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# *In vitro* and *in vivo* evaluation of the multifaceted physiological roles and biochemical pathways of lignin- and morin-based formulations

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Owing to the inherent chemical complexity of natural bioactive compounds, the identification of their molecular targets and biochemical mechanisms of action has always been a challenge in front of biomedical sciences. The present study reviews recent approaches, which combine *in vitro* evaluation and *in vivo* screening of potential physiological activities of the plant-derived bioactive molecules lignin and morin and formulations on their basis. The aim is to assess the potential of the hetero polymer and the flavonoid as platforms for the design of portfolios of innovative pharmacological products aimed for therapy and supportive care. Collectively, the significance of advanced research in this area with special emphasis on the main challenges associated with toxicity issues and other undesirable health consequences resulting from the direct administration of both natural substances was also advocated.

***Keywords***: lignin, morin, biological activity, *in vitro*, *in vivo*

## INTRODUCTION

Natural bioactive compounds play a crucial role for human well-fare due to their diverse pharmacological activities such as antidiabetic, antipyretic, anticancer, antidiuretic, anti-atherosclerotic, antioxidant, antimicrobial, etc., which determine their therapeutic applicability for treatment of many diseases [1]. Flavonoids are the most abundant and ubiquitously found phenols in nature [2, 3].

The benefits of natural polymers in the biomedical field have been thoroughly investigated. Various homogeneous and heterogeneous biopolymers and their biocomposites have attracted much attention for biomedical applications including wound healing, drug delivery [4], tissue engineering, and biosensors due to their unique features and bioactivities [5].

Lignin is one of the most common structural biopolymers in plant cells [6]. It is produced by oxidative coupling of three phenolic alcohols: coniferyl, p-coumaril, sinapyl alcohol in the cell walls. The process is catalyzed by the enzyme peroxidase [7]. In recent years, lignin has been applied as a source for the development of: nanoparticles, nanotubes, hydrogels, sunscreen lotions and 3D printed materials used in biomedicine [8]. The heterobiopolymer is characterized by low cytotoxicity, good stability, biocompatibility, biodegradability, presence of phenolic and aliphatic hydroxyl groups in its structure [9], ability to be modified by targeting molecules [7, 10], antioxidant activity, antimicrobial potential, low proinflammatory effects, nonhemolytic activity [8, 11-13], and reducing serum cholesterol activity [14].

Morin belongs to the class of plant flavonoids, which represent a family of natural plant-derived dietary bioactive compounds with polyphenolic nature found in vegetables, fruits, juices and herbs. They have different biological functions like antioxidant properties and oxygen radical scavenging potential [15-18]. Morin hydrate is a bioflavonol characterized with a hydroxyl group at the 3-position and comprised of a flavone (2-phenyl-1-benzopyran-4-one) backbone. It is a 7-hydroxyl flavonol that contains three additional hydroxy substituents at positions 2′, 4′, and 5. Morin in pure form is a bitter, yellow-colored stable compound that is relatively neutral and insoluble in water. It is an isomeric form of quercetin. Both natural flavonoids could be differentiated from each other based on the B-ring's hydroxylation pattern, which is "ortho" in quercetin and "meta" in morin [19]. Morin belongs to the *Rosaceae, Moraceae* and *Fagaceae* families. Major sources are *Morus alba* L. (white mulberry), *Psidium guajava* (guava), *Maclura pomifera* (guava leaves), *Maclura tinctoria* (old fustic), Osage orange), *Allium cepa*

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(onion), *Psidium guajava* L. (guava leaves), *Maclura tinctoria* (old fustic), *Malus pumila* (apple skin), *Prunus dulcis* (almond), *Chlorophora tinctoria* (figs), *Castanea sativa* (sweet chestnut), *Artocarpus heterophyllus* (jack fruit), tea, red wine, coffee, seaweed and cereal grains [15, 19]. It possesses various physiological properties, such as anti-allergic, anti-arthritis, anti-cancer, anti-mutagenic, anti-diabetic, anti-inflamatory, anti-microbial, hepatoprotective, neuroprotective, antioxidant, hypouremic, nephroprotective, gastroprotective, cardioprotective and others [15, 20]. Morin also performs a systemic protective activity, reducing the negative side effects of some drugs without interfering with their action. It is suitable for long-term administration at low doses without exhibiting toxicity [20].

In this respect, the present study reviews recent approaches which combine *in vitro* evaluation and *in vivo* screening of potential physiological activities of the plant-derived bioactive molecules lignin and morin and formulations on their basis. The aim is to assess the potential of the hetero polymer and the flavonoid as platforms for the design of portfolios of innovative pharmacological products aimed for therapy and supportive care.

### *Novel lignin-based nanoformulations and their physiological activities*

Alkali lignin nanoparticles (ALNP) and dioxane lignin nanoparticles (DLNP) were synthesized by Kamal *et a*l., 2022 [21] from two different lignin sources, namely alkaline softwood lignin (AL) and hardwood dioxane lignin (DL) extracted from subabul stems using a nanoprecipitation method. It was established that DLNP and ALNP possess higher antioxidant activity compared to DL and AL. DLNP offers higher protection for *E. coli* from UV rays, compared to ALNP. A good anticancer effect was observed in various cytological tests and animal experiments with alkaline lignin, the bioactive molecule resveratrol (RSV) and $Fe_3O_4$ magnetic nanoparticles [22]. *In vitro* experiments show increased release of resveratrol from the magnetic RSV-loaded lignin nanoparticles (AL/RSV/$Fe_3O_4$ NPs), reduced tumor size and higher accumulation of administered drugs. There is a reduction of side effects of the administered drugs, compared to the freely administered dosage forms. This formulation is potentially useful for the administration of poorly soluble drugs.

Another scientific group synthesized synthetic lignin which was applied to two human cell lines (breast adenocarcinoma, MCF7 and normal fetal lung fibroblasts, MRC5). Inhibition of cell growth was observed in both cell lines after 4 hours, while a better sensitivity of cancer cells compared to fetal lung fibroblasts was observed after 72 hours of administration in lower doses [23].

Alqahtani *et al*. (2019) [24] established that curcumin-loaded lignin nanoparticles show high mechanical strength and significant encapsulation efficiency (92%). *In vitro* tests proved stability of the nanoformulations in gastric fluid and slow release of the formulations in intestinal conditions. Besides, *in vivo* pharmacokinetic experiments displayed that curcumin-encapsulated nanoparticles increase the bioavailability of curcumin ten times as compared to free curcumin and proved the applicability of the lignin nanoformulations for oral administration of drug molecules with poor bioavailability and low solubility.

Our research team studied the antimicrobial activity of two- and three-component morin/ chitosan/lignin formulations. The combination of morin-chitosan and morin-lignin showed a 100% increase in the inhibitory activity against *S. aureus* compared to the pure components. The combination of morin-chitosan-lignin showed an inhibitory effect on all the tested bacterial strains, with the highest antimicrobial potential against *S. aureus*, and the lowest against *B. cereus* and *E. coli*. The hypothetical mechanisms by which chitosan–morin–lignin combined systems can inhibit microbial growth include: electrostatic interactions between microbial cell surfaces causing cell wall disruption and intracellular component leakage; adhesion and penetration of the morin–polymer combinations into the cell membrane imposing sequential negative effects on protein synthesis processes; chelation of fundamental nutrients and essential metals; ROS production and cell surface pH reductions resulting in mediated bacterial apoptosis [25].

Toxicity of chitosan-coated lignin nanoparticles was tested by Stine *et al*. (2021) [26] and Ravishankar *et al*. (2019) [27]. Binding of chitosan to nanoparticles enhances their adsorption to biological samples through electrostatic adhesion. The newly synthesized nanoparticles were applied directly to the developing zebrafish embryo with the enzymatically removed chorion, and to the chorionic membrane of the embryo. Embryo lethality and sublethal endpoints were monitored. Higher mortality and sublethal endpoints were observed in embryos treated with chitosan-lignin nanoparticles compared to plain lignin and control groups in the concentration of 320 μg/ml. The study proved that chitosan-loaded lignin nanoparticles were more toxic than regular lignin particles [26]. It was established that gels based on chitosan and alkaline

lignin are non-toxic to mesenchymal stem cells *in vitro* and to zebrafish up to 100 μg/ml *in vivo*. When the gel was applied to NIH 3T3 mouse fibroblast cells, they showed good cell migration, suggesting that the gel could be used in wound healing [27].

The bioactivities of binary hydroxypropyl methyl cellulose (HPMC) /lignin) and three-component systems (HPMC/lignin/chitosan) studied by Alzagameem *et al*. (2019) were affected by lignin concentration. The systems displayed higher antioxidant and antimicrobial activity at 5% concentration, while the biopotentials decreased at 30%. Besides, the antimicrobial activity of lignin depending on its source increased in the order: softwood organosolution > softwood kraft > grass organosolution. Testing the films against spoilage bacteria that grow at low temperatures, revealed the activity of HPMC/lignin 1 (extracted by $H_2SO_4$ at pH = 2 for 90 - 180 min at room temperature) and HPMC/lignin/chitosan films against both *Brochothrix thermosphacta* and *Pseudomonas fluorescens* [28].

*Cardioprotective activity of morin*

The cardioprotective effects of morin have been proven by numerous studies. One of these was done by daily administration of morin (40 mg/kg) for 30 days. Albino Wistar rats were then injected subcutaneously with isoproterenol (85 mg/kg) every 24 hours for 2 days. The study of Al-Numair *et al*. (2014) [29] established that isoproterenol (ISO)-induced myocardial infarction (MI) in rats led to increased levels of lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS) in plasma and heart and a decrease in catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), reduced levels of glutathione (GSH) and vitamin E and C. The treatment of the rats with morin provoked a significant return to approximately normal levels of the above parameters.

Deoxycorticosterone acetate (DOCA)-induced salt hypertension in male Wistar rats and antihypertensive and antioxidant effects of morin were studied by Prahalathan *et al*., 2011 [30]. Rats showed significant increase in systolic and diastolic blood pressure, heart rate, and in thiobarbituric acid and lipid hydroperoxide levels. Reduction of SOD, CAT, glutathione peroxidase, glutathione, vitamin C and vitamin E was observed. The administration of morin (50 mg/kg) daily for six weeks returned the parameters to approximately normal levels.

Another scientific team investigated the interactions between morin and different drugs. The combination of diltiazem (15 mg/kg, oral) and morin (1.5–7.5 mg/kg, oral) in rats increased the bioavailability of diltiazem by 1.4 to 1.8 times. The combination of etoposide (6 mg/kg, oral) and morin (15 mg/kg, oral) in rats increased the bioavailability of etoposide by 1.4 times. It was established that in isoproterenol-induced myocardial infarction and doxorubicin cardiac fibrosis, morin restored the mitochondrial function, improved the antioxidant and mitochondrial enzymes and decreased apoptosis [17].

*Neuroprotective effects of morin*

Morin executes a significant neuroprotective action by affecting various mechanisms. There are studies, which provide evidence that morin ameliorates neuroinflammation and oxidative stress in Alzheimer's Wistar rats. *In vitro* studies show that morin can exert anti-amyloidogenic activity reversibly and specifically by binding to the amyloid fibril structure of Aβ instead of to the monomers of Aβ. Morin has been shown to form complexes with Cd (II) and exhibit strong antioxidant activity *in vitro* [18].

Thangarajana *et al.* (2018) induced lead acetate (PbAc) intoxication and subsequent oxidative stress in the rat brain. Oxidative stress, memory impairment, and motor deficits were observed. Rats, that were treated with morin, showed significant recovery of the abnormal changes. Histopathological sections of the cerebral cortex, hippocampus and cerebellum showed neuronal loss in PbAc rats and their recovery with the morin administration [31].

Khamchai *et al*. (2020) administered 30 mg/kg morin by intraperitoneal injection before ischemia, during ischemia, and at reperfusion in rats in brain injury and blood-brain barrier (BBB) disruption. The results showed a significant reduction in the production of reactive oxygen species, lipid peroxidation, blood-brain barrier damage and neutrophil infiltration, inflammation and apoptosis by reducing brain infarct size, ameliorated cerebral damage and increased protein expression. The data obtained displayed that morin exerts protective effect against cerebral and blood-brain barrier damage by attenuating inflammation, oxidative stress and apoptosis [32].

Sharma *et al*. (2017) investigated the effect of intranasal delivery of morin hydrate-loaded microemulsion for the management of Alzheimer's disease. After intranasal delivery, the brain and blood drug concentrations were found to be higher for morin-loaded microemulsion as compared to pure morin. Significant reduction was observed in the pharmacodynamic parameters after intranasal

administration of the flavonoid-loaded micro-emulsion as compared to control group. 21 days of morin-loaded microemulsion treatment resulted in significantly enhanced memory in Wistar rats [33].

Zhang *et al.* (2010) induced Parkinson's disease in mouse. They studied the neuroprotective effects of morin on 1-methyl-4-phenylpyridinium ion (MPP+)-induced apoptosis in neuronal differentiated PC12 cells and a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. Application of morin (5-50 μmol/L) significantly reduced the loss of cell viability, apoptosis and ROS formation. Morin (20 to 100 mg/kg) attenuates behavioral deficits, dopaminergic neuronal death and striatal dopamine depletion. The obtained data suggest that morin has neuroprotective actions *in vitro* and *in vivo* and may be used as a therapeutic agent for the treatment of neurodegenerative diseases [34].

*Hepato-protective effects of morin*

Hepato-protective effects of morin could be attributed to increase in superoxide dismutase, hemoxygenase-1, glutathione-S-transferase, catalase, glutathione peroxidase and decrease in liver transaminase, hepatocyte death, total cholesterol, triacylglycerol, formation of ROS, etc.

Li *et al.* (2019) induced acute hepatotoxicity with $CCl_4$ in pathogen-free male C57BL/6 mice, which provoked reduced liver transaminase levels and hepatocyte death, attenuated liver histopathological changes and inflammatory response. The administration of morin orally lead to hepatoprotective effect by blocking TREM-1-mediated inflammatory response in macrophages and reducing oxidative stress by modulating the regulator of cellular resistance to oxidants Nrf2/HO-1 pathway in the liver [35].

Mondal *et al*. (2022) compared the hepatoprotective effect of free administered morin and morin-encapsulated chitosan nanoparticles in chronic arsenic poisoning of the liver. The flavonoid-loaded nanoformulation was four times more effective compared to pure morin. Administration of the nanoaprticles reduced serum levels of ALT, AST, ALP and tissue arsenic deposition, inhibited the formation of ROS and increased the levels of SOD), GSH, CAT, GST, (GPx), hemoxygenase-1 (HO-1) and NADPH quinone oxidoreductase 1 (NQO1) [36]. Kamal *et al.* (2022) reported that morin significantly protected the rats against paracetamol-induced hepatotoxicity associated with alterations in the plasma total cholesterol, triacylglycerol, and HDL-C levels, as well as liver ALT, AST, ALP, LDH, protein thiol, GSH, SOD, CAT, MDA, and tumor necrosis factor-alpha levels. The histological results showed that morin protected the liver tissues against the toxic effect of paracetamol [37].

*Photoprotective properties of morin*

Tran *et al*. (2022) encapsulated morin in liposomal vesicles to improve the photoprotective properties of the flavonoid. It was established that morin was a strong scavenger of DPPH radicals, displayed a remarkable ROS inhibitory ability, and protected keratinocytes against dust particles by downregulating the expression of matrix metalloproteinase-1 (MMP-1). Water solubility of liposomal morin was significantly improved as compared to the free form of morin due to a reduction in the particle size of the liposomes, which increased dermal absorption. Based on the findings obtained, it was suggested that morin-loaded liposomes can be used for various topical antiaging applications [38]

*Potential of morin against induced lung injury*

Tianzhu *et al*. (2014) studied the anti-inflammatory activity of morin on acute lung injury using lipopolysaccharide-induced acute lung injury mouse model [39]. The experimental results displayed that morin treatment significantly depressed inflammatory cell numbers in the bronchoalveolar lavage fluid, decreased lung NLRP3 inflammasome protein level, and improved SOD activity and inhibited myeloperoxidase activity. Histological investigations established that morin considerably inhibited neutrophils in lung tissue compared with control group, which proved the protective effect of the flavonoid on lipopolysaccharide-induced acute lung injury in mice [39]. The potential of morin against induced lung injury from cigarette smoke was investigated by Cai *et al.* (2018). The obtained results showed that morin significantly inhibited lung pathological changes, myeloperoxidase (MPO) activity and MDA levels. The levels of total cells, macrophages, neutrophils and the production of inflammatory cytokines were also suppressed by the flavonoid. The results indicate that morin may be used as a potential drug for cigarette smoke-induced lung injury [40].

Obviously, morin exhibits advantageous therapeutic effects against acute lung injury, however, the mechanism of its action remains unclear. Recently, a scientific team established considerable increase of inflammation and pyroptosis in the lung tissue of mice with lipopolysaccharide-induced acute lung injury.

According to the study, the flavonoid blocked the activation of the TLR4/TRAF6/NF-κB pathway, synergically inhibited the entry of p65 into the nucleus, inhibited caspase-1 activation and protected the GSDMD protein from cleavage [41].

*Anti-diabetic effects of morin*

Streptozotocin (STZ)-induced diabetes mellitus has been studied by many scientists. Naziret *et al*. (2021) conducted an *in vivo* experiment displaying a significant restoration of changes in fasting blood glucose levels and body weight loss, as well as lowering cholesterol, LDL, triglyceride levels and increasing HDL after morin administration. Significantly increased antioxidant enzymes such as GPx, GSH and % DPPH inhibitory activity, while reduced level of lipid peroxidation Malondialdehyde (MDA) values in pancreatic homogenates of diabetic rats were established. Morin demostrated high antioxidant, antidiabetic and antibacterial potential, making it a suitable therapeutic agent for the treatment of diabetes mellitus and bacterial infections [42]. The study of Vanitha *et al.* (2014) reported that morin reduced blood glucose and improved serum insulin levels, decreased glucose-6-phosphatase and fructose-1,6-bisphosphatase, increased hepatic hexokinase and glucose-6-phosphate dehydrogenase activity in diabetic rats. The bioflavonoid was found to be effective in preserving the normal appearance of pancreatic islets, as well as in preserving insulin-positive cells in STZ-rats. These findings indicate that morin has beneficial effect in diabetes by regulating carbohydrate metabolic enzyme activities [43]. Protective effect of morin on trabecular bone of healthy and diabetic rats with diabetic osteopenia was investigated by Abuohashish *et al*. (2013). Significant bone loss, damage to trabecular bone microarchitecture, density and other morphometric parameters were associated with the disease. Serum levels of glucose, IL-1β, IL-6, and TNF-α were significantly increased, while the levels of insulin and GSH were decreased in diabetic rats. These serum changes returned to normal values after 5 weeks of morin treatment. These results reveal the protective effect of morin against diabetes-induced osteopenia [44]. Antiosteoarthritic properties of morin *in vitro* and *in vivo* were studied by Chen *et al*. (2012). Morin inhibited the expression of matrix metalloproteinase MMP-3 and MMP-13 and increased the expression of tissue inhibitors of metalloproteinase TIMP-1 in interleukin-1b-induced rat chondrocytes. *In vivo* study in a rat model demostrated that morin suppressed cartilage degradation [45].

*Profit of morin/lignin combinations*

Our team created different formulations based on morin. Combination of morin-chitosan and morin-lignin showed a 100% increase in inhibitory activity against *S. aureus* compared to the pure components. The combination of morin-chitosan-lignin showed an inhibitory effect on all the tested bacterial strains, with the highest degree occurring against *S. aureus*, and the weakest against *B. cereus* and *E. coli*. The hypothetical mechanism of action includes electrostatic interactions between microbial cell surfaces, adhesion and penetration of the morin–polymer combinations into the cell membrane, chelation of fundamental nutrients and essential metals. The lower rate of *in vitro* release of morin from flavonoid-encapsulated lignin microparticles, as well as the lack of gastric mucosal irritation, proved the advantages for p.o. application of our newly synthesized microparticles compared to directly applied morin. A significant antioxidant and radical scavenging ability of the particles has been demonstrated [46].

## CONCLUSIONS AND FUTURE PROSPECTS OF LIGNIN- AND MORIN-BASED FORMULATIONS BIOMEDICAL APPLICABILITY

After extensive research we can conclude that lignin is characterized with low cytotoxicity, biodegradability, good stability and low pro-inflammatory effect. As disadvantages of lignin, we can point out the variability in its composition, poor binding and delivery of hydrophilic drugs. In conclusion, based on new scientific research on the antioxidant, antimicrobial, antiproliferative, anticancer, antiviral and antifungal activities of lignin, we may say that it can be used as a vehicle of various agents in biomedicine.

Direct administration of morin has a number of disadvantages, such as poor water solubility, resulting in low oral bioavailability, very short plasma half-life (30 min) after intravenous injection, rapid metabolism, degradation and elimination from the body, hindering its potential contribution to the prevention and treatment of various diseases. It is sensitive to the solvent used and pH, being highly unstable at basic conditions and metastable in acidic and neutral media. Diverse scientific studies have reported *in vitro* and *in vivo* cardioprotective, neuroprotective, nephroprotective, hepatoprotective, anticancer, gastroprotective, pulmonary, and antidiabetic activities of the natural bioflavonoid morin.

Future perspectives for modern scientific research are related to challenges of designing

natural delivery systems for polyphenols, ensuring effective integration of bioactive molecules and their protection from degradation along their physiological distribution pathways and reaching their area of therapeutic action. Another important task for scientists is the determination of a safe and effective dose for the treatment of various acute and chronic diseases.

In summary, to date the available information on lignin and morin and their overall impact on experimental animals is still scarce, but it is a prerequisite for subsequent trials proving their effectiveness against various diseases.

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