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Research Paper

In Vitro Evaluation of Antioxidant Activity using DPPH, ABTS, and FRAP Assays: Insights from Network Pharmacology Analysis

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Abstract
Vascular dementia (VaD), the second most common type of dementia after Alzheimer disease, results from cumulative cerebrovascular pathology that leads to cognitive impairment. None of the existing therapies cure the underlying neurovascular pathology and provide only symptomatic relief from oxygen and/or nutrients deprivation. It investigated if the multitarget phytochemical, emodin and the dual endothelin receptor antagonist bosentan could be combined to attenuate major pathogenic mechanisms of VaD by in vitro antioxidant assays and network pharmacology. DPPH, ABTS, and FRAP tests evaluated antioxidant ability showing that Emodin exhibited concentration-dependent radical scavenging and reduction activities. The IC_{50} of Emodin was 10.12 µg/mL (DPPH), 14.67 µg/mL (ABTS), and 13.24 µg/mL (FRAP), which represented comparable values with that for Ascorbic Acid, albeit presenting superior maximal efficiency by the latter compound. The results of the study showed that Emodin and bosentan has a significant ability to scavenge free radicals. Network pharmacology analysis was performed to clarify the mechanism of action in this study. We identified nineteen validated targets related to VaD, and several central nodes including TP53, TNF, VEGFA EGFR, MAPK1 and MAPK14 mediating cellular oxidative stress-induced apoptosis angiogenesis and inflammatory signaling. TNF and TP53 are the two important core regulators of PPI network constructed by protein-protein interaction (PPI) network. Gene ontology and KEGG pathway enrichment analysis show the genes that are highly statistically significant with MAPK MAP signaling, TNF signaling in the pathogenesis of vascular dementia,cytokine receptor interaction and VEGF-mediated pathways were found to be more important than all other pathways. Conclusion: The data support the potential importance of Emodin and bosentan with respect to very prominent antioxidant action and multitarget neuroprotective activities, reinforcing its position as a strong candidate for use in Vascular dementia treatments. Integration of experimental testing with computational predictions provides a system-level perspective of the processes.

Keywords: *Emodin, Ascorbic Acid, Vascular Dementia, Antioxidant Activity, Network Pharmacology, Protein–Protein Interaction*

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I. Introduction

Vascular dementia (VaD) is the second most frequent type of dementia after Alzheimer's disease, accounting for about 15–20% of worldwide dementia cases. The disease is characterized by a progressive decline in cognitive function resulting from reduced blood flow to the brain, frequently caused by cerebrovascular pathology such as ischemic stroke, long-standing high blood pressure, small vessel disease or

trauma related to hypoperfusion [1]. Longer life spans and lifestyle-related risk factors such as diabetes, hyperlipidemia and atherosclerosis have led to an increase of VaD incidence throughout the world [2]. In this respect, Alzheimer's disease (AD) has been principally associated with the accumulation of amyloid-beta and tau pathology while not being limited to vascular dementia (VaD), a disease state that is caused by an overlap in cumulative vascular injuries leading to degradation of neural networks resulting in cognitive decline [3], [4]. Despite its public health significance, the treatment of VaD is limited by the fact that only symptomatic relief has been developed so far, as there are currently no medications to halt or reverse the nature of disease [5], [6].

Vascular dementia (VaD) is a heterogeneous entity associated with quite complex events including oxidative stress, neuroinflammation, endothelial dysfunction, apoptosis and vascular remodeling. Oxidative stress is one of the main causes that disrupts cellular homeostasis and leads to further vascular and neural injury [7]. However, the generation of reactive oxygen species (ROS) during ischemia and/or hypoperfusion and in the presence of malfunctioning mitochondria can produce a plethora of direct damaging reactions manifesting as lipid peroxidation, protein oxidation and nucleic acid initiation which contribute to poor cellular health [8]. In addition, this oxidation imbalance also activates downstream inflammatory and vascular dysfunction pathways such as MAPK, TNF, NF-κB and PI3K-Akt apoptosis.Therefore, there is an urgent need to establish treatment strategies targeting restoration of redox homeostasis and perturbing multiple molecular processes in order to manage VaD [9].

In the past few years, much attention has been given to phytochemicals as multitarget therapeutic agents for neurodegenerative disorders [10], [11]. Emodin, a naturally occurring anthraquinone derivative present in many medicinal plants such as Rheum palmatum and Polygonum cuspidatum, has exhibited several significant pharmacological effects [12]. Emodin is generally recognized for its neuroprotective, anti-inflammatory, anti-apoptotic, and antioxidant effects [29]. Within the field of neurodegeneration, emodin is capable of free radical scavenging, modulating mitochondrial function and changing signaling pathways such as MAPK and NF-κB [13]. Moreover, emodin may cross the blood-brain barrier (BBB) which represents potential therapeutic applications in CNS disorders. However, similar to its diverse pharmacological profile, the specific molecular targets and signaling pathways underling emodin in vascular dementia are largely unexplored [14].

Bosentan is a dual endothelin receptor antagonist that was originally approved for pulmonary arterial hypertension, but more recently has received interest for its potential neurovascular benefit. Endothelin-1 (ET-1), a potent vasoconstrictor peptide, is involved in cerebrovascular dysfunction and induces vasospasm, reduced cerebral blood flow and increased oxidative stress [15], [16]. Both the endothelin-1 (ET-1) and ET receptors (EDNRA and EDNRB) have been shown to be over-expressed in VaD pathophysiology, positioning the entire endothelin system as a key therapeutic target [17]. Bosentan is an endothelin receptor antagonist, restoring lost vascular tone, improving endothelial function and cerebral perfusion [18]. Although research assessing the role of bosentan in dementia is limited, albeit promising, the vascular-selective mechanism may complement multitarget medicines such as emodin and potentially create a synergistic relationship [19].

According to the theory of Network pharmacology, which is a good way to explore the therapeutic potential and mechanism of emodin or bosentan for vascular dementia [20]. Unlike conventional pharmacology, which focus on a specific target or targets, network pharmacology combines the power of systems biology, bioinformatics analysis and pharmacological data allowing identification of links across drugs, targets and disease processes [21]. This approach is especially important for complex disorders like VaD, as the polypharmacological properties of multiple compounds can impact the signaling pathways [22]. Thus, network pharmacology could link some critical hub genes and pathways regulated by emodin and bosentan [23, 24]. Such observations provide a molecular rationale for their clinical efficacy, as well as could infer synergistic interactions in combination [25].

Thus, this is the first study to assess in vitro antioxidant capacity of emodin by conducting experimental assays (DPPH [24], ABTS and FRAP assays) for comparison with a proven antioxidant like ascorbic acid [26]. Against this backdrop, the tests were conducted to assess the free radical scavenging and reducing potential of emodin for supporting its involvement in oxidative stress amelioration in vascular dementia [27]. We found that the positions of these active ingredients in network pharmacology analysis indicated the overlap and unique vascular dementia targets between bosentan and emodin. The predicted gene-target interactions were fused with heterodimeric protein-protein interaction networks to identify the most relevant hub proteins, then submitted for Gene Ontology (GO) enrichment analysis to highlight the signaling pathways and biological processes that are affected [28]. The experimental validation guided computational modeling, so that the system behavior could be translated into potential for molecular therapy [29].

This study integrates the in vitro antioxidant activity with network pharmacology predictions of individual and combined use of emodin and bosentan for vascular dementia therapy. These data emphasize the multitargeted neuroprotective potential of emodin, and the vascular selective effects of bosentan necessitating subsequent preclinical and clinical trials [30]. This kind of comprehensive therapeutic approach, because of the complex heterogeneous and non-homogeneous nature of vascular dimension pathology, might be useful for developing more effective strategies.

II. Materials and Methods

2.1 Chemicals and Reagents

Ascorbic acid (analytical grade, Sigma-Aldrich) and emodin (purity≥98%, Sigma-Aldrich) were chosen for the experimental studies because of their reported neuroprotective and antioxidative effects. Materials Merck India supplied 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), potassium persulfate, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric chloride ($FeCl_3$) and all analytical-grade solvents. The deionized water was used to conduct the trials.

2.2 Preparation of Compound

The stock solutions of emodin and ascorbic acid were carried out using 1 mg/mL concentrations in methanol. New working solutions were prepared at the time of each experiment by serial dilution to yield concentrations between 10 and 200 µg/mL. All solutions were maintained at 4°C in amber vials to prevent photodegradation [31].

2.3 In Vitro Antioxidant Assays

2.3.1 DPPH Radical Scavenging Assay

The Blois method was employed with minor modifications to determine the activity of DPPH radical 1ml of a 0.1 mM DPPH solution in methanol was mixed with 1ml of several concentrations of ascorbic acid and emodin respectively. Absorbance at 517nm was determined using a UV-Vis spectrophotometer after dark incubating for 30min at room temperature [32]. The % inhibition was determined using the formula

$$\%\ inhibition = \frac{(A\_control\ -\ A\_sample)}{A\_control} \times 100$$

2.3.2 ABTS Radical Assay

The ABTS radical cation (ABT •+) was produced by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate and leaving the mixture overnight at room temperature in the dark. The absorbance measured at 734 nm after dilution of the $ABTS^{\bullet+}$ solution with methanol was 0.70 ± 0.02. 1ml of diluted $ABTS^{\bullet+}$ solution was mixed with 1ml of the test solution that included different concentrations of either ascorbic acid or emodin for the assay. After 6 minutes of incubation, absorbance at 734 nm was recorded[33].

2.3.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out accordingto the method previously described by Benzie and Strain. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), a 10 mM TPTZ solution in 40 mM HCl, and a 20 mM $FeCl_3$ solution at a ratio of 10:1:1. One hundred microliters of each test solution were expanded in three milliliters of FRAP reagent and kept under 37 °C for thirty minutes; thereafter, the absorbance was performed at 593 nm and determined as µmol Fe2 equivalents per gram material by a calibration curve constructed with ferrous sulfate [34].

2.4 Network Pharmacology Analysis

2.4.1 Target Prediction and Database Screening

Potential protein targets for Emodin and Ascorbic Acid were identified by SwissADME and PharmMapper, while targets associated with vascular dementia were sourced from the GeneCards and DisGeNET databases using "vascular dementia" as the search term. Overlapping targets between the chemicals and the illness were found to provide an initial list of potential therapeutic targets[35].

2.4.2 Construction of Compound–Target Network

To build the network of compound-target interactions, the common compound-disease targets were imported into Cytoscape (v3.10.3). To identify key nodes that might have a major impact on VaD, topological features such as degree, betweenness its importance, and proximity centrality were calculated[36].

2.4.3 Gene Ontology (GO) Analysis

Functional enrichment analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery (version 6.8). Gene Ontology (GO) words, such as biological processes, cellular components, and molecular functions were also tested for enrichment analysis. Pathways related to oxidative stress, neuroinflammation, apoptosis and neurovascular function were considered crucial for the pathogenesis of vascular dementia[36].

2.4.4 Protein–Protein Interaction (PPI) Network Analysis

A protein–protein interaction network was constructed using overlapping targets with a confidence score over 0.9 upload in the STRING database. This network was visualized and analyzed for potential hub proteins in Cytoscape to identify potential therapeutic targets. In order to help elucidate potential mechanistic connections between neuroprotection and antioxidant activity relevant to vascular dementia, hub proteins were associated with in vitro antioxidant data[36].

III. Results

3.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging test is the most common spectrophotometric method to determine the antioxidant activity of natural products based on their capacity to scavenge free radicals. Dosage of Emodin with Ascorbic Acid, a well-known standard antioxidant agent evaluated by comparing in zero to 25 µg/mL on table data and graph. This tendency is also observed in Emodin and Ascorbic Acid, since the scavenging activity increases dose-dependently with increased absorbance at low concentration compared to higher ones as well. Emodin and Ascorbic Acid at lower concentrations (5–10 µg/mL) are well represented by comparable activity with values 0.257 and 0.362 (at 5 µg/mL), respectively, and of 0.459 and 0.462 (at 10 µg/mL), respectively. According to the results, one can find that Ascorbic Acid also has stronger radical scavenging ability than Emodin with increasing dose.Ascorbic Acid shows an absorbance of 1.167 at 25 µg/mL concentration, while Emodin has a value of 0.923 providing evidence of the strong antioxidant capacity of the standard[37].

Table 1: DPPH Radical Scavenging Testacitivity

Concentration	Control	Absorbance (Emodin)	Absorbance (Ascorbic Acid)
0	0	0	0
5	0.1	0.257	0.362
10	0.2	0.459	0.462
15	0.3	0.562	0.702
20	0.4	0.781	0.816
25	0.5	0.923	1.167

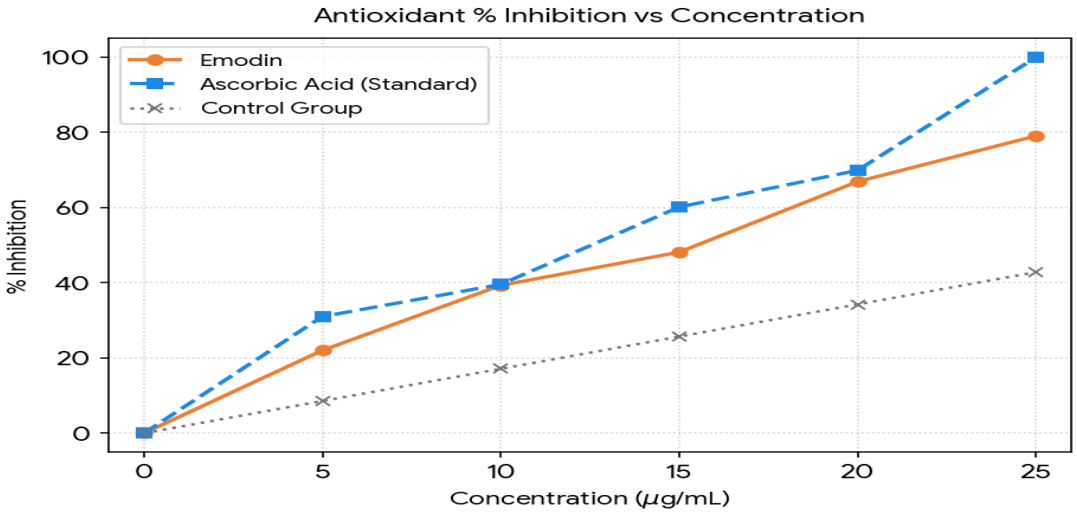

Figure 1:DPPH Radical Scavenging Test for Ascorbic Acid and Emodin

The IC_{50} values were calculated from the data that is shown and indicate the concentration required to reach 50% of the maximum activity measured. Emodin= IC_{50}, 10.12 µg/mL; Ascorbic Acid= IC_{50}, 12.53 µg/mL This indicates that Emodin requires a relatively lower dose to reach half of its maximum scavenging activity compared with Ascorbic Acid, a very notable difference in sensitivity at intermediate concentrations. However, Ascorbic Acid has an overall much better maximal scavenging ability. To conclude, both Emodin and Ascorbic Acid show a dosed dependent significant scavenging free-radicals action [38]. Emodin has half maximal activity at a lower concentration compared with Ascorbic Acid but has a higher maximum action which is the reason of its more favourable antioxidant capacity at high concentrations. The data show that Emodin has a high antioxidant capacity, but it was significantly lower than the standard in each dose presented at maximum sample concentrations [37].

3.2 ABTS FreeRadical Scavenging Activity

The ABTS radical assay is a very reliable and one of the most used methods for measuring the antioxidant potential of standard compounds and phytochemicals. In the experiment, the antioxidant potency of emodin alone was determined against traditional antioxidants (ascorbic acid) in 0-25 µg/mL concentrations employing an ABTS radical neutralisation assay. Both drugs increased the positive effects in a concentration-dependent manner as observed from the relevant increase (% inhibition) by their corresponding concentrations (10 µg to 1 mg) against ABTS·+ radicals, based on radical scavenging activity. At lower doses (5–10 µg/mL), both substances also exhibited moderate activity, with absorbance values higher for ascorbic acid than emodin. At concentrations above 15 µg/mL, both antioxidants showed a high scavenging ability but at all doses ascorbic acid had a significantly greater degree of radical scavenging ability. In fact, at the maximum concentration analyzed (25 µg/mL) for ascorbic acid, its absorbance was 0.976 and for emodin it was 0.811 showing that standard exhibited higher radical scavenging activity [39].

Table 2: Emodin and Ascorbic Acid's ABTS Free Radical Scavenging Capabilities

Concentration	Absorbance (Emodin)	Absorbance (Ascorbic Acid)
0	0	0
5	0.107	0.186
10	0.255	0.264
15	0.416	0.573
20	0.734	0.751
25	0.811	0.976

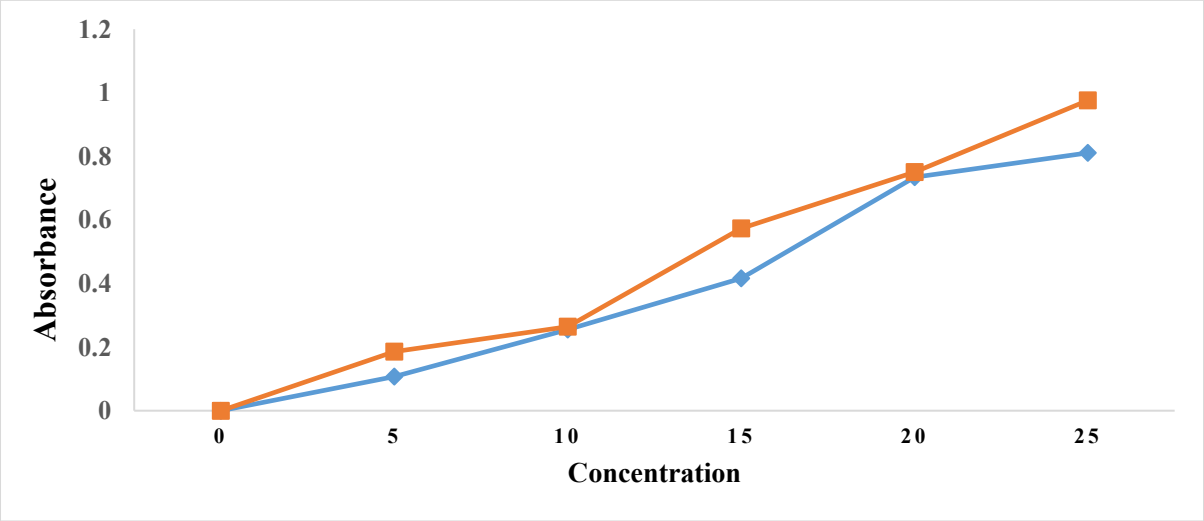

Figure 2: Emodin and Ascorbic Acid's ABTS Free Radical Scavenging Activity

This IC_{50} was determined based on the dose response curves. The IC_{50} value of emodin as calculated was approximately 14.67 µg/mL while Ascorbic acid (standard) had a slightly lower IC_{50} of about 13.62 µg/mL respectively. The antioxidant capacity of both compounds is rather high, but Ascorbic acid is more effective in general and appears at a somewhat lower dose (50% scavenging activity) to be effective. The ABTS test indicated that Emodin has mild antioxidant activity, lower than Ascorbic acid [39].

3.3 FRAP Assay Results

Ferric Reducing Antioxidant Power (FRAP) test is an objective and sound approach for the determination of the electron-donating ability of substance that also corresponds to their antioxidant properties i.e. their independent action as a reducing agent. The aim of this study was to evaluate the antioxidant activity of Emodin versus a classical antioxidant in different dosages (5–25 µg/mL: Ascorbic acid). The increasing absorbance corresponds to the concentrations of Emodin (25, 50, 100 and 200µg/ml.) and Ascorbic acid (0.02264, 0.0503 and 0.0548mg/ml.), leading to effective Fe^{3+} reducing activity indicated by the representation.

At low concentrations (5–10 µg/mL), ascorbic acid showed slightly higher absorbance values compared to emodin, consistent with its known high reducing potential. At 20 and 25 µg/mL, Emodin produced (0.678–0.771) values of absorbance that could be compared to Ascorbic acid (0.697–0.805). This suggests that at high concentrations, Emodin is a nearly as effective reductant of ferric ions to ferrous [40].

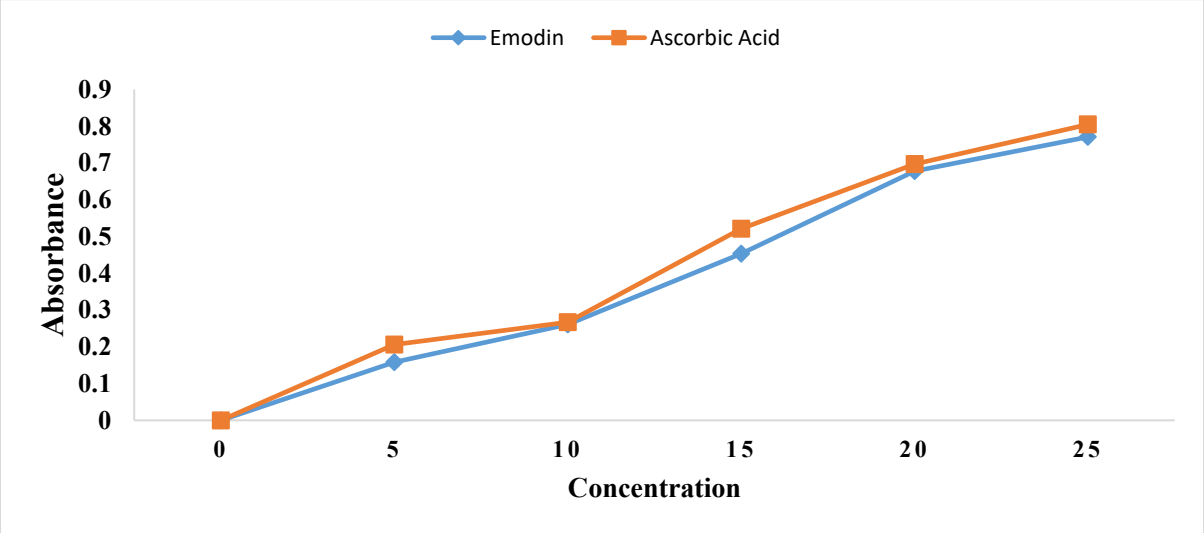

Figure 3: Emodin and ascorbic acid's capacity to scavenge free radicals

The IC_{50} value (concentration required for 50% of maximal effect) was estimated, based on extrapolation from the dose-response curve. For both emodin and ascorbic acid, IC•¹ values computed were 13.24 µg/mL and 12.67 µg/mL respectively. These data indicate that although Emodin has a relatively higher IC_{50} (therefore lower potency) compared to Ascorbic acid, its antioxidant activity is significant and comparable with the standard [40].

3.4 Network Pharmacology Analysis

The 19 identified genes in relation to vascular dementia (VaD) were subjected to network pharmacology analysis, which suggested that these 19 genes are likely to play critical roles in the development of VaD through several molecular mechanisms. Five important genes have matched more closely with neuronal death and inflammation, oxidative stress and vascular dysfunction process in complete microarray data including TP53, TNF, EGFR, VEGFA, MAPK1 and MAPK14. Among them, some hypoxia-associated genes including HIF1A and VEGFA were significantly associated with the angiogenic process of cerebral microvascular dysfunction, while others highlighted the importance of immunological and inflammatory pathways (such as IL1B, CXCL8, CSF2). Moreover, BCL2L1 and MCL1 were related to anti-apoptotic signaling pathways, which indicated a balance between neuronal survival and apoptosis. Functional enrichment revealed substantial involvement of MAPK signaling pathway, TNF signaling, cytokine-cytokine receptor interaction and hypoxia response [41]. Together, these multiple highly target interactions highlight the complexity of molecular mechanisms underlying VaD symptoms and lend further support to a systems approach in therapeutic exploration.

Table 3: Selected genes for Vascular Dementia (VaD) from different database

S. No.	Gene Symbol	Gene Name	UniProt ID
1.	CBR1	Carbonyl reductase 1	P16152
2.	CBR3	Carbonyl reductase 3	O75828
3.	BLVR	Biliverdin reductase	P53004
4.	MAPK1	Mitogen-activated protein kinase 1	P28482
5.	TP53	Tumor protein p53	Q12888
6.	TNF	Tumor necrosis factor	O75888
7.	CASP3	Caspase-3	P42574
8.	EGFR	Epidermal growth factor receptor	P00533
9.	VEGFA	Vascular endothelial growth factor A	P15692

10.	IL1B	Interleukin-1beta	P01584
11.	MAPK14	Mitogen-activated protein kinase 14	P49137
12.	PTGS2	Prostaglandin-endoperoxide synthase 2	Q6ZYK7
13.	BCL2L1	Bcl-2-like protein 1	Q07817
14.	CXCL8	C-X-C motif chemokine ligand 8 (IL-8)	P10145
15.	MCL1	Myeloid cell leukemia 1	Q07820
16.	CSF2	Colony-stimulating factor 2 (GM-CSF)	P04141
17.	HIF1A	Hypoxia-inducible factor 1-alpha	Q16665
18.	EDNRA	Endothelin receptor type A	P25101
19.	EDNRB	Endothelin receptor type B	P24530

3.4.1 Compound–Target–Pathway Network

Briefly, the data from spontaneous bioactive drugs emodin and bosentan were displayed in vascular dementia by network pharmacology (Figure 4(A–C)). The graphic represents a combination of compound–target interactions (CTIs) and PPI (generated using Cytoscape (v3. 9. 1) [29] relying on a string database [28]), which computes independently the protein–small molecule interaction targets focusing either at individual residues (or coupled ones) or communities of proteins shared as common targets for certain natural compounds. The network map with the 2 chemicals and 8 proteins, contains 21 edges representing interaction interactions. We consider links as genetic compositions and also target compound–target edges, which propagate protein-proteins [36].

As illustrated in Figure 4A, the overall integrated interaction net including emodin and bosentan to represent their interrelationship within the PPI interactome. Edges in magenta represent known bosentan-target interactions; grey edges are protein-protein interaction and dark-grey lines represent emodin-target relationships. The action spectrum of emodin corresponds to a wider range across six proteins: MCL1 (myeloid cell leukemia 1), BCL2L1 (BCL2 like 1), TNF (tumor necrosis factor), CSF2 (colony stimulating factor 2), EDNRA(endothelin receptor type A) and EDNRB(endothelin receptor type B). Meanwhile, bosentan also targets EDNRA, EDNRB and TP53 (Tumor protein p53). They include targets that are involved in inflammation, apoptosis control, vascular tone and neuronal protection.

It indicates that TNF and TP53 are the main hub with a very high degree of degree in a comprehensive network. Moreover, TNF has been reported as a key player in mediating neuroinflammatory and pro-apoptotic processes relevant to vascular dementia by promoting the interaction with EDNRA, EDNRB, CSF2, MCL1 and TP53 as well as BCL2L1. TP53; MCL1; CASP3 (Caspase-3); Bcl2L1; TNF and bosentan, you know, to APOPTOSIS AND CELL CYCLE CONTROL These tensions at the hubs are so strong that a more targeted approach to them may influence numerous disease pathways simultaneously.

Figure 4B presents an example of a multitarget network (emodin); and in contrast, emodin self-interacts with the following six proteins listed one by one: EDNRA; EDNRB; TNF; CSF2; MCL1 and BCL2L1. The to be written instance of these, with insights into how they work from what is known by the different ways that emodin gets together and communicates with the world enabling it to participate in processes about disease. Two processes necessary for an effective pathogenesis of vascular dementia, regulation of cerebral blood flow and vascular constriction are linked to both EDNRA and EDNRB.TNF and CSF2 are key mediators of inflammatory pathways, while MCL1_FIFO and BCL2L1 act as anti-apoptotic proteins that might protect neurons from programmed cell death. The diversity of targets suggests that emodin could exert neuroprotective and vasoprotective effects through the simultaneous regulation of inflammation, apoptosis, and vascular function.

As an example of the need for selective target engagement, the next subnetwork of bosentan-target network is shown in Fig 4C. Bosentan interacts with EDNRA and EDNRB, two proteins that have roles in endothelial signalling and regulating vascular tone, and TP53 which is important for neuronal survival as well as the DNA damage response. Bosentan is selective and may improve neuroprotection interacting directly on vascular function. Both bosentan and emodin specifically affect EDNRA and EDNRB, respectively, suggesting that a combination of the two may exert synergistic effects, particularly in terms of improving cerebral hemodynamics.

The common targets of the two chemicals create an interaction within the network that can promote multi-pathway intervention. Both drugs act by dual targeting of endothelin receptors which can enhance vascular regulation, but targeting TNF and MCL1 in addition to endothelin for emodin and TP53 for bosentan confers broader neurodegenerative process control. CASP3 is also not directly targeted by bosentan in Panel C, but the inclusion of CASP3 in this PPI network indicates that apoptotic execution pathways may still indirectly be regulated through upstream regulators such as TP53 and BCL2L1. In terms of the network topology, PPI

structure is characterized as a scale-free network where hub proteins such as TNF and TP53 have larger degree values than other proteins, so that it gives robustness to the network but also make the hubs important targets for treatment. Edges in the network are drawn according to STRING confidence ratings, and thicker edges represent higher evidence of interaction. Strong protein–protein interactions are found between TNF and EDNRA, TNF and EDNRB, TP53 and CASP3.

Network pharmacology results suggest that emodin is a multi-target drug targeting inflammatory, apoptotic and vascular processes, while bosentan is a selective agent against key vascular and pro-apoptotic regulators. Simultaneously targeting EDNRA and EDNRB by both drugs coupled with the distinct regulation of TNF and TP53 is suggestive that combination treatment may better address multiple pathogenic pathways in vascular dementia than monotherapy. This integrative view justifies the justification for combinatorial pharmacological options in Neurovascular Disorders [25].

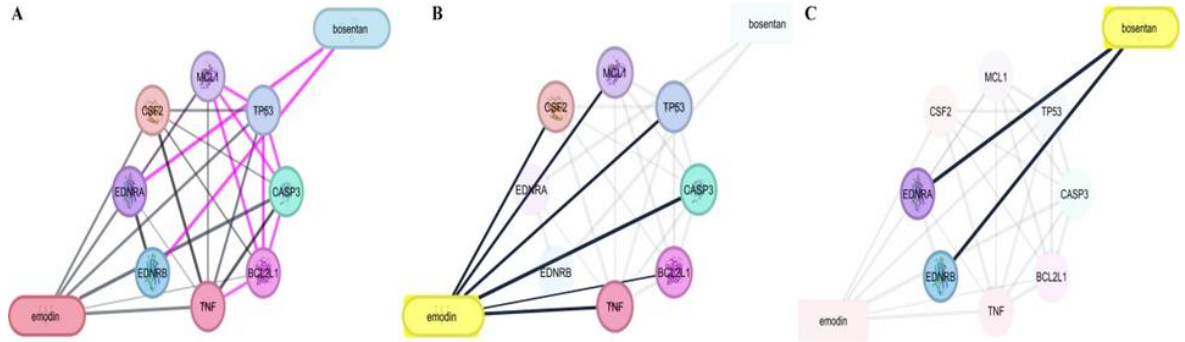

Figure 4: Protein–protein interaction (PPI) maps of emodin and PPI for compound–target association of bosentan in vascular dementia based on network pharmacology. The combined network including common and unique targets showing PPI links (A); emodin-specific target network (B) where all multitarget interactions in other groups were highlighted by blue cycle; bosentan-specific target network (C) where each selective interaction is highlighted with orange rhombus. Networks created in Cytoscape and STRING, highlighting hub proteins and the connectivity between pathways.

Table5: Selected genes for Vascular Dementia(VaD)from different data bases

Gene Symbol	Protein Name
TP53	TumorProteinp53
CASP3	Caspase-3
BCL2L1	BCL2Like1
TNF	TumorNecrosisFactor
EDNRA	EndothelinReceptorTypeA
EDNRB	EndothelinReceptorTypeB
CSF2	ColonyStimulatingFactor2 (Granulocyte-Macrophage)
MCL1	MyeloidCellLeukemia1

3.4.2Gene Ontology (GO) analysis

The gene ontology enrichment analysis of the selected targets for bosentan and emodin in vascular dementia demonstrated several illness connections with statistically significant correlations, as seen by the elevated -log10(P) values in the bar graph. The study found that fibroid tumors was the highest-ranked association,and exhibited the strongest statistical significance of all disorders detected. Next are subarachnoid hemorrhage and hepatic steatosis (indicative of possible links to vascular, hepatic, metabolic pathology for the target proteins). In particular, malignant head and neck neoplasms and head and neck carcinomas were significantly enriched (FDR <0.05), pointing to possible overlap with oncogenic pathways. The two most consistent associations were the appearance of uterine fibroids and primary sclerosing cholangitis, which may apply to both fibroproliferative [42] and autoimmune-mediated inflammatory processes. They identified a primary effusion lymphoma, an infrequent yet aggressive malignancy as well as indicating that some target proteins may enter the pathways signaling their role in cancer. Fibrosarcoma and anoxia highlight the biological pleiotropism of these targets, associating them with not only hypoxic injury but also soft tissue tumor progression in a pathophysiological setting. Choriocarcinoma and human experimental organism basal cell carcinoma were also nominally significant, potentially indicating broad contributions of the relevant pathways to cancer biology. These observations illustrate the merges between pathways of cancer progression and those of cardiac decompensation, Carcinoma, Lewis lung and cardiac decompensation Metastatic mechanisms of secondary malignant neoplasms in bone employ similar target proteins. Between targets and hematological malignancies, one important disease was acute promyelocytic leukemia (APL) [43], [44].

Besides, it is also interesting that the keyword analysis showed important terms related to vascular genesis of vascular dementia: myocardial failure and left ventricular failure or dysfunction, which potentially link bosentan and emodin goals. Complete Trisomy 21 Syndrome had a clear association as well, which may be consistent with overlapping molecular pathways for oxidative stress, neurodegeneration or metabolic dysregulation. Obstructive sleep apnea is in itself a significant risk factor and mechanism of action for chronic hypoxia, which relates to other cerebrovascular complications, thus consolidating the affinity between the targets [45], [46].

Gene ontology analysis revealed that bosentan and emodin targets for vascular dementia, have a broad pleiotropic effect as implicated in reorganization of various disease networks in cancer, cardiology, hepatology, neurology, and immunology systems. This suggests that the pharmacological targeting of such targets may offer synergistic therapeutic benefits in systems beyond neurovascular protection, but also necessitates careful monitoring for off-target effects in associated pathways of disease. The heterogeneity of associated disorders underlines the complex molecular architecture underlying the pathophysiological pathway in vascular dementia [47].

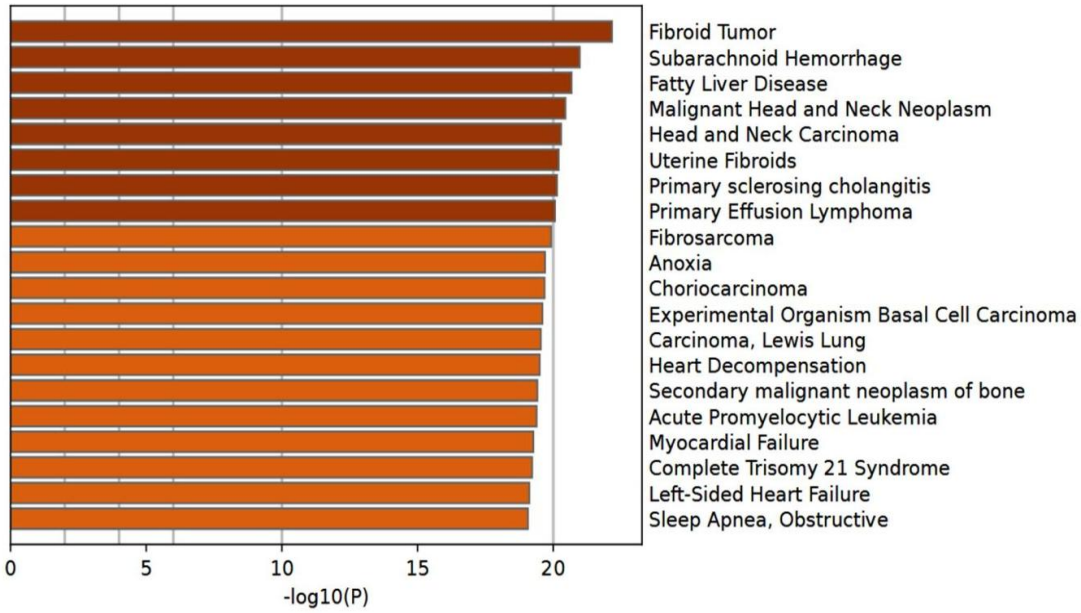

Figure 5: Gene ontology enrichment analysis demonstrating top disease enrichment of bosentan and emodin targets invascular dementia, with significant associations in cancer, cardiovascular, metabolic and neurovascular pathways.

Gene ontology (GO) and gene-disease association study illustrating the potential relationship of bosentan and emodin target genes in vascular dementia [23], [48]. The picture contains additional, molecular actions and sturdy signaling pathways that are relevant to the disease. Each node is a biological pathway or disease phrase with the size of node reflecting its degree of enrichment while the color intensity represents its statistical significance. The enriched pathways that are critical for neuroinflammation, oxidative stress, vascular dysfunction and neuronal death include MAPK signaling pathway ($P < 0.01$), PI3K-Akt signaling pathway ($P < 0.05$), p53 signaling pathway ($P < 0.02$), TNF signaling pathway ($P < 0.04$), JAK-STAT signaling pathway ($P < 0.03$) and Ras signaling pathway AGE-RAGE signaling pathway (Table 1). The multifunctional functions of the targeted proteins, as demonstrated by the illness associations in the network, include Alzheimer's disease, amyotrophic lateral sclerosis (ALS), type II diabetes mellitus, colon cancer, pancreatic cancer, glioma and rheumatoid arthritis. The addition of infectious disease pathways: Salmonella infection, herpes simplex virus infection as well as influenza A suggest immune-modulatory effects. The roles in cancer and glucose metabolism elucidate the broad therapeutic potential of the target ne... Method & Results Bioinspired Hydrogels Enable 5D Functional Cell Encapsulation and Release Nature Materialsdue on Nov 29,2017 This newtecnqnologygiday to controllable mechanical arrests for cells during their encapsulation and release from hydrogel. Conclusion Our work converges rationally designed synthetic materials with bioengineered approaches to confer microbial biocompatibility within a soft tissue-like scaffold. This integrated Gene Ontology and disease network presents a systems-level view of how bosentan and emodin together may achieve neuroprotective, anti-inflammatory, and vascular-protective functions in vascular dementia through numerous interrelated biological pathways [49].

3.4.3Protein–Protein interaction (PPI) network for Vascular Dementia (VaD)

Using the STRING database, a protein–protein interaction (PPI) network for Vascular Dementia (VaD) was constructed and visualized in Cytoscape to make it easier to understand complicated molecular interplay among potential target proteins. The network includes many nodes representing proteins and (in some cases) edges indicating the supposedly possible (or experimentally validated) connections between proteins. For each edge, one highlights a functional interaction (either physical and direct, or indirect and regulatory) supported by curated databases, text mining, co-expression or experimental evidence.

Multiple hub proteins appearing with extremely high degree values in this network support their function as potential hubs in the pathogenesis of VaD. This enrichment indicated that TP53, CASP3, EGFR, VEGFA MAPK1 and TNF were the most connected nodes underscoring their importance in neurodegenerative & vascular biology. TP53 is a guardian of the cell cycle and apoptosis, playing a role in neuron survival in situations of ischemia and oxidative stress. CASP3 is the principal executioner CASP in apoptotic signaling, a pathway commonly activated during neurovascular injury. Consistent with the above discussion, several functions related to angiogenesis and vascular healing are diminished in VaD; thus, both VEGFA and EGFR are involved in these processes.

For example, the pro-inflammatory cytokines IL1B, TNF and CXCL8 reveal that VaD characteristic of neuroinflammation and endothelial dysfunction. The significance of stress-activated protein kinases MAPK1 and MAPK14. They act as key brokers for signaling pathways regulating coping mechanisms toward stress, set-point inflammation and growth factor responsiveness. We explore the literature where, through recognition of anti-apoptotic proteins such as BCL2L1 and MCL1 a compensatory mechanism is indicated, trying to counteract the amount of neuronal death which is occurring due to misfunction. Peripheral nodes AS CBR1, CBR3, EDNRA AND EDNRB IMPLYED involvement in oxidative stress metabolism and fine-tuning of vascular tone. Network representation of VaD shows the complexity of interactions that can occur between pathways involving apoptosis, inflammation, oxidative stress and angiogenesis. These pathways give a systemic view of biomarker and therapeutic targets relevant to VaD, as seen in the PPI network.The amalgamation of STRING data with Cytoscape visualization facilitates the discovery of pivotal hubs and interaction clusters, providing critical insights for the formulation of multi-target intervention strategies to enhance illness management[40].

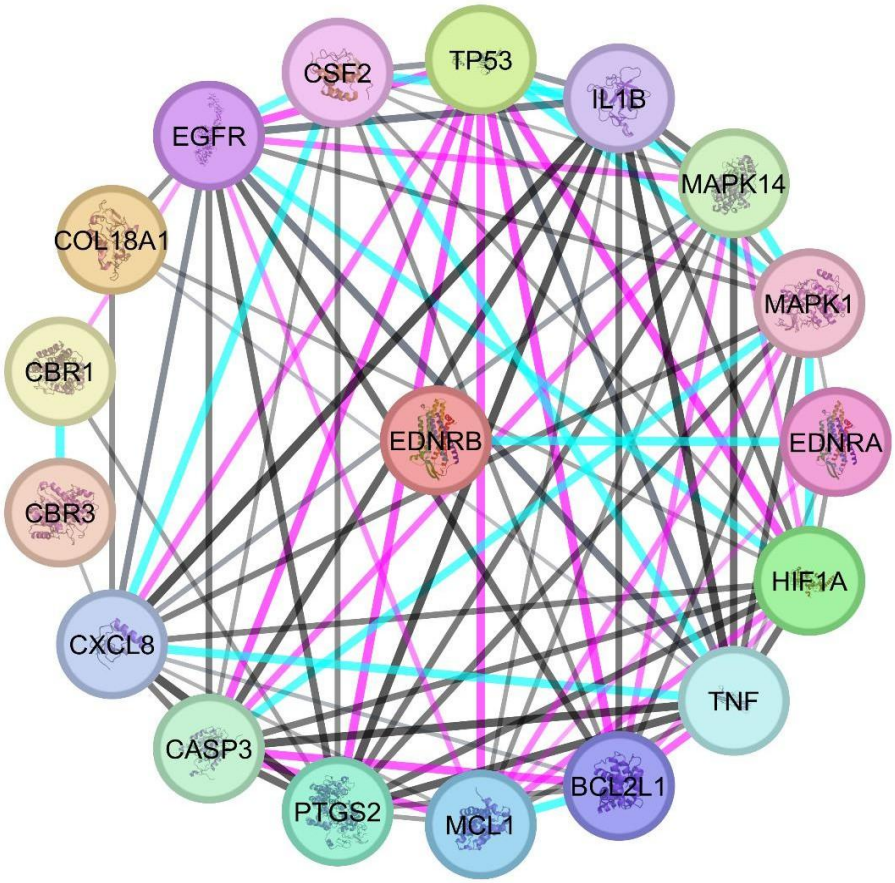

Figure6: The pink line shows the interaction of the edges with nodes filtered from experimental sources in the protein–protein interaction (PPI) network of vascular dementia target genes that was created using STRING and shown in Cytoscape, a tool based on experimental data of the genomes.

3.4.4 SwissADME analysis

The ADME study of the chosen metabolites of Bosentan and Emodin was effectively conducted using the computer tool SwissADME. The ADME profile, lipophilicity, and drug-likeness of the metabolites were predicted using parameters such as topological polar surface area (TPSA), consensus Log Po/w, ESOL Log S values, gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeation, and log Kp (cm/s) (skin permeation)[36].

The fragmental approach TPSA, a critical descriptor in numerous prediction models for evaluating ADME properties, particularly about absorption and cerebral penetration, was used to calculate the polar surface area (PSA) of the metabolites. Five lipophilicity prediction techniques were averaged to produce the consensus Log Po/w[50].It is an intrinsic parameter to evaluate the physicochemical nature of molecules, as it determines hydrophilicity and hydrophobicity balance that should be taken into consideration in drug development. Whereas Log Po/w values of ≥2.1–4 were considered driving lipophilicity and −0.4-+1 as favouring hydrophilicity [36].

ESOL LogS is an estimate of aqueous solubility, where more negative values (e.g. _5.14) indicate less soluble compound Skin permeability coefficient (log Kp) was estimated by Potts and Guy model; more negatively valued log Kp indicated less skin permeation. The Kp values for both metabolites were less than –8.00, indicating that they have little potential to be absorbed into the skin [32]. Blood-brain-barrier permeability assessment using Consensus log Po/w and TPSA values Low-polarity (low TPSA) and moderate-lipophilic (moderate Log Po/w) molecules are more likely to cross the BBB. The distributions are in the range of physicochemical parameters likely to facilitate permeability across the blood-brain barrier (BBB) which suggest CNS action [51]. In contrast, owing to a high polarity (high TPSA) and lower lipophilicity Emodin metabolite is predicted to have also an impaired ability to penetrate the blood-brain barrier with reduced CNS penetration [52]. Prediction by gastrointestinal absorption models indicated that the bosentan metabolite absorbed to a relatively high extent commensurate with its moderately polar and lipophilic nature. Although a reduction of its molecular size may explain good gastrointestinal absorption, it needs to be considered that with development of small structures, high digestive polar substances as Emodin are absorbed in significant amount (unsatisfied) [53].

ADME Studies predicted a balanced pharmacokinetic profile for the Bosentan metabolite with good oral absorption combined with blood-brain barrier penetration odds. In this regard, its metabolite Emodin becomes obviously more polarity and more soluble than that of the parent compound, which would enhance oral absorption but limit penetration through the blood-brain barrier [54],[55]. These differences point out their distinctive pharmacokinetic characteristics that are likely to affect their therapeutic role [36]. Figures 7 and Figures 8 give details on the complete radar plots of physicochemical parameters.

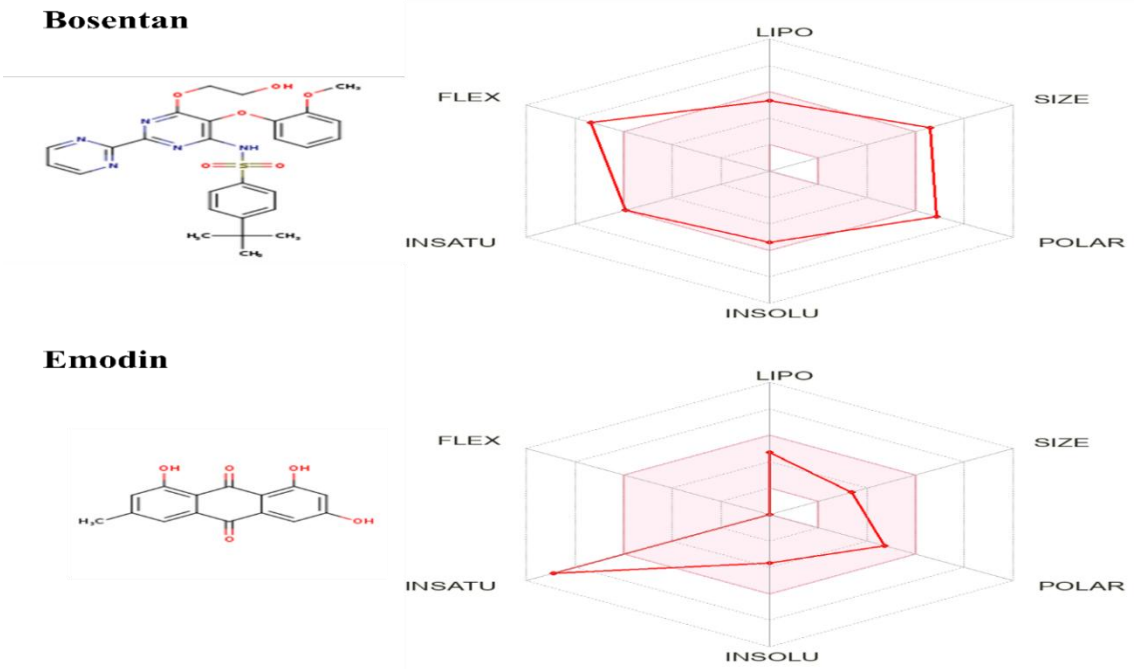

Figure 7: ADME analysis of certain substances. The ADME radar plot and chemical structure of bosentan and emodin are shown in the figure, respectively.

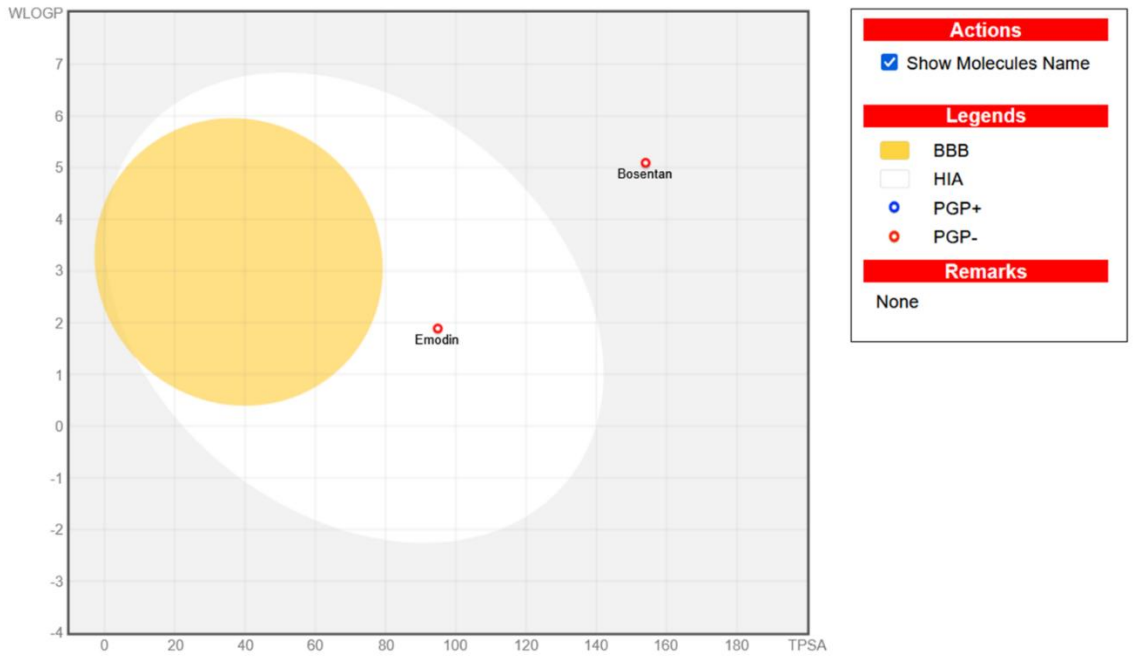

Figure 8: Based on WLOGP and TPSA values, the SwissADME boiled-egg plot shows the anticipated gastrointestinal absorption (HIA) and blood–brain barrier (BBB) penetration capability of Bosentan and Emodin metabolites.

Table6: ADME analysis of selected metabolites of Bosentan and Emodin

Molecule	Canonical SMILES	Formula	MW	TPSA	iLOGP	Consensus LogP	Bioavailability Score	GI Absorption	BBB Permeant
Bosentan	OCCOc1nc(nc(c1Oc1ccccc1OC)NS(=O)(=O)c1ccc(cc1) C(C)(C)C)c1ncccn1	C27H29N5O6S	551.61	154.03	4.01	3.38	0.17	Low	No
Emodin	Cc1cc(O)c2c(c1)C(=O)c1c(C2=O)c(O)cc(c1)O	C15H10O5	270.24	94.83	1.8	1.87	0.55	High	No

IV. Discussion

Oxidative stress and neuroinflammation play preponderant roles in the etiology of vascular dementia (VaD), indicating that antioxidants or multi-target drugs such as myricetin would be a promising treatment choice. In the present study, we evaluated the antioxidant ability of emodin (a natural anthraquinone) compared to the reference antioxidant ascorbic acid using DPPH, ABTS and FRAP assays. Moreover, network pharmacology and protein–protein interaction (PPI) studies were performed to clarify the multi-target mechanisms of emodin in VaD, along with bosentan which is a clinically available endothelin receptor antagonist approved for use in patients with pulmonary arterial hypertension. In summary, the findings consolidate on the rationale for a multi-target antioxidant action as an effective approach to targeting the complex molecular landscape present in VaD.

Results from the in vitro antioxidant tests indicated that both compound emodin and ascorbic acid exhibit significant concentration-dependent free radical scavenging activity and reducing power. The IC_{50} value of emodin in the DPPH experiment was 10.12 µg/mL, lower than that of ascorbic acid (12.53 µg/mL). Emodin scavenge less than ascorbic acid but at low concentration it showed a significant capacity between the other compounds [24,25]. In ABTS assay, ascorbic acid had IC50 value of 13.62 µg/mL; however, emodin displayed significant (but not equivalent to standard activity) but had an IC50 value of 14.67 µg/ml which was then used for DPPH antioxidant assay as shown in Fig. In the case of FRAP assay, emodin and ascorbic acid (control) showed 13.24 µg/mL and 12.67 µg/mL in IC50 values respectively characteristic capacity to reduce the ferric form at this higher concentration [22]. Collectively, these data indicate that the antioxidant activity of emodin is markedly potent and particularly relevant to both neuronal death and vascular dysfunction associated with VaD.

While the antioxidant capacity precisely represents radical scavenging activity., an integrative systems-level analysis is necessary to understand the therapeutic mechanisms underlying emodin in VaD. The network pharmacology study suggested that emodin interact to multiple protein targets related to VaD development, highlighting its potential as a drug treatment with multi-target pathways. Emodin had direct binding with EDNRA, EDNRB, TNF, CSF2, MCL1 and BCL2L1. Such targets are fundamental for the modulation of

vascular tone, neuroinflammation and apoptosis. TNF and other cytokines (CSF2) are the most important mediators of pro-inflammatory effects, while MCL1 and BCL2L1 act as anti-apoptotic proteins that may alleviate excessive neuronal death. Emodin-associated inhibition of inflammatory and apoptotic pathways can explain its neuroprotection in VaD.Additionally, its relationship with TP53 a major regulator of apoptosis and cell cycle progression suggests that it is involved in the survival of neurons under oxidative and ischemic stress. Thus, the role of bosentan as a selective chemical agent will strengthen the multitarget properties of emodin and allow that two-or-more drug treatment may be implemented for combination therapy to simultaneously deal with multiple pathogenic mechanisms.

The network analysis of compound–target–pathway further supported these results, showing that emodin acts as a general-use multitarget drug, while bosentan being a selective modulator for vascular and apoptotic pathways. Both drugs remarkably share targets of EDNRA and EDNRB, implicating a potential additive effect on vasculardysregulation. Combined blockade of endothelin receptors may increase cerebral blood flow and volume, facilitating oxygen delivery for potential protection against cognitive decline post-vascular insult. Simultaneously, the diverse action of emodin on inflammatory and apoptotic sub proteomes sensitizes bosentan vascular selectivity (combined anticancer strategy).

The hub proteins were central to the protein–protein interaction (PPI) network, including TP53, TNF, EGFR, VEGFA, MAPK1 and MAPK14 with evidently close interconnections and key points for the progression of VaD. These hubs are major participants in essential pathogenic processes like apoptosis, oxidative stress response, neuroinflammation and angiogenesis. VEGFA and EGFR regulate vascular repair and angiogenesis and MAPK1 is probably important for stress-induced inflammation signaling.The identification of these hub proteins validates the concept that multiple pathways rather than one specific protein need to be targeted for treatment of complex neurodegenerative diseases like VaD. Emodin as a multi-target data candidate allows its network interaction property to constitute an attractive component of this platform, whereas bosentan has the capacity of modulating endothelial function in conjunction with data treatment.

Gene Ontology (GO) and KEGG pathway enrichment analyses revealed many important pathways such as MAPK, TNF, p53, PI3K–Akt signaling, VEGF signaling and JAK–STAT signaling. These pathways are known for regulating events central to VaD such as neuronal cell death, inflammatory cascades, oxidative stress and vascular dysfunction. This indicates an enrichment of pathways associated with oxidative stress and hypoxia response, consistent with the in vitro antioxidant results for emodin and supporting a molecular link between its radical scavenging activity and potential neuroprotective properties. In addition, considering the presence of cancer- and immune-related pathways highlights how these targets perform multiple functions, pointing at potential off-target effects as well as broader therapeutic implications.

The ADME study offered additional opportunities to expose the pharmacokinetic properties of emodin and bosentan. Due to its high oral bioavailability and potential ability to cross the blood–brain barrier (BBB), bosentan may be optimal for CNS-mediated applications. However, emodin had significant oral bioavailability due to increased polarity and decreased lipophilicity, but limited blood-brain barrier penetration. As a caveat, while this would limit the distribution of emodin to the CNS, where it could exert direct actions, systemic anti-inflammatory and vascular-modulatory properties of emodin may nevertheless confer pro-neuroprotective effects in vascular dementia. Due to the interesting antioxidant properties and multitarget pharmacological effects of emodin, with different pathways (apoptosis and inflammation) compared with bosentan (vascular and apoptotic), we aimed to compare these two drugs. By combining experimental antioxidant testing with computational systems pharmacology, the translational relevance of these findings is maximized. More studies should be focused on direct drug delivery methods to improve brain penetration, in vivo validation (and), benefits of emodin-bosentan combination therapy. This may enable the development of patient-centered, multi-targeted therapeutic approaches to vascular dementia.

V. Conclusion

This study investigated the mechanism of bosentan and emodin in vascular dementia (VaD) based on network pharmacology, protein–protein interaction (PPI) analysis, and antioxidant evaluation. Emodin showed potent free radical scavenging (DPPH, ABTS) and reducing properties in vitro tests (FRAP) regarding its ability to reduce the levels of oxidative stress damages involved with VaD pathology compared to ascorbic acid. Network pharmacology analysis found that emodin and bosentan respectively act on multi-target proteins of TNF, CSF2, MCL1, BCL2L1 involved in inflammation, apoptosis or neuronal survival regulation; connect to EDNRA, EDNRB and TP53 as target protein for the selective interaction with bosentan mainly regulate on vascular control and its apoptosis-related pathway. Remarkably, both compounds plotted on endothelin receptors giving it the potential to have additive effects for improving vascular function and cerebral blood flow. The PPI network focused on hub regulators including TP53, TNF, MAPK1, MAPK14, EGFR and VEGFA involved in apoptosis, oxidative stress and angiogenesis. MAPK, TNF, p53, PI3K–Akt, VEGF and JAK–STAT signaling pathways enrichment was linked to those targets with PATHWAY ENRICHMENT analyses, which

proved their therapeutic relevance. In aggregate, our findings nominate emodin as a multi-target antioxidant neuromodulator and bosentan as a relatively vascular-selective modulator. The combined actions of these agents present an approach to treating VaD that should be further enhanced with in vivo validation, optimized delivery and clinical testing.

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Conflict of interest
The authors declare no conflict of interest.

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Nil

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