



Received on 06 January 2025; received in revised form, 28 January 2025; accepted, 30 January 2025; published 31 January 2025

LC-MS/MS GUIDED ISOLATION AND IDENTIFICATION OF NEURO POTENT PHYTOCONSTITUENTS FROM CHLOROFORM FRACTION OF METHANOLIC EXTRACT OF *LAGENARIA SICERARIA* (BOTTLE GOURD) FRUITS AND EVALUATION OF OBSESSIVE-COMPULSIVE DISORDER IN MURINE MODELS

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Keywords:

Fluoxetine, *Lagenaria siceraria*, Marble-burying behavior, Obsessive-compulsive disorder, Preliminary phytochemical screening, LC-MS/MS analysis

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ABSTRACT: Context: Obsessive-Compulsive disorder (OCD) is one of the most common neurological illness worldwide. Marble burying behavior in murine models is a unique and precise model for the assessment of anti-OCD like action of the tested drug. *Lagenaria siceraria* (Molina) Standley (LS), popularly known as “bottle gourd”, is a potent indigenous vegetable plant with high therapeutic value.

Objective: The aim of this study was to evaluate the anti-OCD (anti-compulsive) effects of the methanolic extract of LS fruits (MLSF) and the phytosterol rich Chloroform fraction of methanolic extract of LS fruits (CFMLS) using the marble-burying test in mice. **Materials and Methods:** The screening of anti-OCD action was carried out on Swiss Albino mice. Moreover LC-MS/MS analysis was carried out for the isolation and identification of the bioactive phytoconstituents responsible for the anti-OCD like effect of CFMLS. **Results and Conclusions:** MLSF and CFMLS, at doses of 100, 200, and 400 mg/kg, *p.o.*, decreased the total number of buried marbles in dose dependent manner. At dose of 400 mg/ kg, CFMLS exhibited the most significant ($P < 0.001$) effect, which was comparable to that of the fluoxetine (10 mg/kg, *i.p.*), reference standard drug. Moreover, none of the tested drug produced any kind of overt motor dysfunction. The results of phytochemical screening of MLSF and CFMLS showed the presence of phytoconstituents like sterols, terpenoids, and traces of flavonoids. Further LC-MS/MS analysis of the CFMLS revealed the presence of a total of 4 bioactive phytocompounds viz. Luteolin (I), β -sitosterol (II), Gamma sitosterol monohydrate (III), and Pregnane-3, 20-diol (IV). Among them Gamma-sitosterol monohydrate (III) and Pregnane-3, 20-diol (IV) were reported for the first time in LS fruits.

INTRODUCTION: OCD is a chronic neurological sickness, which is characterized by unrelenting and distress causing thoughts (obsessions), which are egodystonic and apparently purposeful behavior (compulsions) ^{1, 2}.

The World Health Organization (WHO) has identified OCD as one of the top ten neurological disabling illnesses in the world ³.

Obsessions are characterized by recurrent and constant thoughts, senses or metaphors that are experienced as inappropriate and that cause noticeable anxiety or distress, while compulsions are characterized by the monotonous behavioral actions like repeated hand washing, ordering, checking something, or mental actions like murmuring particular words mutely, praying,

QUICK RESPONSE CODE 	DOI: 10.13040/IJPSR.0975-8232.IJP.12(1).13-21 Article can be accessed online on: www.ijpjournal.com
DOI link: https://doi.org/10.13040/IJPSR.0975-8232.IJP.12(1).13-21	

counting, that the affected person feels determined to perform in response towards an obsession⁴. Marble-burying behavior is a unique model for the screening of anti-OCD agents due to its high analytical and good face validity⁵. According to this model, the rodents impetuously utilize the bedding material to bury unpleasant sources of embarrassment and uneasiness present in their home environment⁶. Certain types of critical and deleterious stuffs like food containing unpleasant taste and odour; the presence of small predators or electrified prod can provoke the marble burying behavior as a defensive behavior indicating the anxiety like condition of animals^{7,8}. The medicinal agents, which used to inhibit this marble burying behavior in mice, are believed to exhibit the anti-OCD action.

Traditional plant-based medicines have always contributed a crucial role in the management of psychological and neurological disorders. Therefore, the complementary and alternative natural therapies and remedies have been well accepted today across the globe. *Lagenaria siceraria* (Molina) Standley syn. *L. leucantha* Rusby; (Family: Cucurbitaceae) is an ethnomedicinal vegetable plant. It is also believed as a rich source of vital nutrients and therapeutically active phytoconstituents, which are utmost needed for maintenance of quality health⁹. Since from ancient period of time, LS fruits have been used for its cardioprotective, hepatoprotective, diuretic, aphrodisiac and purgative properties^{10,11}.

They are used to cure pain, ulcers, fever, cough, bronchitis, leucorrhoea, vaginal and uterine disorders¹⁰. Phytochemical reports on LS fruits suggest the presence of essential nutrients like proteins, vitamin C, vitamin B-complex, β -carotene, pectin, choline, dietary fibers, fatty acids like, palmitic acid, steric acid, and oleic acid. They are also found to have therapeutically potent group of phytoconstituents, like flavonoids, polyphenols, terpenoids, saponins, and tannins^{12,13,14}.

Literature claims that the LS fruits were traditionally used as nerve tonic. But very few investigations have been carried out so far to investigate the influence of LS fruits on neuroprotective action. Moreover, our previous investigations on neurobehavioral screening of LS

fruit reported its anti-depressant effects in animal experimental models of depression¹⁵. Therefore as a part of further research; the present study was intended to scrutinize the marble burying behavior in murine models for the evaluation of anti-OCD like action of LS fruits and phytosterol rich chloroform fraction of LS fruits (CFMLS). Further the separation, isolation and identification of neuroactive phytocompounds were also carried from the chloroform fraction through LC-MS/MS analysis.

MATERIALS AND METHODS:

Collection and Authentication of Plant Material:

Fresh LS fruits (20 kg) were purchased from the local vegetable vendor of Surat, Gujarat, India. Dr. Sumita Dasgupta, Dept. of Botany, Bhagwan Mahavir College of Science & Technology, Surat, identified and authenticated the plant material. A specimen voucher (SU/DPS/Herb/05) of the plant has been deposited and maintained at Herbal Museum, Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, India for future reference.

Extraction of Plant Material and Fractionation of the Crude Extract:

Properly cleaned LS fruits were cut into round slices and then air dried. The dried slices were pulverized into coarse powder. Further, the coarsely powdered dried fruits of LS (20 gm) were subjected to Soxhlet extraction process for 4 hr by using methanol as menstrum. At completion of extraction process, the supernatant was collected and concentrated *in-vacuo* to yield a brown-coloured viscous concentrate.

This concentrate was preserved at freezing temperature (4 °C). Further, the crude methanolic extract of LS fruits was suspended in 250 ml of distilled water in a separating funnel and partitioned successively with petroleum ether, chloroform, acetone, and n-butanol (250 ml each) to obtain fractions in these solvents. All the fractions were collected, concentrated and preserved at 4°C for further studies.

Preliminary Phytochemical Screening: The crude methanolic extract and all the sub-fractions were screened through qualitative phytochemical tests for the detection of various phytoconstituents¹⁶.

Qualitative Determination and Identification of Flavonoids: Phytosterols were detected more accurately by performing the following confirmatory phytochemical tests¹⁷. The test solution was prepared by boiling about 50 mg of CFMLS in 50 ml of distilled water for 5 min and then filtered to obtain a clear solution.

Salkowski Reaction: A few drops of concentrated sulphuric acid were added to the solution both MLSF and CFMLS formed a reddish color in the upper chloroform layer indicating presence of steroids¹⁷.

Gamma-sitosterol Monohydrate: Few drops of concentrated sulfuric acid were added to it followed by the addition of 2-3 drops of acetic anhydride. In this case both MLSF and CFMLS turned to violet blue and finally formed green color which indicates the presence of steroids¹⁷.

Preparation of Test Drug Samples and Treatments: MLSF, CFMLS and fluoxetine were suspended in 1%w/v Sodium carboxy methyl cellulose (SCMC) in distilled water and administered via *p.o.* route. Test samples were administered at dose levels of 100, 200, and 400 mg/kg, *p.o.* Control group animals received only vehicle (1% w/v SCMC) and standard group animals received reference standard drug, fluoxetine (10mg/kg, *i.p.*). Fluoxetine (the standard Selective Serotonin Reuptake Inhibitor drug) was procured as gift sample from Torrent Pharmaceuticals, Ahmedabad, India. All the solvents used in study were of analytical grade.

Experimental Animals: Adult Swiss female albino mice (Body wt.: 25-30 gm) were grouped (n=6) and maintained under standard conditions (temperature: 25-27 °C, relative humidity: 55-65% and 12 h light-dark cycle).

Experimental mice had access to standard rodent chow (Purchased from Pranav Agro Sales, Ahmedabad, Gujarat, India) and water *ad libitum*. Mice were acclimatized to laboratory conditions for at least 8 to 10 days prior to experiments. All the experiments were conducted in a noise-free laboratory compartment during the day light phase (between 08:00 and 16:00 hr) and the mice were not fasted prior to drug treatments. Separate groups (n=6) of mice were utilized for each experimental

model. The animal trials were approved by Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi and permitted too (Protocol approval no. 1521/ac/07/CPCSEA).

Acute Toxicity Study: As per the OECD guidelines-420, MLSF and CFMLS were administered orally at gradual doses of 5, 50, 300, 400 up to 1000 mg/kg and the animals were investigated for the development of toxicity symptoms. During the observation period, there was no death or any kind of toxicity symptom, observed during the study. Therefore, the fractions were classified in Category 5 as per annexure 4 of Globally Harmonized System (GHS) and LD₅₀ was determined as 1000 mg/kg, and 10%, 20% and 40% of the LD₅₀ were selected to perform pharmacological screening study (OECD, 2000)¹⁸.

Assessment of Marble-burying Behavior: The marble-burying model in mice was selected for the evaluation of anti-compulsive effect of MLSF and CFMLS^{19,20}. In this method, a plastic cage with the size of 21×38×14 cm³ was used. At the bottom of the cage 5-cm thick bedding was prepared with the help of sawdust. Further on the bedding floor, twenty small glass marbles (diameter 10-12 mm) were arranged in four different rows at uniform distance **Fig. 1**.

Then each mouse was placed individually in the cage and its marble burying behavior was observed for the time period of 30 min. After completion of each experiment, the number of unburied marbles was counted. The marble was considered as “buried”, when at least two-third of its size was found to be embedded in sawdust bedding.



FIG. 1: MARBLE BURYING MODEL

Spontaneous Locomotor Activity: It is assumed that locomotor activity influences marble burying behavior in the experimental animals. Therefore, the locomotor activity was evaluated and measured by using the actophotometer separately from the marble burying experiment.

Actophotometer records the total counting of the infrared photo beams, which were broken during the locomotion of mice inside the cage. During the experiment, the mouse of each group was individually placed in the central cage area of the actophotometer and its locomotor activity was observed for a total of 15 min at the interval of 5 min of time period²¹.

Statistical Analysis: All data were expressed as mean $\pm$ SEM ($n=6$) and analyzed by one-way analysis of variance (ANOVA), followed by Student Newman-Keuls test. The groups treated with test extract, fraction and fluoxetine were compared with the respective vehicle group. P values <0.001 were considered statistically significant.

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis: Liquid chromatography - Mass Spectrometry (LCMS) UHPLC (UltraHigh performance Liquid Chromatography) system was equipped with an auto sampler, Shimadzu SIL-20AC and the employed column was a Gemini C18, (150mm x 4.6mm, 5 μ m).

The mobile phases were 0.1% Formic in water (A) and 90% acetonitrile in water (B) at a flow rate of 1.000 ml/min. The LC conditions were 5% B during 0.01 min; 90% B during 40 min; and then 5% B followed by 5 min of maintenance.

A Thermo Electron LTQ-Orbitrap XL mass spectrometer equipped with a nano electrospray ion source (Shimadzu, Japan) and operated under Lab Solution Software V 5.91 supplied by Shimadzu software, was used in positive and negative ionization modes for the MS analysis using data-dependent automatic switching between MS and MS/MS acquisition modes.

Identification of Phytochemicals: Interpretation of the mass-spectra obtained from LC-MS analysis was performed by using the database of Mass Bank of North America (MoNA). The spectra of the

separated phytochemicals were compared with the spectra of known standard phytochemicals archived in the Mass Bank library. Further the tentative identification of the separated phytochemicals was carried out.

RESULTS:

Fractionation of the Crude Extract: Total four fractions were prepared and tested for their total yield and organoleptic characters. The results obtained are as per **Table.1**.

Preliminary Phytochemical Screening: The phytochemical screening of CFMLS revealed the presence of phytosterols, terpenoids, and traces of flavonoids, while alkaloids and glycosides were not detected **Table 2**.

Acute Toxicity Study: Results of the acute toxicity studies showed that the LD₅₀ of CFMLS in mice was 1000 mg/kg by *p.o.* route. So accordingly, three dose levels 100, 200, and 400 mg/kg, *p.o.* body weight, corresponding to 10, 20, and 40% of LD₅₀ value (1000 mg/kg, *p.o.*) were selected to perform pharmacological screening studies.

Assessment of Marble-Burying Behavior in Mice: MLSF **Fig. 2** and CFMLS **Fig. 3**, at doses of 100, 200, and 400 mg/kg, *p.o.*, reduced marble-burying behavior in mice in dose dependent manner and the reduction was quite significant ($P<0.001$) as compared to control group. CFMLS significantly reduced the marble-burying behavior. Fluoxetine, at a dose of 10mg/kg, *i.p.*, showed significant ($P<0.001$) results.

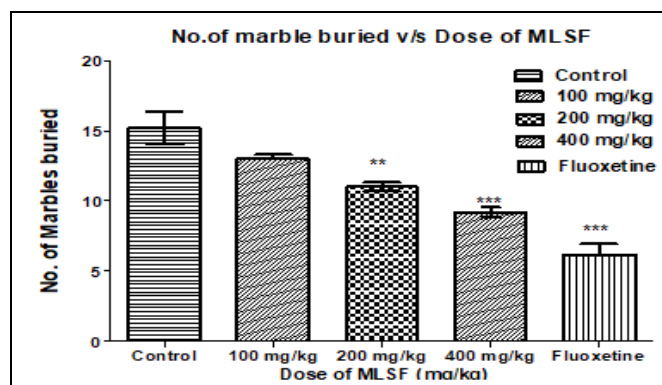


FIG. 2: EFFECT OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS AND FLUOXETINE ON MARBLE-BURYING BEHAVIOR IN MICE^A. ^aValues are expressed as mean $\pm$ SEM ($n = 5$). ** $P<0.01$, *** $P<0.001$; compared with control (one way ANOVA followed by Student Newman-Keuls test).

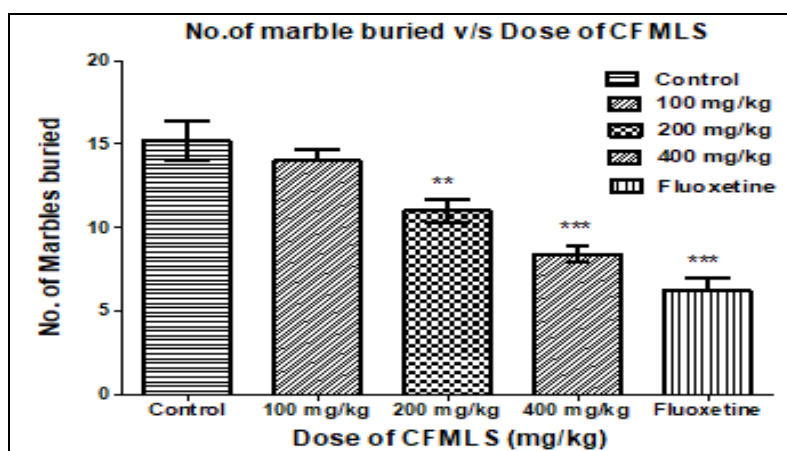


FIG. 3: EFFECT OF CHLOROFORM FRACTION OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS AND FLUOXETINE ON MARBLE-BURYING BEHAVIOR IN MICE^A. ^aValues are expressed as mean ± SEM (n = 5). ***P*<0.01, ****P*<0.001; compared with control (one way ANOVA followed by Student Newman-Keuls test).

Assessment of Spontaneous Locomotor Activity:

At all the selected dose levels, neither MLSF **Fig. 4** nor CFMLS **Fig. 5**, exhibited noticeable reduction in locomotor activity. Diazepam (0.5 mg/kg, *i.p.*) was used as standard drugs, which exerted marked

reduction in locomotor activity. However, it can be concluded that MLSF and CFMLS did not severely affect the ambulatory activity in mice after the drug treatment.

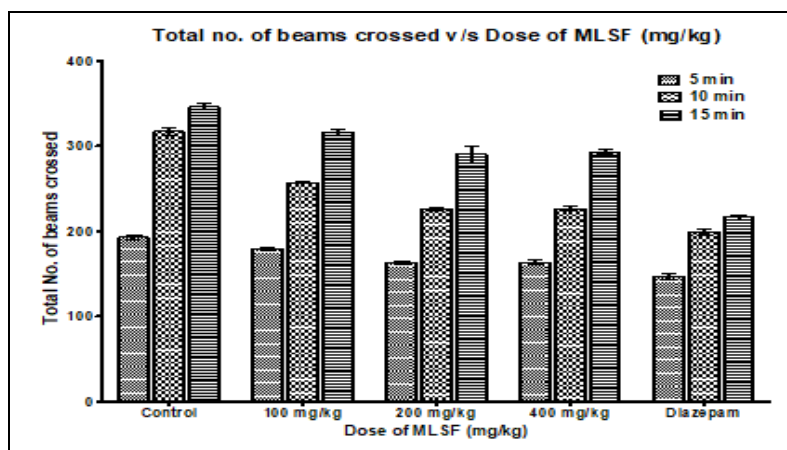


FIG. 4: EFFECTS OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS (MLSF) AND DIAZEPAM ON LOCOMOTOR ACTIVITY IN MICE

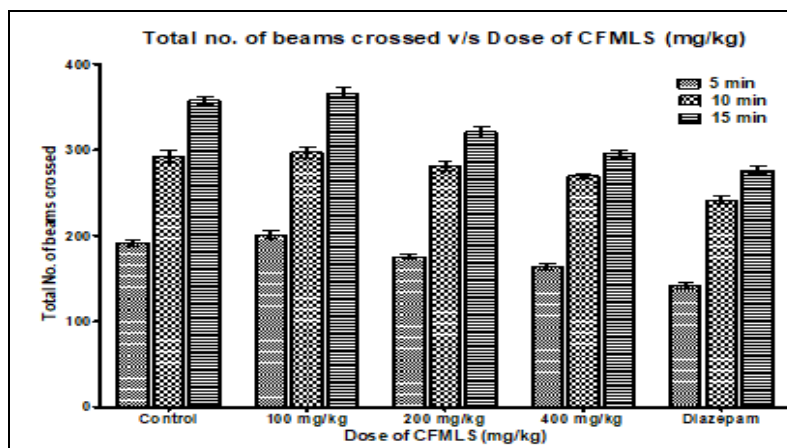


FIG. 5: EFFECTS OF CHLOROFORM FRACTION OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS (CFMLS) AND DIAZEPAM ON LOCOMOTOR ACTIVITY IN MICE

Liquid Chromatography-Mass Spectrometry (LC-MS) Studies: LC-MS chromatogram analysis of the CFMLS showed twelve peaks which indicated the presence of around twelve phytochemical constituents. On comparison of the mass spectra of the constituents **Fig. 7** with the

Mass Bank library, the four compounds were characterized and tentatively identified **Table 3**. The chemical structures of the identified compounds were shown in **Fig. 6**. Among them, γ -sitosterol monohydrate (III) and Pregnane-3, 20-diol (IV) were reported first time in LS fruits.

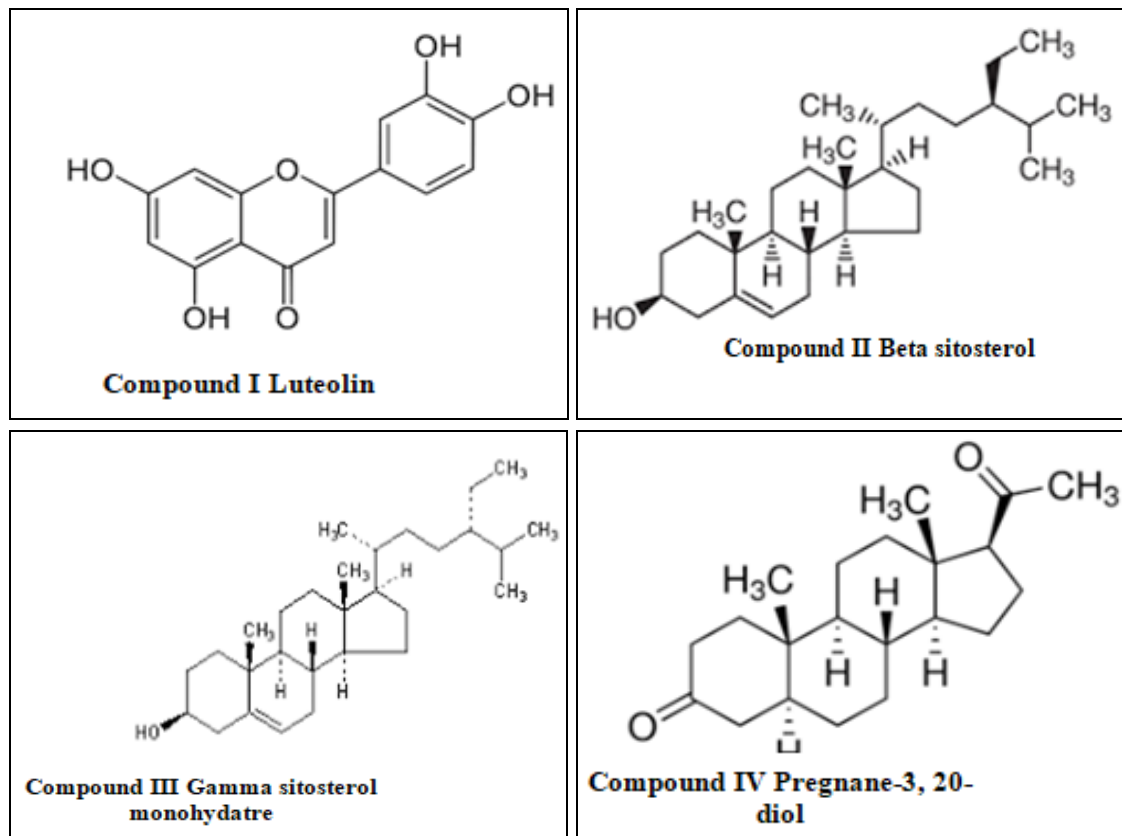


FIG. 6: CHEMICAL STRUCTURES OF THE SEPARATED COMPOUNDS FROM CFMLS

TABLE 1: RESULTS OF ORGANOLEPTIC AND QUANTITATIVE EVALUATION OF VARIOUS FRACTIONS OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS

Sr. no.	Fraction	Colour	Consistency	Yield (%)
1	Pet. ether	Light hazy yellow	Sticky	1.72
2	Chloroform	Dull brown	Non sticky	3.35
3	Chloroform	Brown	Sticky	9.46
4	n- Butanol	Brown	Sticky	3.72

TABLE 2: RESULTS OF PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT OF *L. SICERARIA* FRUITS AND THEIR FRACTIONS

Sr. no.	Test	Pet. Ether	Chloroform	Chloroform	n-butanol
1	Alkaloids	-	-	-	-
2	Carbohydrates	-	-	+	+
3	Phytosterols	+	+	-	-
4	Fixed oils and fats	+	+	+	-
5	Saponins	-	+	+	-
6	Terpenoids	-	+	-	-
7	Phenolic comp. & tannins	-	-	+	+
8	Proteins & amino acids	-	-	-	+
9	Gums and mucilage	-	-	-	-
10	Volatile oil	-	-	-	-
11	Flavonoids	-	-	+	+

TABLE 3: LIST OF SEPARATED AND TENTATIVELY IDENTIFIED BIOACTIVE PHYTOCOMPOUNDS FROM CFMLS THROUGH LC-MS/MS ANALYSIS

Sr. no.	R _T (min)	Molecular weight	Precursor type	Precursor m/z	Molecular formula	Tentative assignment	Mass bank References
1	7.356	286.24 g/mol	[M - 2H] ⁻	284.3	C15H10O6	Luteolin	MoNACCMSLIB0000077233
2	10.647	414.7 g/mol	[M + H] ⁺	415.2	C29H50O	Beta-Sitosterol	HMDB0000852_ms_ms_1203
3	15.657	432.7 g/mol	M	432.2	C29H52O2	Gamma sitosterol	PubChem (133082557)
4	31.657	320.5 g/mol	M	320.2	C21H36O2	monohydrate Pregnane-3, 20-diol/ Pregnandiol	PubChem (4901)

DISCUSSION: In the present study, the methanolic extract of LS fruits and its sterol-enriched chloroform fraction were evaluated for their anti-OCD like action by employing marble-burying behavioral test in mice. Marble-burying behavior is considered as a definite type of defensive behavioral response in rodents. It is not linked with any kind of physical hazard to animal¹⁹. In this behavior, the animal impulsively tries to bury harmless objects, such as glass marbles present in its cage area^{6, 22}, which gives indication of its defensive response. Such kind of behavior is considered as compulsive behavior, as no change in the intensity of the marble-burying behavior is found to be occurred during the repeated testing. Typical anti-depressant agents such as, tricyclics and SSRIs suppress the marble burying behavior^{19, 23} and so exert anti-compulsive action. These observations demonstrate that the marble burying behavior in murine models can be a useful model for the assessment of compulsive behavior rather than anxiety²³. The present study revealed that LS fruit extract and its flavonoid-enriched chloroform fraction (100, 200, and 400 mg/ kg, *p.o.*) reduced the marble-burying behavior and the effect was comparable to fluoxetine, 10 mg/kg, *i.p.*, a standard SSRI drug.

According to pathophysiology of OCD, neurobiochemical, immunological, and genetical factors play critical roles in the development of OCD symptoms²⁴. The specific regions of brain like orbito-frontal cortex, anterior cingulate cortex, and dorsolateral prefrontal cortex are reported to be involved in causes of OCD symptoms^{25, 26}. Certain specific type of neurotransmitters and their levels at nerve endings are concerned in the development of OCD

symptoms. Deficiency of serotonin (5-HT) and lack of availability of serotonin transporter (5-HTT) have been reported in symptomatology of OCD²⁷. Additionally, the rise in glutamate, glutamine and Gamma aminobutyric acid (GABA) levels in the caudate nucleus and the rise glutamate levels in the cerebrospinal fluid have been observed in OCD patients^{28, 29}.

Moreover, the glutamatergic ionotropic, N-methyl, D-aspartate (NMDA) receptors are also found to be involved in cause of OCD³⁰. Thus, it is hypothesized that the serotonin receptors of the OCD patients may be comparatively under stimulated, whereas the glutamate, glutamine and GABA receptors may be over stimulated. This hypothesis is reliable with the fact that the symptoms of OCD can be suppressed by the use of SSRIs, a class of anti-depressant drugs. These SSRI drugs can elevate the level of serotonin, which can be readily available to other nerve endings³¹. And therefore it can be assumed that MLSF and CFMLS may have a similar action to SSRI or some kind of inhibitory effect on serotonergic, glutamatergic or GABAergic neurotransmission. In the present study, the phytochemical investigations on LS fruits revealed the presence of flavonoids, steroids, saponins, terpenes, and phenolic compounds. Similarly, CFMLS showed the presence of sterols, saponins, terpenoids, and traces of flavonoids. Therefore, it can be hypothesized that one or more of these phytoconstituents may be accountable for the anti-OCD like effect of MLSF and CFMLS. Literature suggests that the flavonoids potentiate GABAergic neurotransmission particularly GABA_A receptors expressed in cortical neurons and also to selectively transform GABA_A receptor subtypes^{32, 33, 34}.

The flavonoids isolated from several medicinal plants like, valerian, chamomile, and kava-kava have exerted neuroprotective effects by showing positive allosteric modulation of GABA_A receptors³⁵. Moreover, it has been investigated that the flavonoids can exhibit anti-OCD like effects by modulating the GABA_A-Cl-channel complex, as they are structurally similar to benzodiazepines³⁶. Some experimental studies have also reported that flavonoids have the ability to block NMDA receptors in a concentration dependent manner and so can relieve the symptoms of OCD. In addition to this, it has been also proved that the consumption of flavonoid-rich functional foods can enhance the cortical blood flow and so increase the cerebro-vascular functions, especially in the hippocampus region of brain, which may facilitate neurogenesis and exerts neuroprotective effect³⁷. All these findings point out that the anti-OCD potential of MLSF and CFMLS may be due to the flavonoid compounds present in it^{38,39}.

Moreover, through the brain imaging study, it has been proved that the consumption of sterol-rich foods enhances the cortical and peripheral blood flow in brain and so elevates and maintains the cerebro-vascular functions and neurogenesis process⁴⁰.

In the present study, a total of four phytocompounds **Fig. 6** were separated and identified from bioactive fraction, CFMLS through LC-MS/MS technique. Thus, it can be positively assumed that one or more of these phytocompounds are responsible for neuroprotective action of the LS fruits. Though further investigations need to be scheduled for the determination of accurate and precise molecular mechanism of the neuropotent phytocompounds present in LS fruits.

CONCLUSION: In the current era, plant-based phytochemicals cover more than 50% of all the medicinal substances used in clinical treatments worldwide. Such phytochemicals present in plant-based extracts and their bioactive fractions may serve as lead molecules for the development of novel, effective and safe drug substances because of their structural diversity and wide spectrum of therapeutic effects in contrast to common synthetic

drugs used in the treatment of neurological disorders.

ACKNOWLEDGMENT: We are grateful to the Head, Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India, for providing the facilities during the course of this study. Special thanks to Dr. Sumita Dasgupta, Dept. of Botany, Bhagwan Mahavir College of Science & Technology,

Surat, for identification and authentication of the plant. We are very thankful to Dr. Trupesh Pethani and Mr. Kaushik Zala for helping us with LC-MS data analysis and interpretation.

CONFLICT OF INTEREST: The authors declare they have no conflict of interest.

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How to cite this article:

Prajapati RP, Kalariya MV and Parmar SK: LC-MS/MS guided isolation and identification of neuro potent phytoconstituents from chloroform fraction of methanolic extract of *Lagenaria siceraria* (bottle gourd) fruits and evaluation of obsessive-compulsive disorder in murine models. Int J Pharmacognosy 2025; 21(1): 13-21. doi link: [http://dx.doi.org/10.13040/IJPSR.0975-8232.IJP.12\(1\).13-21](http://dx.doi.org/10.13040/IJPSR.0975-8232.IJP.12(1).13-21).

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