAA was administered at a dose of 200mg/kg and 400mg/kg. Animals were observed individually during the first day and periodically on weekly basis, with special attention given for a total of 28 days (4 weeks).

Preparation of high fat diet (HFD)

The HFD consists of beef tallow – 35% (315 kcal), casein – 20% (80 kcal), corn starch – 15% (60 kcal), sucrose – 20% (80 kcal), corn oil – 5% (45 kcal), mineral mixture – 4% and vitamin mixture – 1% [19, 20]. The dietary ingredients were homogenized in warm distilled water and pellets were prepared. These pellets were dried in hot air oven before feeding. The diets were prepared freshly once in a week. The diet was prepared in such a way that fat accounted for around 62% of the total energy values of the HFD, while carbohydrates accounted for 24% and proteins for 14%.

5.1.8 Induction of Obesity

A group of 15 Wistar albino rats weighing 100–125 g were selected and obesity was induced in them by feeding the HFD for a period of 7 days. After the induction period, the animals weighing around 200–225 g (considered obese prone) were selected for anti-obesity studies [21].

5.1.9 Experimental Procedure

The rats were divided into 5 groups each containing 3 animals

Group I (Normal): Rats fed with normal pellet chow

Group II (Control): Rats fed with HFD 8mg/gm MSG

Group III (Standard): Rats fed with HFD 8mg/mg MSG + Orlistat (50mg/kg)

Group IV (Test1): Rats fed with HFD 8mg/gm. MSG + Ethanolic extract test drug (200mg/kg)

Group V (Test2): Rats fed with HFD 8mg/gm. MSG + Ethanolic extract test drug (400mg/kg)

All medications were administered orally. Three rats of each sex were randomly distributed to each of the 5 groups. Following the introduction of monosodium glutamate for 7 days, a total of 28 days were required to assess the effectiveness of anti-obesity measures. The changes in weekly body mass, locomotion and behavior were examined and recorded. Following the completion of the 28-day research, each rat has its tail chopped in order to obtain its blood for biochemical estimation.

5.2. Evaluation Of Anti Obesity Activity:

5.2.1 Body weight

Every rat's body weight (gm.) was monitored every week for a total of 28 days (4 weeks). An electronic weighing balance is used to determine the weight.

5.2.2 Locomotive activity

Every week for 28 days (4 weeks), locomotive activity was measured using an actophotometer with 5-minute observation duration after drug administration.

5.2.3 Biochemical Analysis

On the 29th day, all of the rats' blood is taken, by tail vein method, and subjected to centrifugation at 3000rpm. The Serum samples were carefully collected and stored for biochemical examination. Levels of LDL-C, total cholesterol, and blood glucose to determine HDL-C, VLDL-C, and triglyceride, researchers employed standard chemicals and a ROBONIK biochemical analyzer.

5.3. Evaluation Of In Vitro Antioxidant Activity

To assess antioxidant activity, the following antioxidant approaches were used:

- Nitric oxidant scavenging activity
- Hydroxyl radical scavenging assay
- Phosphomolybdenum antioxidant assay

Chemicals and instruments: Gallic acid, phosphate buffer, potassium, methanol, sulfuric acid, sodium phosphate, ammonium molybdate ferrous sulfate, hydrogen peroxide. Analytical graded substances were employed throughout the study. pH meter, digital balance, test tubes beakers UV-visible spectrophotometer, electronic weighing balance.

5.3.1. Nitric oxide scavenging activity

Specific nitric oxide synthases produce NO in biological tissues by metabolizing citrulline from arginine via a five electron oxidative reaction, which ultimately resulted in NO.

Principle: At physiological pH (7.2), the solution, sodium nitroprusside breaks down, generating NO. NO reacts with oxygen in aerobic settings to produce stable products (nitrate and nitrite), the levels of which can be measured.

Preparation of standard solution: The standard gallic acid 50µg/ml stock solution in methanol was prepared in further dilution 1µg/ml, 2.5µg/ml, 5µg/ml in water was prepared for the study.

Preparation of test solution: The extract 1mg/ml was prepared in water and further dilutions 100, 200 and 400µg/ml was prepared for the study.

Procedure: In 0.5 ml, phosphate buffer saline (pH 7.4), 2 ml of sodium nitroprusside was dissolved and were combined with 0.5 ml of sample at varied quantities (0.2-0.8 mg per ml). After that, At 25 degrees Celsius, the mixture is incubated. After 150m of incubation, 0.5 mL of the incubated solution is removed and mixed with 0.5 ml of griess reagent [1.0 mL sulfanalic acid reagent (0.33 percent in 20% glacia acetic acid at room temperature for 5 minutes with 1 ml naphthylethylenediamine dichloride (0.1 percent w/v)]. After 30 minutes of incubation at room temperature, the absorbance of the liquid poured into a cuvette is measured at 546nm [22].

The amount of nitric oxide radical inhibition is calculated following the equation

$$\% \text{ inhibition of NO radical} = (A_0 - A_1) / A_0 \times 100$$

Where

A₀ = Absorbance before reaction.

A₁ = Absorbance after reaction has been taken place with griess reagent.

5.3.2. Hydroxyl radical scavenging assay

Preparation of standard concentration: The standard gallic acid 50µg/ml stock solution in methanol was prepared and further dilution 1 µg/ml, 2µg/ml and 5µg/ml in methanol were prepared for study.

Preparation of test sample: the leaf extract 1mg/ml was prepared in distilled water and further dilutions of 50, 100, 300, 500µg/ml were prepared for studies.

Procedure: The hydroxyl radical scavenging ability of the sample extract was measured using the method outlined with some modifications. Individual sample extracts (1ml) were added to a reagent containing 1ml 1, 5mM FeSO₄, 0.7ml mM H₂O₂ and 0.3ml mM sodium salicylate at various concentrations (50, 100, 300, 500 µg/ml). After incubation for 1hr at 37°C, absorbance of the reaction mixture was read at 562nm. [23]

The scavenging ability on hydroxyl radical was calculated using the following equation:

$$\text{Scavenging ability on hydroxyl radical (\%)} = \{(A_0 - A_1) / A_0\} \times 100$$

Where

A₀ = Absorbance of the control reaction (containing all reagent except the sample extract).

A₁ = Absorbance of the sample extract.

5.3.3. Phosphomolybdenum antioxidant assay

According to the procedure, the antioxidant activities of the sample were assessed using the phosphomolybdenum method. The assay is based on the sample reducing Mo (VI)-Mo (V) and then forming a green phosphate/Mo (V) complex at an acid pH. [24]

Preparation of standard concentration: The standard, gallic acid 50µg/ml stock solution in methanol was prepared and further dilution 1µg/ml, 2µg/ml, and 5µg/ml in methanol were prepared for the study.

Preparation of test sample: The leaf extracts 1mg/ml prepared in distilled water and further dilution 50, 100, 300, 500µg/ml was prepared for study.

Procedure: 3ml of reagent solution was mixed with 0.3ml of sample (mg/ml) (0.6M H₂SO₄, 28 mM Na₃PO₄ and 4mM ammonium molybdate). The reaction solution was incubated for 90 minutes at 95 ° C. in a tube. As soon as it reaches room temperature, the absorbance of the solution was determined using a spectrophotometer at 695nm against a blank.

Statistical analysis

The data were expressed as Mean ± SEM. The statistical analysis was carried out using One-way Anova. A p value <0.05 was considered as statistically significant.

Author contributions: Concept – S.A.O.; Design – S.A.O, U.A.O, K.R.S; Supervision – K.R.S.; Resources – S.A.O., Materials – S.A.O; Data Collection and/or Processing- S.A.O, U.A.O, A.P.O.; Analysis and/or Interpretation – S.A.O, K.R.S, U.A.O, A.P.O.; Literature Search – S.A.O, U.A.O, A.P.O.; Writing – S.A.O.; Critical Reviews – S.A.O, K.R.S U.A.O, A.P.O

Conflict of interest statement: The authors declared no conflict of interest" in the manuscript.

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