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RESEARCH PAPER

Mechanistic insights into the anti-ulcerative effects of Avipattikar churna: a network pharmacology and experimental approach

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Abstract

Introduction Avipattikar Churna is prescribed for gastric conditions in Ayurveda. The herbs present in churna have anti-inflammatory, antioxidant, and cytoprotective activities and the sugar part of churna is responsible for acid neutralization. We investigated the mechanism of action of Avipattikar churna against peptic ulcer using network pharmacology, in-vitro, ex-vivo and in vivo studies**.**

Methods Network pharmacology of churna was performed to identify the protein and pathways targeted by phytoconstituents of churna. In the phytochemical analysis the quantification of phenols, flavonoids, tannins, in vitro studies including acid neutralizing capacity, ex-vivo studies including proton pump inhibition and antioxidant activities of three extracts of churna (aqueous, alcoholic and hydro-alcoholic) and in vivo studies were performed.

Results Network analysis revealed Quercetin, Ellagic acid, Turpetholic acid, Copaene, and Eugenol having activity in peptic ulcer. The quantitative estimation of total phenols, flavonoids and tannins were found to be $12.467 \pm 0.273\%$ w/w, $4.734 \pm 0.091\%$ w/w, and $8.0368 \pm 0.138\%$ w/w respectively. The proton pump inhibition activity of all three extracts was concentration-dependent, with the hydroalcohol extract being the most effective at 250 ug/mL. Aqueous and hydroalcoholic extracts were found to improve levels of SOD, catalase and glutathione. Additionally, *in-vivo* studies revealed decrease in NFκB, TNF-α, and IL-6 post treatment. The ulcer protective effect was supported by histopathology results.

Conclusion From the experimental data we conclude that Avipattikar churna may act via various mechanisms including acid neutralization, proton pump inhibition and antioxidant activity. Further in vivo studies suggest that churna may suppress the NF-κB signaling pathway, which is associated with significant reductions in TNF-α and IL-6 levels, decreased inflammatory cell infiltration, promotion of ulcer healing, and induction of apoptosis.

Keywords Peptic ulcer · Antacid · Proton pump · Antioxidant · Network pharmacology · Inflammatory markers · Avipattikar churna

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Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AKT1	Protein kinase B
ALC	Alcoholic
AQ	Aqeoues
ATP	Adenosine-5′-triphosphate
CACO-2	Colon adenocarcinoma
CHUK	Conserved helix-loop-helix ubiquitous kinase
CNSK2B	Casein kinase II subunit beta
DTNB	5, 5′-Dithiobis (2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid

EGRF	Estimated glomerular filtration rate
FDR	False discovery rate
FPR	Formyl peptide receptor 1
GIFtS	GeneCards Inferred functionality scores
GSH	Glutathione
H. pylori	Helicobacter pylori
H + /K + -ATPase pump	Hydrogen potassium ATPase
H2O2	Hydrogen peroxide
HA	Hydroalcoholic
HCl	Tris–hydrochloric acid
HIF-1	Hypoxia-inducible factor
HSP90AB1	Heat shock protein 90 alpha family class B member 1
IMPPT	: Indian medicinal plants, phytochemistry and therapeutics
KCl	Potassium chloride
KEGG	Kyoto encyclopedia of genes and genomes
MAP3K5	Mitogen-activated protein kinase kinase 5
MAPK	Mitogen-activated protein kinase
Meq	Milliequivalents
MgCl2	Magnesium chloride
mM	Millimetre
Na2CO3	Sodium bicarbonate
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NFκB1	Nuclear factor NF-kappa-B 1
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
OMP	Omeprazole
PG	Prostaglandins
PI3K-Akt	Phosphatidylinositol 3-kinases/protein kinase B
PIK3R1	Phosphatidylinositol 3-kinase phosphorylates 1
PPI	Proton Pump Inhibitors
PPI	Protein–protein interaction
PTGS1	Prostaglandin-endoperoxide synthase 1
PTGS2	: Prostaglandin-endoperoxide synthase 2
PUD	Peptic ulcer disease
Rap1	Repressor/activator protein 1
ROS	Reactive oxygen species
SMILE	Simplified molecular input line entry system
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
STRING	Search tool for the retrieval of interacting genes/proteins
TNF-α	Tumor necrosis factor-α
TTC	Total tannin content
USP	United States pharmacopeia
VEGF	Vascular endothelial growth factor

Introduction

A scourge of nineteenth and early twentieth centuries, peptic ulcer disease persists in posing a risk of premature mortality, with majority of the impact in developing nations (Malfertheiner and Schulz 2020; Mohsen Naghavi et al. 2015). It is an acid peptic injury to digestive tract, causing mucosal damage greater than 3–5 mm and extending to the muscularis propria or submucosal lining (Lanas and Chan 2017; Narayanan et al. 2018; Xie et al. 2022). Primarily involving the stomach and proximal duodenum, peptic ulcer disease (PUD) leads to an annual incidence of 0.1–0.3% with a prevalence of 5–10% across the globe (Lanas and Chan 2017). The aetiology of PUD is multifaceted, characterized by imbalance between protective and aggressive factors (Dimaline and Varro 2007). The protective factors encompass mucus, nitric oxide (NO), bicarbonate, prostaglandins (PG), antioxidants, and blood flow (Yandrapu and Sarosiek 2015; Galura et al. 2019). In contrast, endogenous aggressive factors encompass bile reflux, hydrochloric acid, abnormal motility, pepsin, and reduced blood flow (Masclee et al. 2014, Datta De and Roychoudhury 2015, Sverdén et al. 2018, Serafim et al. 2020). PUD is additionally linked with Reactive Oxygen Species (ROS). Numerous clinical findings suggested that the mechanisms by which alcohol, Non-Steroidal Anti-inflammatory Drugs (NSAIDs), and Helicobacter pylori cause PUD involve ROS (Moss et al. 1992).

The current management of PUD includes antacids, antisecretory drugs (Proton Pump Inhibitors (PPI), H_2 receptor blockers), potassium-competitive acid blockers and cytoprotective agents (Lanas and Chan 2017; Scally et al. 2018). Several adverse effects, including mineral, vitamin B12 and vitamin C deficiency, have been linked to prolonged PPI use. In a few instances, it may also result in cardiovascular risks, bone fractures, infections, and rebound hyper acid secretion (Koyyada 2021).

Avipattikar churna, a polyherbal Ayurvedic preparation, is commonly used for treating peptic ulcers. Each constituent of churna shows different effects, including cytoprotection, reducing gastric secretion, increasing mucosal resistance, potentiating gastric mucosal defence

factors, preserving basal gastric mucosal blood flow, and increasing mucus secretion (Al-Yahya et al. 1990; Khandhar et al. 2010; Santin et al. 2011; Gyawali et al. 2013). Even though it is widely prescribed for PUD, but its mechanism of action in not fully elucidated. Hence, the present investigation was undertaken to investigte various mechanisms (anti-secretory, antacid and antioxidant properties) of different extracts of Avipattikar churna using network pharmacology, in-vitro, ex-vivo and *in-vivo* studies.

Methods

Preparation of churna

Avipattikar Churna was prepared as per the Ayurvedic Pharmacopoeia of India (Committee et al. 2003). All the herbs were purchased from Ahmedabad's local market and were verified at Gujarat University's Botany Department (GU/BOT/2022). It contains ginger (*Zingiber officinale* -1 part*)*, black pepper (*Piper nigrum* -1 part*)*, Long pepper (*Piper longum* -1 part*)*, Amla (*Embelica officinalis*-1 part*)*, Behda (*Terminalia bellirica* -1 part*)*, Harde (*Terminalia chebula* -1 part*),* Vidang (*Embelia ribes* -1 part*)*, Vaj (*Cyperus rotundus*-1 part*)*, Cardamom (*Elettaria cardamomum*-1 part*)*, cinnamon leaf (*Cinnamomum tamala*-1 part*)*, Clove (*Syzygium aromaticum* -11 part*)*, Jalap (*Ipomoea turpethum*-44 part), lavana (Ammonium salt-1 part) and candy sugar (*Saccharum officinarum*-66 parts). All the ingredients were powdered individually in a pulverizer and passed through sieve (#85). Each powder was weighed separately and mixed in a specified ratio. To create a homogeneous mixture, the finished powder was run through sieve number 44.

Preparation and evaluation of extracts

Phytochemical screening

Various chemical tests were performed to validate the presence of metabolites such as primary and secondary phytoconstituents during qualitative phytochemical screening (Evans 2009; Gokhale et al. 2009).

Estimation of total phenolic, total flavonoids and total tannin contents

Total phenolic content (TPC), expressed as gallic acid equivalent, was quantified using the Folin-Ciocalteu method (Singleton and Rossi 1965; Pundarikakshudu et al. 2023). Aluminium chloride was employed for estimating total flavonoid content and Rutin was utilised as standard (Olajire and Azeez 2011). Using the titrimetric method, tannic acid content (TTC) was determined.

Acid neutralizing capacity by USP method

The acid neutralizing capacity (ANC) of water, alcohol, and hydroalcoholic Churna extracts was determined using Digene as the standard (Anonymous 2009).

Analysis of Avipattikar churna by network pharmacology

Avipattikar churna phytoconstituent mining and target prediction

Phytoconstituents of Avipattikar Churna (12 plants) were mined from the Indian Medicinal Plants, Phytochemistry and Therapeutics (IMPPT) and Dr. Duke's database. Drug-likeness of phytoconstituents were predicted using Molsoft. Phytoconstituents were screened based on drug-likeness scores and F30% bioavailability. Protein targets were predicted using SuperPred with $\geq 80\%$ probability. ADMET profiles were generated using ADMET Lab 2.0 and visualized in a heat map (Khanal et al. 2019).

Disease target mining

Peptic ulcer targets were extracted from GeneCards using keywords peptic ulcer, oxidative stress, H^+/K^+ ATPase, H_2 receptor, and H. pylori. After deduplication, genes with Inferred Functionality Scores (GIFtS) above 45 were selected.

Protein–protein interaction

Common targets of Churna phytoconstituents and peptic ulcer were extracted using Venny 2.1 and analysed in the STRING 11.5 database for protein–protein interaction (PPI). A high-confidence network (0.700) was constructed for Homo sapiens and simplified by hiding disconnected nodes (Shannon et al. 2003).

KEGG analysis and network construction

Based on PPI data, we analysed KEGG pathways to find out pathways involved in peptic ulcer modulated by phytoconstituents. To prepare the final network of active constituents-target proteins-pathways, cytoscape 3.7.2 was used. For interpretation of network, node colours and size were used i.e. dark colour, large size indicates high edge count (degree) (Shannon et al. 2003).

Ex Vivo *studies*

Preparation of extracts

Avipattikar churna (100 mg) was extracted using distilled water, hydroalcoholic mixture (70:30), and alcohol, refluxed at 60 °C for 30 min, filtered, and diluted to 100 mL. Stock solutions (1 mg/mL) were prepared, with dilutions of 50–250 µg/mL.

H^+/K^+-ATPase assay

Preparation of parietal cells The parietal cell suspension was prepared using goat stomach mucosa. The tissue was cleaned, scrapped, homogenized with Tris–HCl buffer (200 mM; pH 7.4). The content was centrifuged for 10 min at 5000×g and the supernatant for 20 min to obtain parietal cell sediment, used for H^+/K^+-ATPase activity. The goat stomach was obtained from a slaughterhouse, Ahmedabad (Reyes-Chilpa et al. 2006).

Determination of H^+/K^+-ATPase activity ATPase activity was measured calorimetrically by phosphate release from ATP (1 mM) at 400 nm. Parietal cell sediment was incubated with omeprazole and Avipattikar Churna extracts (50–250 µg/mL) for 60 min at 37 °C. The reaction was initiated with Tris–HCl (20 mM), ATP (2 mM), $MgCl_2$ (2 mM), and KCl (2 mM) and terminated after 30 min with trichloroacetic acid (10%) post 30 min incubation at 37 °C. Omeprazole was used as a positive control. Experiments were performed in triplicate (Fiske and Subbarow 1925).

Antioxidant activity

Preparation of tissue homogenate The fresh goat liver was obtained from the slaughterhouse, cleaned (using ice cold buffer) and cut into 1 mm slices using sterile scalpel. One gram of tissue was suspended in 5 mL phosphate buffer (saline homogenate) and 100 µl of H_2O_2 (200 µM) was added. This mixture was shaken and incubated at 37 °C for an hour. The H_2O_2 treated homogenate was incubated at 37 °C with different extracts of Avipattikar churna including alcoholic, hydoalcoholic and aqueous at concentration ranging from 50 to 250 µg/mL.

- Positive control: Untreated tissue homogenate
- Negative control: Tissue homogenate + H_2O_2
- Treatment 1: Tissue homogenate + H_2O_2 + Aqueous extract
- Treatment 2: Tissue homogenate + H_2O_2 + Alcoholic extract
- Treatment 3: Tissue homogenate + H_2O_2 + Hydroalcoholic extract

All the above-mentioned mixtures were centrifuged at 15,000 rpm for 1 h. SOD activity was measured in the supernatant, while the activities of catalase and glutathione were measured in the homogenate.

Superoxide dismutase assay The superoxide scavenging activity was based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan. To the reaction mixture consisting of 1 mL sodium bicarbonate (50 mM), 0.4 mL NBT (24 µM), 0.2 mL EDTA (0.1 mM) various concentrations of extracts and 0.5 mL of liver homogenate were added and incubated at 25 °C for 5 min. Then, 0.4 mL hydroxylamine hydrochloride (1 mM) was added to initiate the reaction. At 560 nm, the absorbance was noted on zero and five minutes. The absorbance of negative control was also recorded. SOD activity was represented as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was measured as units per milligram of protein (Beauchamp and Fridovich 1971).

Catalase To measure Catalase activity, to tissue homogenate (0.2 ml), 1.2 ml of 50 mM phosphate buffer (pH 7.0) was added. To initiate the reaction 1.0 ml of the H_2O_2 (30 mM) was added. The absorbance was measured for 3 min at an interval of 30 s at 240 nm. The enzyme blank was also performed in parallel. The enzyme activity was measured in mmoles of H_2O_2 decomposed/min/mg protein (Nandi et al. 2019).

Reduced glutathione In 0.5 ml homogenate, 2.5 ml of 5% TCA was added and centrifuged for 10 min. Using sodium phosphate buffer (0.2 M, pH 8.0) 0.2 ml supernatant was made up to 1 ml. DTNB (2 mL) was added to the mixture, and the absorbance was recorded at 412 nm after 10 min. The amount of reduced glutathione was expressed as µg of GSH/mg of wet tissue (Moron et al. 1979).

In vivo studies

Selection of animals

Adult male Wistar rats (180–200 g) were procured from Torrent Research Centre, Ahmedabad, India, and used for the study. The study was approved by the Institutional Animal Ethics Committee of L. J. Institute of Pharmacy, with protocol number LJIP/IAEC/22–23/14. Throughout the study, animal care was conducted in accordance with the guidelines of the Committee for Control and Supervision

of Experiments on Animals, India (CCSEA). The rats were allowed to acclimatize in the animal house of our institute for three weeks prior to the study. Temperature (25 ± 2 °C) and humidity (55 ± 5%) were maintained, along with a 12-h light/dark cycle. Standard diet and water ad libitum were provided to the rats.

Study design and peptic ulcer induction

Randomly, 36 rats were divided in six groups (n = 6). After 24 h of fasting, peptic ulcer was induced with a one oral dose of Indomethacin (50 mg/kg). DMSO, Omeprazole (50 mg/kg, p.o.), and hydroalcoholic extract of AVC at doses of 250, 500, and 750 mg/kg were administered for 7 days to the control, standard and treatment groups respectively. On day eight, rats were euthanized using isoflurane and cervical dislocation. Stomachs were excised, opened along the greater curvature, rinsed with ice-cold water and saline, and ulcer scoring was performed. Homogenates were prepared at 10,000 rpm for 20 min at 4 °C and stored at − 20 °C for biochemical analyses (NFκB, IL-6, and TNF-α). Histopathology was conducted using hematoxylin–eosin staining.

Normal control: Healthy rats were given 2 ml CMC orally once in a day for 7 days.
Disease control: Indomethacin (50 mg/kg was dissolved in 2 ml CMC, p.o.)
Standard: Indomethacin (50 mg/kg, p.o.) and Omeprazole (50 mg/kg, p.o.). Both the drugs were dissolved given 2 ml CMC.
Treatment 1: Indomethacin (50 mg/kg, p.o.) and hydroalcoholic extract of AVC dissolved in DMSO (250 mg/kg, p.o.)
Treatment 2: Indomethacin (50 mg/kg, p.o.) and hydroalcoholic extract of AVC dissolved in DMSO (500 mg/kg, p.o.)
Treatment 3: Indomethacin (50 mg/kg, p.o.) and hydroalcoholic extract of AVC dissolved in DMSO (750 mg/kg, p.o.)

Gross gastric lesion evaluation and ulcer Index

After excision, the stomach was opened from greater curvature and washed with ice-cold saline. Ulcer of gastric mucosa appeared as bands and scars of haemorrhagic lesions. Such lesions were photographed and analysed using image J (Wayne Rasband, MD, USA). The ulcers were scored using the method as follows: 0 score for almost normal mucosa; 1 for vascular congestion; 2 for one or two lesions; 3 for severe lesions; 4 for very severe lesions; and 5 for mucosa with full of lesions (Reddy et al. 2012). The ulcer index and % of ulcer inhibition were calculated employing following formula (Szabo and Hollander 1989).

$$\text{Ulcer index} = \frac{\text{Ulcerated Area}}{\textit{Total Stomach Area}} \times 100$$

$$\text{Ulcer inhibition (\%)} = \frac{(\textit{UI in disease control} - \textit{UI in test})}{\textit{UI in disease control}} \times 100$$

Determination of inflammatory markers

NF-ƙB, IL-6, and TNF-α levels were measured in gastric tissue using ELISA kit (SARD Biosciences). The stomach sections were homogenized in cold phosphate buffered saline (pH 7.4) and centrifuged (R-8C Remi Revolutionary Laboratory Purpose Centrifuge) at 10,000 rpm for 20 min at 4 °C. The supernatants were used for the determination of inflammatory markers.

Histopathology

Histopathological analysis was done using Hematoxylin and eosin dye. The effect of treatment was evaluated through assessment of presence of various layers and damage to mucosal layer. For evaluation, sample from each group was fixed in 10% formalin for 6 days, embedded in paraffin and sectioned (4–5 mm), stained with Hematoxylin and eosin dye, and evaluated under light microscope.

Statistical analysis

Data are expressed as Mean ± SEM. Statistical significance between control and treated groups was determined using one-way ANOVA, followed by post hoc analysis with Dunnett's test. Analyses were performed using GraphPad Prism v8.0.2 (GraphPad Software, Inc., California, USA), with $p < 0.05$ considered statistically significant.

Results

Preparation and evaluation of extract

Phytochemical screening

In-house Avipattikar churna showed carbohydrates, terpenoids, tannins, proteins, flavonoids, phenols, steroids, and resins.

Total phenolic, flavonoid and tannin contents

The total phenolic contents of the aqueous, alcoholic and hydroalcoholic extract of Avipattikar churna, calculated from

the regression equation ($Y = 2.5402x - 0.0512$, $R^2 = 0.9936$) was found to be $4.353 \pm 0.116\%$ w/w, $12.467 \pm 0.273\%$ w/w and $5.53 \pm 0.027\%$ w/w (gallic acid equivalents) respectively. The total flavonoid contents of the aqueous, alcoholic and hydroalcoholic extract of Avipattikar churna, calculated from the regression equation ($Y = 0.0006x - 0.0050$, $R^2 = 0.9968$) was found to be $3.8226 \pm 0.180\%$ w/w, $4.735 \pm 0.091\%$w/w, $4.249 \pm 0.845\%$w/w (Rutin equivalents) respectively. The aqueous, alcoholic and hydroalcoholic extract of avipattikar churna contained $5.958 \pm 0.138\%$ w/w, $8.0368 \pm 0.138\%$ w/w, and $9.976 \pm 0.24\%$ w/w of total tannins respectively.

Acid neutralizing capacity by USP method

The acid neutralizing capacity (meq) of all three extracts (aqueous, hydroalcoholic extract, alcoholic extract) and Digene were 11.98 ± 0.2887, 5.067 ± 0.0667, 3.067 ± 0.1437 and 12.33 ± 0.333 respectively. The aqueous extract showed prominent results comparable to the marketed antacid formulations.

Analysis of Avipattikar churna by network pharmacology

Avipattikar churna phytoconstituent mining and target prediction

In total, 701 phytoconstituents of Avipattikar churna were retrieved using IMPPT and Dr. Duke's phytochemical and ethno botanical database. Keeping druglikeness score and F30% oral bioavailability as screening criteria, 69 active ingredients were retrieved (Fig. 1). Amongst these Desmosterol, Beta-sitosterol, and Bisabolol were found to have the highest drug likeness score. Majority of phytoconstituents were identified as terpenoids (38%), flavonoids (13%), polyphenols (9%) and alkaloids (9%). Post target fishing using Superpred, 246 targets, with 80% or above probability, were predicted to be regulated by 69 phytoconstituents. Compound target network showed 314 nodes, arranged based on degree. The ADMET profile of phytoconstituents is shown in Fig. 2.

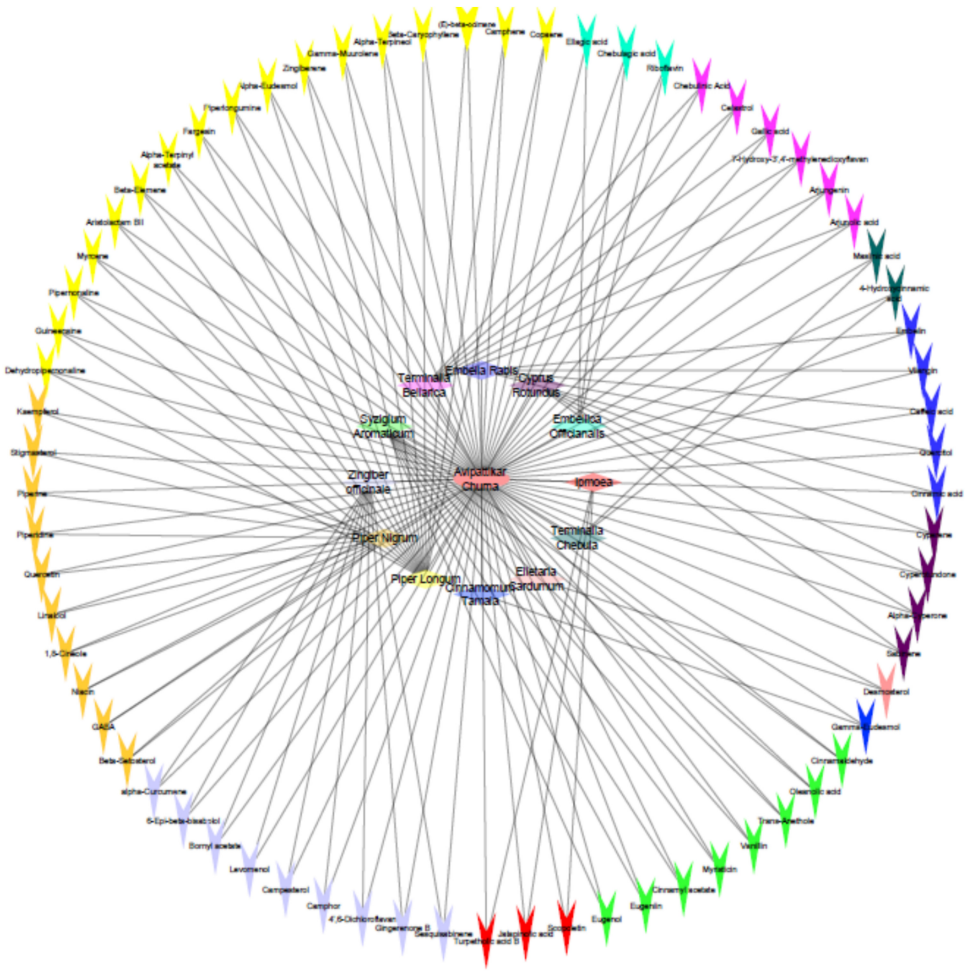

Fig. 1 Plant-Phytoconstituent Network

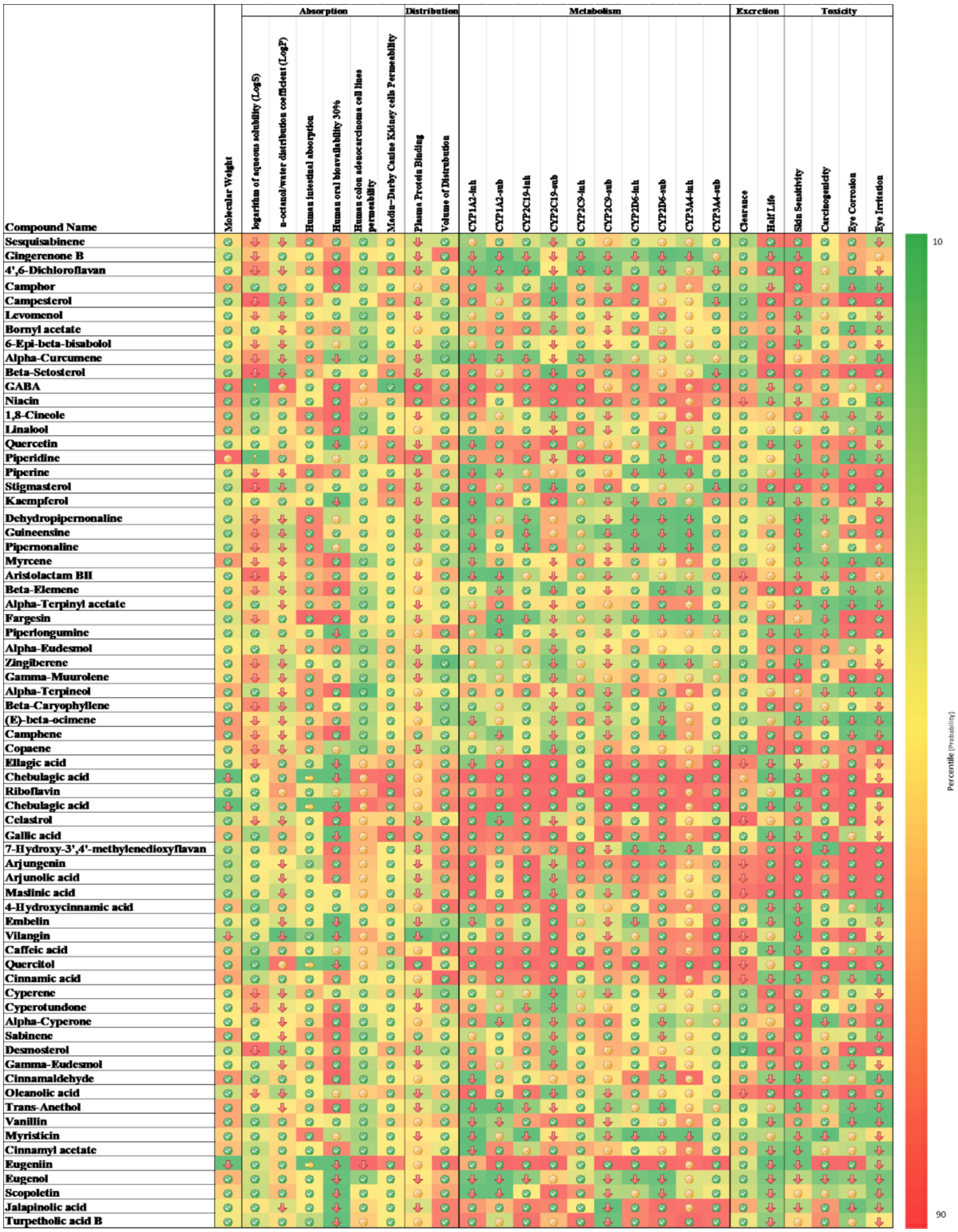

Fig. 2 ADMET Prolife (Color of square indicate value of ADMET property; Marks in the square indicated empirical decision: near to acceptable range of property: within acceptable range of property: unacceptable range of property)

Disease target mining

Using various key words including peptic ulcer (1326), oxidative stress (13,169), H^+/K^+ ATPase enzyme (702), H. Pylori (2987) and H_2 Receptor (4301) in genecards, we found 22,528 genes. The genes were further screened based on GIFtS. We found 3702 genes playing role in peptic ulcer.

Protein–protein interaction

Using Venny 2.1, common target proteins of phytocontituents and disease were found to be 213. The common target proteins were subjected to STRING to construct protein–protein interaction (PPI) network. With high confidence level (0.7) and concealing disconnected nodes in the network, we obtained 119 proteins and 507 interrelations. The network was visualized in cytoscape 3.7.2. Additionally, degree of top 20 proteins was also presented in form of bar graph. TP53 was found to have the highest degree (74), indicating that it could act as hub in the network. Besides, many other proteins including AKT1 (68), SRC (60), STAT3 (56) were found to have high degree, indicating their role in peptic ulcer and their modulation by churna phytoconstituents (Fig. 3).

KEGG analysis and network construction

Thirty-eight pathways that are directly related to peptic ulcers were found by the peer interpretation of protein interaction under the KEGG pathway analysis. Among them PI3K-Akt signalling pathway, MAPK signalling pathway, HIF-1 signalling pathways, Rap1 signalling pathways and Focal adhesion were recognized to mark the highest gene set counts with lowest FDR (false discovery rate).

Finally, cytoscape 3.7.2 was used to build the phytochemical-target protein-pathway network. The constructed network has 353 nodes representing 69 phytoconstituents, 246 targets and 38 pathways. The total edge count was found to be 594 among which 346 were pathway-target interactions and 248 were phytoconstituent-target interactions. Quercetin, Ellagic acid, Turpetholic acid, Eugenin, Aristolactom BII, Maslinic acid, Copaene, Jalapinolic acid, Celastrol, Arjunolic acid and Eugenol were having higher degree compared to other phytoconstituents. NFKB1, MAPK1, AKT1, PIK3R1, EGRF, PTGS1, HSP90AB1, CHUK, CNSK2B, and FPR1 have highest edge count (Fig. 4).

Ex-vivo models

H^+/K^+-ATPase assay

H^+/K^+ ATPase inhibition was evaluated using goat homogenate. Extracts were compared to one another and to omeprazole for their inhibitory efficacy. Hydroalcoholic extract at dose 150 and 200 ug/mL showed significantly high inhibition compared to omeprazole (Table 1). The activity of all three extracts were concentration-dependent, with the hydroalcoholic extract being the most effective at 250 ug/mL. In comparison to aqueous and alcoholic extracts, hydroalcohol was found to be more effective at all concentrations.

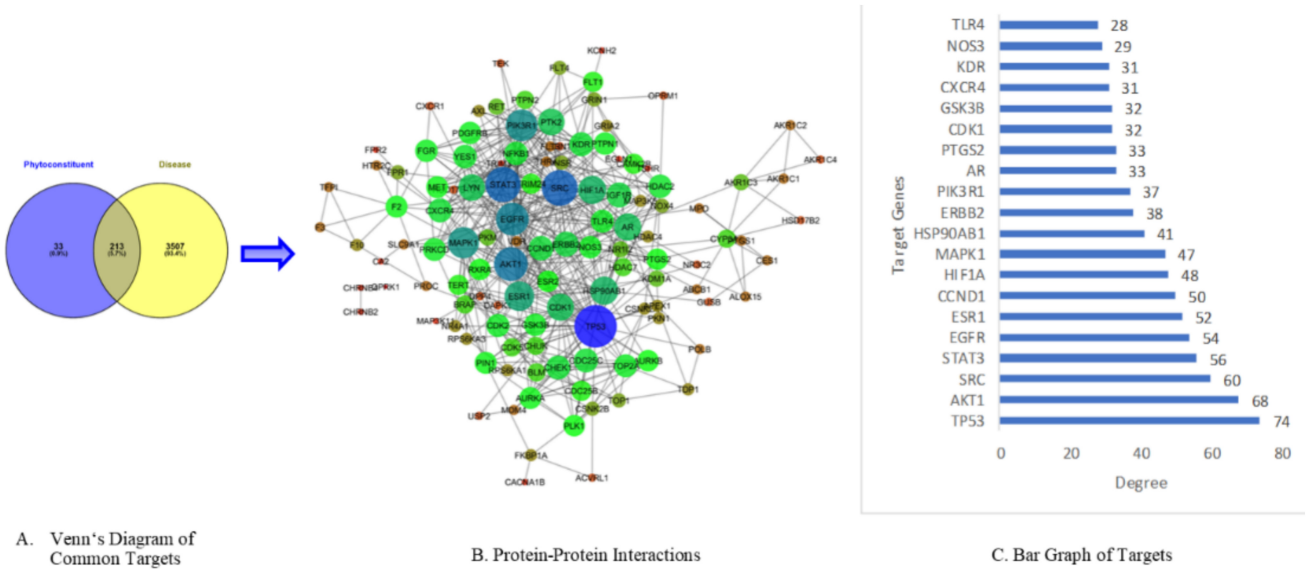

Fig. 3 Protein–Protein interaction visualization

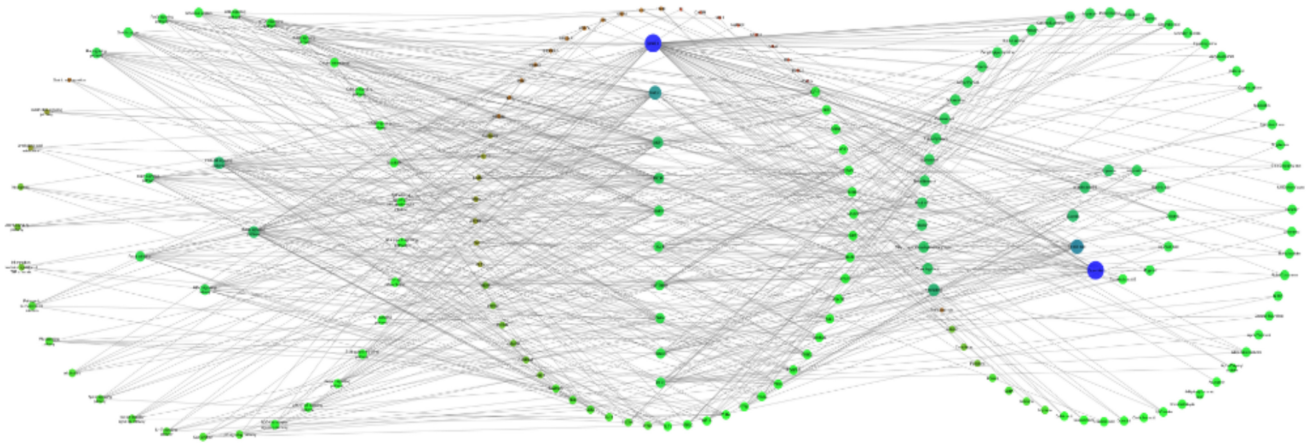

Fig. 4 Phytoconstituent-target-pathway network (First circle: Pathways, Second Circle: Target Proteins, Third Circle: Phytoconstituents; the central circles and line represents nodes with high degree)

Table 1 H + /K + ATPase Inhibition activity

Concentration (µg/mL)	AVC AQ	AVC ALC	AVC HA	OMP
50	40.65 ± 1.45	18.82 ± 1.46$^{***\ a}$	39.48 ± 2.10^{c}	35.92 ± 1.67
100	52.24 ± 1.17	31.97 ± 2.02$^{***\ a}$	55.5 ± 1.19^{c}	49.95 ± 1.79
150	59.96 ± 1.20	52.77 ± 1.59	66.83 ± 1.46$^{*\ c}$	58.48 ± 1.76
200	64.65 ± 1.45	67.59 ± 1.19	74.52 ± 1.50$^{*\ \#}$	66.28 ± 1.53
250	73.75 ± 1.44	70.07 ± 1.04*	83.69 ± 2.12$^{b\ c}$	78.26 ± 1.05

AVC Avipatikar Churna, *AQ* Aqueous, *HA* Hydroalcoholic, *ALC* Alcoholic. Data are presented as Mean ± SEM. Analysis was done by two-way ANOVA with Post-hoc comparison by Tukey's test. ***p < 0.001, vs Omp, *p < 0.05 vs Omp; ^{a}p < 0.001 vs Aqueous, ^{b}p < 0.01 vs Aqueous, ^{c}p < 0.001 vs Alcoholic, #p < 0.05 vs Aqueous

Table 2 Antioxidant activity of Avipattikar churna

Extracts	Positive Control	Negative Control	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL	250 µg/mL
SOD							
Aqueous	15.53 ± 0.68***	09.07 ± 0.84	11.88 ± 00.55**	12.43 ± 00.22***	13.09 ± 00.28***	12.22 ± 00.30***	12.74 ± 00.15***
Alcohol			11.19 ± 00.49	10.92 ± 00.51	12.19 ± 00.34***	10.96 ± 00.20	10.50 ± 00.42
Hydroalcohol			12.52 ± 00.36***	13.43 ± 00.17***	13.69 ± 00.26$^{***\ a}$	13.85 ± 00.36$^{***\ b}$	15.11 ± 00.29$^{***\ cd}$
Catalase							
Aqueous	73.89 ± 0.33***	48.26 ± 03.32	66.39 ± 01.09$^{***\ c}$	67.33 ± 02.24$^{***\ b}$	65.75 ± 02.57***	71.96 ± 01.76***	72.84 ± 00.75$^{***\ a}$
Alcohol			51.41 ± 02.19	52.98 ± 01.87	57.87 ± 01.71	67.45 ± 02.98***	60.99 ± 01.35**
Hydroalcohol			68.71 ± 01.75$^{***\ c}$	69.26 ± 02.02$^{***\ c}$	72.04 ± 01.90$^{***\ b}$	73.37 ± 02.90***	75.14 ± 02.07$^{***\ b}$
Reduced glutathione							
Aqueous	00.30 ± 0.02***	0.17 ± 00.01	0.24 ± 0.0019$^{***\ c}$	0.25 ± 0.0018$^{***\ c}$	00.26 ± 0.0032$^{***\ c}$	00.29 ± 00.0040$^{***\ c}$	0.31 ± 0.0023$^{***\ c}$
Alcohol			0.18 ± 0.0023	0.19 ± 0.0025	00.21 ± 0.0032*	00.21 ± 00.00229**	0.24 ± 0.0035***
Hydroalcohol			0.26 ± 0.0041$^{***\ c}$	0.26 ± 0.0061$^{***\ c}$	00.29 ± 0.0044$^{***\ c}$	00.30 ± 00.0058$^{***\ c}$	0.31 ± 0.0038$^{***\ c}$

Data are presented as Mean ± SEM. Analysis was done by One Way ANOVA following post hoc comparison by Tukey's test. (***p < 0.001, **p < 0.01, *p < 0.05 vs DC; ^{a}p < 0.05, ^{b}p < 0.01, ^{c}p < 0.001 vs AL; ^{d}p < 0.05 vs AQ)

Antioxidant activity

Superoxide dismutase Using goat liver homogenate, we quantified the superoxide dismutase enzyme. SOD levels produced by different extracts were compared with each other and with a negative control group. When the results were compared to the negative control group, a significant difference in SOD levels was seen in the positive control

group. Aqueous and hydroalcoholic extracts showed highly significant results at all doses compared to negative control group. At 250 ug/mL hydroalcoholic extract showed significant results compared to aqueous and alcoholic extract (Table 2) (Misra and Fridovich 1972).

Catalase Catalase levels of different extracts were compared with each other and with a negative control group. In our experiments, we observed that the levels of catalase in the positive control group were noticeably greater than those in the negative control group. It was also noted that all aqueous and hydroalcoholic extracts have shown significant catalase activity than negative control group (H_2O_2). Alcoholic extract has comparatively lesser activity than aqueous and hydroalcoholic (Table 2).

Reduced glutathione We noted a significant reduction in glutathione levels in the positive control group when compared to the positive control group. All the aqueous and hydroalcoholic extracts showed significantly high level of glutathione compared to negative control group. At concentration of 50 and 100 µg/mL, alcoholic extract did not exhibit any notable activity whereas 150–250 µg/mL showed significant activity compared to negative control. The results revealed that aqueous and hydroalcoholic extracts have significantly higher and similar activities which are superior to that of alcoholic extract (Table 2).

In vivo studies

Effect of Avipatikar churna on indomethacin induced gastric lesions

The macroscopic examination of the gastric mucosa revealed no lesions in the normal control group. The mucosal layer exhibited normal thickness without evidence of inflammation. However, a minor hemorrhagic lesion was observed in two animals, yielding an ulcer score of 0.33 ± 0.21. In contrast, the mucosal layer of the disease group displayed pronounced congestion and severe lesions of varying diameters distributed across the surface, with an ulcer score of 3.8 ± 0.30. The standard treatment group exhibited vascular congestion along with a few lesions and mild hemorrhagic changes, resulting in an ulcer score of 1.5 ± 0.34. All treatment groups demonstrated mild congestion with hemorrhagic lesions ranging from mild in the high-dose group (1.16 ± 0.16) to moderate in the low-dose (2.33 ± 0.21) and medium-dose (1.66 ± 0.21) groups. The

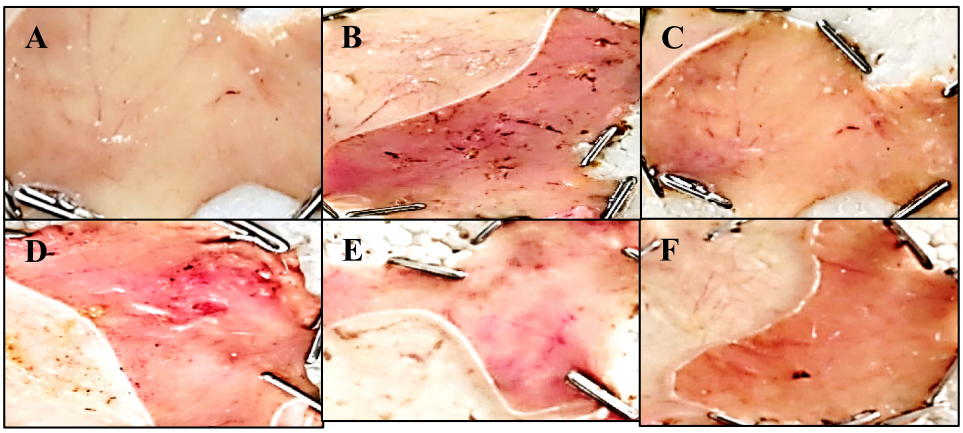

Fig. 5 Effect of Avipattikar churna on Gastric Mucosa (**A** Control; **B** Disease Control; **C** Standard; **D** Treatment 1; **E** Treatment 2; **F** Treatment 3)

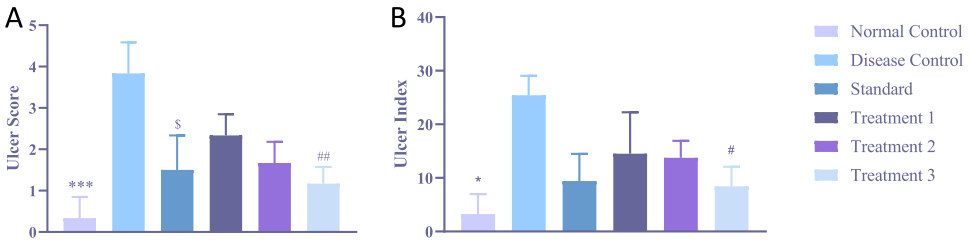

Fig. 6 Effect of Avipattikar churna on gastric lesions (**A** Ulcer Score, Indomethacin treatment resulted in severe mucosal damage, characterized by extensive hemorrhagic lesions. In contrast, treatment with Avipattikar Churna led to a reduction in the ulcer score, indicated by smaller number of lesions; **B** Ulcer Index: Indomethacin treatment caused significant mucosal ulceration, leading to a high ulcer index. Conversely, pre-treatment with a high dose of Avipattikar Churna significantly reduced the ulcer index, demonstrating its protective effect against mucosal damage)

indomethacin treatment group showed a significantly higher ulcer index (25.43 ± 2.57) compared to the control group (3.23 ± 2.64). Treatment with standard (9.40 ± 3.59), low-dose (14.53 ± 5.46), and medium-dose (13.76 ± 2.24) led to a reduction in ulcer index compared to the disease group; however, these differences were not statistically significant. Notably, the high-dose treatment group exhibited a significantly reduced ulcer index (8.42 ± 2.57) compared to the disease group (Fig. 5; Fig. 6).

Effect of Avipattikar churna on inflammatory markers

To assess the anti-inflammatory effects of Avipattikar Churna, we measured inflammatory marker TNF-α, IL-6, and NF-κB. Indomethacin treatment significantly increased TNF-α levels (disease control: 0.227 ± 0.038) compared to the normal control group (0.118 ± 0.016). Treatment with low-dose (0.138 ± 0.011) and medium-dose (0.130 ± 0.006) Avipattikar Churna reduced TNF-α levels compared to the disease control, with the high-dose treatment (0.118 ± 0.008) achieving a significant reduction. Similarly, indomethacin treatment elevated IL-6 levels in the disease control group (0.236 ± 0.001) relative to the normal control group, while low-dose (0.174 ± 0.012), medium-dose (0.181 ± 0.006), and high-dose (0.195 ± 0.002) treatments of Avipattikar Churna significantly decreased IL-6 levels compared to the disease control. NF-κB levels were also elevated in the disease control group (0.161 ± 0.02) compared to the control. Notably, the high-dose Avipattikar Churna treatment significantly reduced NF-κB levels (0.090 ± 0.006) relative to the disease control (Fig. 7).

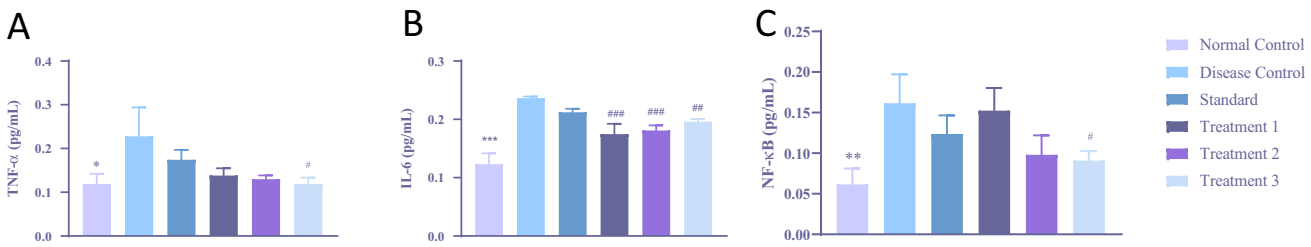

Fig. 7 Effect of Avipattikar Churna on inflammatory markers (**A** Levels of TNF-α: Significant reduction of TNF-α was noted in treatment group compared to disease control. **B** Levels of IL-6: All treatment groups showed significantly low IL-6 compared to disease control. **C** Levels of NF-κB: Effect of Avipatikar churna lead to significantly reduce NF-κB in high dose treated group compared to disease control.)

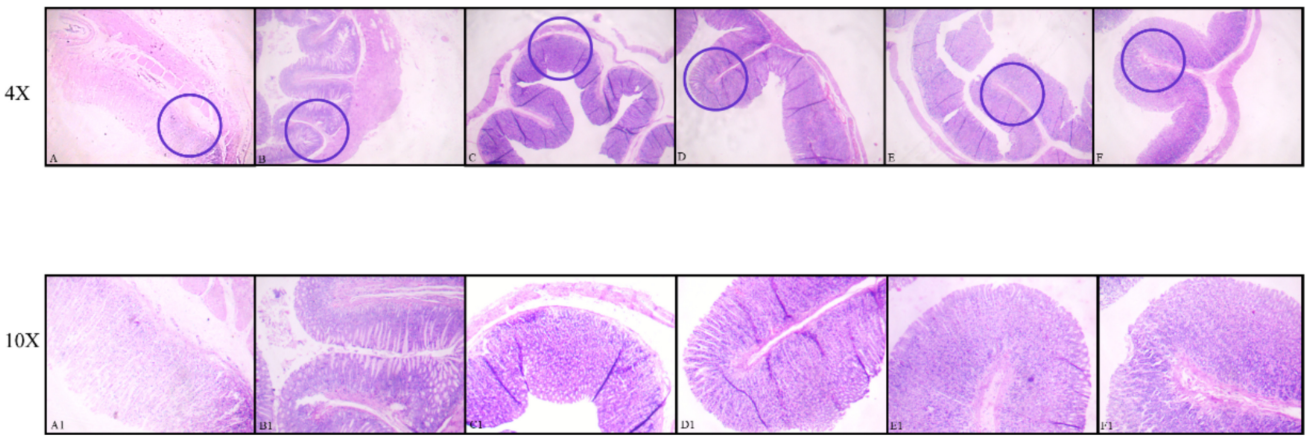

Fig. 8 Effect of Avipattikar churna on histopathological analysis (At 4X magnification, the control group **A** displays a normal mucosal layer, whereas the disease control group **B**shows significant damage to both the mucosal and submucosal layers. The standard treatment group **C** presents with slight rupture of the mucosal and submucosal layers. Treatment 1 **D** reveals minor damage to the mucosal layer, while Treatments 2 and 3 **E** and **F** demonstrate intact mucosal and submucosal layers. At 10X magnification, the control group **A1** features healthy mucosal and normal parietal cells. In contrast, the disease control group **B1** exhibits necrotic tissue. The standard treatment group **C1** displays healthy submucosal cells with a reduction in necrotic cells. Treatments 1 (**D1**), 2 (**E1**), and 3 **F1** all show healthy mucosal and submucosal cells with no signs of inflammatory cell infiltration.)

Effect of Avipatikar churna on histopathology of gastric mucosa

Histopathological examination of normal gastric tissue revealed various layers, including the mucosa and submucosal layer. The administration of indomethacin caused considerable stomach injury, evidenced by pronounced necrotic alterations in the gastric mucosa and the infiltration of inflammatory cells. The pre-treatment with Avipattikar churna markedly diminished the degenerative alterations in the stomach mucosa of all treated groups, showing the beneficial effect of the churna (Fig. 8).

Discussion

As peptic ulcer is a multifactorial disease, a multi-target approach including proton pump inhibition, cytoprotectivity, antioxidant activity, radical scavenging potential, mucus secretion and acid neutralization may help to heal as well as prevent complications. Recently, various phytoconstituents have been studied and used for their potential against peptic ulcer. Avipattikar churna, an Ayurvedic preparation, is a polyherbal formulation with highly active phytoconstituents acting against various endogenous factors responsible for peptic ulcer. So far, work has been done on the use of Avipatikar churna against peptic ulcer, yet the specific mechanism of action is not elucidated anywhere. Therefore, to elucidate the mechanism of churna, network pharmacology, in-vitro, ex-vivo and in-vivo studies were conducted in this investigation.

Excessive acid secretion leads to inflammation and ulceration at the stomach lining (Orwa Jaber Houshia 2012). One of the effective treatments of acidity is antacids. We confirmed antacid activity of Avipattikar chruna by acid neutralizing capacity (Adinortey et al. 2013). Aqueous extract of churna was found to be as potent as Digene. As sugar is highly soluble in aqueous extract it may have helped in antacid activity of chruna. The results can therefore be used to anticipate chruna's activity as an antacid in treatment of peptic ulcer.

Zingiberene (Yamahara et al. 1988), Oleanolic acid (Rodríguez et al. 2003), 1–8 Cineole (Takayama et al. 2011), beta Caryophyllene (Tambe et al. 1996), alfa Terpineol (Souza et al. 2011), Myrcene (Bonamin et al. 2014) and beta Sitosterol (Xiao et al. 1992) are some of the drugable terpenoids with anti-ulcerogenic potential. Terpenoids reduce acid secretion, inflammatory mediators, stomach emptying, oxidative damage, and gastric mucosal defence factors to treat ulcers (Awaad et al. 2013; Sharifi-Rad et al. 2018). Ellagic acid, Gallic acid, Caffeic acid (Kolgazi et al. 2021), Vanillin (Ciciliato et al. 2022), and Eugenol (Shah et al. 2022) are anti-ulcer phenols and polyphenols in churna (Beserra et al. 2011; Zhou et al. 2020; Longo et al. 2021). Polyphenols fight ulcers through antioxidant, cytoprotective, and anti-secretory mechanisms (Sumbul et al. 2011). Quercetin, Kaempferol, and Vilangin are anti-ulcer flavonoids.

Damage to cell lipids, proteins, and DNA by digestive tract ROS is a major cause of mucosal injury (Zhou et al. 2022). Compound-target-pathway network suggests most phytoconstituents target various pathway to reduce oxidative stress. Further studies suggest that Quercetin (Weng et al. 2011), Eugenol (Zhang et al. 2021), Ellagic acid (Aslan et al. 2020), and Kaempferol (Rajendran et al. 2020) have powerful antioxidant properties. The finding was supported by experimental data where we found antioxidant properties of churna extract.

Enhanced TNF-α and IL-6 expression contributes to ulcer aggravation and long-term non-healing, as evidenced by their positive correlation with ulcer severity (Zhou et al. 2022). KEGG analysis revealed TNF-α signaling pathway involvement through PIK3R1, AKT1, CHUK, NFKB1, MAP3K5, and PTGS2 genes. Network pharmacology suggested that phytoconstituents, including Quercetin, Ellagic acid, Aristolactum BII, Copaene, Jalapinolic acid, and Arjunolic acid, target these genes and may benefit them (Shakeri et al. 2018; Carrasco-Pozo et al. 2016). According to network pharmacology data, Avipattikar churna is a promising treatment for peptic ulcer and related disorders.

The H^+/K^+-ATPase pump along with reactive oxygen species as an endogenous factors play central role in peptic ulcer. We evaluated H^+/K^+ ATPase inhibition activity of Avipattikar churna with omeprazole as positive control. As evidenced from Table 1, hydroalcoholic extract exhibited maximum H^+/K^+ inhibitory activity. Maximum inhibition at 250 ug/mL of hydroalcoholic extract and omeprazole was found to be 83.69% and 78.26% with an IC_{50} value of 75.50 and 94.05 µg/mL respectively. Phenols and Flavonoids with hydroxyl group present at various position may have produced enzyme inhibition activity. As According to Murakami et al. 1999, hydroxy groups at the flavonoid backbone's 3, 5, and 7 positions are important for inhibiting H^+/K^+ ATPase (Murakami et al. 1999). The phenolic compounds including Ellagic acid, Gallic acid, Eugenol, Epicatechin, Gingerol and flavonoids including Biflorin, Kaemferol and Quercetin present in churna have hydroxyl group at various positions. The results are further supported by the total phenol ($5.53 \pm 0.027\%$, Gallic acid equivalents) and flavonoid ($4.249 \pm 0.845\%$, Rutin equivalents) of churna hydroalcoholic extract.

We investigated antioxidant enzyme activity of Avipattikar churna ex-vivo. SOD, by scavenging superoxide anion, play an important role in scavenging the ROS. As represented in Table 2, aqueous and hydroalcoholic extract showed significantly high SOD compared to

H_2O_2 control. At highest concentration, hydroalcoholic extract showed significantly high SOD than aqueous extract. Catalase converts hydroxyl group to water. We found saturation effect of aqueous and hydroalcoholic extract. Hydroalcoholic extract showed the best effect at the highest dose compared to other extracts. We also studied effect of churna on reduced glutathione levels. We found that aqueous and hydroalcoholic extract showed significantly high effect compared to H_2O_2 control, as represented in Table 2.

Indomethacin is known to induce gastric ulcers by inhibiting cyclooxygenase (COX) enzymes and generating ROS, contributing to gastric mucosal inflammation. Consequently, an indomethacin-induced ulcer model was employed to evaluate the protective effects of Avipattikar Churna (Ko et al. 2020; Das et al. 1997). Our results demonstrate that indomethacin treatment significantly increased the ulcer index, characterized by haemorrhagic bands, scar lesions, ulcerative inflammation, gastric degeneration, and necrosis consistent with previous findings (Abu-Baih et al. 2024). Conversely, pre-treatment with Avipattikar Churna resulted in a dose-dependent reduction in both ulcer index and ulcer score, indicating its protective effect. Notably, the high-dose treatment group showed an ulcer index comparable to the control group, reflecting the preservation of healthy mucosal cells.

The activation of NF-κB, a critical transcription factor involved in immune and inflammatory responses, was markedly induced by Indomethacin, as evidenced by elevated NF-κB levels in the disease control group compared to the control. Indomethacin triggers NF-κB activation through the degradation of IκB-α, mediated by ROS. Once activated, NF-κB translocate to the nucleus, promoting the expression of inflammatory cytokines such as TNF-α and IL-6 (Akanda and Park 2017; Zhang and Feng 2019). Avipattikar Churna treatment effectively reduced NF-κB levels, likely due to its antioxidant properties that scavenge ROS (Ukil et al. 2006; Suzuki et al. 2001). Increased levels of TNF-α and IL-6, which attract neutrophils and mononuclear cells to the gastric lamina propria, were observed with indomethacin treatment, correlating with heightened gastric inflammation (Sidahmed et al. 2015). Avipattikar Churna's suppression of the NF-κB signalling pathway was associated with significant reductions in TNF-α and IL-6 levels, as well as decreased inflammatory cell infiltration. These observations were corroborated by histopathological analyses, which revealed necrotic tissue with inflammatory infiltration in the disease control group and intact mucosal cells in the treatment groups. These findings underscore Avipattikar Churna's role in mitigating gastric inflammation.

Conclusion

Peptic ulcer is a multifactorial disease requiring a multi-target therapeutic approach. This study demonstrates that Avipattikar Churna exhibits significant anti-ulcer activity through multiple mechanisms, including acid neutralization, proton pump inhibition, antioxidant effects, and anti-inflammatory activity. It effectively inhibits H^+/K^+ ATPase, comparable to omeprazole, and its phytochemical constituents—polyphenols, terpenoids, and flavonoids—contribute to its anti-inflammatory and antioxidant properties. In the indomethacin-induced ulcer model, Avipattikar Churna significantly reduces the ulcer index and score, preserving gastric mucosal integrity. Additionally, it suppresses NF-κB activation, thereby attenuating gastric inflammation. These findings suggest that Avipattikar Churna is a promising therapeutic candidate for peptic ulcer treatment due to its multi-faceted protective effects.

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Author contribution PS: Data curation, investigation, methodology, formal analysis, writing- original draft preparation; KP: Supervision, conceptualization, writing review and editing; VK: Software, data curation, writing- original draft preparation, methodology, formal analysis; MZ: Conceptualization, supervision, validation, writing – review & editing. All authors have approved the final manuscript.

Data availability The author confirms that this published paper contains all of the data produced or analysed during this investigation Research.

Declarations

Conflict of interest The authors do not have any conflicts of interest to disclose. All co-authors have reviewed and agree with the content of the manuscript. Our work is not influenced by any financial interests that could potentially affect the outcome.

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