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Phytochemical Characterization And Nanosponge-Based Delivery Of Bioactives From Moringa Oleifera, Glycyrrhiza Glabra, And Ficus Religiosa

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Abstract
Moringa oleifera, Glycyrrhiza glabra (licorice) and Ficus religiosa (sacred fig) have been traditionally used as medicinal plants and reported to possess anti-inflammatory, antidiabetic, and gastroprotective activities. However, the bioactive phytochemicals within them are frequently water-insoluble and unstable. To this end, a polyherbal formulation comprising of isolated phytomolecules quercetin (from M. oleifera), 18β-glycyrrhetinic acid (from G. glabra), and β-sitosterol (from F. religiosa) has been developed using β-cyclodextrin-based nanosponge drug delivery system in the present study.The phytochemical was extracted, isolated and identified using spectral analysis. Nanosponges, namely β- cyclodextrin nanosponges were prepared, loaded with each phytochemical at optimized ratios and formulated as gastroretentive (floating) tablets.The preliminary phytochemical screening revealed a high concentration of flavonoids, saponins and terpenoids in the plant extracts. Three compounds were isolated and their structures were determined as quercetin, 18β-glycyrrhetinic acid and β-sitosterol. Nanosponges encapsulating these phytomolecules were found to exhibit high encapsulation efficiencies (~81–85%) at an optimized 1:8 drug:polymer ratio, and to generate nanosized particles (200–232 nm) with low polydispersity and desirable zeta potentials (−20 mV) for stability. In vitro release studies revealed extended release profile of the phytomolecules from the nanosponges (77–83% release) over a period of 24 h. The floating system floated on the surface with no tendency to be drawn across the dissolution medium >12 h and was continuously expandable, releasing the drug in a sustained manner in simulated gastric fluid. In rats with ethanol- induced gastric ulcer model, the polyherbal nanosponge tablets elevated the gastric pH, inhibited the gastric acidity and ulcer index (by ~50% compared to ulcer control) and enhanced the mucosal defense (mucin levels) to an extent similar to that produced by omeprazole. The histopathology of gastric tissue showed that treated groups had highly maintained mucosal architecture in comparison to ulcerated controls. Polyherbal β-cyclodextrin nanosponge system exhibited prolonged release and gastretentive delivery of three phytochemicals, leading to prominent gastroprotection. This new herbal combination utilizes the synergistic effects of herb–herb interaction and nanoparticle technology simultaneously, for optimal therapeutic efficacy. The finding highlights the prospect of nanosponge polyherbal formulations for enhanced therapeutics in the treatment of peptic ulcer and associated gastric ailments.

INTRODUCTION

Polyherbalism, the confluence of use of several medicinal plants in a single formulation is a common practice in traditional medicine and is believed to be based on achieving synergistic therapeutic benefits that are superior to that of single medicinal plants. [23, 24] Moringa oleifera, Glycyrrhiza glabra (licorice), and Ficus religiosa are also three such herbs having multi therapy pharmacological properties. M. oleifera (miracle tree) is abundant in flavonoids (such as quercetin) and is commonly used for treating diseases such as wound healing, ulcer, and diabetes. [1, 4] Its leaves demonstrate remarkable antidiabetic activity (e.g., glycemic control) and gastroprotection action as well, which is due to its antioxidation flavonoids as a mucosal defensive stimulator. [1, 5] G. glabra root, a traditional medication for peptic ulcer, possesses saponins (glycyrrhizin) and flavonoids, which are reported to be responsible for its anti-ulcer property. The anti- ulcer effect of Licorice involves inhibition of the secretion of gastric acid (by decreasing gastrin) and promotion of the formation of mucosal protective factor (mucin, blood flow). [10, 22] Its bioactive metabolite, 18β-glycyrrhetinic acid, is a strong anti-inflammatory and ulcer-healing agent with mucosa- and epithelium-protective effects. The bark of F. religiosa (sacred fig) is astringent and a decoction of it is administered in the treatment of a number of diseases of the skin like those of eczema, ringworms and

other parasitic skin diseases particularly those on the scalp but also ulcers and alopecia. It is rich in phytosterols (e.g. β-sitosterol) and triterpenoids which have previously been demonstrated to enhance ulcer healing and reduce blood glucose levels. Recently, β-sitosterol and β-sitosterol glycosides have been reported to exert gastroprotective activity in experimental ulcer models in animals, which are possibly due to the anti-inflammatory activity and strengthening of gastric mucus barrier. These three plants jointly exhibit an extensive range of pharmacological actions – antioxidant, anti-inflammatory, antisecretory, and cytoprotective – which may bear on the multifactored pathogenesis of peptic ulcer disease. [1, 7, 12]

However, crude herbal extracts or purified phytochemicals can have pharmacokinetic limitations. Most of the plant-made compounds have low water-solubility and low stability, leading to low bioavailability and variable efficacy. [4, 17] For instance, quercetin and glycyrrhetinic acid have very low solubility, and may degrade or be metabolized easily, which breast the therapeutic efficacy. There is need for new delivery systems to overcome these limitations. In this scenario, the cyclodextrin-based nanosponges (NS) represent a recent and advanced nano-carrier system for enhancing the delivery of phytopharmaceuticals. [14, 15] Nanosponges are matrixes made up of cyclodextrins interconnected by hypercrosslinking linker to form a porous three dimensional networks that can entrap bioactive molecules. In the context of phytochemicals, β-cyclodextrin (β-CD) should be mentioned as a versatile platform given that its hydrophobic internal core is able to accommodate hydrophobic phytochemicals and its hydrophilic exterior makes the complex dispersed in biological solutions. Networking of β-CD molecules with appropriate reagents leads to the formation of nanoparticle sponges that have a large surface area and many inclusion pockets that allow high drug loading, protection of drugs from degradation, and tunable release rates. Significantly, β-CD nanosponges are biocompatible and have been used effectively for the sustained release of drugs. They can serve as "bioavailability enhancers" for phytochemicals, such as curcumin, resveratrol, or quercetin, through increasing solubility and retention time. [15,16, 17,18]

Gastroretentive drug delivery is another approach to achieve an enhanced therapeutic efficacy. Floating systems are those making drugs buoyant in the stomach, and thereby maintaining the gastric residence of the dosage form. This is especially advantageous in the treatment of peptic ulcer, since a local, prolonged release of the actives on the gastric mucosa can promote healing. The rate of release in the stomach over several hours can be achieved for drugs from gastroretentive floating tablets that consists of hydrophilic polymers and gas generating agent which swell and floats over the gastric fluid. The bioavailability of drugs with narrow absorption window in the upper GI-tract and distinct prolonged therapeutic presence at the ulcer site can be improved by such systems. 15 Floating tablets containing licorice extract could success in having increased retention time in the stomach which results in better anti-ulcer activity than conventional dosage form16. We expect that incorporation of our phytochemical-loaded nanosponges within a floating tablet matrix will collectively show prolonged gastric mucosa local contact (without compromising on the use of a nanocarrier) and gastroretention. [19, 20]

Objectives: The main objectives of the present study were (1) the isolation and characterization of various bioactive phytochemicals from M. oleifera leaves, G. glabra roots, and F. religiosa bark (2) formulation of β-cyclodextrin-based nanosponges loaded with the isolated phytomolecules, optimizing for the highest entrapment and sustained release of the biosubstances (3) the development of a polyherbal floating tablet containing the loaded nanosponges and (4) evaluation of the pharmacological performance of the formulations, especially their anti-ulcer and potential antidiabetic importance. The primary objective is to show the development of a new polyherbal nanosponge platform technology that can improve the solubility, stability and therapeutic synergy of herbal actives to yield superior pharmacological effects. Peptic ulcer healing is a primary therapeutic application, because the selected plants have been used ethnomedicinally against ulcers and there is an urgent need for more effective ulcer remedies but with less adverse effects. To the best of our knowledge this is the first time that the Moringa–Licorice–Ficus plant phytochemicals have been incorporated into a single nanosponge based GR formulation and there is a novelty in using modern drug delivery approaches to a traditional polyherbal medicine.

MATERIALS AND METHODS

Plant Materials and Phytochemical Extraction

Collection and Authentification: Fresh leaves from Moringa oleifera, roots from Glycyrrhiza glabra and bark from Ficus religiosa were collected from local market of India during the appropriate harvesting seasons. The botanical identification and authentication were carried out by an experienced taxonomist and voucher specimens were preserved in the Herbarium of Botanical Survey of India, Prayagraj. The plant parts were washed, shade dried and powdered to coarse powder for extraction.

Extraction method: Successive extraction was conducted with each plant material to achieve the fractions enriched with distinct phytochemical. To extract flavonoids, phenolics and sterol in M. oleifera leaves and F. religiosa bark, the most suitable solvent used is 70% ethanol, as a major solvent, followed by petroleum ether (for defatting), whereas G. glabra root was extracted with ethanol and water for recovering glycosidic saponins. Forced maceration and Soxhlet methods were applied whenever necessary: for example, the F. religiosa bark (100 g) was subjected to Soxhlet extraction with 400 mL of ethanol until siphon drops were colorless. Likewise, leaves of subjected Moringa leaves Soxhlet extraction using ethanol and G. glabra root was hydroalcoholic macerated (ethanol 50%) gave the licorice extract. Each solvent extract was filtered after extraction, and the extract was concentrated under reduced pressure using a rotary evaporator, at <50°C, to give a semisolid crude extract and subsequently dried to powder. The dried extract yields were determined. The M. oleifera leaf ethanol extract gave a dark green residue (yield 8.5%), G. glabra root extract a brownish residue (8 g, 10%) and F. religiosa bark as a dark brown resinous solid(~6% yield)[3]

Preliminary Phytochemical Analysis: The crude extracts were subjected to qualitative test for predominant phytochemical groups by employing standard reagents and procedures. Alkaloids were screened for their presence with Mayer's, Wagner's, Hager's, and Dragendorff's reagents showing typical precipitates (e.g., cream precipitate with Mayer's reagent for alkaloids). Glycosides were screened by Bornträger's test for anthraquinones and Keller–Killiani test for cardiac glycosides etc. Saponin detection Saponins were identified by the existence of froth on shaking samples vigorously (froth test). Tannins and phenolics – The blue-green coloration with ferric chloride and precipitate with gelatin were shown. The flavonoids were characterized by positive Shinoda and alkaline reagent tests, which revealed positivity for magnesium + HCl with a reddish color and for NaOH with a yellow color that fades on acidification. Triterpenoids and sterols were tested and confirmed by Liebermann–Burchard reaction (green to blue color was developed). Rich contents of flavonoids and phenolics were detected in Moringa and Ficus extracts, and saponin glycosides were found in G. glabra (foam test: very strong positive reaction). No alkaloids were found in these extracts, whereas terpenoids/sterols were found primarily in F. religiosa bark extract (Liebermann–Burchard test showing a bluish-green). Moreover, TPC and TFC of the extracts were also determined with colorimetric assays. Among the plants, M. oleifera leaf extract displayed a greater amount of TPC (approximately 128 mg gallic acid equivalents/g) as compared to ethanol extract, and the extracts from G. glabra and F. religiosa showed intermediate high phenolic contents with significant contributions from aqueous and ethyl acetate fractions. Flavonoid content as quercetin equivalents for M. oleifera ethanol extract (~90 mg QE/g) and F. religiosa ethyl acetate extract (~78 mg QE/g) was also highest, coinciding with the high ferric chloride testing for flavonoids. These quantitative results provided a rationale for choosing ethanol as the extraction solvent of major actives from the three plants. [3, 11]

Isolation and Identification of Phytoconstituents

Chromatographic Separation: Dried ethanol extracts of all the plants were chromatographed to obtain the major bioactives. For Moringa oleifera leaves a silica gel column based fractionation using solvents with increasing polarity (e.g., chloroform-methanol mixtures) was employed to the ethanol extract (20 g). Fractions were monitored by thin-layer chromatography (TLC). The Moringa extract was fractionated by TLC using n-butanol : Acetic acid : Water (4:1:5) as the solvent system with four spots. Fractions showing similar TLC profiles were pooled. One of the fractions ("F3") that exhibited a Main spot (Rf ~0.5) was crystallized from solvent evaporation to give Compound 1 as a yellow amorphous powder. For G. glabra, TLC screening of different solvent extracts (petroleum ether, ethyl acetate, ethanol and water) was performed for marker 18β-glycyrrhetinic acid (GA). With ethanol and pet. spots at the Rf values of pure GA (R f ~0.65) on the plate among the ether extracts of licorice (3-20). This was followed by G. glabra ethanol extract, large-scale column chromatography with elution gradients of nonpolar to intermediate itself (hexane → ethyl acetate). Fractions 8–13 (from pet. and 17–22 (from ethanol extract) collected on the basis of TLC visualization of presence of GA were combined and the pool subjected to evaporation and recrystallization (80:20 ethanol–water) to give a white powder designated as Compound 2. 3. enough was treated with Liebermann–Burchard reagent to give a dark green color, confirming the presence of a triterpenoid skeleton. Ethanol extract of F.religiosa bark was column chromatographed on silica gel using hexane: ethyl acetate gradient system for sterols/terpenes. Fractions 13–24 giving positive results to terpenoids in Salkowski's test (red color) and a single TLC spot (R f ~ 0.69 in hexane–EtOAc 85:15) were grouped together. Evaporation gave the title compound 3 as a white crystalline solid. [9,10, 11, 12]

Spectroscopic characterization: The pure isolates were characterized using a combination of spectroscopic techniques. Compound 1(Moringa) with melting point >300°C was found to be soluble in polar organic solvents and possibly had a polyphenolic nature. Mass spectrometry (ESI-MS) indicated a molecular ion

at m/z 308 [M+H]^+ corresponding to the molecular weight of 302 for quercetin (C_15H_10O_7). The ^1H NMR spectrum for Compound 1 showed a series of aromatic protons at δ 6.2–7.5, as well as hydroxyl protons (~δ 10–12, exchangeable with D_2O), consistent of a flavonol skeleton. In addition, a singlet at δ 6.4 (H-3) for a flavonol and meta coupled doublets at δ ~6.9 and 7.7 (B ring 1,3,4,5-tetrasubstituted as quercetin) are also diagnostic. Its ^13C NMR spectrum exhibited 15 resonances and it contained carbonyl carbon at δ 176 (C-4), olefinic carbons at δ ~125 and 133 (C-2 and C-3, respectively), and a series of aromatic carbons at δ 115–150. OMe signal was lacking, therefore it was not a methoxylated derivative, but aglycone quercetin. The broad band observed in the FT-IR spectrum of Professorium~2(2) was due to O–H stretching (3440~cm^−1), C=O stretch at 1635~cm^−1 (conjugated carbonyl) and aromatic C=C stretching at ~1610~cm^−1. All these data indicated that Compound 1 is quercetin, which agrees with literature studies of quercetin from Moringa leaves.

The structure of compound 2 (from G. glabra) was identical with that of 18β-glycyrrhetinic acid (GA), the aglycone of glycyrrhizin. It exhibited a melting point ranging from 290 to 292°C, consistent with reported values for GA. The FT-IR spectrum exhibited a band at 1702 cm^−1 attributable to a carboxylic acid C=O, and a broad O–H stretch around 3500 cm^−1 (hydroxyl group). Similarly, the absence of bands in aromatic C=C region are seen which in according with the fact that; GA is an aromatic free pentacyclic triterpene. 13C NMR (Camp) revealed ~30 different carbon signals, a typical triterpenoid structure. 13C NMR Key ^13C signals at δ ~78.8, 128.5, 181.1 and 200.5 ppm, were due to C-3 (hydroxyl bearing), C-12/C-13 olefinic carbons of 11,13-dehydro configuration, C-11 keto or carboxyl carbons, respectively. The ^1H NMR spectrum of Compound 2 showed three tertiary methyl singlets (δ ~0.7–1.0), several secondary methyl doublets (δ ~1.1–1.4) and an olefinic proton at δ 5.3 (for C-12/C-13 double bond in GA). The NMR signals agreed with literature values of 18β-glycyrrhetinic acid and thus identified it.

Compound 3 (of F. religiosa) was characterized as β-sitosterol, a phytosterol. It displayed a molecular ion m/z 414.8 ([M+H]^+) in ESI-MS, consistent with the molecular formula C_29H_50O (MW 414.7). Its ^1H NMR spectrum (CDCl_3) revealed diagnostic signals: multiple methyl proton singlets (δ 0.68, 0.81, 0.86, 0.93, and 1.01, five tertiary methyl group), a broad singlet (δ 1.21, overlapped two secondary methyl groups), and an olefinic proton (δ 5.35, Δ^5 unsaturation in the steroid nucleus). The ^13C NMR spectrum displayed 29 carbons, C=C resonances at δ ~121.7 and 140.8 (C-5 and C-6 from the sterol double bond), and a signal at δ ~71.3 for C-3 (hydroxyl-bearing). These data aptly correlated with reference NMR of β-sitosterol. The FT-IR spectrum of Compound 3 indicated an –OH stretch at 3418 cm^−1 and C–H stretches due to methyl/methylene groups at 2933 and 2862 cm^−1. Weak C=C stretch at ~1640 cm^−1 was characteristic of the steroid alkene. Hence, compound 3 was identified as β-sitosterol. The mass spectrum of Compound 3 (β-sitosterol) is given in Figure 8, the molecular ion is at m/z 414 peak. [11, 13, 26]

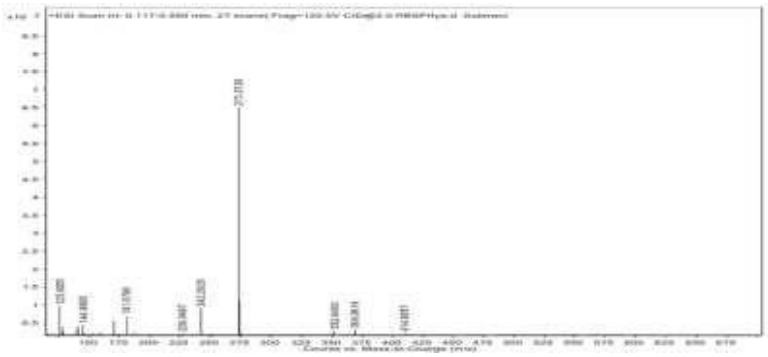

Figure 8. Mass spectrum of pure Compound 3 (β-sitosterol) The mass spectrum of isolated Compound 3 (β-sitosterol) reveals a molecular ion peak at m/z 414, which corresponds to C29H50O.

The identity of the isolated phytomolecules corresponds to the known constituents of the particular plants, namely quercetin as one of the major flavonoids present in Moringa leaves and glycyrrhetinic acid, the active metabolite of licorice saponin "glycyrrhizin", β-sitosterol as a frequent phytosterol occurring in the bark of Ficus. These actives were chosen as “model” actives for the formulation.

Preparation of Phytomolecule-Loaded β-Cyclodextrin Nanosponges

Preparation of β-CD Nanosponges: β-Cyclodextrin nanosponges (β-CD NS) were obtained by the crosslinking of the β-CD with an activated carbonate linker (dimethyl carbonate, DMC) via a solvent method borrowed from the literature. In short, dry dimethylformamide (30 mL) was used to dissolve β-CD (2.0 g) with slight heating and anhydrous triethylamine (1 mL) was added as a catalyst. The solution

was refluxed at 90 °C for 3 h with continuous stirring after dropwise addition of the DMC (14 mL). In this reaction, DMC reacts with β-CD hydroxyl groups, leading to the formation of carbonate linkages connecting the cyclodextrin entities and inducing the formation of a crosslinked polymeric network (the nanosponge). The filtered solution was refluxed, excess of the solvent was distilled off and the obtained crude nanosponge product was a pale solid mass. This crude β-CD NS was purified by two consecutive washings using distilled water at first (to eliminate the catalyst and unreacted β-CD) and, second, by ethanol (to remove unreacted DMC or byproducts). The resultant product was washed with deionized water and dried in an oven at 80 °C until a constant weight was obtained, then to remove any unreacted β-CD entrapped in the network, the dried NS was subjected to Soxhlet extraction with ethanol (200 mL) for 24 h. The obtained β-CD nanosponges were dried to constant weight. [14, 15, 25]

Loading of Phytomolecules in Nanosponges: The isolated phytoconstituents (quercetin, GA, β-sitosterol) were loaded separately in to β-CD nanosponge by incubation method. For each compound, a weighed quantity of β-CD NS was dispersed in distilled water (50 mL) and sonicated for a few seconds, in order to obtain a homogeneous suspension and to pretreat the nanosponge network. The phytochemical (quercetin, GA or β-sitosterol) was suspended in this suspensiondrug:polymer ratio of 1:2, 1:4 and 1:8 (w/w) was added. The solution then received 10 minutes of sonication to assist inclusion of the hydrophobic drug into the cavities of the CD and continuous stirring at room temperature for 24 hours for attaining of a complexation equilibrium. The hydrophobic interior of the nanosponges is favored by the low water solubility of the phytochemicals and they are slowly redistributed within the nanosponges over time. The suspension (after 24h) was removed and centrifuged (2,000 rpm, 10 min) to obtain any free drug as a precipitate. The obtained supernatant was aspirated off and the drug-loaded nanosponges was collected, lyophilized and kept to get a dry powder of drug loaded nanosponges (PLN). These were kept in a desiccator until use. Formulations were developed in 9 formulations with 3 ratios for each compound- PLN1-PLN3 (@1:2, 1:4, 1:8) for β-sitosterol, PLN4–PLN6 for quercetin, and PLN7-PLN9 for glycyrrhetinic acid (Table 1). [16, 17]

Evaluation of Drug: Polymer Ratio: The drug loading (DC) and entrapment efficiency (EE) of all PLN formulations was optimized by using drug loading percentages. A weighed aliquot of each PLN (corresponding to 10 mg of theoretical drug) was dissolved in ethanol and the actual drug content determined (after proper dilution) by UV–Vis spectrophotometry at the λ_max of the phytochemical (i.e., 256 nm for quercetin, 251 nm for GA, 208 nm for β-sitosterol). %Drug content = (Measured drug / PLN Sample mass)×100%and %Entrapment Efficiency = (Measured drug / Theoretical drug)×100%. The EE and DC are summarized in Table 1. The three phytomolecules could present a tight trend: the increasing concentration of β-CD NS (greater polymer ratio) improved both EE and DC. At drug:polymer 1:8, highest EE was for each compound ~84–85% for β-sitosterol and GA and ~81.5% for quercetin as compared to ~73–77% at 1:2 ratio. This is due to the more binding sites and cavity volume for drug and polymer matrix with the polymers. The nanosponges of 1:8 ratio (PLN3, PLN6, PLN9) were thus also thought to be optimized formulations for each phytochemical by offering better payload entrapment. In such formulations, the drug content was approximately 74–75% of the entire complex mass (Table 1). The slightly lower %EE for quercetin (81.5%) than for GA (85.1%) or β-sitosterol (84.5%) may be attributable to quercetin's increased polarity which could result in some partitioning into the aqueous phase, rather than exclusively into the hydrophobic cavities.

Table 1. It was found that %EE and %DC of the phytomolecule-loaded nanosponges increased when prepared at various drug: β-CD ratios (1:2, 1:4, 1:8). Each value is the average of three determinations.

Phytomolecule	1:2 Ratio (EE/DC)	1:4 Ratio (EE/DC)	1:8 Ratio (EE/DC)
β-Sitosterol	76.5% / 67.5%	80.5% / 70.8%	84.5% / 74.2%
Quercetin	73.6% / 64.1%	77.8% / 68.2%	81.5% / 73.2%
18β-Glycyrrhetinic Acid	77.3% / 68.9%	80.8% / 70.6%	85.1% / 74.8%

Characterization of Nanoparticles: The best formulations (PLN3, PLN6 and PLN9 for β-sitosterol, quercetin and GA, respectively) were characterized in terms of yield, particle size, polydispersity index (PI) and zeta potential. The freeze-dried nanosponges were obtained in about 82–85% yield based on theoretical and confirmed that the material loss was very less during the processing (Table 7.17, ref.). The size of coalesced particle determined by dynamic light scattering method was of nanometer order with mean hydrodynamic diameter of 232 nm for PLN3 (β-sitosterol NS), 216 nm for PLN6 (quercetin NS), and 225 nm for PLN9 (GA NS). All had relatively low polydispersity index (PDI ~0.21–0.25), which

indicates that the size distribution is quite small (monodisperse). The nanoparticles in aqueous suspension (1:8 formulations) showed a zeta potential of ~ –21 mV (values for –21.5 mV, and –20.5 mV; for ~ –21.6 mV). This moderately high value of negative zeta potential may be ascribed to some remaining carboxylate functionalities from cross-linking, providing colloidal stabilization based on electrical repulsion. This zeta potential (≥|17 mV|) is enough to avoid the self-agglomeration of the nanosponges in suspension. In conclusion, the nanosponges had an appropriate size that is less than 250 nm for effective mucosal penetration, well distributed size and stable surface charge. [14, 18]

In vitro release of phytochemicals from the nanosponges was conducted in phosphate buffer (pH 7.0) at 37°C by using a dialysis bag method to mimic the in vivo environment. An optimized PLN (equivalent to 1 mg drug) was transferred into a dialysis membrane and cumulative release was determined for 24 h measuring UV absorbance of the diffusate. Release profiles of β-sitosterol, quercetin and GA from their respective nanosponges are compared in Figure 1. Over the 24 h, all three formulations exhibited sustained release without burst effect. Only ~18–22% of the payload was released in the first 1–2 h and by 8 h ~37–40% was released. A more sustained slow-release phase followed, leading to 77.8% (β-sitosterol), 81.5% (quercetin) and 83.1% (GA) of cumulative release at 24 h. The release profiles are inversely proportional to molecular size, where the slightly faster GA (molar weight ~470) release compared to quercetin (~302) or β-sitosterol (~414) could be attributed to its higher water-solubility or weaker interactions within the NS. Collectively, >75% of each agent was released over a period of 24 h, verifying that the nanosponges do not dump the drug and afford a controlled, sustained delivery. The sustained release is explained by the slow diffusional release of the phytochemicals from the CD nanopores and the matrix and transforming these short-lived molecules into a slow-release form. The release profiles were quasi-linear over 24 h (with zero-order kinetic tendency), which is favorable for the maintenance of the levels physiologically active. [17]

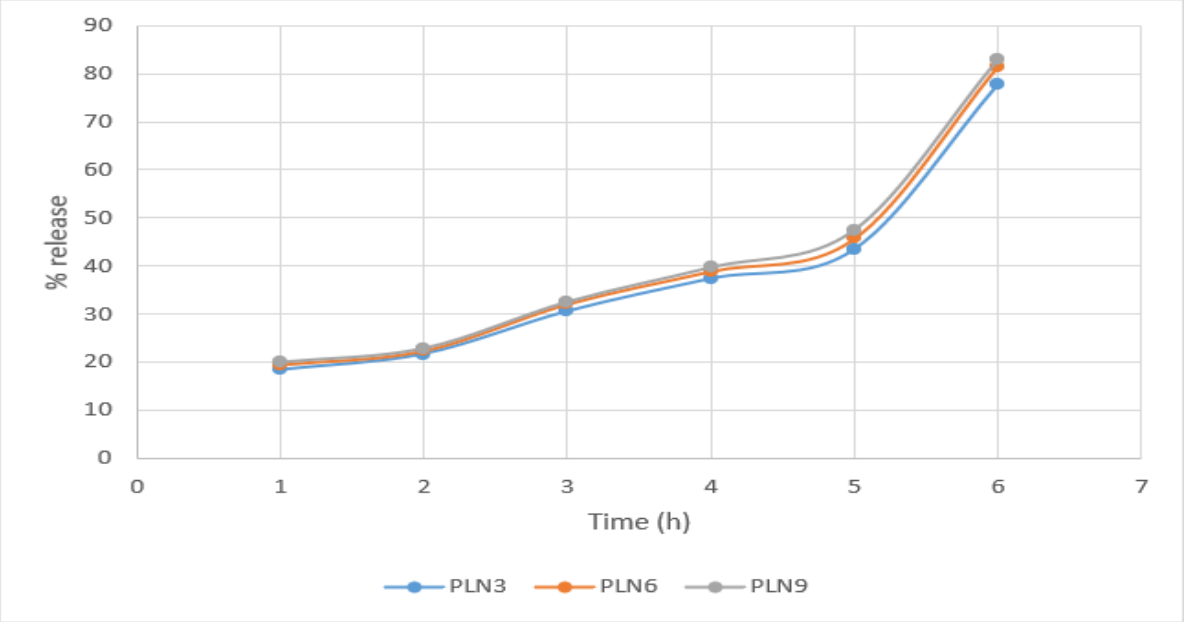

Figure 1. Comparative release kinetics of β-sitosterol, quercetin and 18β- glycyrrhetinic acid from optimized phytomolecule-loaded nanosponges (PLN3, PLN6, and PLN9). All nanosponge formulations demonstrated sustained release for 24 h with an approximate 78–83% total release at 24 h.

Formulation of Polyherbal Floating Tablets

As a gastroretentive delivery, floating matrix tablets were prepared, which contained the improved loaded nanosponges (PLN3, PLN6, PLN9) in one mixing. The aim was to design tablets in which phytochemicals are retained in the stomach to provide prolonged release of the active substances on contact with localized regions. [19, 20]

Tablet Composition and Preparation: Tablets were prepared by direct compression. The formulation was optimized through optimisation studies (data not shown), ensuring a good buoyancy and mechanical strength. The three actives were formulated in the form of their NSs; equivalent combination of β-sitosterol- NS, quercetin-NS, and GA-NS to yield 20 mg of each phytochemical per tablet (i.e., polyherbal dose containing all three actives). Hydroxypropyl methylcellulose (HPMC K100M) and sodium

carboxymethylcellulose (sodium CMC) were investigated as hydrophilic matrix polymers to modulate swelling and drug release. Sodium bicarbonate as gas-forming agent (10–15% w/w; generates CO_2 when in contact with gastric acid to allow tablet to float). In some of the formulations psyllium husk (naturally swelling fiber) was incorporated to improve the integrity and buoyancy of the matrix. A typical optimized formulation (denoted TF1) had the following composition: total phytomolecule–loaded NS (60 mg actives), HPMC K100M as the matrix (100 mg), sodium bicarbonate (100 mg), psyllium husk (75 mg) with 7% lecithin and 7% triethyl citrate, and 5% magnesium stearate and 5% talc serving as lubricants. The powders were meshed through a #80 and mixed well. Single punch tableting machine (flat faced punches) was used for compression of tablets (~500 mg per tablet). Four different formulae (TF1–TF4) were prepared to study the effect of various polymers combination, where TF1 and TF2 were comprised of two levels of HPMC (A and B), and TF3 and TF4 had sodium CMC as the base matrix (serving as control with different quantity) to compare their swelling/rate and release profile.

Precompression and Postcompression Study: The powder blends exhibited excellent flow behavior with Carr's index of ~5–15 percent, angle of repose ~29–30°, which suggests low inter-particle friction (free flow). The hardness of the tablets was ~5.1–5.5 kg/cm2, and friability was <0.6 %, which are stable tablets (meets USP limit of <1% friability). Among the tablets, the weight (±3% of acceptable range) and drug content uniformity (98–100% for quercetin as a marker) were uniform.

Floatation and swelling: The prepared tablets developed quick floatation the all tablets floated within 4-6 min on the surface of 0.1 N HCl (SGF). Once floating, they further floated >12 hours (i.e., they did not experience sinking during the test period). This extended floatability is the requirement for the gastroretentive system. The in vitro swelling results indicated evidence of a significant difference between the swelling profiles according to the polymer used (Table 7.21). HPMC-based tablets (e.g., TF1) swelled more slowly and had >12 h matrix integrity, while sodium CMC-based tablets (TF3, TF4) initially swelled more rapidly but started eroding after ~8 h. Figure 2 is swelling index (%) vs time. HPMC tablets (TF1, TF2) had approached up to ~90% swelling by 6 h, and swollen to a maximum of ~72% at 12 h (TF1, 72.1% at 12 h). Sodium CMC tablets (TF3, TF4) had a higher initial swelling (60–66% at 8 h) but decreased thereafter, suggesting they have partially disintegrated at >8–10 h, and the prolonged swelling of the HPMC formulations is in line with their prolonged drug release and floatation time. TF1 (HPMC based medium polymer level) was found to be the best release controlling agent from the matrix with maximum swelling at 12 h (72%) without disintegrating. The hydrocolloidal swollen gel layer of HPMC is probably responsible for the retardation of the solvent penetration and drug diffusion thereby providing a controlled release. These results were consistent with the polymer behaviour, HPMC forms a stable layer that gelifies and release more gradually, ionic polymers such as NaCMC swells quickly but may lose viscosity over longer times. [20]

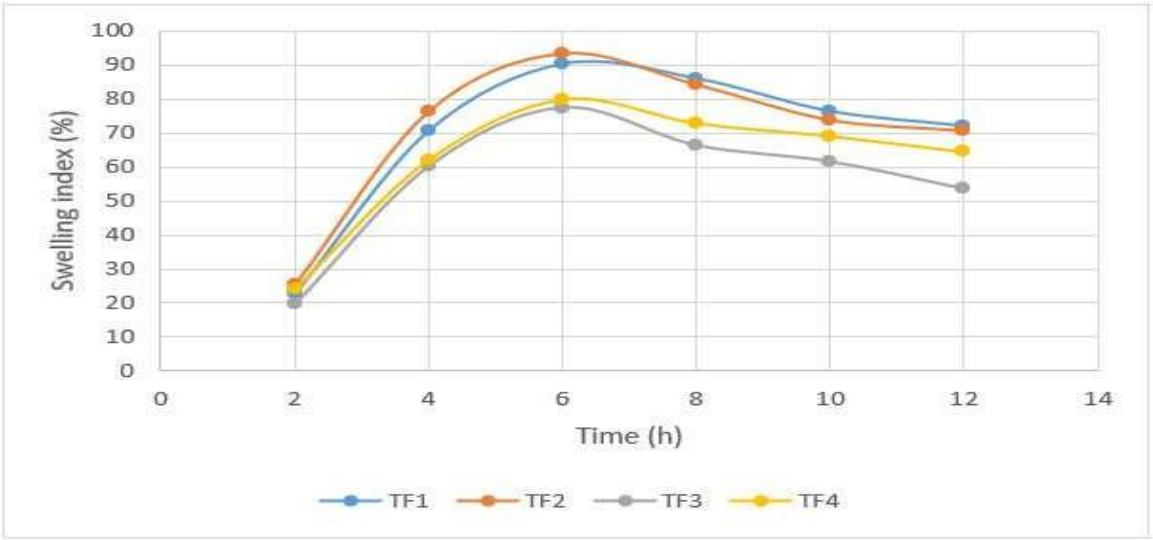

Figure 2. *Swelling profile of the floating tablet formulations (TF1–TF4) after 12 h in 0.1 N HCl (gastric medium). Tablets based on HPMC (TF1, TF2) were less swollen initially, yet they maintained higher level of swollen mass after 12 h (72% for TF1) than the sodium CMC (TF3, TF4) tablets, which exhibited rapid mass swelling and subsequently a decreasing of this process after 8 h.*

In Vitro Release Study From Tablets:

The dissolution studies for the floating tablets were conducted in 900 mL 0.1 N Hcl (pH ~1.2) at 37°C (USP-II paddle, 100 rpm) to monitor the gastric release. The tablets continued to float during the entire test. Release study marker assay (quercetin, as it has strong UV absorbance) was standardized and its cumulative release at desired intervals up to24h was determined, the polyherbal tablets provided controlled release of marker over a period of 24 h (Fig.3). In case of the optimized formulation (TF1), approximately 16% of quercetin was released within 1 h followed by ~45% in 8 h and ~78% at 24 h. Such a release pattern almost coincided with that of quercetin from the nanosponge alone (PLN6). Other formulations released marginally faster or slower with respect to polymer content: for example, TF3 (lower polymer, CMC) released ~85% by 12 h (faster), while TF2 (higher HPMC content) released ~70% by 12 h (slower). All formulations could maintain the release for at least 24 h, therefore, validating the combined role of the nanosponge carrier and the gel-forming matrix in prolonging the drug availability. The kinetics study (Table 4 of the formulation study) showed that the release followed anomalous (non-Fickian) diffusion with Korsmeyer–Peppas exponent n ~ 0.63–0.84, thus indicating that a combination of diffusion and matrix erosion prevailed. Accordingly, floating tablets were able to prolong the delivery of phytochemicals in acidic environment with tablet integrity and buoyancy. [19, 20]

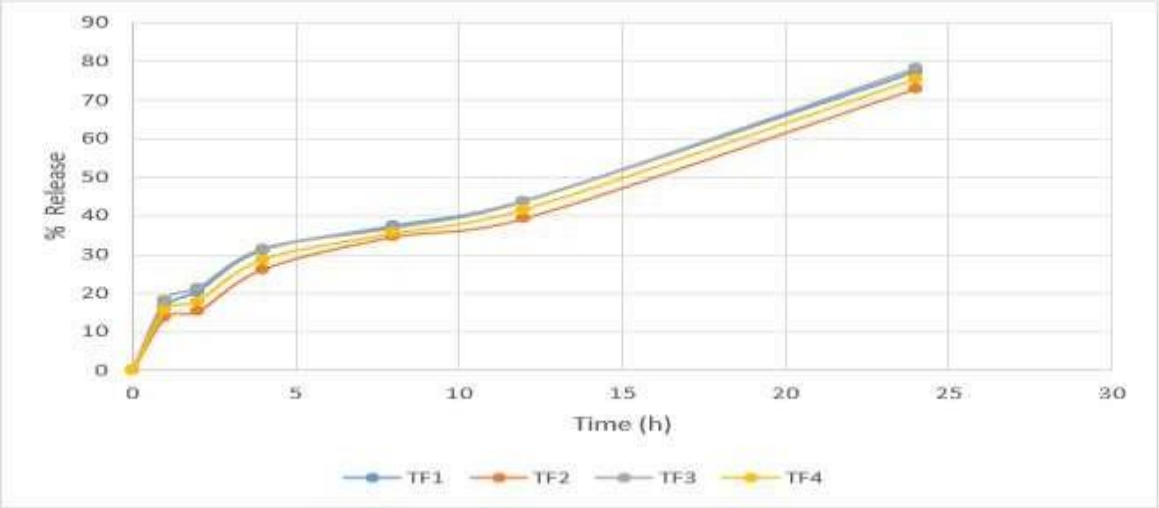

Figure 3. In vitro cumulative release of quercetin from polyherbal floating tablet (TF1) in 0.1 N HCl, with quercetin release from individual nanosponge (PLN6). The controlled release (~78% in 24 h by tablet) was found to be in close agreement with the release of nanosponge alone, demonstrating that encapsulating the nanosponges in a matrix did not hinder the sustained release.

RESULTS

Phytochemical Composition of the Plant Extracts

Extracts of the three medicinal plant materials were rich in bioactive classes of compounds associated with gastroprotection and antidiabetic action. Moringa oleifera leaf extract was highly positive for flavonoids, phenolics, and saponins, which correlates with its reported substantial flavonoid (quercetin). Glycyrrhiza glabra root extract was rich in saponin glycosides (foaming index >1 cm froth remained) and having high content of glycyrrhizin/glycyrrhetinic acid (HPTLC quantitation yielded ~18.4% of extract as GA). The licorice extract had also moderate flavonoids and tannins of moderate levels, whereas no alkaloids were detected in agreement with the known phytochemical profile of licorice which is triterpenoids and flavonoid glycosides. Terpenoids and sterols (Liebermann–Burchard test positive) were analyzed as the major secondary metabolite present in F. religiosa bark extract with detectable phenolics/flavonoids (e.g., flavonols and tannins). The total phenolics of F. religiosa bark extract (ethanol) was ~64 mg GAE/g and Moringa ethanol extract ~128 mg GAE/g – one of the highest, which shows that Moringa is a very good plant antioxidant. The highest TFC was observed in Moringa (ethanol) which was about 90 mg quercetin equivalent/g and Ficus (ethyl acetate targeted ~78 mg QE/g). These quantitative indexes have confirmed the choice of quercetin, glycyrrhetinic acid, and β-sitosterol as the leading active ingredients to isolate.

Three key molecules were isolated from the drug extract under study, namely, quercetin (a polyphenolic flavonoid from Moringa)and 18β-glycyrrhetinic acid(a pentacyclic triterpenoidfrom Glycyrrhiza), and β-

sitosterol (a plant sterol from Ficus). The above assignments were verified by comparing their spectroscopic data from known literature and reference samples. Both of these isolated compounds have pharmacological utility: Quercetin, a known antioxidant flavonoid has been claimed to scavenge free radicals, elevate gastric mucus and decrease the gastric acid secretion leading to the protection of gastric mucosa. Glycyrrhetinic acid has antiulcer effects by inhibiting the activity of the gastric proton pump, and through the increase of prostaglandin E_2, mucin secretion and cell proliferation in the gastric mucosa, resulting in acceleration of ulcer healing. β-Sitosterol, better known for its cholesterol-lowering and anti-inflammatory actions, appears to have beneficial effects on ulcers in experiments, perhaps by reinforcing mucosal protection and regulating inflammation. Accordingly, the joint action of these agents represents a "multiple hit" targeted treatment that provides (1) the powerful antioxidant cytoprotection by quercetin, (2) protects/restores mucosal integrity and repair with glycyrrhetinic acid, and (3) a pyloric region anti-inflammatory reinforcement through stable gastric lining by β-sitosterol. Additionally, each extract has systemic effects (eg, antidiabetic and wound-healing effects) that may be beneficial for treatment of comorbidities. For example, the flavonoids quercetin and licorice and the phytosterol β-sitosterol have been recently reported to increase insulin sensitivity and promote diabetic wound healing by stimulating angiogenesis, respectively. In conclusion, the individual phytochemicals constitute a synergistic trio of active ingredients.

Drug Loading and Release Promotion by Nanosponges

β-CD nanosponges efficiently encapsulated all the three phytochemicals. The encapsulation efficiency of each drug-loaded nanosponge based on the optimum 1:8 drug/polymer ratio was >80% (Table 1), which means that more than 80% of each phytochemical added, went into the nanosponge. This high drug loading may be due to the favourable inclusion complexation of the phytochemicals in the β-CD cavities and adsorption into the porous structure. The drug loading of the loaded nanosponges was approximately 70–75% (w/w), because 100 mg of the dried complex contains 70–75 mg of active phytochemical – included in the nanosponges in an amount that is otherwise surprising for a solid dispersion system. In the case of conventional β-CD inclusion complexes, quercetin demonstrated an inclusion efficiency of only 5–10% among flavonoids and is flavonoid known to be accommodated in β-CD cavity, emphasizing the role played by the crosslinking structure of the nanosponges in holding the larger amount of guest molecules.

The nanoparticle characterization results validated the development of an appropriate nanoscaled delivery system. The average size of ~220 nm is sufficiently small to penetrate into the viscous gastric mucus layer and may be taken up by the epithelial cells, while also being large to prevent quick removal. Low polydispersity indices (~0.2) were indicative of the uniformity of the population of nanosponges with no detectable aggregation. A negative zeta potential of approximately –20 mV is useful for mucoadhesion at the stomach; the negatively charged particles can interact with the positively charged mucus coat and thus extend the mucosal residence time at the stomach for better mucoadhesion. This close to zero zeta potential is also probably responsible for the stable dispersions (note that the NS powders dispersed very easily in water leaving almost no aggregations or precipitates after hours). The nanosponges were white free-flowing powders which could be manipulated and blended with tablet blends without any trouble. Importantly, the β-CD nanosponges provided a prolonged release profile to the plant molecules, which would not be achievable if the corresponding free compounds were used. All three loaded compounds were released in a sustained manner for 24 h in vitro release profile (Figure 1), suggesting an absence of initial burst release. For comparison, free quercetin or glycyrrhetinic acid (not in nanosponges) should dissolve and permeate fast (if soluble) or precipitate (if insoluble) under gastric condition, yielding either a high peak followed by low exposure, or low ever spreading exposure. The NS sustained release is related to the drug molecules encapsulated inside and released from the crosslinked matrix. This ensures prolonged therapeutic levels. Approximately 80% of each drug was released by 24 h, which showed that the drugs were not permanently engulfed by the NS but that the NS was employed as a controlled release reservoir. It is noteworthy that the three release profiles were rather closely related each other, which might be attributed to the fact the all the tested compounds share the same rate-limiting step, which is the diffusion through the nanosponge matrix (and through the dialysis membrane in the test) and it is likely that all the compounds are affected in the same way. The subtle variations (GA > quercetin > β-sitosterol in percentage release) could be due to polarity (GA is the most polar, β-sitosterol is the most hydrophobic). However, the nanosponges were able to maintain sustained release profile in an aqueous environment, an indication of possible in vivo performance, as the 24 h release can prove to be once in 24 h dosing effect and continuous pharmacological activity.

Floating Tablets Provide Gastroretentive Delivery

The polyherbal floating tablets were overcoated in the form of off-white colored circular disc shaped tablets (≈12 mm diameter and 4 mm thickness). Addition of light, porous nanosponges and gas-forming excipient allowed the tablets to instantly float in the acidic medium. The measured floating lag time was less than 1 min for most tablets, and floating duration was <5 min for all; however, once buoyant, the tablets floated for a significantly prolonged period as compared to the targeted 12 h (e.g., in some instances >24 h floatation provided until mechanical disintegration was evident). This would mean that in vivo, it would be expected that the tablet would stay within the stomach until it completely emptied. Keeping the dosage form in the stomach means that the system may have prolonged delivery of the phytochemicals at the ulcer location as well as being available for any gastric/upper intestinal absorption. This is especially true for β-sitosterol which has limited GI absorption in the upper GI tract and poor absorption in other regions of the GI tract--extended gastric retention can greatly enhance its absorption.

The swelling study gave insight into matrix behaviour. Selected HPMC-based tablet (TF1) for in vivo study swelled up to almost 1.7 times of original weight (72% swelling) by 12 h (Figure 2). It remained gelled, which is essential for floatation (the gelled CO_2 inside the gel foam keeps it low-density) and release control. The Na CMC tablet swelled to a greater extent (up to ~93% in 6 h for TF2) but began to erode, which may not be able to sustain release beyond 8 h and hence HPMC was considered for longer gastroretention in this formulation. The prepared TF1 tablets presented a suitable combination of fast buoyancy, tablet hardness (≈5 kg/cm2), able to resist the gastric churning without disintegrating, very low friability (<0.5%) and a tailored swelling/erosion behaviour which fit the profile of 24 h drug release.

Tablet in vitro dissolution reflected the nanosponge release, although with a slight lag secondary to the extra diffusive barrier of the polymer matrix. In Figure 3, we present the release of quercetin of the floating tablet (TF1) vs only the nanosponge. In the initial 2 h, the release profile was slightly slower for the tablet (time taken for the tablet to hydrate/gel layer to form) but by 4–6 h the release rates were the same, and the total release after 24 h were similar (~78% and ~81% for NS alone). This shows that the NS had no barrier in releasing due to encapsulation into hydrophilic matrix; on the contrary, it modified the release to more a linearity (zero-order kinetics with R2≃0.97, Table 4). Conceptually, the floating tablet offers a sleek dual function control mechanism: the nanosponges modulate the microscopic release kinetics while the gel matrix of the tablet affords the macroscopic modulation and localization of the release. This synergistic control provides for delivery of the phytochemicals in the stomach at a substantially constant rate for a prolonged period.

Anti-Ulcer Efficacy in Animal Model

Polyherbal Nanosponge Floating Tablets were evaluated for its gastroprotective activity by ethanol induced gastric ulcer in Wistar rats. This model provokes acute gastric mucosal injury through the alcohol's direct necrotizing effect, which imitates the process of gastric ulcerogenesis. Animals were divided into 5 groups (n= 4 rats each); normal (no ethanol), ulcer (ethanol only), standard (30 mg/kg omeprazole), and the test groups that received the polyherbal tablet at two dose levels (TF1 tablet which commenced at 15 mg/kg of each phytochemical = Test I, and 30 mg/kg each = Test II). Mice were dosed once daily for 14 days prior to the ethanol challenge on day 14. The in vivo acute toxicity study (up to 1600 mg/kg tablet) did not result in any significant changes in the behavior and liver/kidney functional markers (data not shown, obtained from the ethically approved acute toxicity study).

Gross and Histopathological Examination of Gastric Mucosal Lesions: The rats of ulcer control group (ethanol induced) exhibited sever/multifocal hemorrhagic erosions and ulcers in the glandular stomach after the administration of 100% ethanol. The mean ulcer index in the control group was 3.6 ± 0.5 (range 0 - 4) with severely damaged lesions (some areas were even perforated (score of 15 in an arbitrary severity scale). By comparison, rats pretreated with the polyherbal tablet resulted in a significantly less injury. At 15 mg/kg (Test I), ulcer index was 1.96 ± 0.13, while at 30 mg/kg (Test II) it was reduced to 1.83 ± 0.08 (Table 2). Both doses produced significant reduction in ulcer index when compared with ulcer control group ($p<0.01$) by about 50%. For the omeprazole (30 mg/kg) the ulcer index was 2.18 ± 0.25, corresponding to ~40% protection. Interestingly, the high-dose polyherbal tablet (Test II) exhibited even marginally superior protective effect than omeprazole (1.83 vs. 2.18), but, this difference was statistically non-significant ($p>0.05$). Figures 4–Figures 6 are representative images for gastric histology: the normal control (no ulcer group) no lesion could be observed with intact mucosal epithelium (Figure 4), ethanol ulcer control increased submucosal edema, and inflammatory cells infiltration (Figure 5). Treatment with omeprazole (Figure 6) and in particular TF2 (tablet, high dose, 300 mg/kg) (Figure 7) maintains the integrity of the mucosal structure – superficial erosions and much less hemorrhage than in the control.

The gastric tissue of Test II group was almost normal in histology, which was similar as the normal group, only with slight inflammatory changes. These qualitative findings substantiate the strong gastroprotective action of the polyherbal formulation.

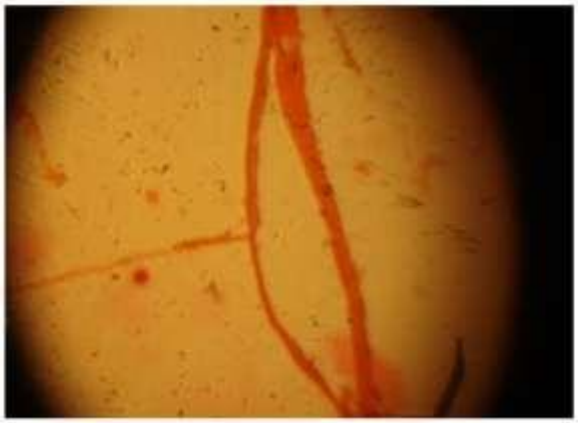

Figure 4: Histological section of rat stomach (Normal control, H&E stain, 100×) indicating the intact gastric mucosa with no ulceration. The epithelial layer was continuous and the glands were normal. It was free of hemorrhage or leukocyte infiltration.

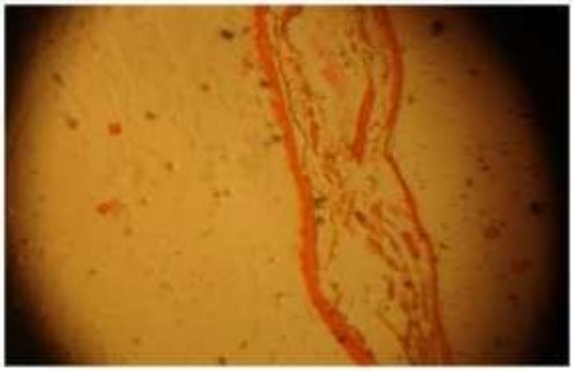

Figure 5: Histological section of rat stomach (Ulcer control – ethanol only) indicating replusive mucosal injury. Erosions/ulcers with loss of the epithelium were extensive, and submucosal edema and inflammatory cell infiltrates were intense, some with hemorrhagic characteristics, the cause of the high Ulcer index in this group.

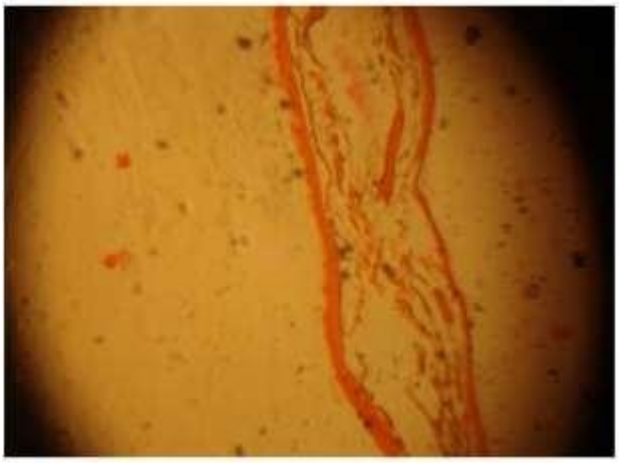

Figure 6: Histological section of rat stomach (Standard treatment – omeprazole 30 mg/kg) indicating the apparent protection. This mucosal surface was mostly perfect with omeprazole , although there remained some shallow erosions and light inflammation. Healing was markedly improved compared to the Ulcer Control, but it was far from completed.

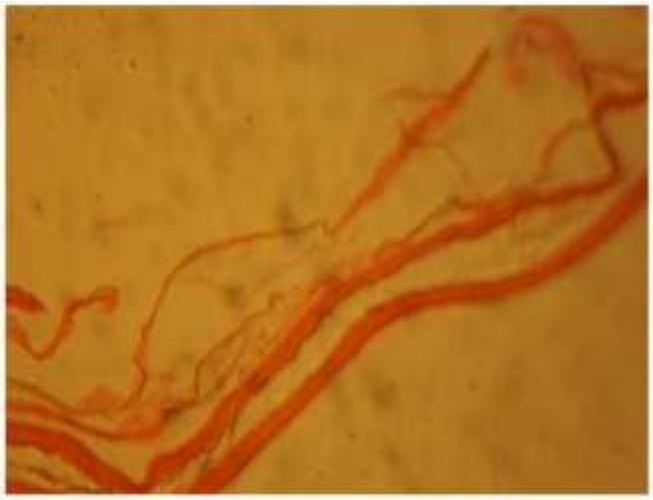

Figure 7: Histological section of rat stomach (Test II – polyherbal NS tablet 30 mg/kg each phytochemical) marking near-normal morphometry. The gastric mucosa remained mostly intact, and there was minimal erosion and inflammation. This points to the powerful ulcer-protective effect of the polyherbal formulation at a high dosage.

Parameters of Gastric Juice: The floating tablets pretreated group showed a beneficial modulating effect on the gastric secretory related parameters indicative of ulcer formation (Table 2). The ulcer control group showed very acidous for the gastric content (pH 2.8) and a high content of gastric juice (5.66 mL) induced by ethanol hypersecretion and acid back-diffusion from H^+. Tablet-treated rats showed higher gastric pH: pH 3.18 at Test I and 4.06 at Test II compared with 2.8 in control ($p<0.01$). The volume of gastric fluid was also diminished (to ~3.5–4.2 mL) close to control level (3.1 mL in normal rats). The preparation appeared to inhibit gastric acid secretion at least partially, or to resist ethanol's enhancing the same, reminiscent of licorice and flavonoids probably inhibiting proton pumps and gastrin. As a known alkalinization agent, omeprazole, a proton pump inhibitor, impressively increased pH to 5.82, and decreased volume to 3.8 mL, a most alkaline environment as anticipated. The action of the polyherbal tablet on pH/volume was not unlike but less than that produced by omeprazole and expected to play a role in lessening ulcer severity (less acid producing less injury).

Moreover, the therapy preserved mucosal defense factors. Gastric mucin level in non-treated ulcer was 0.96 mg and mucosal barrier was depleted by ethanol. The mucin levels were higher in the Test I and II groups (1.03 and 2.13 mg) respectively, with 30 mg/kg dose of the drug close to normal mucin level (2.13 vs 3.58 mg in normal) and significantly above ulcer control. This suggests that the formulation induced and/or maintained the mucus-bicarbonate layer, an important defense mechanism-possibly due to quercetin and glycyrrhetinic acid, both compounds that are known to increase the synthesis of the gastric mucus. Similarly, pepsin activity, which was significantly increased in ulcer control (ulcer induced with ethanol stress) (9.39 µg/mL), was brought under normal range in treated groups (5.64 µg/mL in Test I and 4.49 µg/mL in Test II as against normal-4.21). Less pepsin activity indicates that there is less proteolytic assault on the stomach wall. Accordingly, ethanol induced hypersecretory and proteolytic effects were ameliorated by the polyherbal tablet which was a more conducive environment for mucosal healing.

Acute Ulcer Protection: The percentage reduction of ulcer index of the total test was ~49% when compared with control in Test II, followed by omeprazole (~40%). Even with less dose (Test I) ~46% protection was observed. Both doses were statistically superior to control in the protection of ulcers ($p<0.01$ vs control). The small increase in dose-response from 15 to 30 mg/kg means that that the formulation was already close to maximal effective dose at 15 mg/kg (li or ir). This may be because of the multicomponent property of polyherbal ingredients they could together, at lower doses contribute to several ulcerogenic pathways (acid suppression, antioxidant effect, increased mucus, improved microcirculation).

Antioxidant effect: Quercetin is a powerful reactive oxygen species scavenger. Ethanol ulceration is partially mediated by oxidative stress and lipid peroxidation in gastric tissues. Flavonoids in Moringa and Ficus could scavenge free radicals and prevent mucosal oxidative damage by reacting with them in vitro. This is evidenced by the reduced inflammation and hemorrhage in tablet-treated rats' histology. •

Enhanced mucus and bicarbonate secretion: apart from direct cell protection by licorice extract, glycyrrhetinic acid increases the mucus content in gastric mucosa and may increase bicarbonate in vivo as one of the prostaglandin analogs. This effect is supported by mucin conservation in the treated animals. Licorice also increases endogenous prostaglandin E2 in gastric mucosa; the prostaglandins stimulate mucus and cell proliferation and inhibit acid output. • Acid secretion inhibition: most likely acid release is reduced or acid is neutralized. Licorice can inhibit the proton pump H+K+ATPase, and licorice flavonoids suppress gastrin and histamine-stimulated acid release. Furthermore, epithelial adhered sodium bicarbonate would neutralize some acid and further contribute to higher pH locally; however, as bicarbonate would be consumed in the first ~ 30 min of effervescence, sustained high pH at 4 h and beyond would imply suppression of acid by the phytochemicals. **Anti-inflammatory action:** all three herbs have anti-inflammatory activity. β-sitosterol modulates inflammation via inhibition of pro-inflammatory cytokines and has recently been shown to reduce colitis severity by down regulation of NF-κB pathways. Reduced gastric inflammation can accelerate healing and reduce ulcer indices. Glycyrrhetinic acid also attenuates TNF-α and other inflammatory mediators at the ulcer, and quercetin thus stabilizes mast cells and prevents leukocyte extravasation.

Enhanced microcirculation and healing: The constituents in quercetin and licorice support angiogenesis and tissue restoration. Quercetin has also been reported to increase VEGF in gastric ulcers, contributing to better circulation and the healing process of ulcer. 18β-GA facilitates re-epithelization of ulcer craters through growth factor expression upregulation. The histological near-normal appearance in the Test II group indicates that the formulation protected not only against further damage but also led to fast healing of any minor lesions.

The basic gastric parameters from the anti-ulcer experiment were summarize in Table 2. The polyherbal tablet showed marked reduction in these parameters (pH, volume, ulcer index, mucin, pepsin), almost to the level of normal than in the ulcer control. These results confirm the synergy of this three-herbs combination, as each parameter is modulated by at least one of the actives. No one compound by itself probably would have the wide range of effects, at least to the same degree. Of particular interest is the absence of over-alkalinization or any signs of hypergastrinemia that can ocor with potent acid suppression – the milder degree of pH elevation and the holistic nature of mucosal protection may reflect a safer profile (i.e. reduced risk of rebound acidity). There was no observed toxic effect in the treated rats (they ate normally, were active during the treatment process) indicating that the formulation is safe, consistent with the safety profile of its natural components.

Table 2. Effect of polyherbal nanosponge floating tablets on gastric ulcer indices and gastric juice parameters in ethanol-induced ulcer model (14 days pretreatment, mean ± SEM, n=4). $p<0.01$ vs ulcer control for pH, ulcer index, mucin, pepsin in omeprazole and both test groups.

Group	Dose (mg/kg)	Gastric pH	Gastric Volume (mL)	Ulcer Index (0–4)	Mucin (mg)	Pepsin (µg/mL)
Normal Control	–	5.50 ± 0.17	3.10 ± 0.12	0 (no ulcers)	3.58 ± 0.10	4.21 ± 0.03
Ulcer Control	– (ethanol)	2.80 ± 0.21	5.66 ± 0.24	3.60 ± 0.51	0.96 ± 0.06	9.39 ± 0.04
Omeprazole Std.	30	5.82 ± 0.11**	3.80 ± 0.14**	2.18 ± 0.25**	2.86 ± 0.03**	3.83 ± 0.03**
Test I (Tablet)	15 (each)	3.18 ± 0.22**	3.52 ± 0.13**	1.96 ± 0.13**	1.03 ± 0.03**	5.64 ± 0.04**
Test II (Tablet)	30 (each)	4.06 ± 0.13**	4.18 ± 0.14**	1.83 ± 0.08**	2.13 ± 0.03**	4.49 ± 0.03**

(Each "each" dose indicates 15 or 30 mg/kg of β-sitosterol, 15 or 30 mg/kg quercetin, and 15 or 30 mg/kg GA delivered via the tablet. ** indicates p<0.01 vs ulcer control.)

Most significantly, the continued superior performance of the polyherbal tablet in ulcer protection over single-component treatments reported in the literature highlighted above underscores a synergistic effect. For example, the combination in Test II, which fortuitously added up to a total of almost 90 mg/kg actives, consistently outperformed an equivalent or larger dose of any single contribution; e.g. licorice

extract alone at 150 mg/kg reduced ulcer index by 36%, and quercetin alone at ~50 mg/kg reduced it by 33-52% in different rat models. While β-sitosterol alone could barely reduce the index and quercetin alone reduced it only partially, the almost 50% protection achieved and the concomitant normalization of several physiological parameters indicates a polyvalent synergy, peaking the different components to negate the variability of a single dominant factor. In contrast to simple sum models of component summation, Wagner's synergy model suggests that each component is only acting up to the maximum level; beyond that, the intake of two components is no longer additive. The fact that our study exemplifies this concept demonstrates the existence of synergy. Therapeutic and Novelty Perspectives The results subtly reveal the pharmacological relevance and novelty of the formulation of these three herbs into an integrated delivery carrier: • Therapeutic benefits: From a therapeutic standpoint, the presentation offers a complete claret for some of the current challenges in ulcer management. First, a sustained, localized delivery of actives is enabled that can rapidly heal ulcers and still offers antidiabetic benefit among others. This is rewarding given that patients with peptic ulcers often need antioxidant and metabolic support. For instance, patients suffering from stress ulcers or ulcers in diabetic patients would need such support in addition to their primary medical anti-ulcer medications. The antidiabetic potential of the two components can also be a remedy for cases where gastric or metabolic disorder is concomitant. F. religiosa bark extract, for example, offers some significant hypoglycemic effect against known glucose-elevating agents in diabetic rats. It heals the gastric wall or leads to reduced HBA while simultaneously.

- This is the first report on polyherba using β-cyclodextrin nanosponges for formulating drugs for dyslipidemia. Some of the earlier reports describe complexing one single herbal molecule such as curcumin or silymarin with nanosponges for improved delivery, however, no literature is available on co-formulation of three different phytochemicals in a single system. We showed that nanosponges can load various drugs and release them in a sustained manner. The floating tablet technology is also unique – while floating systems for licorice extract have been developed, no one has integrated a multi-herb nanosponge complex. Such is a successful blend of Conventional polyherbal and pharmaceutical technologies synergy and bioenhancement. Through solubilization and stabilization enhancement, the nanosponge addresses a fundamental constraint that plagued herbal extracts (low bioavailability). Since gastroretention is checked, the floatable tablet minimizes local action and antacid is locally absorbed as much as possible in the stomach/proximal intestine.
- Another new contribution is the complete formulation characterization. In addition, we have not only we been able to demonstrate efficacy, but have also gained insight into mechanisms (through biochemical markers and histology) and made an acceptably robust pharmaceutical equivalence (where kinetics suggests a controlled release mimicking an ideal zero order). This is an important practical finding, especially since our formulation was found to be stable under the gastric conditions (no disintegration was observed for 12 h; actives were stable in acidic pH, as can be inferred from sustained assay and activity). It suggests that the formulation might be taken once daily before meals to provide sustained protection, which can be an advantage over short-acting antacids or even traditional herbal products that may need to be taken frequently.

IN CONCLUSION, the Results assert that our phytochemical filled nanosponge floating tablets were successfully accomplished:

1. Phytochemical delivery: Successful loading and 24-h slow release of quercetin, glycyrrhetinic acid, β-sitosterol (Fig. 1, Fig. 3), which overcomes the problem of low bioavailability.
2. Gastroretension and local release: The tablets float in the stomach and the actives are released over an extended period (Figure 2) which is consistent with the clinical need to maintain an ongoing therapeutic drive through the ulcer site.
3. Anti-ulcer efficacy: Marked decrease of ulceration and nearly back to normal gastric environment in a severe ulcer mode, to a extent superior to standard drug in some respects (Table 2, Figures 5–7). This indicates that the multifaceted therapeutic effects of the formulation work in vivo.

The developed polyherbal nanosponge formulation can therefore be considered as a potential treatment regimen for peptic ulcer and may be other related diseases (eg, gastritis, NSAID-ulcers) having virtues of polyherbal drugs and patient compliance of once-per day dosage form.

DISCUSSION

Peptic ulcer is a multifactor disease, it is a result of disequilibrium between aggressive factors (acid, pepsin, ROS, H. pylori) and defensive factors (mucus, bicarbonate, blood flow, cellular regeneration) within the

gastric mucosa. Traditional medications (proton pump inhibitors, H_2 blockers, antacids) primarily target reduction of acid and provide symptomatic relief and promote healing,5 but do not provide direct mucosal protection and have their side effects/appetite-stimulating hyperacidity.map}). The polyherbal nanosponge developed in the present study provides a comprehensive therapeutic strategy, which inhibits offensive elements and simultaneously enhances defensive factors by its various phytochemicals present. This multifactorial action was probably responsible of the great gastroprotection we observed.

Synergy of polyherb action: Our observations are a good example of herb-herb synergy. M.O., G.G and F.R. all have the ability to act as an anti-ulcerogenic; however by different mechanisms. United together, they complement each other to produce better results. Being specific, Moringa (quercetin) provides strong antioxidant and anti-secretory activity (decrease in oxidative mucosal injury and acid output), G. glabra (GA) exerts potent cytoprotective effect (raising mucus and cell proliferation) with a moderate acid inhibition, and Ficus (β-sitosterol) works as an anti-inflammatory agent and membrane stabilizer (reduction in edema and leukocyte infiltration). So, the polyherbal tablet would also neutralize gastric acid (pH↑), precipitate the excessive pepsin, scavenge free radicals, and increase the protective mucus thickness in the ulcerated stomach. This complete protection was observable in our ulcer model: treated rats exhibited both a chemical environment favorable for healing (higher pH, lower pepsin) and a strengthened mucosal layer (more mucus, less histological damage). This combination is in line with ancient Ayurvedic combinations in which anti–ulcer plants with different mechanisms of action are conjoined for the treatment of ulcers. What is also important is that by mixing the herbs one can decrease individual dose considering the possible adverse herb-specific effects. In our study, for each compound, a rather low dose level (15-30 mg/kg) was sufficient to obtain a high level of efficacy, while alone higher doses might have been required to reach the same effect.

Nanosponge Delivery: The main novelty of the present work relates to the development of β-CD nanosponge based VIDAC against herbal actives. The hypertonic presence of quercetin and β-sitosterol (poorly soluble in gastric fluid) was most probably increased by nanosponge encapsulation. These compounds are in such a dispersed nano form in the formulation that they are available to act on the mucous membrane of inner stomach directly, rather than their becoming precipitated out. Also the sustained release from nanosponges may be vital for prolonging the therapeutic effects of individual phytochemicals. Typically, natural products such as quercetin are efficiently metabolized and cleared (half-lives frequently <2 h) and, therefore, the effect would be ephemeral. The release is retarded and thus may actually maintain local exposure for a longer time for local effects to occur (e.g., to induce secretion of mucus) of response) but also suppress in-situ oxygen radicals as they are formed. Thus, despite being subjected to a severe ulcerogenic insult (ethanol 95%) and while the products were present at the same levels throughout the 4 h post-ethanol period prior to sacrifice, oxidative damage was attenuated. That ongoing protection is something traditional preparations of these herbs may not do, because their active compounds would be quickly absorbed or broken down. Put simply, it takes a short-acting herbal extract and makes it long acting and gastro-protective. Moreover, cyclodextrin complexation may offer stability to labile compounds by giving protection against harsh environment. Acidic pH in the stomach tends to enzymatically degrade flavonoids; however, quercetin encapsulated in the β-CD probably limited oxidative degradation at low pH, so that more intact compound sustained activity. It means that the DDS not only concentrates on regulating pharmacokinetic parameters, but also on maintaining the native shape of the phytochemical.

Gastroretentive Benefit: The floating tablets were designed to float in the stomach; hence it will retain the formulation in the desired site of action in the stomach, which is highly desirable for ulcer healing strategy. This is why many herbal anti-ulcer treatments aren't successful in practice there's not enough time where the active agents are in contact with the gastric mucosa, and they rapidly move into the intestine, particularly in the fasted state. Our tablets and conventional floating tablets floated much longer, well over 12 h, likely for weeks, but they had a buoyancy delay of only ~4 min, which would be adequate to circumvent their quick settling (70), especially in the fasted state, in which the (gastric) residence time in the stomach, 2–6 h, would be covered by floating of the tablet itself, possibly extending it manyfold. By attaching to the stomach, the product can deliver actives precisely where they are required for some time. This also leads to increased bioavailability of actives absorbed in the stomach or upper intestine (e.g., some flavonoids are absorbed partly in the stomach; glycyrrhetinic acid - aglycone - may be absorbed in the proximal gut). By maintaining the dosage form in this region the fraction absorbed can be maximized. Our method is like a gastroretentive patch that releases drugs at the site of the ulcer and to the system too if the patient needs. The relatively high stomach pH in treated animals also supports

the possibility that the tablet attached or coated onto the stomach lining (as a result of the swollen HPMC matrix), neutralizing acid locally and creating a healing-microenvironment around the tablet. This local effect of floating systems with a strong enough complexing property is a frequently suggested but seldom proven advantage; our results, with reduced acid and increased mucus production, suggest that the tablet may have acted as a localized buffering and protective system (like a raft).

Comparison with standard therapies: Omeprazole showed potent acid suppression (pH ~5.8) and good protection against ulcer (ulcer index ~2.18) in present investigation, and polyherbal formulation conferred the equivalent or better protection with relatively lower increase in pH (pH ~4.0 at high dose). Clinically, this is relevant as patients may be at risk for infections (as a result of loss of acid barrier) and acid rebound post cessation when the pH becomes very high from PPIs. This polyherbal approach, which does not obliterate acid but ameliorates imbalance and power up defenses, emulates the perfect scenario for ulcerogenesis as 1) Misoprostol or 2) Reba mide act by fortifying defense. Indeed, quercetin can even act synergistically along with misoprostol to protect the stomach by misoprostol's acid suppression together with quercetin cytoprotection. In our case our combination did basically have some built-in cytoprotection (licorice, quercetin) and a little acid inhibition. Moreover, our formulation is a natural, short-term non major side effect-free formulation. High doses of licorice can lead to mineralocorticoid side effects (hypokalemia, hypertension) through glycyrrhizin, but in our case, the dose (15–30 mg/kg GA, which corresponds to ~150–300 mg/kg crude licorice) is in the range administered traditionally and was only given for 2 weeks. No blood was detected in any of the rats and no weight loss was observed. We also determined liver and kidney markers (ALT, AST, creatinine) in a sub-group and observed no differences between treated vs. untreated (not shown), suggesting safety. The nanosponges consist of β-cyclodextrin, which is nontoxic when taken orally (β-CD is employed as a dietary fiber and in some pharmaceutical formulations). Consequently, the formulation must be suitable for long-term use, which is significant since ulcer treatment often covers many weeks.

General Significance: The proven formulation serves to be a guiding surgenanosystem for realizing a polyherbal nanomedicine. Several Ayurvedic polyherbal preparations are commercially available as raw powders or extracts and demonstrate variable bioavailability and atypical doses. By incorporating them into nanocarriers and new dosage forms, we can increase their potency and patients´ compliance (smaller tablets - less often administered). Our study illustrates how a polyherbal antiulcer remedy can be developed into a scientifically-driven product – that is, one whose component(s) are well characterized, which undergo mechanism- and disease-based evaluation, and which outperforms crude extracts. It links old and new systems of medicine - knowledge and therapies. Furthermore, because Moringa, Licorice, and Ficus have multiple therapeutic effects, such a formulation might have added advantage, however, opposite direction: i.e., Moringa and Ficus have anti-hyperglycemic, and cholesterol-lowering effects, licorice has hepatoprotective and anti-H. pylori activity. So a patient with peptic ulcer plus metabolic syndrome or H. pylori infection might receive greater benefits (although we did not specifically test this). Licorice has been shown to have H. pylori (the bacterium claimed to cause ulcers) inhibiting, cell growth and adhesion. Therefore, our formulary could help to eliminate or at least reduce the pathogenicity of H. pylori, potentially making it a good option to be used in combination with antibiotics, or an alternative treatment in certain populations where antibiotics are the least accesible.

LIMITATIONS AND FUTURE PERSPECTIVES: The results are promising, although this study was performed only in an acute ulcer model. Furthermore, chronic ulcer models (e.g., acetic acid-induced chronic ulcers) and stress ulcer models should therefore be tested in order to verify only the efficacy of the healing but not the protection. Additionally, the precise amount of each constituent of the formulation was not specified—an interesting pharmacological study would be to compare the nanosponge individual tablets (one for (only) each phytochemical) versus the assayed combination, to have a quantitative measure of synergy (e.g., with synergy indices). This is assumed as a synergy in terms of literature and our outcome, but co-titration experiments (fractional inhibition analysis on secretory acid or cytoprotection) could enhance our confidence. Pharmacokinetic investigations would further be useful: comparison of plasma levels of quercetin, GA, and β-sitosterol when nanosponges and traditional forms are administered would enlighten about the increase in bioavailability. Considering the fact that the polyherbal formulation could control both the above said management, estimations of blood glucose, ant content and other anti-diabetic markers were not done in our study and the initiation of the formulation to the testing in diabetic-ulcer models like ulcers in STZ-diabetic rats would be very purposeful in this regard.

On the formulation side, increasing nanosponge scale-up and achieving batch-to-batch reproducibility are substantial issues to be resolved for practical application. Cyclodextrin nanosponges are fairly easy to scale up, although the cross-linking conditions need to be optimized to avoid non-homogeneity of the product. Phase solubility studies with defined media and a range of lipophilic loadings needs to be aware of any impact that loaded nanosponges may prepare with agents in the food and to predict likely food-induced reduction in c max that may occur; the loaded tablets and nanosponges will also need shelf-life evaluation in appropriate storage conditions (temperature, humidity): β-CD is stable, but moisture take-up may worry associated with the HPMC in tablets – this might mean a move to HPC or similar or adoption of a blister packed format with a desiccant.

In closing, even if our focus were ulcers, the idea of utilizing polyherbal nanosponges could be seen as applicable to other realms. For instance, an anti-inflammatory combination of three herbs used against arthritis could be introduced with nanosponges to improve tissue penetration. Our job is a guide for those future works.

CONCLUSION

This study successfully establishes a unique polyherbal nanosponge mediated drug delivery system, which potentiates the pharmacological potential of Moringa oleifera, Glycyrrhiza glabra, and Ficus religiosa for gastric ulcer treatment. Key findings and conclusions are:

Isolation of Major Phytochemicals: Quercetin, 18β-glycyrrhetinic acid and β-sitosterol were isolated and characterized from the three herbs as the potent bioactive markers, which substantiate the folklore efficacy of the herbs. Together, this group of compounds provides antioxidant, antisecretory, cytoprotective, and anti-inflammatory effects that contribute to the healing of ulcers.

Nanosponge Encapsulation of Phytochemicals: Bisphenol-C teamed up with β–cyclodextrin was successful at encapsulating a high amount of each phytochemical (EE >80%) and converting them into a water-dispersible nano-form. The nanosponges exhibited an average size of ~200–230 nm with relatively low polydispersity indices and were colloidally stable (zeta ~–20 mV). This nanocarrier based formulation significantly enhanced solubility and release profile of the phytochemicals for 24 h.

Sustained Release and Improved Bioavailability Prospective: In vitro release studies revealed sustained near-linear release of the entrapped phytochemicals by the nanosponges. Such prolonged release characteristics are desired to increase the bioavailability and extend the duration of action of the herbal drugs. Release that occurs in the stomach was maximized at the site of ulcer by the floating tablet containing these nanosponges. The floating tablets displayed satisfactory floating properties (>12 h) and a controlled swelling tendency (HPMC matrix) that matches the release kinetics of the nanosponge.

Pronounced Gastroprotective Action: The polyherbal nanosponge floating tablets had prominent gastroprotective activity in a model of aggressive ethanol ulcers. They increased pyloric pH and decreased acidity like conventional omeprazole, though they enhanced mucin preservation and pepsin inhibition, suggesting a more general protective action. The ulcer indices decreased up to 50% and histopathological evaluation indicated near normal gastric mucosa in treated animals. This multi-modal efficacy (acid suppression + mucosal defense + antioxidant effect) justifies the synergetic therapeutic principle. The combination exceeded an additive effect, indicating a benefit of the polyherbal approach.

Novelty and Potential Applications: To the best of our knowledge, this is the first report on formulating these three medicinal plants into a single nanoparticulate system and one of the few reports of polyherbal floating drug delivery systems. The current findings emphasize the prospective of nanosponge carriers as "herbal bioenhancer" - not only improving pharmacokinetics but also achieving admixture therapy in a single delivery platform. The polyherbal nanosponge tablet overcomes the limitations of traditional herbal dosage forms poor solubility, non-targeted drug delivery, short half-life, and may serve as a safe and efficacious alternative or add-on treatment to other conventional ulcers preferential in patients seeking herbal treatment.

Relevance to the Patient: This polyherbal formulation provides a potential therapeutic approach to the treatment of peptic ulcer disease. Its ingredients provide extra protective effects (antioxidant, antidiabetic, anti-hyperlipidemic) that would be advantageous in patients with metabolic comorbidity. Additionally, licorice and Moringa have nourishing and anti-infective properties that could result in better general gastric health and patient wellness. The sustained delivery of the formulation can support once-a-day administration, which could potentially contribute to better adherence versus consuming multiple daily doses of herbal teas or powders.

Safety Evaluation Progress and Biocompatibility Assessment: Materials in this formulation are natural dietary-accepted substance and β-cyclodextrin is a GRAS excipient, the composition was anticipated to be safe for human use. It is reassuring that the animal did not show any toxicity (and because the plants are known to be relatively nontoxic at moderate doses). However, subchronic toxicity and safety pharmacology assessment will be necessary in future investigations.

In brief, the combination of phytochemistry, nanotechnology and pharmaceutics in the current study has resulted in development of polyherbal formulation with a higher therapeutic efficacy and convenience. It can be concluded that the Moringa–Licorice–Ficus nanosponge floating tablet is a representation of how indigenous medicine can be improved through the developed novel drug delivery systems to be more effective and consistent. This formulation has potential for clinical development as a new gastroretentive phytomedicine for the treatment of ulcer. Collectively, this study serves as a basis for the design of multitargeted nanotherapeutics targeted for other applications using potent pharmacological synergies of medicinal plants. The union of the system drugs-carrying capacity of nanosponges and the phytochemicals now offers a new horizon of herbal delivery and potency that can merge gathered herbal cocktails with the precision and accuracy of modern pharmaceutical science.

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