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System-Level Biological Evaluation of Flaxseed Intake on Sleep Regulation

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ABSTRACT

Flaxseed (Linum usitatissimum L.) is a valuable functional food known for its rich content of omega-3 fatty acids, lignans, α-linolenic acid, and high-quality proteins. The primary goal of this research was to explore the potential sleep-enhancing effects of flaxseed through network-based analysis. Differentially expressed genes (DEGs) associated with flaxseed intake were obtained from the GSE36422 dataset in the Gene Expression Omnibus (GEO) repository. Genes linked to sleep were retrieved from the GeneCards database and subjected to undirected protein-protein interaction (PPI) network evaluation to identify central hub genes. Overlapping DEGs and hub genes were identified as key targets influenced by flaxseed. These hub genes were further examined using directed PPI networks to determine the roles of overlapping genes and their immediate interactors. Among 212 hubs related to sleep, RAF1 emerged as a prominent hub gene. Evaluation of sleep-associated hubs revealed that MAPK family members, CCND1, KRAS, RAF1, PIK3CA, and EGFR acted as major regulators, while HRAS, AKT1, IL6, RHOA, TNF, SRC, FOXO3, CDC42, PRKACG, PRKACB, MAPK8, and JAK2 were key downstream targets. The MAPK family, KRAS, and PIK3CA were highlighted as essential direct interactors of RAF1. Results suggest that RAF1, a central sleep-related hub gene, is downregulated following flaxseed intake. This downregulation of RAF1, along with modulation of MAPK family members, KRAS, and PIK3CA, may contribute to enhanced sleep quality.

Keywords: Flaxseed, Gene expression, Protein, Sleep

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Introduction

Flaxseed (Linum usitatissimum L.) is a widely recognized oilseed crop frequently utilized in the food sector. It is abundant in omega-3 fatty acids, lignans, α-linolenic acid, and easily digestible proteins [1]. Research has shown that flaxseed oil serves as an excellent source of unsaturated fatty acids with significant functional and health benefits [2]. Evidence from studies indicates that compounds in flaxseed may offer complementary therapy or preventive effects against myocardial infarction [3]. Flaxseed exhibits multiple beneficial activities, including anti-inflammatory, antioxidant, laxative, estrogen-like, and antimicrobial effects. Its consumption has been linked to protection against conditions involving oxidative stress, cardiovascular issues, cystic fibrosis, neurodegenerative diseases, dry eye syndrome, diabetes, irritable bowel syndrome, obesity, liver and kidney disorders, polycystic ovary syndrome, osteoporosis, postmenopausal symptoms, and various cancers [4]. Notably, flaxseed is rich in ω-3 fatty acids and tryptophan, compounds that elevate serotonin levels—a key regulator of sleep—and this has drawn interest for managing insomnia [5].

Analyzing gene expression profiles is a standard approach for uncovering molecular mechanisms underlying biological processes and disorders. This technique has been applied to examine the effects of various plant-based compounds. For instance, Morshedzadeh *et al*. used gene expression profiling to demonstrate reduced systemic inflammation and symptom severity in ulcerative colitis patients treated with ground flaxseed or flaxseed oil [6].

Genomics, as a comprehensive high-throughput tool, enables the identification of numerous differentially expressed genes. Combining herbal remedies with genomic approaches has addressed challenges in medical research and drug development [7, 8]. Given the intricate nature of genomic data, bioinformatics and computational tools are essential for interpreting results effectively [9].

PPI network evaluation provides an effective interactive framework for genomic data analysis. Rostami-Nejad *et al*. applied PPI networks to investigate the anticancer and neuroprotective effects of curcumin [10]. Similarly, Arjmand *et al*. elucidated the anti-stress mechanisms of saffron using this method [11]. In this investigation, the impact of flaxseed intake on sleep enhancement was examined through PPI network analysis. Data sourced from the GEO database were analyzed using both undirected and directed PPI networks to pinpoint essential genes targeted by flaxseed.

Materials and Methods

Ethical considerations

The study received approval from the Ethics Committee of Shahid Beheshti University of Medical Sciences under code IR.SBMU.RETECH.REC.1403.526.

Data collection

To evaluate flaxseed's influence on sleep, gene expression data from mouse lung tissue were accessed from the GEO database. Profiles comparing flaxseed-supplemented and control groups were obtained from GSE36422 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36422>). The control samples followed a semi-purified AIN-93G diet, whereas the flaxseed group received the same diet with an additional 10% (w/w) flaxseed for three weeks. Significant DEGs between groups were identified using the GEO2R tool. GeneCards, a comprehensive human gene resource, offers detailed insights into genes, diseases, variants, proteins, cells, and pathways [12]. Sleep-associated genes were queried and compiled from GeneCards. Overlaps between significant DEGs and sleep-related genes were then examined to highlight shared elements.

Undirected PPI network evaluation

To identify key genes associated with sleep, the sleep-related genes retrieved from GeneCards were incorporated into an undirected protein-protein interaction (PPI) network using Cytoscape software. The network was built with a stringent confidence score threshold of 0.9. Network properties were examined through the “Network Analyzer” plugin in Cytoscape to pinpoint central nodes. Hub genes were defined using a threshold of mean degree plus two standard deviations. Genes shared between the significant differentially expressed genes (DEGs) and these hub genes were designated as primary targets of flaxseed that play a role in sleep regulation.

Directed PPI network evaluation

The identified hub genes were further investigated in a directed PPI network to determine the immediate interactors of the overlapping genes. These hubs were integrated into a directed regulatory network using the CluePedia plugin in Cytoscape, incorporating actions such as expression, activation, and inhibition. The overlapping genes along with their direct neighbors were isolated from the network for detailed examination. To assess the most influential nodes, the largest connected component of this directed network was evaluated using edge directionality via the “NetworkAnalyzer” tool in Cytoscape. The network layout was organized according to out-degree and in-degree parameters.

Statistical analysis

Differentially expressed genes were deemed significant if their adjusted p-value was less than 0.05. The undirected PPI network was generated with a confidence score of 0.9. Hub genes were selected based on a cutoff of mean degree plus two standard deviations.

Results and Discussion

A total of 56 significant DEGs were found to distinguish the flaxseed-supplemented group from the control group. From the GeneCards database, 9964 genes linked to sleep were obtained. Overlap analysis revealed three shared

genes—CALM3, F5, and RAF1—between the 56 flaxseed-associated DEGs and the 9964 sleep-related genes. Of these 9964 sleep genes, Cytoscape recognized 7105 for further processing, while 2859 were excluded due to insufficient data. The complete undirected PPI network, encompassing isolated nodes and sub-networks, is illustrated in **Figure 1.** As shown in **Figure 1**, a large number of nodes (2550 genes) remained isolated owing to absence of documented interactions. For the primary connected component, the mean degree was 14 with a standard deviation of 21, establishing a hub cutoff of 56 (mean + 2×SD). This identified 212 hub genes (those with degree >56). Notably, RAF1 was the only gene common to both the 56 flaxseed DEGs and these 212 sleep-related hubs.

To explore interconnections involving RAF1 and the broader hub set, a directed regulatory PPI network was constructed for the hubs. The largest connected component of this regulatory network is displayed in **Figure 2**, with edges representing activation, inhibition, and expression interactions. To emphasize RAF1's centrality, its direct interactors were extracted from this component **(Figure 3)**. **Figure 3** reveals intricate regulatory links to RAF1 through both activation and inhibition. Accordingly, the subnetwork was separated into two distinct clusters: one dominated by activation edges and the other by inhibition edges **(Figures 4 and 5).**

To underscore the influence of specific nodes in the regulatory network, the main connected component was visualized based on out-degree and in-degree centrality measures **(Figures 6 and 7).** Nodes with elevated out-degree were classified as primary regulators (actors), while those with high in-degree were deemed key targets (controlled genes). Node size and color in **Figures 6 and 7** reflect these centrality values. The principal actor genes include members of the MAPK family, CCND1, KRAS, RAF1, PIK3CA, and EGFR **(Figure 6).** In contrast, the main controlled genes comprise HRAS, AKT1, IL6, RHOA, TNF, SRC, FOXO3, CDC42, PRKACG, PRKACB, MAPK8, and JAK2 **(Figure 7).** These actor and controlled genes are summarized in **Table 1.**

Flaxseed, regarded as a nutrient-rich “superfood,” contributes to disease prevention, particularly those linked to diet. Its consumption has been associated with reduced blood pressure, improved lipid profiles, better insulin sensitivity, and favorable effects on fasting glucose levels [13].

Table 1. The critical actor and controlled genes of main connected component of the directed PPI network of the hub genes

No.	Actor gene	No.	Controlled gene
1	MAPK1	1	HRAS
2	MAPK3	2	AKT1
3	MAPK14	3	IL6
4	MAPK11	4	RHOA
5	CCND1	5	TNF
6	KRAS	6	SRC
7	RAF1	7	FOXO3
8	PIK3CA	8	CDC42
9	EGFR	9	PRKACG
-	-	10	PRKACB
-	-	11	MAPK8
-	-	12	JAK2

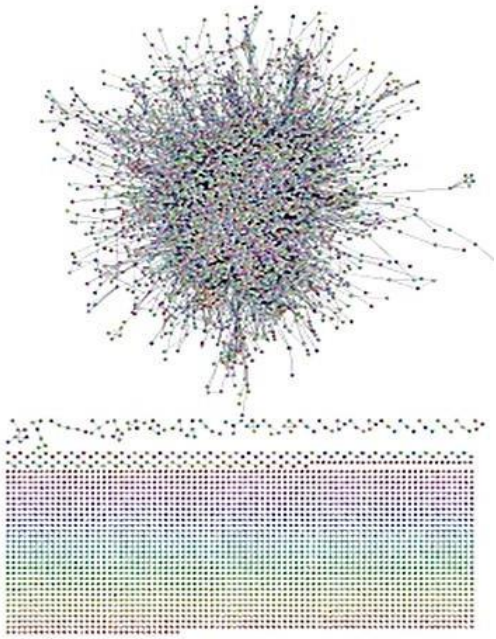

Figure 1. Protein-protein interaction (PPI) network constructed from sleep-associated genes, comprising 7105 nodes and 36,399 edges (built with a confidence score threshold of 0.9). Notably, 2550 genes lacked documented interactions and thus appeared as isolated nodes.

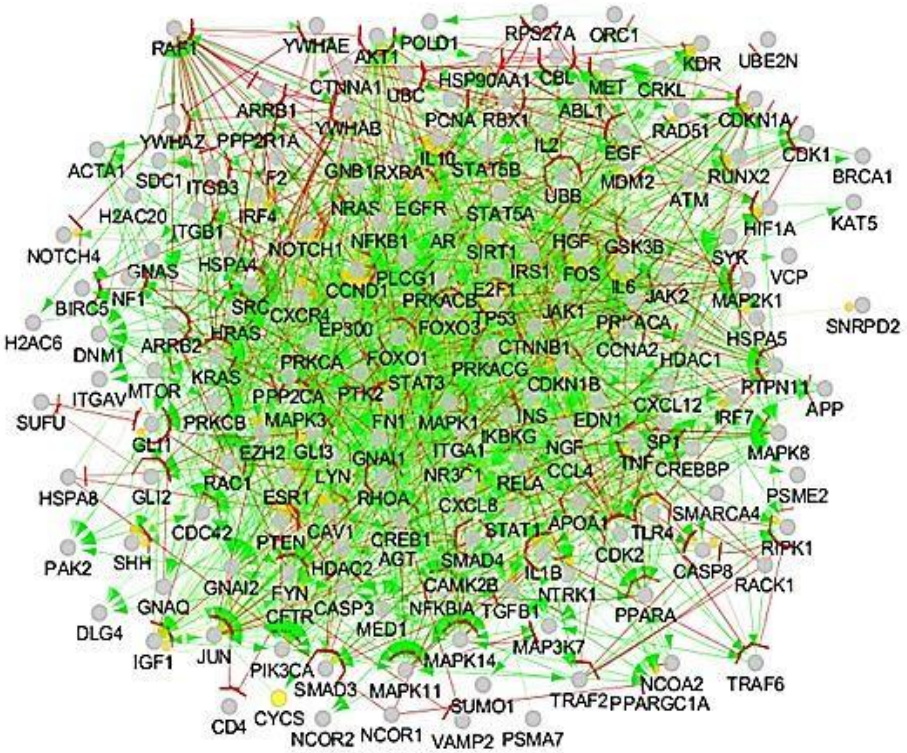

Figure 2. Primary connected component of the directed regulatory network involving the hub differentially expressed genes (DEGs). Nodes colored green, red, and yellow indicate activation, inhibition, and expression interactions, respectively.

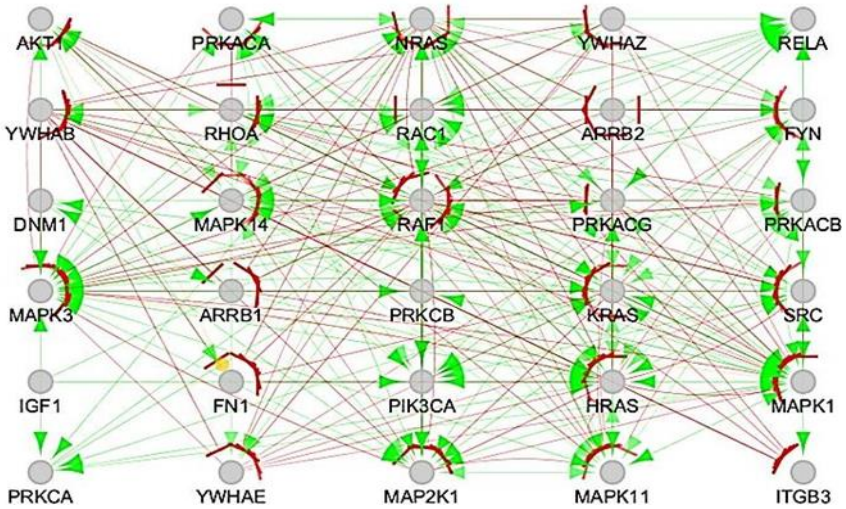

Figure 3. The directed regulatory network centered on RAF1 and its immediate neighbors, with green and red indicating expression, activation, and inhibition regulatory interactions.

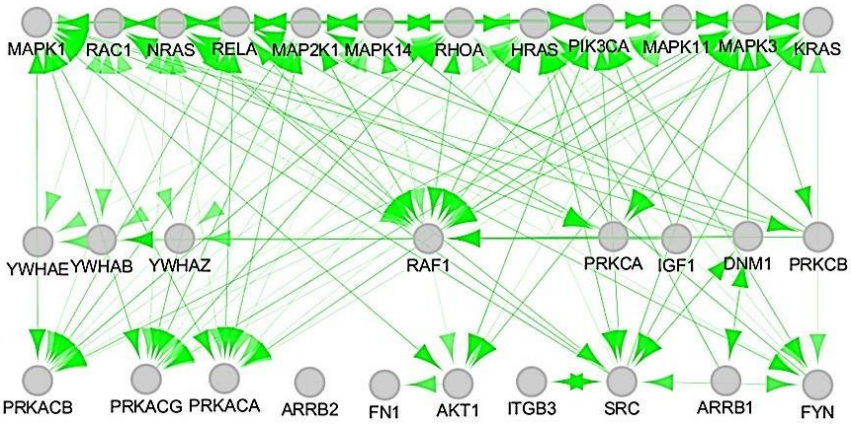

Figure 4. Directed regulatory network highlighting the activation interactions involving RAF1 and its immediate neighbors. RAF1 exhibits bidirectional (reciprocal) activation relationships with the genes positioned above it, whereas the genes aligned below lack mutual regulatory effects with RAF1.

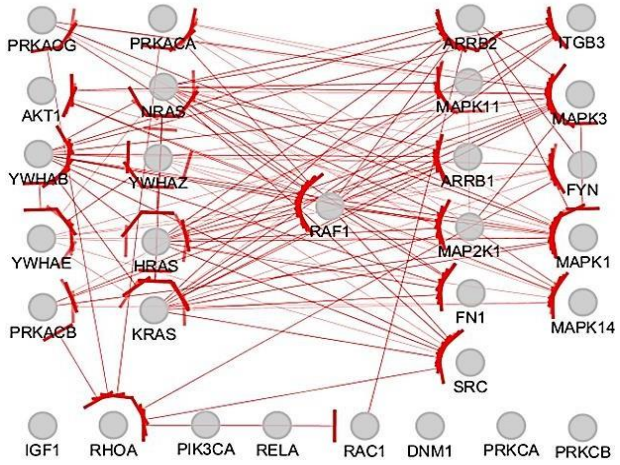

Figure 5. Directed regulatory network illustrating the inhibition interactions involving RAF1 and its immediate neighbors. The two columns on the right represent genes inhibited by RAF1; the two columns on the left depict genes that engage in bidirectional (mutual) inhibition with RAF1; and the genes aligned below have no inhibitory relationship with RAF1 (these genes are included as first neighbors through activation or expression interactions).

In the current investigation, the potential role of flaxseed consumption in ameliorating sleep disorders was explored. Analyses revealed that flaxseed targets sleep-related genes, namely CALM3, F5, and RAF1. A total of 9,964 genes were identified as associated with sleep. To determine the significance of these three genes within the broader set of 9,964 sleep-related genes, 212 hub genes were identified as key players in sleep regulation. Results demonstrated that RAF1 functions as a hub gene. Hub genes are widely regarded as pivotal in elucidating the molecular mechanisms underlying diseases or specific physiological conditions in numerous studies [14-16]. Further refinement was performed to narrow down the hub gene list. A directed protein–protein interaction (PPI) network incorporating the hub genes was built, utilizing expression, activation, and inhibition interactions. Two primary objectives were pursued: identification of RAF1's immediate neighbors and detection of the most central (critical) hubs. Employing directed PPI networks represents an effective approach for evaluating and prioritizing hub genes to pinpoint the most essential ones [17]. As shown in **Figure 3**, 29 hub genes were identified as first neighbors of RAF1. Additional examination indicated that these first neighbors connect to RAF1 exclusively through activation and inhibition interactions, whereas expression interactions serve as the links among the first neighbors themselves.

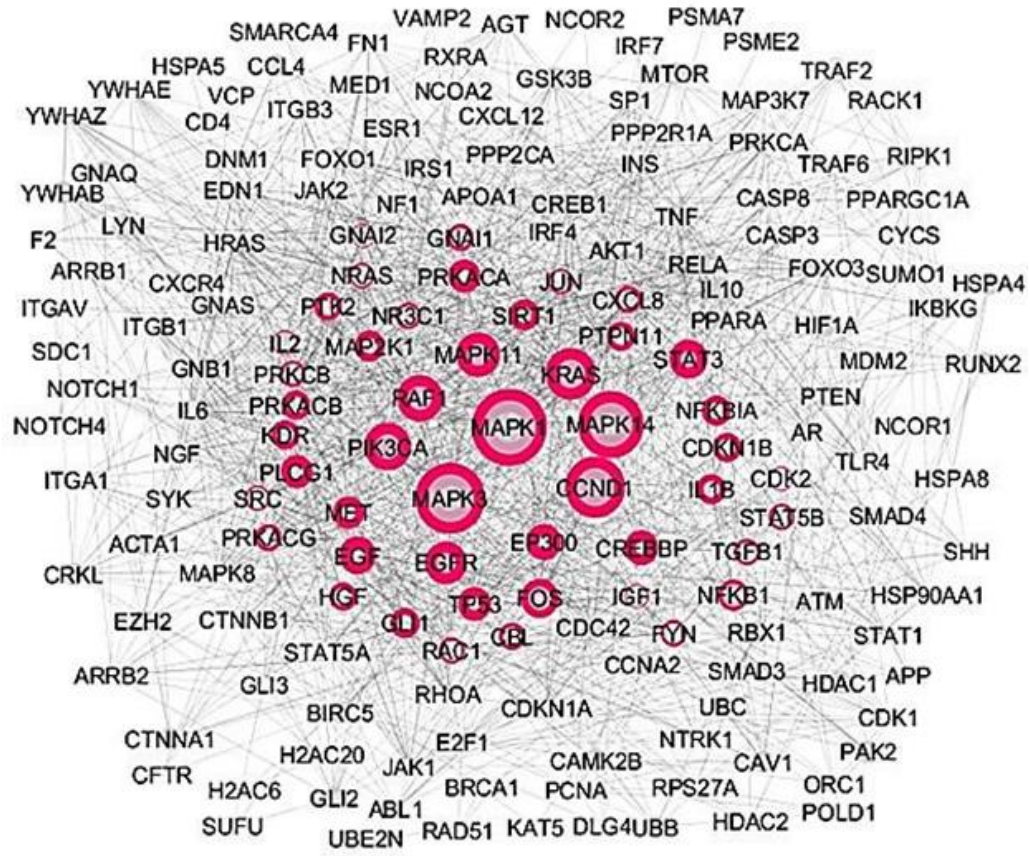

Figure 6. Primary connected component of the directed regulatory network comprising the hub differentially expressed genes (DEGs). Node size is proportional to the out-degree value, with larger nodes indicating higher out-degrees.

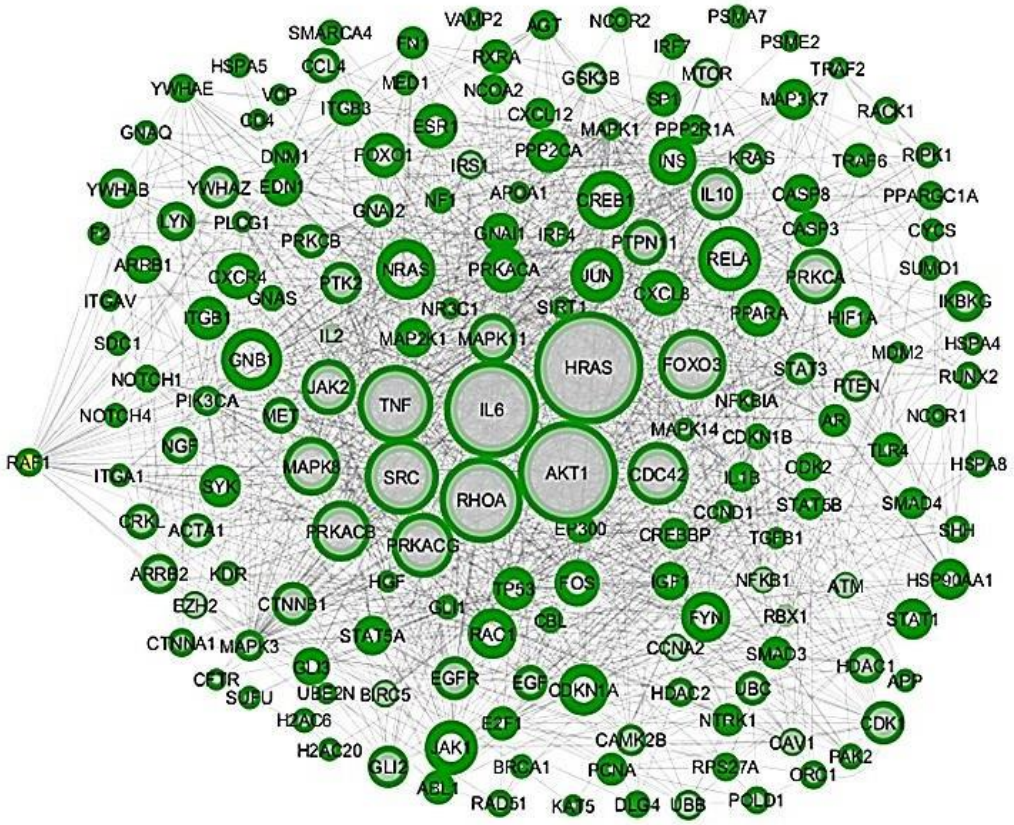

Figure 7. Principal connected subnetwork of the directed regulatory network of hub DEGs, with larger nodes representing higher in-degree values; the location of RAF1 is indicated on the left side of the figure.

According to **Figure 4**, the principal regulators influencing the immediate neighbors of RAF1 through activation mechanisms include MAPK1, RAC1, NRAS, RELA, MAP2K1, MAPK14, RHOA, HRAS, PIK3CA, MAPK11, MAPK3, and KRAS. In contrast, the key immediate neighbors exerting inhibitory effects on RAF1 encompass PRKACG, PRKACA, AKT1, NRAS, YWHAB, YWHAZ, YWHAE, HRAS, PRKACB, and KRAS, as shown in **Figure 5.** Overall, 19 genes—namely MAPK1, RAC1, NRAS, RELA, MAP2K1, MAPK14, RHOA, HRAS, PIK3CA, MAPK11, MAPK3, KRAS, PRKACG, PRKACA, AKT1, YWHAB, YWHAZ, YWHAE, and PRKACB—emerged as pivotal regulators and regulated targets among the direct neighbors of RAF1. When comparing these essential direct interactors of RAF1 with the identified hub genes, members of the MAPK family, KRAS, and PIK3CA stand out as vital regulators, whereas HRAS, AKT1, RHOA, PRKACG, PRKACB, and MAPK8 appear as the most prominently regulated genes among RAF1's immediate neighbors. The activation of MAPK by RAF1 has previously been documented [18]. Additionally, research by Wang *et al*. has highlighted the influence of the MAPK pathway on circadian gene regulation [19]. It appears that RAF1 exerts its effects primarily through regulatory cascades linked to MAPK, KRAS, and PIK3CA activity.

RAF1 (also known as C-RAF) belongs to the family of RAF protein kinases [20]. In this study, RAF1 exhibited downregulation in response to flaxseed intake. Existing literature indicates that the RAF/MEK/ERK signaling pathway plays a complex role in transmitting signals from the cell membrane to the nucleus, thereby promoting cell cycle progression, gene transcription, and apoptosis [21]. Given RAF's prominent involvement in cancer development, inhibiting the RAF/MEK/ERK cascade with RAF-targeted agents represents an established therapeutic strategy for cancer [21-23]. In a study by Zhang *et al*., downregulation of RAF1 was linked to amelioration of insomnia in a rat model [24]. The insomnia model was induced by administering p-chlorophenylalanine (PCPA) suspension, and acupuncture at the Back-Shu point resulted in enhanced appetite, better learning and memory performance, extended sleep duration, improved overall mental condition, and normalized proinflammatory cytokine levels. The mechanism involved suppression of the ERK/NF-κB pathway, leading to reduced expression of RAF-1, MEK-2, ERK1/2, and NF-κB, ultimately alleviating insomnia-related symptoms.

In the current research, it was proposed that the direct neighbors of RAF1 within the network of sleep-related hub genes, modulated by flaxseed consumption, contribute substantially to insomnia relief. Mitogen-activated protein kinases (MAPKs) are enzymes that govern critical cellular processes, including motility, differentiation, proliferation, and survival [25]. Evidence shows that MAPK participates in mechanisms underlying hippocampus-dependent memory consolidation, with elevated activity observed during the rapid eye movement sleep phase [26].

As reported in prior studies, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), KRAS, and BRAF activate both the PI3K-PTEN-AKT pathway and the RAS-RAF-MAPK pathway, which are essential for regulating cell motility, proliferation, and survival [27]. Thus, the regulatory immediate neighbors of RAF1 are implicated in controlling fundamental cellular processes.

Chronic sleep deprivation is linked to increased DNA damage and elevated cancer risk, while disrupted circadian rhythms correlate with dysregulated KRAS signaling, further promoting tumorigenesis. Consequently, insomnia and fatigue may serve as early markers of cancer development [28].

Conclusion

In summary, intake of flaxseed correlates with reduced expression of the RAF1 gene, a central hub among sleep-related genes. Lowering RAF1 levels may alleviate insomnia. Network analyses revealed that RAF1, together with MAPK family members, KRAS, and PIK3CA, represents key sleep-associated genes directly or indirectly influenced by flaxseed consumption. These genes are implicated in major physiological and pathological processes, particularly carcinogenesis.

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Conflict of Interest: None

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Ethics Statement: None

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