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Comparative Evaluation of Antioxidant and Anti-Depressant Activity of Macrotyloma Uniflorum vs. Clitoria Ternatea Methanolic Leaf Extracts

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ABSTRACT

Background: Depression and anxiety are prevalent psychiatric disorders in the modern era, often attributed to the alterations in signalling neurotransmitters or the release of biogenic amines characterized by changes in consciousness, psychomotor functions, and emotional disturbances.

Purpose: Several antidepressant drugs are available; they may cause irreversible adverse effects. Therefore, exploring herbal remedies as an alternative therapy holds potential benefits. *Macrotyloma uniflorum* and *Clitoria ternatea* are among the plants traditionally utilized for their anti-depression activity.

Method: This study aims to compare the antidepressant activity of the methanolic extracts of *M. uniflorum* and *C. ternatea* leaves. Their phytochemical compositions were analysed, and antioxidant activities were evaluated using DPPH and Superoxide radical scavenging assays.

Results: The outcomes of phytochemical screening indicated the presence of secondary metabolites in both extracts, including alkaloids, saponins, glycosides, steroids, flavonoids, phenols, terpenoids and tannins. The antioxidant assays indicated significant antioxidant potential in both *M. uniflorum* and *C. ternatea* extracts. Various biochemical assays, behavioural studies, and histopathological evaluations were conducted using a toluene-induced mice model.

Conclusion: The behavioural study and biochemical estimations confirmed the antidepressant potential of both extracts. However, the *M. uniflorum* extract exhibited better efficacy in alleviating depressive symptoms. In light of future perspectives, it would be valuable to isolate and identify the specific compounds facilitating a more targeted pharmacological approach for depression pharmacotherapy.

1. Introduction

Depression, a neurological condition, stands as one of the most prevalent diseases and, as per the World Health Organization (WHO), could potentially emerge as the leading contributor to disability in the coming years. It is followed by symptoms such as changes in consciousness and psychomotor and emotional disturbances (Strohle, 2003; Sarris, 2007). It is caused by signaling changes in neurotransmitters or the release of biogenic amines (Gupta *et al.*, 2015; Bigoniya *et al.*, 2014). Among the neurotransmitters are adrenaline, glutamate, dopamine, serotonin and nor-adrenaline, with nor-adrenaline and serotonin contributing to the regulation of mood, psychological and emotional behaviors (Seo *et al.*, 2008; Lesch, 2007). One of the therapies for treating depression is to increase serotonin levels and reduce its absorption, leading to an increase in total serotonin activity (Zhu *et al.*, 2018). Synthetic drugs such as diazepam, lorazepam, sertraline, imipramine, etc., are mostly prescribed for treatment or management, but they show side effects such as xerostomia, mydriasis, bowel irregularity, sedation, temporary fatigue, and headache that contribute to poor patient compliance (Luethi & Liechti, 2020). All these factors have been associated with an increased demand for medicinal plants as a safe substitute therapy. Some plants have antidepressant potential and contain various phytoconstituents, which may be explored for new drug development against these disorders, mainly in cases where patients do not respond to existing therapies (Sofowora *et al.*, 2013; Bahramsoltani *et al.*, 2015; Kumar & Kumar, 2018). *M. uniflorum* and *C. ternatea* contain a variety of phytochemicals such as alkaloids, saponins, glycosides, flavonoids, terpenoids, and phenols. They possess various pharmacological activities and act as a tonic, astringent, diuretic etc. From the traditional system of medicines, they

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are extensively utilized in herbal medicines due to their intrinsic activities. They offer multiple health benefits for conditions like bronchitis, asthma, UTIs, heart diseases, and certain brain disorders (Shad *et al*., 2014). Various studies have been conducted till now to explore the pharmacological activities of these plants. Recently *M. uniflorum* leaf extract was studied to explore the anti-obesity activity in rats (Vadivelu *et al*., 2019). *C. ternatea* has been used for ages as a memory enhancer, stimulant, anxiolytic, and sedatives. A study was conducted by Maitry and her colleagues to explore the mechanism behind the wound healing activity of *C. ternatea* leaf extract (Maity *et al*., 2012). Swathi and her team studied the anti-inflammatory and anti-arthritic potential of *C. ternatea* leave extracts (Swathi *et al*., 2020). These scientific investigations are helpful in developing a rationale for future research that aids in establishing a strong rationale for exploring the antidepressant studied on selected plant species. Comparative study helps to understand and evaluate the specific effects of depression to identify the unique mechanism of action. Another important aspect is the evaluation of safety profile of these plant extracts, to be viable alternatives for populations where access to conventional antidepressant medications may be limited. In this study, a comparative evaluation of both plants was conducted to evaluate the antidepressant properties.

2. Materials and methods

2.1. Reagents and Chemicals

Chemicals used in the study had the highest grade purity available. Toluene, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ethanol, hydrochloric acid, methanol, NaOH, sulphuric acid (H_2SO_4), ascorbic acid, Tween 20, hydrogen peroxide (H_2O_2) etc., were purchased from loba chemie Pvt. Ltd.

2.2. Preparation of the Extracts

M. uniflorum (Reference number: BIT/KA-09) and *C. ternatea* (Reference number: BIT/NN-01) leaves were collected in July-August 2021. The plant herbariums were verified and specimens were authenticated by K.K Karthigeyan (Scientist E), Botanical Survey of India, Central National Herbarium, Howrah. The leaves were air-dried in the shade and subjected to subsequent extraction (Cold extraction method) using 80% methanol with intermittent shaking. 15 days later, the solvent was decanted and filtered using filter paper. The solvent was then subjected to evaporation using a vacuum rotary evaporator to obtain the dry product. The dry extracts obtained were kept at 4°C until further experimental works were conducted (Ashenafi *et al*., 2023).

2.3. Preliminary Phytochemical Screening and Subsequent Quantitative Analysis

The percentage yield of the methanolic leaf extracts of *M. uniflorum* (MUM) and *C. ternatea* (CTM) were calculated, and the preliminary detection of phytoconstituents was done using standard screening assays. For the identification of phytochemicals, present predefined tests such as Keller kiliani (glycosides), Shinoda (flavonoids), Ferric chloride test (phenols), Mayer's test (alkaloids), Foam test (saponins), Liebermann-Burchard test (sterols), Braymer's test (tannins) and Salkowski test (terpenoids) were conducted for both the extracts respectively.

To assess the total phenolic content (TPC), briefly, 0.5 milliliters of MUM and CTM extracts at varying concentrations of 200, 400, 600, 800, and 1000 µg/ml were mixed with 1.25 ml of FC reagent. In this, 5 milliliters of 7.5% sodium carbonate (Na_2CO_3) was added, mixed and allowed to leave for 45 minutes. The absorbance of the solution was measured at 765 nm. The Gallic acid standard curve was prepared to determine the TPC and expressed as mg Gallic acid equivalent per gram (GAE/gm.) Folin-ciocalteu method was employed.

The total flavonoid content in MUM and CTM extracts was determined using the Aluminum chloride reagent method. 1 ml of both the extracts at varying concentrations was mixed with 200 µg of 5% aluminum chloride ($AlCl_3$), and the absorbance was measured at 420 nm. The standard curve for quercetin was prepared, expressing the flavonoid content as milligrams of quercetin equivalent per gram (QE/gm.) (Sharma *et al*., 2020).

2.4. Assessment of Anti-oxidative Properties

The antioxidant activity of the MUM and CTM extracts was calculated using DPPH scavenging activity and superoxide radical scavenging activity. For the DPPH assay, the samples were prepared that contained 1 ml of extract at 10, 25, 50, 75 and 100 µg/ml and 1 milliliter of DPPH solution (0.1 ml in methanol). A mixture containing 1 milliliter of methanol and DPPH was taken as control. Solution (Ascorbic acid) at different concentrations (equivalent to extract) was used. The DPPH-scavenging (%) was evaluated using the equation:

$$\%Inhibition = \frac{Ac - As}{As} \times 100 \quad (1)$$

[Where, Ac = absorbance of the control, As = absorbance of the sample. From the obtained values, the IC_{50} was calculated]. (Baliyan *et al*., 2022)

Superoxide radical scavenging assay is based on the principle of the extracts to inhibit the synthesis of formazan, a dye-based artificial chromogenic product. This inhibition

occurs via superoxide radical scavenging generated within the riboflavin-light-NBT- system. It comprised 25 ml (50 mM) phosphate buffer (pH 7.6), 10 µl riboflavin (2.2 mg), 75 µl Triton X-100 and NBT (0.1 mg/3ml), added sequentially. The reaction mixture of the extracts and standard was started for 20 minutes. Right after illumination, the absorbance was assessed at 748 nanometers (Sharma *et al*., 2018). The percentage of scavenged superoxide anion was determined.

2.5. Cell viability Assay

Human neuroblastoma cells (SH-SY5Y cells) were used to study the cell viability which were seeded in 96-well plate at a cell density equivalent to 1.0×10^4 cells per well and was grown and cultured with DMEM (Dulbecco's-Modified Eagle Medium) and 10% foetal calf serum. The cells were placed at 37^0C in a 5% carbon dioxide incubator for 24 to 48 hours. Following the incubation period, the culture media was discarded, and the cells received washing and incubated in a growth media with ascending concentrations of MUM and CTM (1-100 µg/ml) for a duration of 48 hrs. Ten microliters (µl) at 5mg/ml of 3(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) with prepared buffer (phosphate saline) was added prior to the incubation period. Following centrifugation and discarding the supernatant, 100 microliters of dimethyl sulfoxide (DMSO) were then mixed to facilitate the dissolution of the formazan formed. Absorbance measured at 530 nm (Larsson *et al*., 2020).

2.6. Experimental Design and Treatment Schedule

Disease-free Swiss albino mice of weight ranging from 25-30 grams were housed at a temperature (23 ± 1) °C and relative humidity (RH) of 45-55 % under a 12-hour light/dark cycle. The mice were provided access to a standard pellet diet and water ad libitum. All the animals underwent 2 weeks acclimatization period prior to the initiation of the experimental works. The experimentations on animals were planned and executed in accordance with established guidelines of CCSEA and Institutional Animal Ethical Committee at BIT Mesra, Ranchi (Approval No. 1972/ PH/BIT/116/21/IAEC). Five groups were taken, (N=6). Group I-Control group with no stress (treated with saline), Group II- Toluene treated group (500 mg/kg bw, i.p), Group III- MUM (200mg/kg p.o) + Toluene treated group, Group IV- CTM (200mg/kg p.o) + Toluene treated group and Group V- Group III- Imipramine (25mg/kg p.o) + Toluene treated group. Further, the body weight and behavioral changes were observed on the 1st, 4th and 16th days using TST and FST. At the end of the experimental work, mice in each group were euthanized, Brain tissue was isolated, and Biochemical and histological evaluations were carried out.

2.7. Biochemical Estimation

Free radicals are generated due to stress-induced lipid peroxidation, producing a complex mixture of peroxides that leads to carbonyl compounds. Malondialdehyde (MDA) levels in the brain were estimated using thiobarbituric acid. One of the most important antioxidant molecules is Glutathione due to the presence of the thiol group. Within cells, glutathione remains in its reduced form by the action of the enzyme glutathione reductase, reducing other metabolites and enzyme systems. The reduced glutathione (GSH) levels in the brain were estimated using DTNB. Superoxide dismutase (SOD) catalyzes the disintegration of the superoxide ions into oxygen and hydrogen peroxide. The protein content in the brain was estimated by the Bradford protein assay method (Usman *et al*., 2023).

2.8. Histopathological Examination

The fixed brain specimens from control, toluene-treated and extract-treated groups were dehydrated using isopropyl alcohol, followed by clearing and embedding in wax (paraffin). Tissue sections, each 5 micrometers thick, were sliced with the help of a microtome. These sections were placed on the slides using MA solution (Mayer's albumin solution) and subsequently deparaffinized using xylene after warming. The staining of the sections was done using haematoxylin and eosin. The dried and mounted specimens were examined under light microscopy (Kumar *et al.,* 2018).

2.9. Statistical Analysis

The statistical evaluation was done using Graph pad prism version 8 software following one-way ANOVA test and all the results are plotted and presented as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Preliminary Phytochemical Screening

The percentage yield of MUM and CTM extracts were 5.45 % and 4.65 % w/w, respectively. Phytochemical screening of both extracts indicated the presence of flavonoids, alkaloids, glycosides, phenols, terpenoids, tannins, saponins and sterols. The presence of flavonoids, phenols, and anthocyanin in the methanolic leaf extracts of both plants was also confirmed by the literature review. The phytoconstituents levels are mainly affected by factors like the degree of maturity, environmental conditions, genetic differences, etc. The total phenolic and flavonoid content in the MUM extract was approximately 80.12 mg GAE/gm. and 34.02 mg QE/gm., respectively. While, in the CTM extract, the total phenolic

and flavonoid content was approximately 50.10 mg GAE/gm. and 30.06 mg QE/gm., respectively.

3.2. Anti-oxidative Properties of MUM and CTM Extracts

The significant antioxidant activity in MUM and CTM extracts is evident from the DPPH scavenging and superoxide radical scavenging assays that were comparable to the standard (Figure 1). Due to the increase in extract concentration, the percentage of free radical inhibition was also enhanced. In the DPPH scavenging assay, the half maximal inhibitory concentration (IC50) of MUM and CTM extracts was found to be 60.52µg/ml and 291.14 µg/ml (Figure 1A), respectively. Also, in the Superoxide scavenging activity (Figure 1B), the IC50 value of MUM extract was found to be 589 µg / ml, and that of CTM extract was 940 µg/ml. The antioxidant property of both extracts can be due to the phenols which are present, as hydroxyl groups. Research reports have also revealed that both extracts exhibit strong DPPH radical-scavenging and Fe2+ chelating activities (Siddhuraju & Krishnan, 2007; Jeyaraj *et al*., 2022). In comparison, MUM extract shows more antioxidant potential, possibly related to a higher phenolic content.

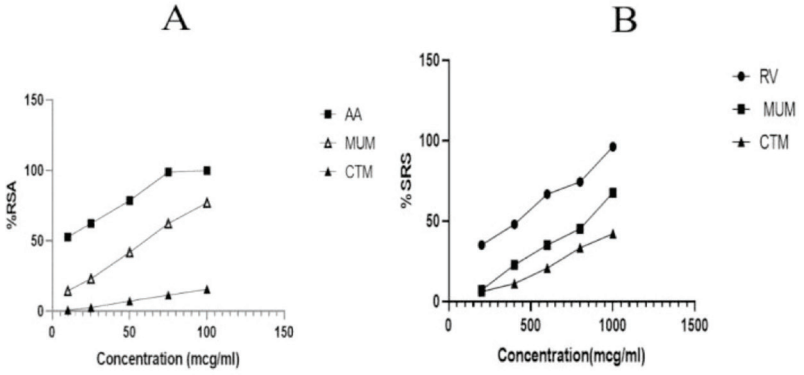

Figure 1: Graph showing antioxidant activity of MUM and CTM extracts. (A) % DPPH free radical scavenging activity of MUM, CTM and ascorbic acid at different concentrations. (B) Superoxide radical scavenging activity of MUM, CTM and Riboflavin at different concentrations. Values are presented as means ± SD. MUM, methanolic extract of *M. uniflorum* leaves; CTM, methanolic extract of *C. ternatea* leaves; AA, ascorbic acid; RV, Riboflavin.

3.3. Cell Viability Assay

Cell viability assay demonstrated no significant cell death at all the varying concentrations of the extracts used (Figure 2). Neuronal protection of human neuroblastoma cells against cell death could offer advantages in defending the cells against cellular stress factors, which is crucial for fostering anti-anxiety activity and providing protection against psychiatric disorders (Karvandi *et al*., 2023).

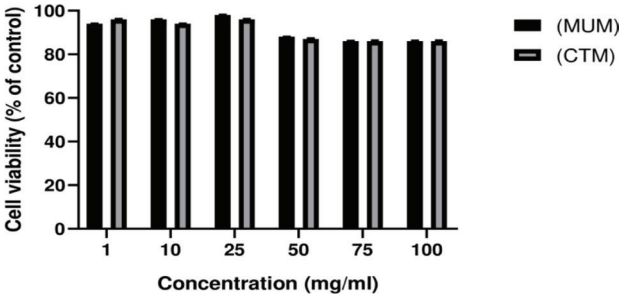

Figure 2: Cytotoxicity assay of MUM and CTM extracts using human neuroblastoma cells (SH-SY5Y cells).

3.4. Antidepressant Activity of MUM and CTM Extracts

For the assessment of the antidepressant activity of MUM and CTM Extracts, depression was induced using toluene and behavioral changes, biochemical changes and histopathological evaluation were carried out (Ekeanyanwu *et al*., 2021).

3.5. Effect on Behavioral Changes

The effect of MUM and CTM extracts on toluene-induced immobility in the TST and another using FST are illustrated in Table 1 and Table 2, respectively. In TST, mice subjected to toluene treatment exhibited an increased duration of immobility on the fourth day compared to those treated in the vehicle-treated group. However, following 16 days of toluene injection, there were no significant differences observed in the immobility of the animals. Both extracts have significantly reduced the toluene-enhanced immobility time ($P < 0.001$ vs disease control). The standard drug, imipramine, at a dose of (1 mg/kg), observed a significant

reduction in the duration of immobility. A similar pattern of results was observed in FST. However, the effect of MUM extract was stronger than that of CTM extract in terms of immobile time induced by toluene.

Table 1: Effect of MUM and CTM extracts on tail suspension test

Days	Vehicle Control (Immobility time, sec)	Disease Control (Immobility time, sec)	MUM treated (Immobility time, sec)	CTM treated (Immobility time, sec)	Standard (IMP) (Immobility time, sec)
1	180.5 ± 9.5	195.7 ± 0.5$^{\#}$	182.5 ± 9.5*	190.5 ± 0.5	170± 0.5 *
4	118.7 ± 3.2	185.3 ± 6.5$^{\#\#\#}$	113.6 ± 3.2***	125.3 ± 6.5***	105± 6.5***
16	90.5 ± 8.5	100.3 ± 15.5$^{\#}$	95.5 ± 8.5	95.3±15.5	95 ± 15.5

Table 2: Effect of MUM and CTM extracts on forced swimming test

Days	Vehicle Control (Immobility time, sec)	Disease Control (Immobility time, sec)	MU treated (Immobility time, sec)	CT treated (Immobility time, sec)	Standard (IMP) (Immobility time, sec)
1	190.5 ± 4.5	195.0 ± 0.4	190.5 ± 9.5	195.7 ± 0.5	185± 0.5
4	180.2 ± 7	215.0 ± 3$^{\#\#}$	190.6 ± 3.2**	200.3 ± 6.5*	175± 6.5***
16	165.0 ± 5.5	205.3 ± 15.5$^{\#\#\#}$	180.5 ± 8.5**	190.3±15.5*	172±15.5**

3.6. Effect on Biochemical Antioxidant Assays

Estimation of antioxidant enzymes (MDA, GSH, SOD and total protein content) using biochemical evaluation were carried out in the brain tissue. The MDA level reduced by toluene treatment (0.658±0.021 nm MDA/ mg protein in the brain) was further increased significantly in MUM and CTM-treated groups. The levels of GSH and SOD were maintained in the presence of both the extracts. The total protein content decreased due to toluene treatment was also enhanced significantly in the presence of MUM and CTM extracts (Figure 3). The results of all the biochemical assays imply that MUM extract has more significantly ameliorated the toluene altered levels of enzymes and total protein content.

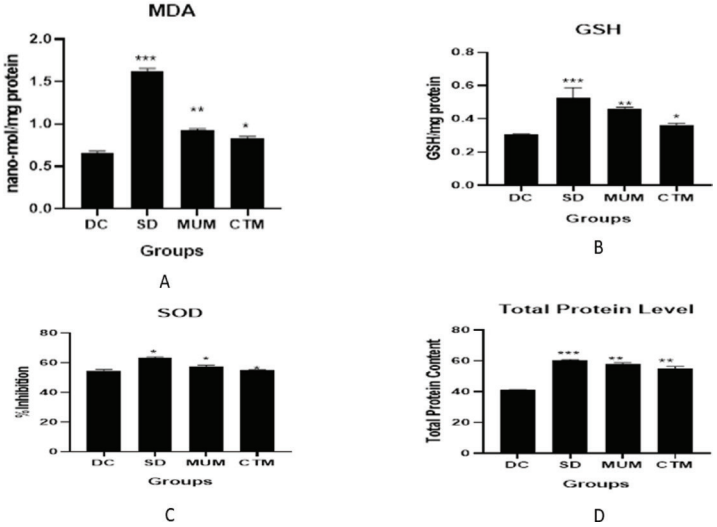

Figure 3: Estimation of Biochemical parameters after the treatment of MUM and CTM extracts in depression-induced animals. (A) Malondialdehyde (MDA) levels, (B) Glutathione (GSH) levels, (C) Superoxide dismutase (SOD) levels, and (D) Total protein levels. Mean ± SD (n=3) analyzed by one-way ANOVA.

3.7. Effect on Histopathological Changes

Histological studies were performed in the forebrain region (cerebral cortex) of the normal, depressed and extract-treated animals. The histology of adrenal glands was also performed to show the expansion of cortical cells in the toluene-treated groups (Figure 4). Various histopathological changes, including Cellular Edema, Degenerative Changes, Gliosis and Infiltration in the perivascular area, were observed in depressed animals. Expansion of adrenal cortical cells was also observed in the depressed group. In the extract-treated groups, fewer and milder changes were observed in the cerebral cortex regions of the brain, which exemplifies the significance of the plant extract as an efficient protective agent and as well as it can reverse degenerative changes and reinstate psychological illness.

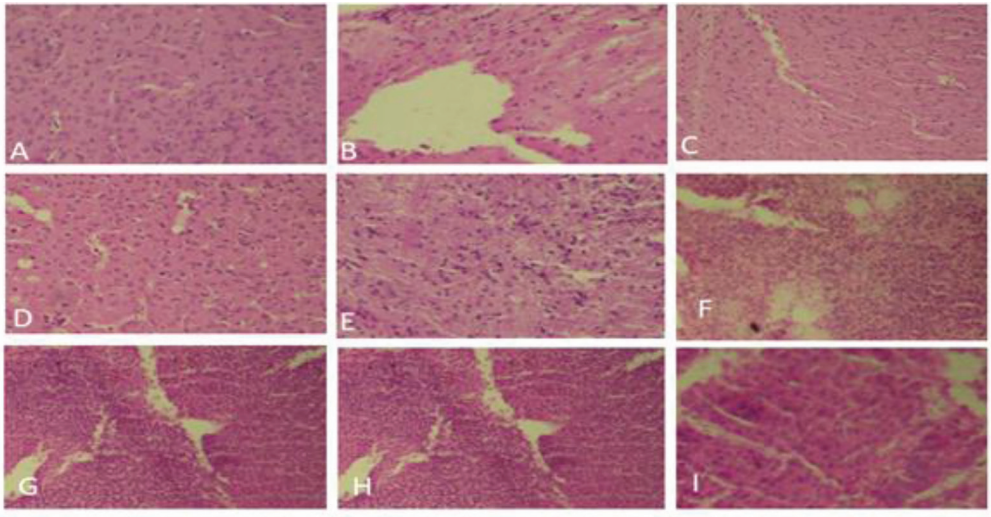

Figure 4: Various histopathological changes. (A) H&E stained Brain cerebral cortex tissue of Control, (B) depression-induced group showing cellular edema and degenerative changes, (C) standard treated group, (D) MUM extract treated group, and (E) CTM extract treated group. (F) H&E stained adrenal gland of toluene treated mice, (G) standard treated, (H) MUM treated, and (I) CTM extract treated group.

4. Conclusion

In conclusion, this study reports the presence of some important phytochemicals in the methanolic leaf extracts of *M. uniflorum* and *C. ternatea*. Plant polyphenols are well known for their anti-oxidant and free radical scavenging activities. This study revealed the presence of a considerable amount of total phenolic and flavonoid content in the extracts, which could be the reason for their anti-oxidant activity. MUM and CTM extracts have shown significant antioxidant properties in DPPH and superoxide scavenging assays. MUM extract has shown IC50 value of 60.52 µg/ml, which is comparatively much lower than that of 291.14 µg/ml calculated for the CTM extract. The superoxide scavenging assay has shown that the MUM extract had an IC50 of 589 µg/ml, while the CTM extract showed a higher IC50 of 940 µg/ml. These results suggest that the MUM extract has a greater antioxidant potential compared to the CTM extract. No cell death was seen in the cell viability at any concentration for both the extracts. Depression was induced with toluene in the animals. Toluene induced mobility was reduced by both the extracts. In TST, immobility time was decreased in both the extract as compared to imipramine, which was also shown in FST results. In biochemical investigation, MDA levels were increased by both the extracts. GSH and SOD levels and enhanced total protein content in brain tissue altered by toluene. Histopathology showed the potent alterations caused by toluene in causing cellular edema, degenerative changes. This comparative study revealed that *M. uniflorum* exhibited superior antidepressant activity compared to *C. ternatea*.

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Authorship contribution

Amit Kumar performed the literature search and prepared the methanolic extract. Sayak Khawas performed the quantitative and qualitative assay. Kumar Anand performed the in-vivo experimental work. All the contributors prepared the manuscript according to their experimental work. Dr. Neelima Sharma designed the study and finalized the manuscript.

Ethical Approval

All the animals were approved by the Institutional Animal Ethics Committee (Approval No. 1972/ PH/BIT/116/21/ IAEC).

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Conflict of Interest

There is no conflict of interest.

Declarations

It is an original article and has neither been sent elsewhere nor published anywhere.

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