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Research Paper

Flaxseed (*Linum usitatissimum*) Modulates Oxytocin Signaling Pathway via CALM3-mediated Calcium Signaling: A Protein-protein Interaction Network and Functional Enrichment Analysis

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ABSTRACT

Introduction: This study aimed to elucidate the molecular mechanism underlying the anti-anxiety effects of flaxseed (*Linum usitatissimum*) by investigating its role in modulating the oxytocin (OXT) signaling pathway through protein-protein interaction (PPI) network analysis.

Methods: Gene expression profiles from the Gene Expression Omnibus (GEO) dataset (GSE36422) were analyzed to identify flaxseed-induced differentially expressed genes (DEGs). Bioinformatics tools, including functional enrichment (KEGG, GO) and PPI network construction (STRING, Cytoscape), were used to map key genes and pathways. Hub and bottleneck proteins were identified using Cyto-Hubba's Maximal Clique Centrality (MCC) and Betweenness algorithms, focusing on genes shared between flaxseed and OXT signaling.

Results: Of 98 initially DEGs, 56 were significantly regulated by flaxseed. Three critical genes *CALM3* (upregulated), *NFATC4*, and *RAF1* (both downregulated) emerged as shared mediators between flaxseed and OXT pathways. *CALM3*, a calcium-sensing hub protein, exhibited extensive network connectivity, interacting with 58% of OXT pathway proteins and functioning as a bottleneck regulator. Pathway analysis revealed flaxseed's influence on OXT signaling, cellular senescence, and the cyclic guanosine monophosphate-activated protein kinase G (cGMP-PKG) pathway. *NFATC4* downregulation suggested disinhibition of OXT signaling, while *RAF1*'s role in MAPK cascades further supported anxiolytic effects.

Conclusion: Flaxseed exerts its anti-anxiety effects primarily through *CALM3*-mediated OXT signaling, leveraging calcium-dependent pathways. These findings provide mechanistic evidence supporting flaxseed as a natural anxiolytic and highlight its potential as a dietary intervention for anxiety disorders.

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Highlights

- Flaxseed exerts anti-anxiety effects by modulating the OXT signaling pathway.

- Network analysis identified CALM3 as the central hub protein, linking flaxseed-induced calcium signaling to enhanced OXT function.

- The anxiolytic mechanism involves upregulation of *CALM3* and downregulation of *NFATC4* and *RAF1*.

- Flaxseed is a dietary intervention targeting neuroendocrine pathways for anxiety management.

Plain Language Summary

Feeling stressed or anxious is the most common mental issue, and many people look to natural foods for relief. Flaxseed, a small brown seed used for centuries, is thought to have calming properties. Our study aimed to discover exactly how it might work in the body to reduce anxiety. Using computer-based analysis of biological data, we mapped out how flaxseed's compounds affect brain and body pathways. We discovered that flaxseed does not act randomly; it specifically targets a well-known "calm and connection" system in the body linked to the hormone oxytocin. Oxytocin (OXT) is famous for promoting bonding and reducing stress. The key finding is a protein called CALM3. Think of it as a central switchboard that uses calcium, a common signaling molecule in cells, to send messages. We found that flaxseed appears to turn up CALM3. This boosts the OXT system's signals while also dialing down other proteins that normally act as brakes on this calming pathway. This research provides the first scientific map showing a direct biological link between eating flaxseed and the brain's natural anti-anxiety systems. It moves flaxseed beyond a general health food to a potential dietary strategy with a clear mechanism. For people interested in non-pharmaceutical methods for managing stress and anxiety, this study offers a scientifically-backed reason to consider adding flaxseed to their diet. It also gives researchers a new target (the CALM3 protein) for developing future natural interventions for mental well-being.

Introduction

Linum usitatissimum (Linn.), commonly known as flax, belongs to the Linaceae family (Watson & Preedy, 2012). This species is known for its striking pale-blue flowers with five distinct petals. The seeds produced by flax plants, referred to as flaxseeds, are characterized by their flat, pointed, oval shapes (Ansari et al., 2019). The entire plant is commercially utilized, either in its raw state or after undergoing processing (Singh et al., 2011). Flaxseed has been incorporated into the human diet since the dawn of the first civilizations. In ancient Egypt and Greece, it was used for medicinal purposes (University of Wisconsin--Extension et al., 1990). Flaxseeds are known for their significant nutritional benefits and are a primary source of omega-3 fatty acids, particularly α-linolenic acid. They are also abundant in short-chain polyunsaturated fatty acids, soluble and insoluble fibers, phytoestrogenic lignans, such as secoisolariciresinol diglycoside, proteins, and a range of antioxidants (Ivanov et al., 2011; Oomah, 2001; Singh et al., 2011; Touré & Xueming, 2010). The properties of these compounds make *L. usitatissimum* a suitable choice for addressing various health concerns, including respiratory issues, neurological disorders, diabetes, gastrointestinal problems, constipation, abdominal pain, urinary infections, and skin inflammation (Al-Madhagy et al., 2023; Mueed et al., 2022). A notable feature of flaxseed, as demonstrated by multiple studies, is its capacity to reduce anxiety (Al-Madhagy et al., 2023; Anis et al., 2016).

Generalized anxiety disorder (GAD) is a persistent and incapacitating condition that impacts approximately 6.1% of people throughout their lives. Sensations of fear, unease, and discomfort define anxiety. It may present physically with symptoms, such as perspiration, restlessness, muscle tension, and an elevated heart rate (DeMartini et al., 2019). Available treatment options encompass psychological therapies and pharmacological interventions (Hoge et al., 2012). Global guidelines for the management of GAD recommend selective serotonin reuptake inhibitors, serotonin and noradrenaline reuptake inhibitors, and pregabalin as primary treatment options, due to their proven effectiveness and favorable safety profiles (Bandelow et al., 2023).

Oxytocin (OXT), a neuropeptide composed of nine amino acids, was the first human hormone to be identified (Carter, 2022). This molecule is particularly known for its role in stimulating uterine contractions. Furthermore, OXT has been proposed as an anti-anxiety agent in various studies (Gully et al., 2024). Although OXT is released under various physiological conditions, research has indicated that its secretion can also be triggered by external stimuli, such as flaxseed extract (Lecová et al., 2024; Sirotkin, 2023).

Natural products constitute a substantial portion of modern pharmaceutical agents, especially in disease treatment (Pal & Shukla, 2003). A unique strategy to deepen our understanding of the therapeutic mechanisms of active compounds present in medicinal plants involves predicting the gene networks they influence (Shao & Zhang, 2013). The analysis of protein-protein interactions (PPI) is beneficial for pinpointing this essential aspect (Tang et al., 2023). PPI network analysis elucidates the PPIs within a biological context (Rezaei-Tavirani et al., 2022). Although the literature has documented the anti-anxiety effects of flaxseed, its molecular mechanism remains poorly understood. This study aimed to demonstrate its molecular mechanism through the analysis of PPI networks.

Materials and Methods

This study examined the gene expression profiles triggered by flaxseed using a thorough bioinformatics methodology. Gene expression data were meticulously gathered from the Gene Expression Omnibus (GEO) dataset GSE36422 (National Center for Biotechnology Information, 2025), utilizing Gene Expression Omnibus (GEO)2R (National Center for Biotechnology Information, 2025) for the comparative analysis of expressed genes among various experimental groups (Subramanian et al., 2023). The initial data processing included a detailed Venn diagram (Figure 1) to clarify the gene expression profiles uniquely linked to flaxseed intervention. Bioinformatics techniques were employed to elucidate the molecular mechanisms underlying flaxseed-induced gene expression.

A comprehensive analytical approach was adopted, beginning with functional enrichment analysis using KEGG pathway and gene ontology (GO) methodologies. To gain a thorough understanding of molecular interactions, the genes overlapping with flaxseed differentially expressed genes (DEGs) and OXT signaling pathways were subsequently analyzed. To validate the targeted genes, a PPI network for the OXT signaling pathway was constructed using STRING (version 11.5), with interaction confidence carefully filtered to a minimum score of 0.4 (Szklarczyk et al., 2023). Subsequently, network analysis was performed. Cytoscape (version 3.10.3) was utilized (Shannon et al., 2023), employing CytoHubba's Maximal Clique Centrality (MCC) algorithm to rank hub proteins; betweenness centrality was used to identify bottlenecks (threshold: Top 5 nodes), and functional analysis was conducted with the ClueGo plugin, utilizing a kappa score of 0.4. Furthermore, the key genes associated with OXT in the context of flaxseed were investigated within the network.

Results

The Venn diagram depicting gene expression induced by flaxseed from GSE36422 shows 98 significant genes linked to flaxseed, of which 56 were retained after data cleaning. The 56 genes were further analyzed. The data were assessed using Venn diagrams comparing Radiation–(flaxseed-radiation), control-radiation, flaxseed-control, and (flaxseed-radiation). The Venn diagram revealed that 98 DEGs were significantly different between the flaxseed and the control groups (Figure 1). Analysis using a volcano plot indicated a significant difference in gene expression profiles between samples fed a 10% flaxseed diet for three weeks (flaxseed group) and untreated samples. The box plot for the flaxseed-control comparison demonstrated that all gene expression profiles were median-centered and comparable. Likewise, the density plot exhibited a consistent pattern in the flaxseed-control analysis. After eliminating duplicate genes and uncharacterized entries, the 98 significant DEGs were reduced to 56, representing genes significantly influenced by the presence of flaxseed.

Pathway analysis identifies three significant genes: *CALM3* (calmodulin3), *NFATC4* (nuclear factor of activated T cells 4), and *RAF1* (Raf-1 Proto-Oncogene), which are prevalent in both the flaxseed and OXT signaling pathways (Figure 2 and Figure 3). KEGG pathway enrichment analysis revealed several pathways characterized by high counts and the most elevated logP values, including the C-type lectin receptor signaling pathway, the OXT signaling pathway, cellular senescence, and the cyclic guanosine monophosphate-activated protein kinase G (cGMP-PKG) signaling pathway. These genes are crucial across all pathways, with *CALM3* up-regulated (overexpressed) and *NFATC4* and *RAF1* down-regulated (underexpressed).

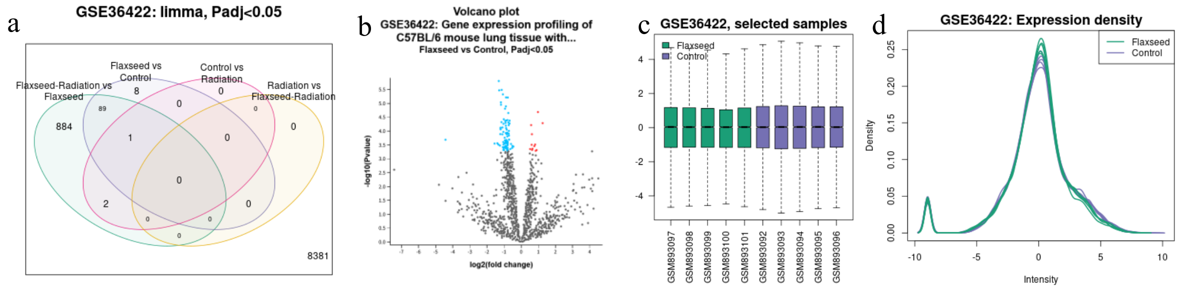

Figure 1. The flaxseed-induced gene expressions of GSE36422

a) Venn diagram for radiation – (flaxseed - radiation), control - radiation, flaxseed - control, and (flaxseed - radiation) - flaxseed analyses, b) Volcano plot of gene expression analysis of the flaxseed group versus samples without any treatment (control group), c) Box plot of flaxseed – control analysis, d) expression density for flaxseed – control analysis

GO analysis, depicted with various colored lines, illustrates distinct biological pathways, where the size of each node reflects the relative significance or connectivity of each gene within the network. *CALM3* is represented as a red node, indicating its involvement in multiple signaling pathways, such as cellular senescence, OXT signaling, C-type lectin receptor signaling, and GnRH signaling, and its associations with viral infection pathways (specifically HIV-1 and cytomegalovirus). Color coding indicates upregulation (red on the log2 fold change scale). *NFATC4*, depicted as a purple node within the network, is linked to pathways associated with long-term potentiation, viral infections, and GnRH signaling. This transcription factor is essential for immune response and cellular development. Color coding indicates downregulation (purple in the log2 fold change scale). *RAF1*, illustrated as a blue node, is significantly connected to various pathways: cGMP-PKG signaling, responses to viral infections, and long-term potentiation. The color coding also suggests it is downregulated (purple in the log2 fold change scale) (Figure 2). The genes related to the OXT signaling pathway included *CALM3*, *NFATC4*, and *RAF1*, with a p-adj value of 0.0017. *RAF1* is involved in the MAPK signaling pathway, *CALM3* regulates calcium channels, and *NFATC4* negatively regulates the OXT pathway (Figure 2). The precise positioning of each gene within the OXT signaling pathway is as follows: *RAF1* is integrated into the MAPK signaling pathway, activated

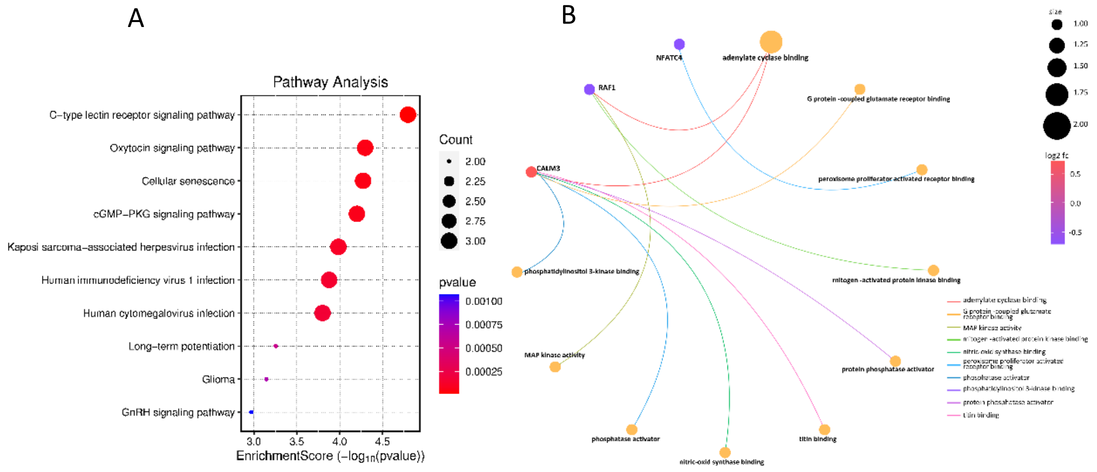

Figure 2. Functional enrichment analysis of 56 selected genes expressions of flaxseed

A) KEGG pathway enrichment analysis for flaxseed genes, B) GO enrichment analysis categorizes biological processes

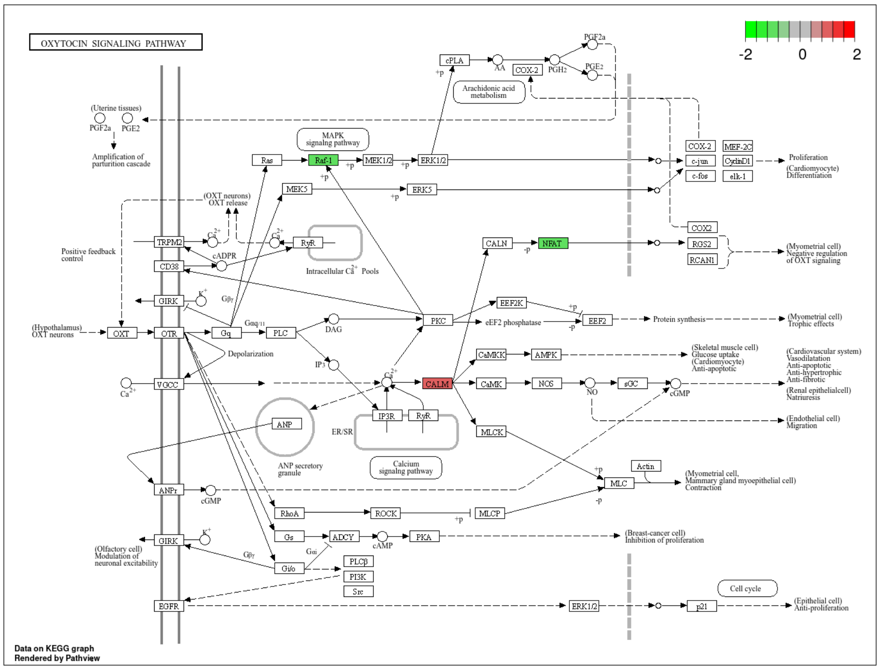

Figure 3. The KEGG graph of the OXT signaling pathway

downstream of rat sarcoma (Ras), which is stimulated by the activation of the OXT receptor. It phosphorylates and activates MEK1/2, which subsequently activates ERK1/2. The activation of ERK1/2 results in the transcriptional regulation of genes crucial for cardiomyocyte proliferation, differentiation, and survival. *RAF1* is vital in enhancing the parturition cascade (the process of uterine contraction during labor). *NFATC4* operates downstream of calcium signaling, activated by calcium-dependent signaling via calcineurin (CALN). Once dephosphorylated by calcineurin, *NFATC4* migrates to the nucleus to influence gene expression. It plays a role in the negative regulation of OXT signaling in myometrial cells by regulating genes, such as *RCAN1* and *RGS2*, that modulate OXT receptor signaling and calcium dynamics. *CALM3* plays a pivotal role in calcium signaling by binding to calcium ions, thereby functioning as a calcium sensor. Additionally, it activates downstream targets, such as calmodulin-dependent kinase and calcineurin (CALN). Through these interactions, *CALM3* regulates myosin light chain kinase, an essential regulator of muscle contraction in both myometrial and mammary gland cells. In summary, *RAF1* is instrumental in mediating MAPK signaling pathways that facilitate proliferation and differentiation. *NFATC4* regulates transcription in response to calcium signaling, thereby modulating OXT signaling. *CALM3* serves as a calcium sensor that governs various downstream pathways, including those involved in contraction and metabolism. These genes are vital for the physiological responses triggered by OXT, which encompass uterine contraction, cardiovascular effects, and cellular proliferation (Figure 3). A collection of 147 proteins associated with the OXT signaling pathway was assembled utilizing STRING and subsequently examined in Cytoscape. Summary statistics of the network: Number of nodes: 147, number of edges: 2172, average number of neighbors: 29.551, network density: 0.202, network heterogeneity: 0.644, network centralization: 0.406. Among these proteins, 86 (highlighted in red) were the primary neighbors of CALM3, and 91 of the 147 proteins were the primary neighbors of the three specified genes. The top five hub and bottleneck proteins were identified using the Cyto-Hubba plugin (Tables 1 and 2). Within the first neighbor nodes, there was one hub protein (denoted by a diamond shape) and five bottleneck proteins, including CALM3 (denoted by a rectangle shape) (Figure 4).

Discussion

Anxiety is recognized as one of the most prevalent mental disorders in society. In the context of Iranian traditional medicine literature, anxiety is perceived differently (Kaplan et al., 2007). The term "Khuf" refers to the unease concerning a potentially adverse situation that may occur in the future (Motovaseliyan et al., 2016). Avicenna identified flaxseed as a valuable therapeutic approach for managing anxiety (Avecinna, 2005). Flaxseed (*L. usitatissimum* L., Linaceae), rich in biologically active compounds, is recognized for its nutritional, physi-

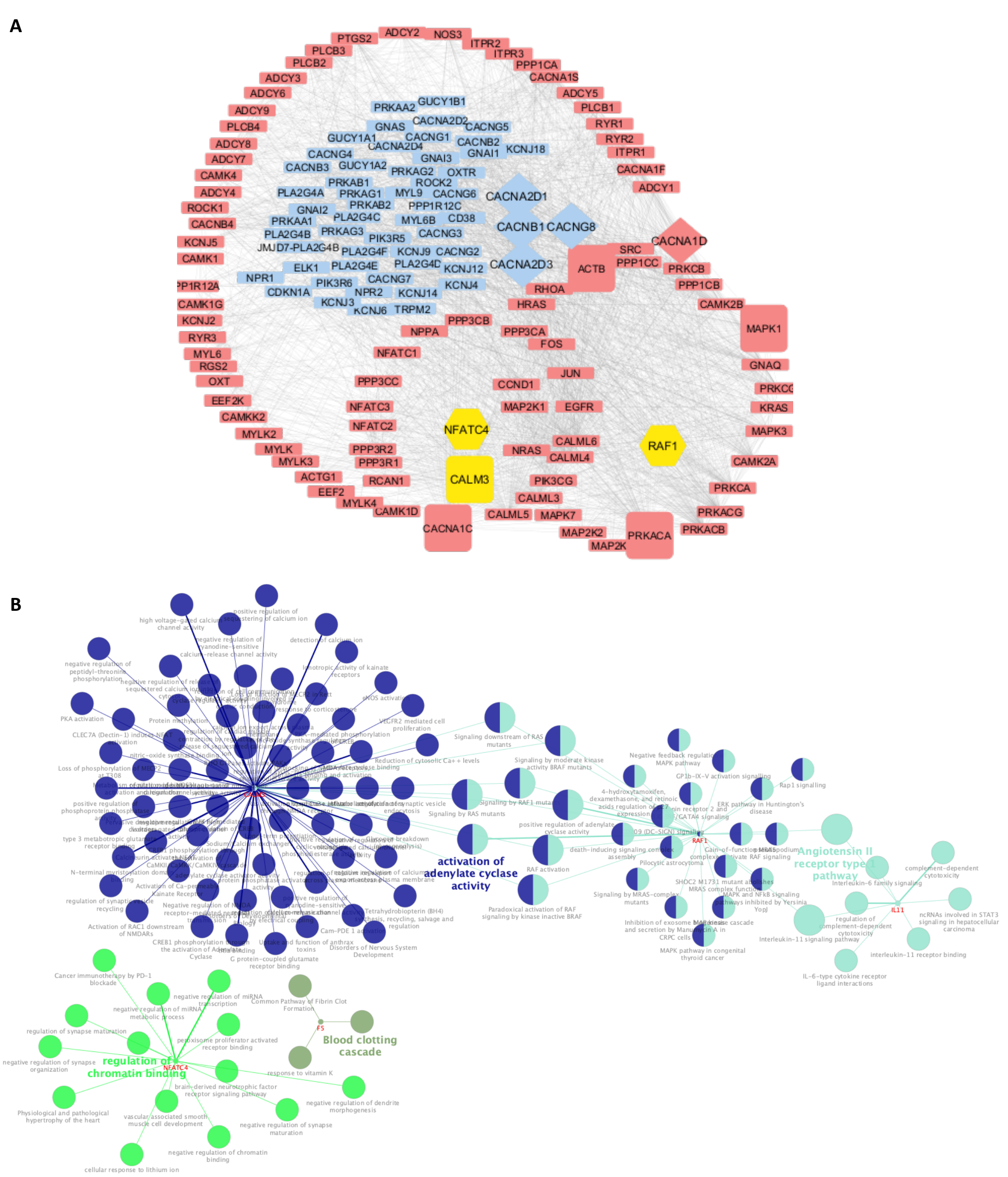

Figure 4. A) The PPIs network of the OXT pathway: The yellow nodes represent the common genes with flaxseed, the red nodes represent the first neighbors of CALM3, the hub nodes are in a diamond shape, and the bottlenecks are rectangular; B) This network illustrates the functional enrichment of biological pathways derived from genes modulated by flaxseed, constructed using the ClueGO plugin: Each node represents a significantly enriched pathway. Edges indicate gene overlap between pathways, highlighting potential mechanistic crosstalk. The green cluster, anchored by the core gene *NFATC4*, highlights pathways associated with the regulation of chromatin binding and modulation of synaptic signaling. The dark blue cluster, dominated by *CALM3*, centers on activating adenylate cyclase activity and calcium-mediated signal transduction. A light blue cluster, merging with dark blue elements and linked by *RAF1*, underscores the involvement of MAPK and RAS signaling cascades, pointing to flaxseed's potential influence on cell proliferation and survival mechanisms. The light cyan cluster, anchored by interleukin-11 (IL11), which also reflects involvement in inflammatory and cytokine-mediated pathways, including the IL11 signaling pathway and angiotensin II receptor type 1 pathway.

Table 1. The top 5 hub proteins ranked by the MCC algorithm

Rank	Name	Score	Betweenness Centrality	Degree
1	CACNA1D	5.40E + 22	0.600823045	54
2	CACNA2D1	5.40E + 22	0.530909091	33
3	CACNB1	5.40E + 22	0.528985507	31
3	CACNG8	5.40E + 22	0.525179856	30
3	CACNA2D3	5.40E + 22	0.506944444	29

Nte: Scores represent MCC values. Higher indicate greater hubness.

ological, and therapeutic properties (Sirotkin, 2023). Research conducted both in vivo and in vitro has provided evidence of hormonal regulation by flaxseed, including stimulation of OXT expression (Sirotkin, 2023; Tou et al., 1999). Lecová et al. (2024) found that pigs fed a diet enriched with 10% flaxseed showed increased OXT levels. Additionally, Vlčková et al. (2022) indicated that flaxseed has a positive impact on OXT 's physiological effects by offering a protective mechanism for OXT receptors in the presence of xylene. In this study, we illustrated the regulation of various biological pathways by flaxseed-induced DEGs, including the C-type lectin receptor signaling pathway, the OXT signaling pathway, cellular senescence, and the cGMP-PKG signaling pathway, as determined by functional enrichment analysis. The anti-anxiety properties of OXT are well-documented and extensively researched in medical sciences. Tang et al. (2024) demonstrated that the intranasal administration of OXT alleviated post-surgical anxiety in mice. Furthermore, Chaipunko et al. (2025) revealed that OXT can modulate the hypothalamic-pituitary-adrenal (HPA) axis and mitigate anxiety.

GO analysis identified three genes (*CALM3*, *RAF1*, *NFACT4*) among 56 flaxseed-induced DEGs as the most significant and relevant common genes linking flaxseed to the OXT pathway. Among these three pivotal genes, *CALM3* stands out as the most crucial, exhibiting distinct characteristics and regulatory behaviors. In contrast to its counterparts, *RAF1* and *NFATC4*, which were downregulated, *CALM3* was upregulated, and its remarkable position within the network designates it as a vital mediator in this molecular cascade. This interpretation is further reinforced by *NFATC4*'s established role as a negative regulator of the OXT pathway. Consequently, its downregulation is anticipated to lead to diminished inhibition and enhanced OXT signaling. The network analysis further highlighted *CALM3* as a bottleneck, sustaining first-neighbor interactions with five other essential proteins (four bottleneck proteins and one hub protein). Furthermore, it exhibited remarkable connectivity with 85 first neighbors, accounting for 58% of all proteins involved in the OXT signaling pathway. This approach implies that *CALM3* functions as a master regulator within the network, orchestrating various signaling cascades and ensuring proper information flow throughout the system. The significant impact of *CALM3* within the network strongly supports its position as the primary molecular mediator, through which flaxseed may exert its anxiolytic effects via the OXT pathway. OXT, a neuropeptide that plays a role in social bonding, reproductive functions, and stress responses, primarily exerts its effects by

Table 2. The top 5 bottleneck proteins ranked by betweenness centrality

Rank	Name	Score	Betweenness Centrality	Degree
1	CALM3	36	0.701923077	85
2	MAPK1	19	0.591093117	55
3	CACNA1C	16	0.610878661	57
4	PRKACA	15	0.712195122	88
5	ACTB	10	0.577075099	45

Note: Scores reflect betweenness values; higher scores show higher bottleneck influence.

activating its receptors, thereby increasing intracellular calcium levels. The rise in calcium activates *CALM3*, which facilitates downstream signaling processes, such as the activation of the MAPK pathway and the RhoA/Rho kinase pathway, both of which are associated with smooth muscle contraction and other physiological responses to OXT (Bakos et al., 2018; Munk et al., 2022; Uvnaes Moberg et al., 2022). Moreover, *CALM3* may also affect cardiovascular functions regulated by OXT, underscoring its potential role in integrating calcium signaling with OXT 's effects on the cardiovascular system (Ayar et al., 2014; Hirasawa et al., 2001; Rabow et al., 2023). Therefore, *CALM3* acts as a vital link between calcium signaling and the OXT 's physiological effects. Additionally, it offers potentially significant insights for therapeutic strategies in anxiety management through dietary interventions.

Conclusion

This study clarifies a new mechanism by which flaxseed influences anxiety via the OXT pathway. In silico analysis identifies three common genes associated with both anxiety and flaxseed. These genes are crucial in the OXT signaling pathway, with *CALM3* identified as a primary calcium-sensing regulator. Simultaneously, the downregulation of *NFATC4* and *RAF1* further facilitates the disinhibition of OXT signaling and the modulation of the MAPK cascade. These results not only affirm the traditional applications of flaxseed but also underscore its potential as a dietary supplement for anxiety disorders. Future investigations should confirm these mechanisms through clinical trials and examine the synergistic effects of conventional treatments.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Supervision: Mostafa Rezaei Tavirani and Maryam Hamzeloo-Moghadam; Methodology: Hajar Sadat Sadeghi and Melika Abrishami; Data collection: Hajar Sadat Sadeghi Sadeghi and Melika Abrishami; Data analysis: Hajar Sadat Sadeghi Sadeghi and Melika Abrishami; Investigation and writing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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