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# ANTI-OBESITY POTENTIAL OF THE ETHANOLIC EXTRACT AND THE ISOLATED COMPOUND (ISOQUERCITRIN) OF AERIAL PARTS OF *LAGENARIA SICERARIA* (MOL.) STANDLEY IN HYPERCHOLESTEROLEMIC ZEBRA FISH

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**KEYWORDS:**
Obesity, Isoquercitrin, Orlistat, Zebra fish.

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## ABSTRACT

Obesity-"a dying weigh of life" is a medical condition in which there is excess fat accumulation to such an extent that it may cause adverse effects leading to reduced life expectancy and increased health problems. Globally about 2.8 million people are dying each year as a result of being obese. Obesity which is the first wave of cluster of non communicable diseases foreshadows its explosion to degenerative disorders. The vegetable gourd (Bottle gourd) belonging to the family Cucurbitaceae as mentioned in scriptures is found to have rich phytoconstituents. The purpose of this paper is to explore the traditionally narrated usage of the plant as a potent agent to treat diseases. Hence various extracts of *Lagenaria* were subjected to anti-oxidant potential evaluation and the best extract was selected and subjected to column chromatography for isolation of phytoconstituent. Anti Obesity activity of extract and isolated compound showed prominent body weight reduction and also decrease in Total cholesterol, Triglycerides and LDL levels when compared with the standard marketed Orlistat drug.

## INTRODUCTION [1-11]:

Obesity, the killer lifestyle disease is the preventable cause of death worldwide. Recently, evidence has begun to emerge that oxidative stress may be linked to obesity. For example, one of the features of obesity is the increased production of fat cells (called adipocytes), and it has been shown that oxidative stress in the environment around the cell can increase this process. Obesity may induce systemic oxidative stress (OS) and, in turn, OS is associated with an irregular production of adipokines, which contributes to the development of the metabolic syndrome. The sensitivity of CRP (C-reactive protein) and other biomarkers of oxidative damage are higher in individuals with obesity and correlate directly with BMI and the percentage of body fat, LDL oxidation, and TG levels. Zebra fish( a small fish with big future) is gaining increasing attention as a vertebrate model for studying human diseases due to its astonishing facts like embryos are see through ,can be easily manipulated. It takes only 24 hours to develop from fertilised egg to that which resembles a tiny fish whereas for mouse it takes 21 days.

India is sitting on a gold mine of well-recorded and well practiced knowledge of traditional herbal medicine. In the developing world the trend is changing from use of synthetic to natural herbal medicine because herbal drugs are considered less toxic, relatively safe and effective in chronic conditions. So nowadays, natural products are believed to be an important source of new chemical substances with potential therapeutic applications. *Lagenaria siceraria* (Mol.) Standley.( is one such plant with promising folklore and traditional claims of medicinal uses Currently there is a greater demand for anti-obesity drugs as most of the drugs act by directly suppressing the hypothalamic centre of appetite and satiety regulation which may result in unpredictable risks. Hence the current work was attempted to establish the Anti-Obesity potential Of the selected Ethanolic extract which showed highest anti-oxidant ability and the isolated compound Isoquercitrin by column chromatography that was characterized by spectral studies.

## MATERIALS AND METHODS:

The plant material was collected from local farms of Dheeran nagar of Thirichirapalli district and was authenticated by Prof. P. Jayaraman, Institute of Herbal botany, Plant Anatomy Research Centre, Tambaram, Chennai. The plant material was certified as *Lagenaria siceraria* of family Cucurbitaceae and the specimen no. is PARC/2012/1344. The aerial parts were separated and shade dried for 15 days and powdered.

### Experimental animals:

Adult zebra fish (*Danio rerio*) were procured from Kolathur, Chennai, Tamilnadu, India

### Chemicals:

All the chemicals used in the study were of analytical grade procured from Supra chemicals, Chennai. Standard drug Orlistat was obtained as a gift sample from Lupin Pharma, Mumbai.

**Preparation of extract [12]**

The powdered material was successively extracted with Petroleum ether, Chloroform, Ethyl acetate and Ethanol by hot percolation method using Soxhlet apparatus. All the extracts were filtered and concentrated by distillation using Rotary vacuum evaporator and the solvents were recovered. The final solution was evaporated to dryness at room temperature. The extracts were stored in the desiccator and were used for further studies.

**Phytochemical Screening [13-15]**

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical tests.

**Anti-Oxidant ability:**

Increased oxidative stress in accumulated fat is an important pathogenic mechanism of obesity. Three methods were carried out to determine the antioxidant ability of various extracts of *Lagenaria siceraria*.

**1. Reducing power ability [16]**

Different concentrations of extracts (1.0 ml) were mixed with 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Then 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml from the supernatant was mixed with 1.25 ml of distilled water and 0.25 ml $FeCl_3$ solution (0.1%, w/v). The absorbance was measured at 700 nm. Increased absorbance values indicate a higher reducing power. The standard used was Vit- C.

**2. Hydrogen peroxide scavenging assay [17]**

Hydrogen peroxide activity of the extract was estimated by replacement titration method .To 1ml of varying concentration of extract, 1 ml of (0.1 M) $H_2O_2$ and 10 ml of (2M) $H_2SO_4$ and 100 µl of (3%) ammonium molybdate, 7 ml of (1.8 M) potassium iodide was added .This reaction mixture was titrated against sodium thiosulphate until the disappearance of yellow colour.

$$\% \text{ inhibition} = [(V_0-V_1) / V_0] \text{ X } 100,$$

$V_0$-Volume of thiosulphate used to titrate blank

$V_1$- Volume of thiosulphate used to titrate against the extract.

3. **Thiocyanate method [18]**

The peroxy radical scavenging activity was determined by thiocyanate method using vit. C (50-800 µg/ml) as standard. Increasing concentration of the fractions (50-800 µg/ml) in 0.5 ml of distilled water was mixed with 2.5 ml of 0.02 M linoleic acid emulsion (in 0.04 M phosphate buffer pH 7.0) and 2 ml phosphate buffer (0.04M, pH 7) in a test tube and incubated in darkness at 37°C.Then 0.1 ml of 30% ammonium thiocyanate solution and 0.1 ml of 20 mM ferrous chloride

in 3.5% hydrochloric acid was added to the reaction mixture. The red colour developed was measured at 500 nm. The percentage scavenging activity was calculated and the $IC_{50}$ values of the fractions were compared with the standard (Ascorbic Acid). The percentage inhibition was calculated using the formula

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

**HPTLC analysis of Ethanolic extract:**

HPTLC was performed on silica gel 60 $F_{254}$ TLC aluminium sheet under laboratory conditions. 5 µl of the sample was applied as 5 mm band using Hamilton syringe with Camag Linomat automated TLC applicator. After the sample application, the plate was developed in a Camag Twin trough glass tank pre saturated with mobile phase Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26) up to 90 mm. After the development, the plate was dried in hot air and the images captured in photo-documentation chamber in white light and in UV (254nm and 366nm). The developed plate was sprayed with ammoniacal silver nitrate and dried at 100 ° C for 10 minutes and documented again in day light and in UV. The retention factor ($R_f$) and % area was calculated using Wincats software.

**Isolation of the active phytoconstituent of Ethanolic extract of Lagenaria by column chromatography and its characterisation by spectral studies [19-24]**

he Ethanolic extract (4gm) was subjected for the isolation of active phytoconstituents to silica gel (60-120 mesh) column chromatography.The extract was gradiently eluted with the solvent ratios as given in table 1.The eluents obtained ( each of 5 ml) was collected at uniform interval and the efficiency of separation was monitored by TLC (silica gel G 60 F254 TLC plates of E. Merck, layer thickness 0.2mm) using solvent system Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26). The developed chromatogram was observed under UV. The fractions 25-31 showed single spot on TLC, afforded the compound I (0.65 gm). These compounds were purified by recrystallization from methanol. Table 1: Column chromatography of Ethanolic extract

| S.NO | Eluent | Solvent Ratio | Fractions |
|---|---|---|---|
| 1 | Petroleum ether | 100% | Fraction-1 |
| 2 | Petroleum ether: Chloroform | 90 : 10% | Fraction-2 |
| 3 | Petroleum ether : chloroform | 80 : 20 % | Fraction-3 |
| 4 | Petroleum ether : chloroform | 70: 30 | Fraction-4 |
| 5 | Petroleum ether : chloroform | 60: 40 | Fraction-5 |
| 6 | Petroleum ether : chloroform | 50: 50 | Fraction-6 |

| | | | |
|---|---|---|---|
| 7 | Petroleum ether : chloroform | 40:60 | Fraction-7 |
| 8 | Petroleum ether : chloroform | 30:70 | Fraction-8 |
| 9 | Petroleum ether : chloroform | 20: 80 | Fraction-9 |
| 10 | Petroleum ether : chloroform | 10: 90 | Fraction-10 |
| 11 | Chloroform | 100 | Fraction-11 |
| 12 | Chloroform: Ethyl acetate | 90: 10 | Fraction-12 |
| 13 | Chloroform: Ethyl acetate | 80: 20 | Fraction-13 |
| 14 | Chloroform: Ethyl acetate | 70: 30 | Fraction-14 |
| 15 | Chloroform: Ethyl acetate | 60:40 | Fraction-15 |
| 16 | Chloroform: Ethyl acetate | 50: 50 | Fraction-16 |
| 17 | Chloroform: Ethyl acetate | 40: 60 | Fraction-17 |
| 18 | Chloroform: Ethyl acetate | 30: 70 | Fraction-18 |
| 19 | Chloroform: Ethyl acetate | 20: 70 | Fraction-19 |
| 20 | Chloroform: Ethyl acetate | 10: 90 | Fraction-20 |
| 21 | Ethyl acetate | 100 | Fraction-21 |
| 22 | Ethyl acetate: Ethanol | 90: 10 | Fraction-22 |
| 23 | Ethyl acetate: Ethanol | 80: 20 | Fraction-23 |
| 24 | Ethyl acetate: Ethanol | 70: 30 | Fraction-24 |
| 25 | Ethyl acetate: Ethanol | 60: 40 | Fraction-25 |
| 26 | Ethyl acetate: Ethanol | 50: 50 | Fraction-26 |
| 27 | Ethyl acetate: Ethanol | 40: 60 | Fraction-27 |
| 28 | Ethyl acetate: Ethanol | 30: 70 | Fraction-28 |
| 29 | Ethyl acetate: Ethanol | 20: 80 | Fraction-29 |
| 30 | Ethyl acetate: Ethanol | 10: 90 | Fraction-30 |
| 31 | Ethanol | 100 | Fraction-31 |

**Acute toxicity studies of the Ethanolic extract and the isolated compound in Zebra fish embyos [25-28]**

Acute toxicity studies were carried out based on the OECD guidelines 203.

**Principle of the test:**

Zebra fish embryos were individually exposed to a range of concentrations of the test substance in 24-well microtiter plates. The test is initiated immediately after fertilization and is continued for 72 hours. Lethal effects, as described by five apical endpoints (Coagulation of fertilized eggs, Lack of somite formation, Lack of detachment of tail, Lack of heart beat and death) are determined by comparison with controls to identify the $LC_{50}$ values. The test method is based on using a minimum of five test concentrations as well as appropriate controls, with 5-10 individual embryos per exposure concentration. Each substance should be tested in parallel in two to three independent replicates. The examination of the eggs was carried out with the aid of a microscope at 24, 48 and 72 hours post fertilization (hpf). Photos of the micro-well plates were taken with a mounted digital camera (Canon Power Shot ProI) and the results were calculated by probit analysis (percentage mortality).

The corrected (%) mortality was calculated by using **Schneider-Orelli's formula**.

$$\text{Corrected} = \frac{\text{(Mortality \% in treated plot - Mortality \% in control plot)}}{\text{100 - Mortality \% in control plot}} \times 100$$

**Anti-Obesity potential [29-30]**

**Feed preparation**

The feed was prepared by mixing the following formula of suitable ingredients with suitable moisture and was moulded to pellet form after drying in oven.

**Table 2: Composition of the feed:**

| Ingredients | Normal Feed (g) | High Cholesterol Diet (g) |
|---|---|---|
| Dry fish | 5 | 5 |
| Fish meal | 10 | 10 |
| Acetes | 3 | 3 |
| Soya | 25 | 25 |
| Wheat gluten | 2 | 2 |
| Wheat | 27 | 25 |
| Broken rice | 22 | 20 |
| Fish oil | 2 | 2 |
| Lecithin | 1 | 1 |
| Vitamin | 2 | 2 |
| Binder | 1 | 1 |
| Cholesterol | 0 | 4 |
| Total | 100 | 100 |

The extract of plant was mixed with the diet at the time of feeding.

Adult zebra fish (*Danio rerio*) were procured from Kolathur, Chennai, Tamilnadu, India and were allowed to adapt to laboratory conditions for 1 week before the commencement of the experiment as per ethical principles.

This was carried out at the Muttukadu research station –Central Institute of Brackishwater Aquaculture, Santhome, Chennai.

Fish were housed in groups of 10 animals/tank were kept in aquaria within the laboratory at 26 ± 1°C and a 12 – h light /dark cycle was maintained. Standardized water (ISO 7346 – 1, 1996) was used for the maintenance of the adult zebra fish. It was prepared from deionised water and the following salts were added: $CaCl_2$. $2H_2O$ (117.6 mg/L), $MgSO_4$. $7H_2O$ (49.3mg/L), $NaHCO_3$ (25.9mg/L) and KCl (2.3 mg/L) (Sigma Aldrich) to produce standardized water.

Fishes were divided into seven groups of ten each.

**Table 3: Experimental set up**

| Animals( n=10) | Treatment |
|---|---|
| Group I | Normal diet group ( feed without 4% cholesterol) 10 mg/day/fish as BID for 4 weeks |
| Group II | High cholesterol diet group (feed with 4% cholesterol) 10 mg/day/fish as BID for 4 weeks |
| Group III | Treated with high cholesterol diet containing 50µg orlistat (Standard drug) HC diet 10 mg/day/fish + Orlistat (50µg) /day/ fish) as BID for 4 weeks |
| Group IV | Treated with high cholesterol diet containing 5% *Lagenaria siceraria* ethanolic extract (LSEE) HC diet 10 mg/day/fish + Crude extract (LSEE) 1 mg/day/ fish) as BID for 4 weeks |
| Group V | Treated with high cholesterol diet containing 7.5% *Lagenaria siceraria* ethanolic extract (LSEE) HC diet 10 mg/day/fish + Crude extract (LSEE) 1 mg/day/ fish) as BID for 4 weeks |
| Group VI | Treated with high cholesterol diet containing 10% *Lagenaria siceraria* ethanolic extract (LSSE) HC diet 10 mg/day/fish + Crude extract (LSEE) 1 mg/day/ fish) as BID for 4 weeks |
| Group VII | Treated with high cholesterol diet containing 50µg of Isoquercitrin HC diet 10 mg/day/fish + Isoquercitrin (50µg) /day/ fish) as BID for 4 weeks |

**Blood collection and biochemical estimation**

After feeding for 4 weeks, the fishes were weighed by taking in a plastic cup or beaker containing water. After tarring the beaker, the fishes were weighed and height was measured .The blood was drawn from the heart of adult fish and combined with PBS, then collected into vaccutainer. Then it was centrifuged for 10 min at 2,500 rpm to get serum which was pipetted off the top. Serum from zebra fish were then subjected for the estimation of biochemical parameters like serum total cholesterol (TC), HDL-cholesterol, and triglyceride (TG), Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) using a commercial semi auto analyser.

**Statistical analysis:**

In biological experiments, the data (results) obtained at the end of the experiment is put to statistical analysis to determine whether the effect produced by a compound under study is genuine and not due to chance. Data are expressed as Mean ± Standard error of mean. Statistical comparisons between groups were done by one way analysis of variance (ANOVA) followed by Duncan's multiple-range test to analyse the differences. A $p<0.005$ was considered to be significant.

## RESULTS AND DISCUSSION:

Phytochemical studies showed the presence of secondary metabolites like alkaloids, flavanoids, tannins, sugars, phytosterols, saponins etc., The maximum constituents were found in Ethanolic extract followed by Ethyl acetate extract.

Anti-oxidant ability

**Reducing Power Ability**

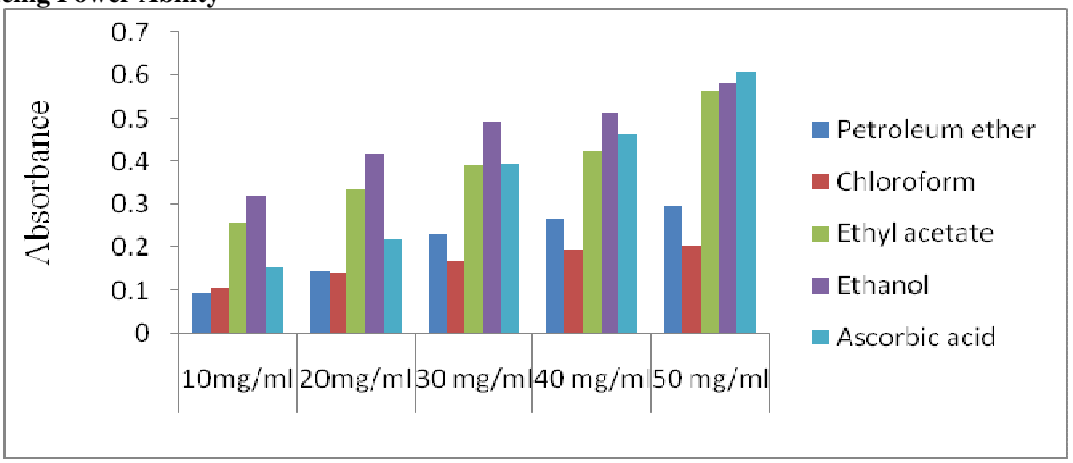



**Fig 1: Reducing power ability**

The reducing power ability of the aerial parts of *Lagenaria siceraria* (Mol.) Standley. The principle involved in this method is the conversion of ferricyanide ($Fe^{3+}$) complex to the ferrous form ($Fe^{2+}$) producing Prussian blue colour which is measured at 700 nm .The absorbance of the reducing power ability of the Petroleum ether, chloroform, Ethyl acetate, Ethanol and the Standard (Vit C) was found to be 0.297, 0.206, 0.566, 0,563 and 0.612 respectively at a maximum concentration of 50 mg/ml.

**Hydrogen Peroxide scavenging assay:**

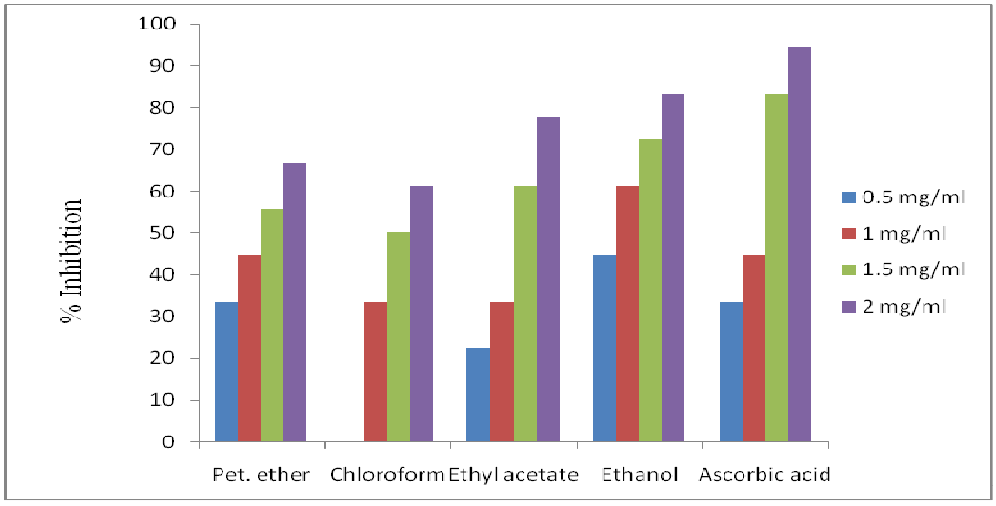



Fig 2: Hydrogen peroxide scavenging assay

**The IC $_{50}$ values of** petroleum ether, chloroform, ethyl acetate, ethanol and standard ascorbic acid was found to be 1.25, 1.60, 1.28, 0.65 and 0.93 mg/ml respectively.

**Thiocyanate method**

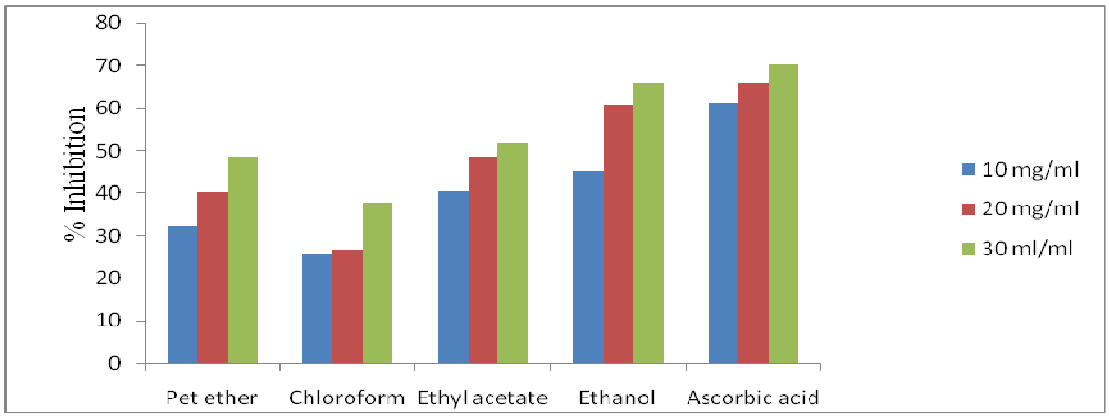



**Fig 3: Thiocyanate method**

**The IC $_{50}$ values of** petroleum ether, chloroform, ethyl acetate, ethanol and standard ascorbic acid was found to be 3.19, 5.28, 2.54, 1.29 and 1.51 mg/ml respectively.

From the Fig 1, 2, 3, it is clear that the Ethanolic extract showed the highest antioxidant ability as it has highest percentage inhibition when compared with the standard ascorbic acid. Hence it was selected for isolation by column chromatography.

**TLC and HPTLC profile of Ethanolic extract:**

**Table 4: TLC of ethanolic extract**

| S.NO | EXTRACTS | SOLVENT SYSTEM | Rf VALUES |
|---|---|---|---|
| 1. | Ethanol | Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26) | 0.88 |
| **2.** | Ethanol | Toluene: ethyl acetate: diethylamine (70: 20: 10) | 0.38 |

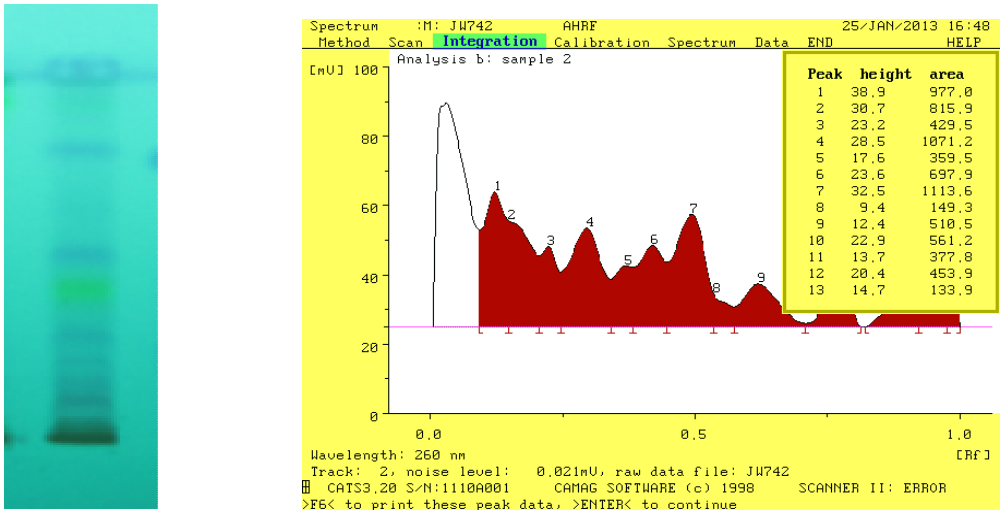


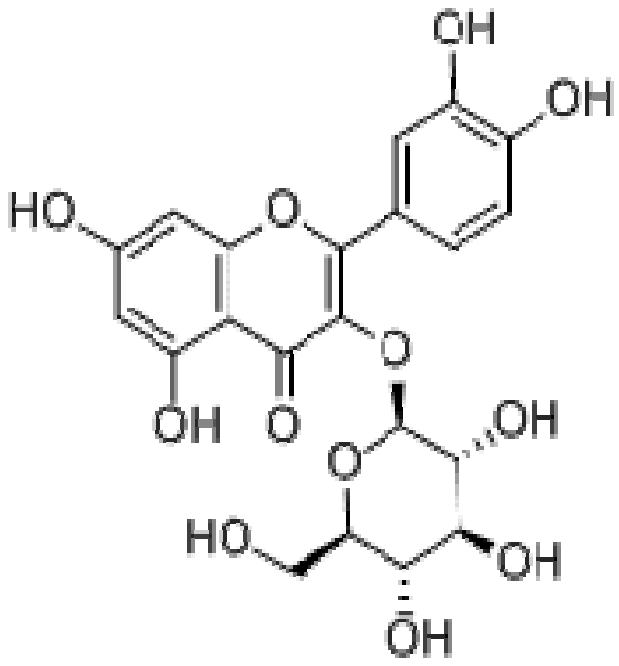

**Fig 4: HPTLC of ethanolic extract**

The TLC profile showed spots for alkaloid and flavanoid solvent systems .From the HPTLC graph ,it was observed that the 13 peaks indicates the presence of various phytoconstituents.

**Isolation:**

**TLC:**

Fractions 25-31 as indicated in Table 1 showed single spot and Rf Value of 0.8. A brownish yellow amorphous powder was isolated from the Ethanolic extract of Lagenaria siceraria aerial parts.IR Spectrum showed a strong absorption band at 1690 and 1720.93 cm-1 which are characteristic of C=O bond. The 1H-NMR spectrum of the compound has the characteristic set of signals that shows the aromaticity of Isoquercitrin moiety.The 13C-NMR spectrum showed the characteristic peaks of flavanoid and sugar moieties. In mass spectra, the characteristic molecular peak was found at 464.4 and an important glucose moiety peak was observed at 301.1. A doublet at 5.11 ppm due to proton of anomeric carbon (C-1") of the sugar moiety indicated the sugar (glucose) of isoquercitrin. The β-position of glucose was revealed from the coupling constant (J=6.9).The 1H-NMR, 13C-NMR, mass data of this compound were consistent with reported data of Isoquercitrin. The TLC profile showed a single spot and the Rf value was found to be 0.8.On the above deliberations, compound I was deduced as Isoquercitrin.




ISOQUERCITRIN

**Acute toxicity studies**

**Acute toxicity study of Zebra fish embryos**

**Table 5: Acute toxicity study of Zebra fish embryos**

| Sr.no | Dose | Coagulation | | Lack of somite formation | | Lack of detachment of tail | | Lack of heart beat | | Death |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hrs | 48hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 72 hrs |
| 1 | Water | - | - | - | - | - | - | - | - | - |
| 2 | DMSO | - | - | - | - | - | - | - | - | - |
| 3 | 1 µg/L of extract | - | - | - | - | - | - | - | - | - |
| 4 | 10µg/L of extract | - | - | - | - | - | - | - | - | - |
| 5 | 100µg/L Of extract | - | - | - | - | - | - | - | - | - |
| 6 | 1mg/L of extract | 1 | 2 | 2 | 3 | 3 | 3 | - | 3 | **3** |
| 7 | 10mg/L of extract | 1 | 3 | 2 | 4 | 3 | 4 | - | 4 | **4** |
| 8 | 20mg/L of extract | 5 | 6 | 6 | 6 | 6 | 7 | - | 8 | **8** |
| 9. | 1 mg/L of Isoquercitrin | 1 | 2 | 2 | 3 | 3 | 3 | - | 3 | **3** |
| 10. | 10 mg/ml of Isoquercitrin | 2 | 4 | 3 | 5 | 2 | 5 | - | 5 | **5** |

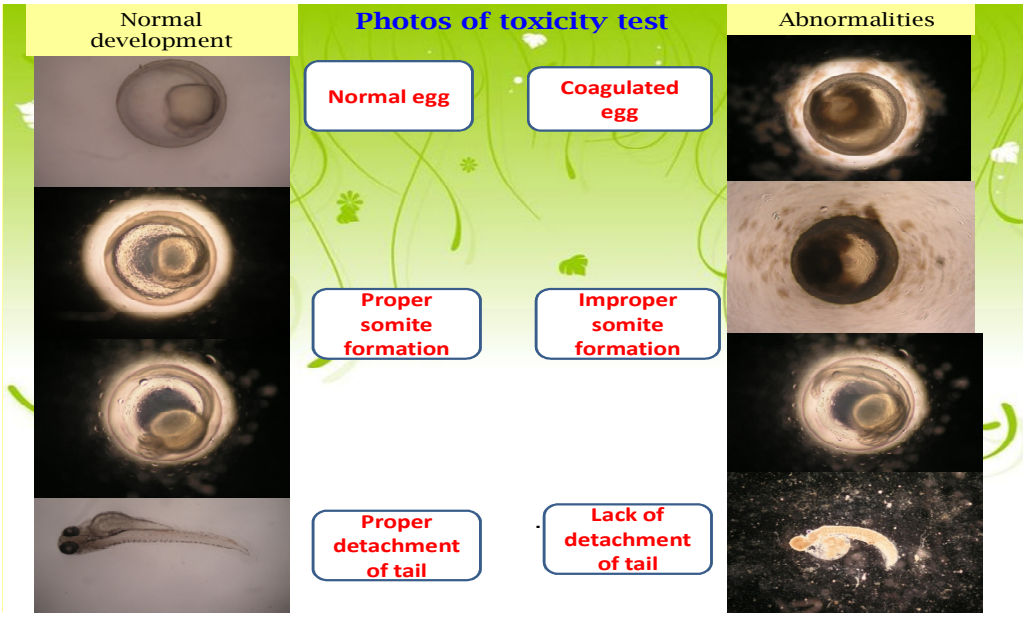



**Fig 5: Photos of acute toxicity studies of Zebrafish embryos**

The survival rate of zebra fish embryos treated with various concentrations of ethanolic extract (DMSO, Water, **1 µg/L, 10 µg/L, 100 µg/L, 1 µg/L, 1mg/L,10mg/L,20mg/L**) were noticed. Significantly decreasing survival rate was observed in when the dose is higher.

According to probit analysis, no mortality was observed in both the water and DMSO medium. It was observed that there was no mortality in 1µg/L and 10µg/L and 100µg/L concentration. But 30%, 40% and 80% mortality was observed at 1mg/ L, 10mg/ L and 20mg/L respectively. The isolated compound Isoquercitrin showed 30 % and 50 % mortality at img/ml and 10mg/ml respectively. According to OECD guidelines the dose which has upto 30% mortality is considered safe.

As a natural product with pharmacological activities, *Lagenaria siceraria* is a promising candidate for drug development with lesser adverse effects or toxicity with effective concentrations. Together, these results indicate that zebra fish are suitable model organisms to study the toxic effects.

**Anti Obesity potential**

**Table 6: Weight gain and body mass index**

| **Body weight** | **Parameters** | | | |
|---|---|---|---|---|
| | Initial body weight (mg) | Final body weight(mg) | Body weight gain (mg/study) | BMI |
| **Normal** | 618±0.57 | 624±1.92 | $6^{a}$ | $14.92\pm0.30^{a}$ |
| **High fat diet** | 514±2.87 | 546±2.67 | $32^{b}$ | $16.35\pm0.43^{b}$ |
| **Orlistat** | 545±4.54 | 554±1.09 | $9^{c}$ | $14.88\pm1.12^{a}$ |
| **5 % LSEE** | 627±0.81 | 653±1.65 | $26^{d}$ | $16.34\pm0.28^{cb}$ |
| **7.5% LSEE** | 487±2.94 | 506±1.27 | $19^{e}$ | $15.83\pm0.70^{ab}$ |
| **10 % LSSE** | 564±3.68 | 578±1.06 | $13^{f}$ | $15.38\pm0.51^{ac}$ |
| **Isolated compound** | 612±1.88 | 620±1.87 | $8^{c}$ | $13.38\pm0.54^{d}$ |

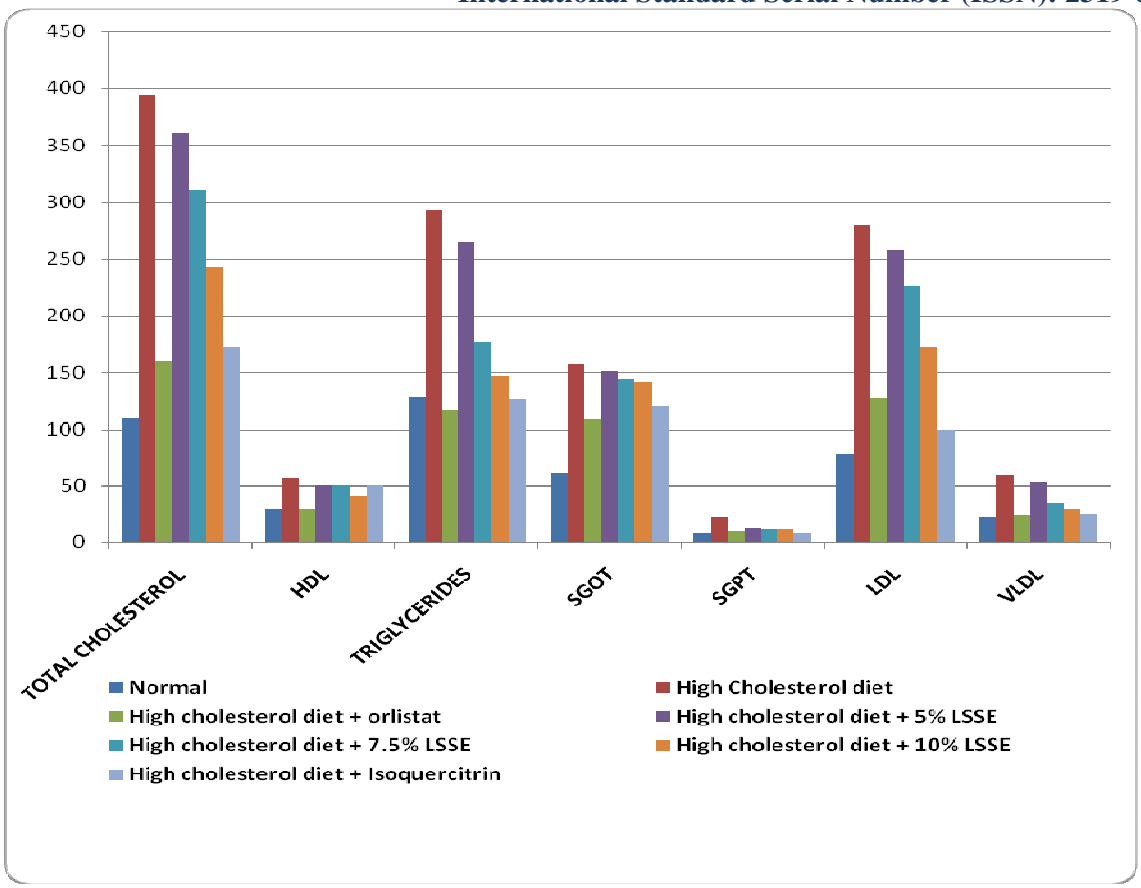



**Fig 6: Biochemical parameters assessed in zebra fish serum**

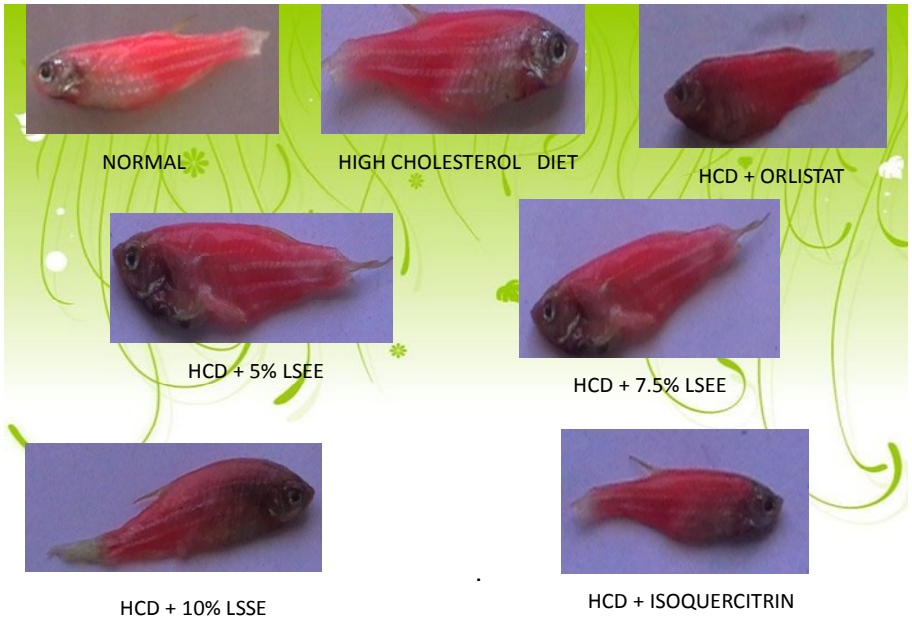



Fig 7: Photos of fish taken at the end of Study period (28 days)

## CONCLUSION:

From the studies it is concluded that the plant *Lagenaria siceraria* (Mol.) Standley. has anti obesity potential. Further studies on other animal species are required to confirm their activity. In addition to the isolated compound postulated to be a flavanoid glycoside (Isoquercitrin), other components with potential anti obesity activity may also present in the plant. These need to be isolated and studied further.

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