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Synergic action of plant derivatives and honey for high efficacy of Dawa-ul kurkum formulation against hepatocellular carcinoma

Seyedeh Sara Kamyab[a], Alpana Moghe[a,*], Manasi Deshpande[b], Narendra Bhatt[b], Ghazalla Mulla[c] & Manjiri Karandikar[d]

[a]Rajiv Gandhi Institute of IT & Biotechnology, Bharati Vidyapeeth Deemed to be University, Pune 411 046
[b]College of Ayurveda, Bharati Vidyapeeth Deemed to be University, Pune
[c]Z. V. M. Unani Medical College & Hospital, Azam Campus, Camp, Pune 411 001
[d]Medical College, Hospital and Research Center, Bharati Vidyapeeth Deemed to be University, Pune 411 043
[*]E-mail: alpana.moghe@bharatividyapeeth.edu

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Hepatocellular carcinoma with high incidence and poor survival stands as a major global health burden. Various challenges involved in drug therapies impede their efficacy for substantial protective effects. They emphasize the necessity for the development of safe and affordable options suitable for long-term treatment. The present study has explored the therapeutic potential of traditional Dawa-ul-kurkum formulation for anticancer effects against hepatocellular carcinoma (HCC). The formulation and its individual ingredients were screened for cytotoxic effects, induction of apoptosis, and cell cycle arrest on HepG2 cell line. Dawa-ul-kurkum was further evaluated for regression of tumor growth in diethylnitrosamine (DEN) induced HCC rat models. Low, medium, and high doses of the formulation were fed orally to the test groups. A Combination of Dawa-ul-kurkum with the anticancer drug Doxorubicin was tested for assessment of adjuvant therapy. The classical dosage form with honey of each ingredient plant drug displayed enhanced cytotoxic effect and pharmacologically significant IC_{50} values. The Dawa-ul- kurkum formulation was superior in inducing apoptosis and arresting cells in the G0-G1 phase of the cell cycle. Significant regression of tumor growth and reversal of biochemical parameters in DEN-induced HCC rat study endorsed its prominent anticancer effect. The promising potential to induce apoptosis, cell cycle arrest, and regression in tumor growth validated the traditional Dawa-ul-kurkum formulation for anti-hepatoma activity. The study convincingly promotes the use of Dawa-ul-kurkum formulation for the development of a cost-effective and safe therapeutic strategy against HCC.

Keywords: Anticancer, Cost-effective, Hepatocellular carcinoma, Honey, Unani medicine

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HCC represents a complex form of cancer that poses resistance to the majority of chemotherapeutic drugs. There has been a concerning increase in its incidence noted in recent years. An estimated 841,080 cases and 781,631 new deaths of liver cancer occurred around the world in 2018[1]. Liver diseases in India accounted for approximately 18.3% of all liver-related deaths globally[2]. Modern treatment modalities for HCC include surgical resection, targeted chemotherapy, image-guided ablation, chemoembolization, and liver transplantation. However, the grim prognosis and the exceedingly low (4%) five-year survival rate of HCC emphasize the constraints in available treatment options. Additionally, restricted availability of advanced medications, cost, lack of awareness, and the emergence of drug-resistant hepatitis virus strains hinder the efficacy of these drugs[3]. They underscore the crucial need for the development of treatment modalities that are highly accessible, cost-effective, and safe for long-term use.

Dawa-ul-kurkum is a traditional polyherbal formulation used in the treatment of liver dysfunction, abdominal pain, ascites, and anorexia. It is one of the most complex formulations covering a broad spectrum of symptoms[4]. It comprises of seven ingredients *viz; Nardostachys jatamanasi*, *Cinnamonum zylanicum*, *Cinnamonum cassia*, *Sousurria lappa*, *Commiphora myrrha, Crocus sativus* and *Cymbopogon jwarankusa* each with proven hepatoprotective activity[5]. The formulation is based on traditional principle of using finely ground powder mixed with honey. It has multiple properties such as diuresis, deobstruction, and astringent. It is prescribed for various acute and chronic liver disease conditions in Unani system of medicine[6]. Hafeez *et al.*[7] have shown its effectiveness in reducing

[*]Corresponding author

symptoms of non-alcoholic fatty liver disease (NAFLD). Reshi *et al.*[8] have reported marked protection against antitubercular drug-associated hepatotoxicity as well as paracetamol-induced liver damage in experimental rats. Their study on ethanol-induced liver damage in rats showed lower levels of reduced glutathione (GSH) as well as antioxidant effect elicited by Dawa-ul-Kurkum[9,10]. Gupta *et al.*[11] reported the standardization of Dawa-ul kurkum employing marker constituents of its seven ingredients using HPTLC and molecular docking techniques. Their findings suggested that Z-guggulsteron emerges as a primary molecule in Dawa-ul-kurkum potentially indicating its therapeutic efficacy[11].

To gain deeper insights into the efficacy of the Dawa-ul Kurkum formulation, we evaluated its anticancer properties against hepatocellular carcinoma through both *in vitro* and *in vivo* studies. The formulation was prepared and standardized in Unani Pharmacy of ZVM Unani Medical College and Hospital, Pune, India. The cytotoxic effect against HepG2 (HCC) cell line was tested. The aqueous extracts and traditional honey extracts of its each ingredient were also tested for effect on HepG2 cell line. They were evaluated for induction of apoptosis using Annexin V – FITC assay and cell cycle analysis. The formulation was further assessed for its effect on tumor growth regression through an *in vivo* study using a DEN-induced rat tumor model. These investigations were undertaken to validate the traditional formulation and explore its potential utilization in the management of hepatocellular carcinoma

Materials and Methods

Procurement of plant material

Seven ingredients of the formulation *viz*; 1) *Nardostachys jatamanasi* (NJ), 2) *Cinnamonum zylanicum* (CZ), 3) *Cinnamonum cassia* (CC), 4) *Saussurea lappa* (SL), 5) *Commiphora myrrha* (CM), 6) *Crocus sativus* (CS), 7) *Cymbopogon jwarancusa* (CJ) were procured from authentic suppliers and/or natural habitat. They were authenticated and analysed by physicochemical, organoleptic parameters, and TLC. The plant samples satisfying the Pharmacopeia guidelines were selected for the present study.

Preparation of 'Dawa-ul-Kurkum'

The Dawa-ul-Kurkum formulation was prepared in Unani pharmacy using National Formulary guidelines[6]. Briefly, 35 g of CM gum powder was soaked in a 1:3 solution of Brandy and water for 24 h. The CS stemen, NJ rhizome, CC, CZ bark, CJ flowers, and SL roots were ground to a fine powder. The suspension of CM and brandy was mixed with 750 (g) g of Honey. Equal parts (35 g) of ingredient powders were added to it progressively with continuous stirring. The prepared product was stored at room temperature. The formulation prepared at three different times was standardized by organoleptic, physicochemical, TLC, and HPTLC analysis[12]. It was diluted with PBS for subsequent use in cellular assays.

Preparation of aqueous extracts and traditional dosage form using honey

The aqueous extracts were prepared by extracting 5 g of powdered plant sample in 50 mL of water for 48 h at room temperature. The extracts were lyophilized after filtration. 10 mg of lyophilized extract was dissolved in 1 mL PBS and used as a stock solution in all cellular assays. The traditional dosage form with honey was prepared by mixing 10 mg of each finely ground powder with 1 mL of honey. Subsequent dilutions were prepared in PBS. The aqueous and honey dosage forms of all drug samples were tested for cytotoxic effects on the HepG2 cell line using MTT assay and successive cellular assays.

Cell culture

A human cancer HepG2 (hepatocellular carcinoma) cell line obtained from the National Animal Cell Repository, Pune was maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 2 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum (Gibco) at 37ºC in 5% CO_2 atmosphere.

MTT Assay

5 x 10^4 HepG2 cells were grown in 96 well microplates and cultured for 24 h. Different concentrations (15.62 µg/mL - 500 µg/mL) of aqueous extracts and honey dosage forms of each plant drug, as well as Dawa-ul Kurkum formulation, were then treated on the cells. An equal amount of PBS was added to the control wells. The Standard drug doxorubicin was used as a positive control. Following the addition of all test samples, plates were incubated for 48 h. Subsequently, 10 µL MTT dye (5 µg/mL, Sigma-Aldrich) was added to the wells and left to incubate for 5 to 6 h. 100 µL of DMSO and 25 µL of glycine buffer were added and the plates were read at 570 nm (BIO-RAD, Model 680). Cell survival

was assessed by comparing the mean absorbance (OD) of replicate wells to that of the control. Graphs and IC50 values were determined using GraphPad Prism 6.0 software.

Induction of apoptosis using Annexin V, FITC assay

$1x10^5$ cells/mL of HepG2 cell line were cultured in 24 well plates for 24 h. They were treated with 62.5 and 125 µg/mL concentrations of the extracts, Dawa-ul-kurkum formulation, and the standard drug doxorubicin for 48 h. Cell suspension from each well was prepared using trypsin EDTA treatment and spun for 10 min at 1500 rpm. The pellet was resuspended in 500 µL of 100 mM HEPES, 1.4 M NaCl, 25 mM $CaCl_2$, and 5 µL of annexin V-FITC conjugate (Sigma-Aldrich) and 10 µL of propidium iodide solution. Tubes were incubated in the dark for 10 min and fluorescence at Ex 488 nm was measured in a flow cytometer.

Cell cycle analysis

$1x10^5$ HepG2 cells were seeded in each well of 24 well plates. Cells were treated with 62.5 µg/mL concentrations of plant extracts, Dawa-ul-kurkum formulation, and the standard drug doxorubicin in triplicate for 48 h. Cells were trypsinized and cell suspension after washing with cold PBS was centrifuged at 1800 rpm for 7 min. Cells were fixed using ethanol and centrifuged at 2000 rpm for 5 min. The pellet was resuspended in 0.5 mL of PBS containing 0.25% Triton x-100 and incubated on ice for 15 min. Cells were spun at 2000 rpm and the pellet was resuspended in 0.5 mL of PBS containing 50 µL of RNase (100 µg/mL) and 200 µL PI (50 µg/mL stock solution). The tubes were read at Ex 405 and Em 440 nm wavelengths in FACS BD Bioscience Canto II.

Effect of Dawa-ul-kurkum formulation on inhibition of tumor growth in Diethylnitroseamine (DEN) induced HCC rats

The use of rats for this study was approved by the Institutional Animal Ethics committee (IAEC BVDU, Pune) and the experiments were performed following CPCSEA guidelines. Wistar male rats of 7 weeks age, (160-200 g) were fed with commercial pellets and free access to water. They were divided randomly into 7 groups. Rats of Group 1 (Normal Control) were fed with a standard diet and pure drinking water. Rats of Group 2 (DEN-control) to Group 7 were administered an intraperitoneal injection of 40 mg/Kg DEN every week from 5^{th} week to 18^{th} week. Rats of Group 3 (Standard Drug) were injected with 4 mg/ Kg doxorubicin from 9^{th} week to 12^{th} week. Rats of Groups 4, 5 and 6 were orally fed with Dawa – ul kurkum formulation in low (200 mg/Kg), medium (300 mg/Kg) and high doses (400 mg/Kg) respectively every day from week 9 to 18. Rats in Group 7 (combination of test drug with standard drug) were injected with 4 mg /Kg of doxorubicin once a week for 4 weeks starting from week 9. They were also fed with a medium dose of test formulation every day from week 9 to week 18 of the experiment. Blood samples were collected from all test rats at weeks 5, 10, and 15 and serum was separated for biochemical testing.

Isolation of liver lobes and preparation of liver homogenate

After 18 weeks, the rats were anesthetized and sacrificed. The liver lobes were isolated and examined for weight and number of tumor nodules. 50% of liver tissue was washed in ice–cold PBS and minced. 10% homogenate was prepared in 0.1 M Tris – HCL buffer (pH 7.4). 50% of the remaining liver tissue was fixed in 10% neutral buffered formalin for histopathology. They were embedded in paraffin and serial (5 µm) sections were stained with hematoxylin & eosin. Neoplastic nodules and HCC were classified based on published criteria. The blood samples and liver homogenate were evaluated for biochemical parameters alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine transferase (ALT), and tumor markers carcinoembryonic antigen (CEA) and alfa-feto protein (AFP) using biochemical enzyme assay kits.

Statistical analysis

GraphPad Prism 6.0 was used for data analysis. Significance was assessed through one-way ANOVA. A p-value less than 0.05 were regarded as statistically significant. In the graphs, *, **, and *** represent significance levels of < 0.05, < 0.01, and < 0.001, respectively.

Results

Procurement of selected plant drugs and processing

The plant samples were authenticated by the Taxonomy Division, Department of Botany, Savitribai Phule Pune University. The parts of the plants were separated, cleaned, and powdered. The photographs of authenticated plant drugs are presented in Supplementary Fig. S1.

Preparation of Dawa – ul Kurkum formulation

The Dawa-ul-Kurkum formulation was prepared as described earlier. It was standardized using organoleptic, physicochemical properties, TLC and HPTLC analysis[13].

Cytotoxic effect on HepG2 cell line

The selected part of each test drug was powdered and mixed with water as well as honey to prepare two separate extracts; i) aqueous extract and ii) powder mixed with honey (classical dosage form)[6]. These extracts, Dawa-ul-kurkum formulation and the standard drug Doxorubicin were assessed for effect on HepG2 cell line using MTT assay. Figure 1 (a-j) presents the percent viability of cells after treatment with various concentrations (15.625 µg/mL – 500 µg/mL) of aqueous (blue) and honey (pink) extracts of each plant drug for 48 h. Figure 1 (h) and Figure 1 (i) depicts the effect of Dawa – ul – kurkum and standard drug doxorubicin.

The IC_{50} values of the test drugs calculated using Graph Pad Prism 6.0 software are compared in Table 1. The results reveal that all test plant drug samples have significant cytotoxic effects on HepG2 cell line. Among the two types of extracts, honey dosage forms demonstrated enhanced cytotoxic effect and

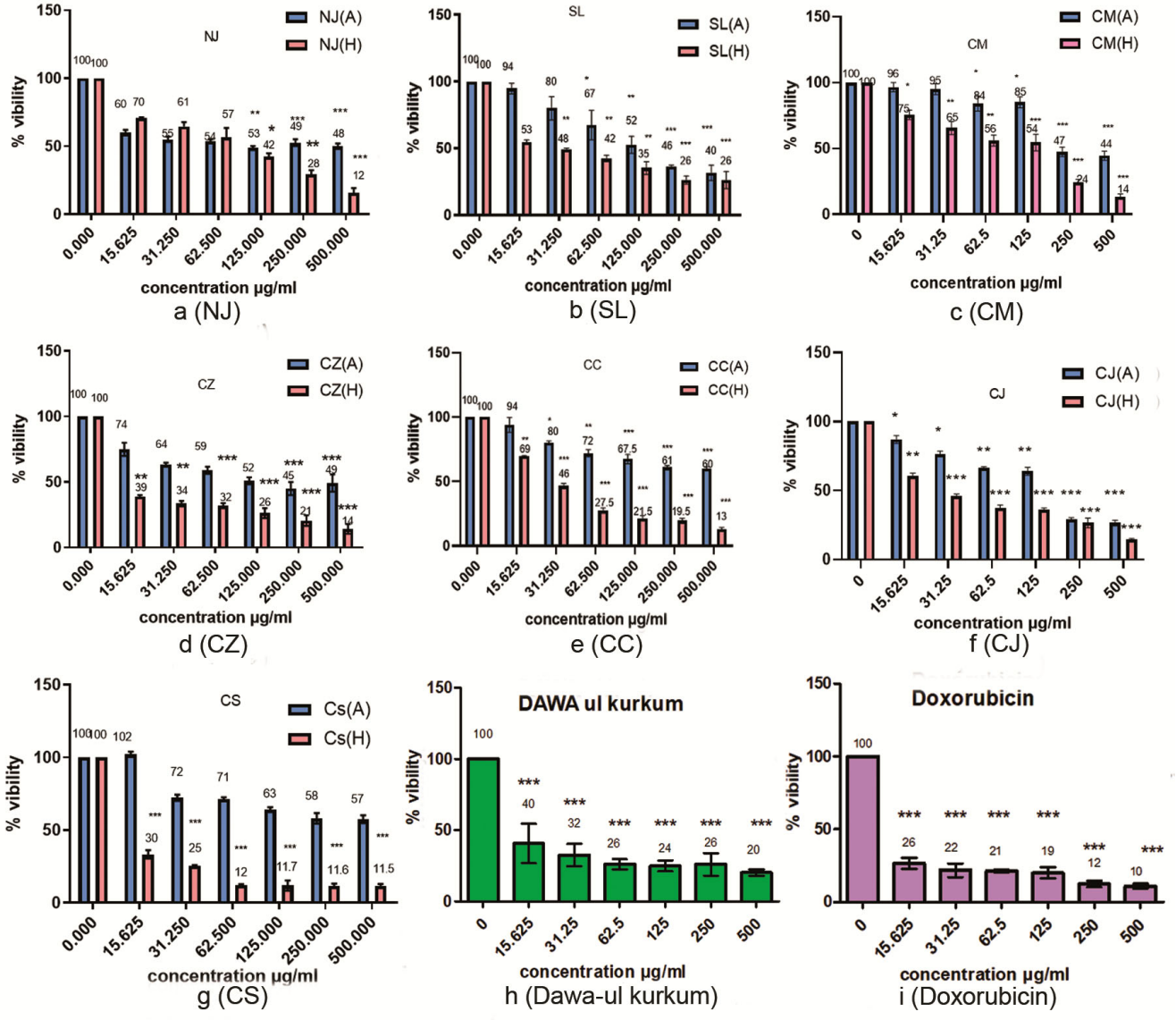

Fig. 1 — (a-g) Bar graph showing the percent viability of cells after treatment with 15.62 µg/mL – 500 µg/mL of aqueous (blue) and honey (pink) extracts of plant drugs. (h) and (i) show the effect of Dawa-ul-kurkum and standard drug doxorubicin on percent viability of HepG2 cells. *, ** and *** in the graphs indicate significance < 0.05, < 0.01 and < 0.001, respectively

pharmaceutically significant IC_{50} values for all the plant samples (Table 1). Maximum effects among the individual test drugs were shown by CS, SL, and CC respectively. The Dawa-ul-kurkum formulation was highly effective in imparting a cytotoxic effect on HepG2 cell line. It displayed a pharmacologically significant IC_{50} value of 8.3 µg/mL which almost matched with 6.8 µg/mL shown by the standard drug Doxorubicin. Thus, a profound cytotoxic effect was displayed by Dawa-ul-Kurkum formulation. The effect was much higher than those shown by each of its ingredients.

Determination of apoptosis using Annexin V staining

HepG2 cells treated with test drug samples were stained with Annexin V, FITC and analyzed by flow cytometry. Figure 2 (a-h) and Figure 3 (a-c) present the histograms of plant drugs (honey extracts), Dawa-ul kurkum formulation, and Doxorubicin respectively. Figure 4 compares the percentage of cells in Q2 (late apoptosis), Q4 (early apoptosis), and Q3 (viable cells) quadrants for all drug samples.

Figure 2 to Figure 4: All drug samples induced apoptosis at 62.5 µg/mL concentration in HepG2 cell line. The drugs CM (68%), CJ (62%), CS (61%) CC (57%), NJ (56%), and CZ (35%) were effective in inducing early apoptosis in the significant number of HepG2 cells. The proportion of viable cells was negligible (1-15%) compared to 96% seen in control wells.

The Dawa-ul -Kurkum formulation (Fig. 3 & Fig. 4) presented maximum effect in inducing apoptosis. At a concentration of 62.5 µg/mL, the maximum number (95%) of cells were observed in the Q2 quadrant, indicating the late apoptosis stage, with only 0.7% of the cells present in the Q3 quadrant, signifying viable cells. The results were very similar to those shown by doxorubicin which induced apoptosis in 96% of cells and showed 0.2% viable cells at the end of the study period. The results clearly displayed a very high potential of Dawa-ul kurkum formulation to induce apoptosis in HCC cells.

Table 1 — IC 50 values for aqueous and honey extracts of the plant drug samples and Dawa –ul kurkum formulation

Sr. No	Sample	IC_{50} value Aqueous extract (µg/mL)	IC_{50} value –Honey dosage form (µg/mL)	p valve
1	*Cinnsmomum zeylanicum*	97.13	24.25	0.001
2	*Nardostachys jatamanasi*	63.43	21.98	<0.001
3	*Cymbopogon jwarancusa*	126.9	26.4	<0.0001
4	*Cinnamomum cassia*	38.18	23.92	<0.0001
5	*Sausurria lappa*	62.95	13.87	<0.0001
6	*Commiphora myrrha*	145	64	<0.0001
7	*Crocus sativus*	36.15	12.86	<0.0001
8	DAWA-UL-KURKUM	--	8.313	<0.0001
9	Standard Drug Doxorubicin	6.8	--	<0.0001

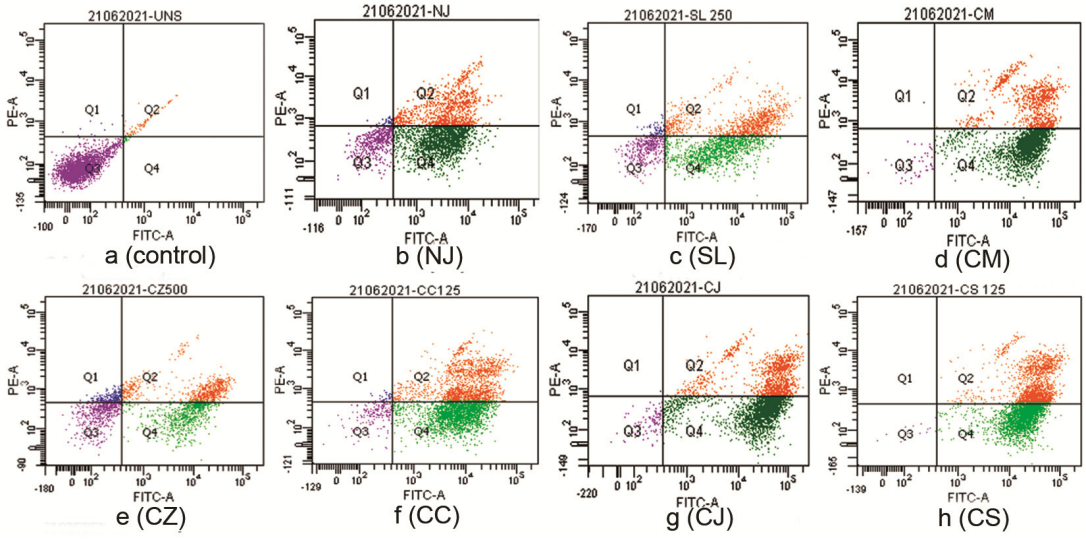

Fig. 2 — (a-h) Histogram of Annexin V-FITC flow cytometry analysis of HepG2 cells treated with NJ, SL, CM, CZ, CC, CJ and CS extracts, respectively

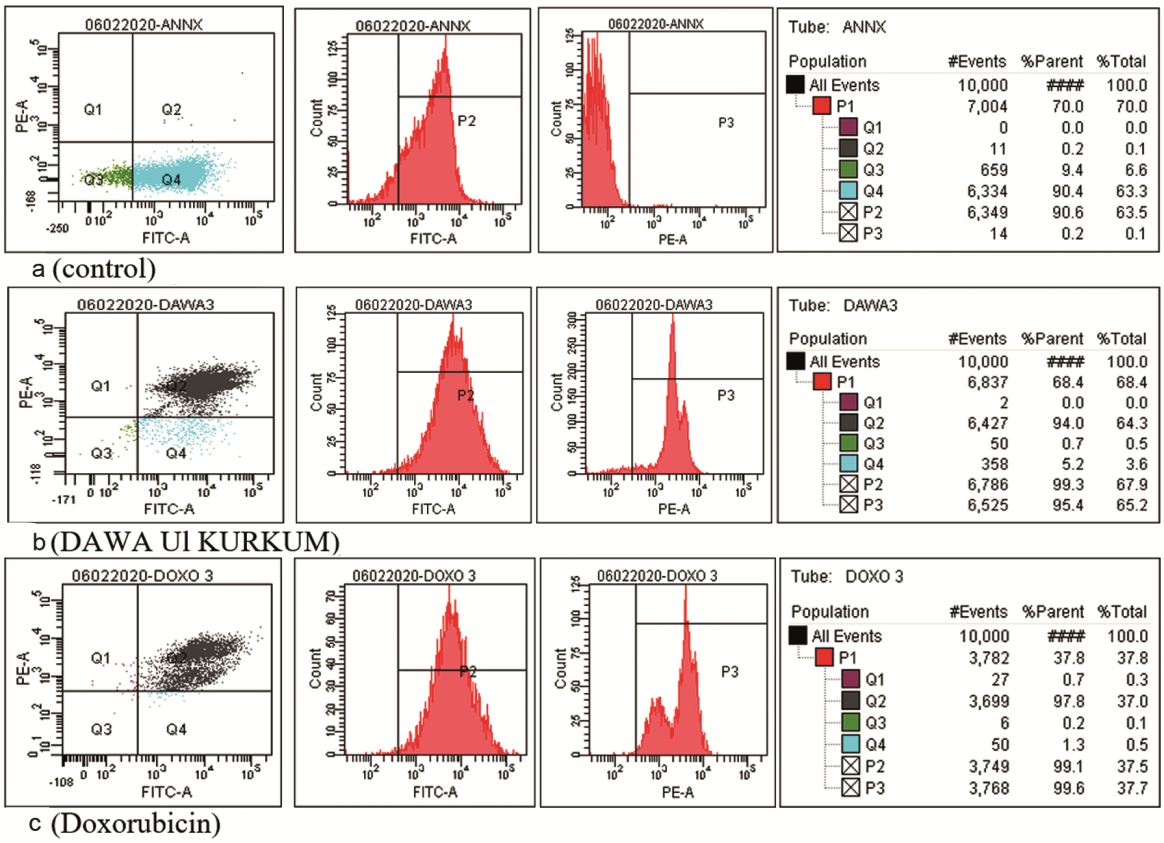

Fig. 3 — (a-c) Shows histogram of Annexin V- FITC staining flow cytometry analysis of HepG2 untreated cells (Annexin control); cells treated with Dawa-ul-kurkum formulation and with standard drug doxorubicin. Annexin control show 0.2% Annexin +ve cells; Dawa-ul-kurkum shows 94% Annexin +ve cells and the standard drug doxorubicin shows 97.8% Annexin +ve cells

Effect on cell cycle analysis

The histogram in Figure 5 (a-j) shows the distribution of cells in G0, G1, S, G2 and M phases of cell cycle after treatment with 62.5 µg/mL concentrations of each test drug and Dawa-ul-kurkum formulation. The untreated control showed 11%, 70%, and 16% of the cell population in G0, G1, S phase and 4% in G2 phase of cell cycle respectively. The cells after treatment with the test drug showed a significant number of cells in G0 phase. The comparison of the percentage of cells arrested in G0 phase by the test drug samples is presented in Figure 5 & Figure 6. Each test drug induced a significant increase in the population of cells in G0 phase of cell cycle. The results confirmed the potential of all test drug samples to arrest a significant number of cells in G0 phase of cell cycle. Among the individual plant drugs, NJ and SL were the maximum effectors in arresting 77, and 70% of cells in G0 phase, respectively. About 83% of cells were arrested in G0 phase by Dawa-ul-Kurkum formulation. The results of the cell cycle analysis supported a high ability of the test formulation

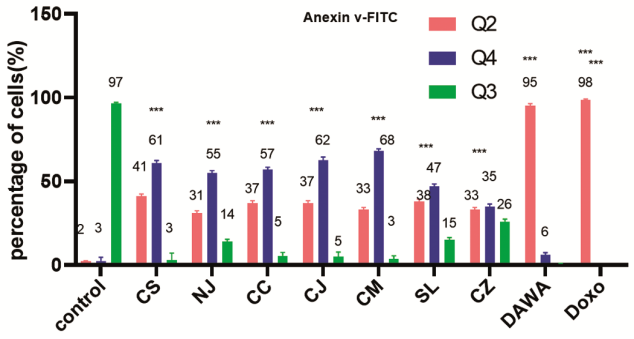

Fig. 4 — Comparison of the percent cells in Q2 (late apoptosis), Q4 (early apoptosis) and Q3 (viable cells) quadrants of histogram of all test drugs

to arrest the HCC cells in the resting phase of the cell cycle.

Overall, the *in vitro* investigations displayed a profound potential of Dawa-ul kurkum formulation to arrest the growth of HCC cells. Pharmacologically significant IC_{50} values and induction of apoptosis suggested its high anti HCC potential with efficiency equivalent to that of the standard drug doxorubicin.

Table 2 — Illustrates the number tumor nodules, weight of animals and liver in rat study groups

Treatment	Total no of nodules	Initial body weight (g)	Final body weight (g)	Liver weight (g)
Control	0	189	334	7.97
DEN	120	200	298	19.03
DEN+Doxorubicin	13	201	302	8.88
DEN+ DUK low dose	73	205	300	16.24
DEN+ DUK medium dose	55	207	308	13.37
DEN+DUK high dose	22	202	319	10.07
DEN + DUK Medium dose + doxorubicin	33	195	320	10.63

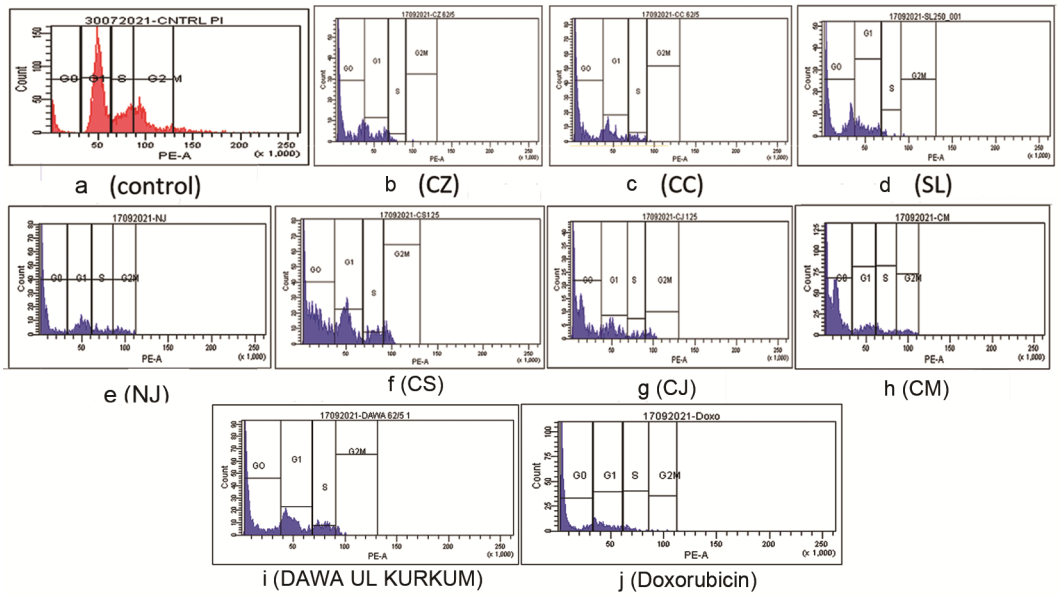

Fig. 5 — (a-j) Shows the cell cycle histograms of HepG2 cells treated with test drugs

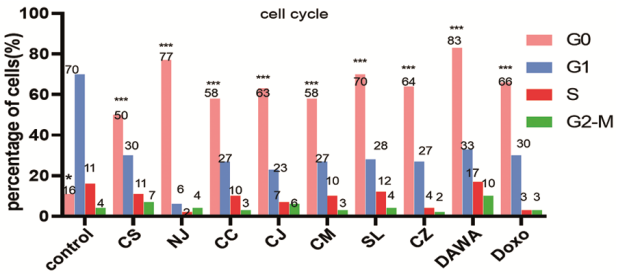

Fig. 6 — Illustrates the percentage of cells in G0, G1, S, and G2-M phases of the cell cycle following treatment of the HepG2 cell line with test drug samples

Comparison of the individual plant drug and the Dawa-ul kurkum formulation revealed a prominent cumulative effect of single ingredients in polyherbal formulation. It suggested an augmenting effect of individual plant drugs in the formulation which appeared to have further enhanced due to use of honey in this formulation.

***In vivo* study**

The Dawa-ul-kurkum formulation was further tested for regression of tumor growth in diethylnitrosamine (DEN) induced HCC male Wistar rats. Rats were orally fed low (200 mg/kg), medium (300 mg/kg) and high (400 mg/Kg) concentrations of formulation from week 9 to week 18. The effect was compared with normal control, DEN control, and standard drug doxorubicin-treated rats.

Results of body weight, liver weight and number of nodules

Table 2 presents data relating to weights of animals, liver, and number of nodules. The average initial body weight of Group 1 normal animals was 189 g which increased to 334 g during 18 weeks study period. Their liver at the end of the study period weighed 7.97 g. Intravenous injections of 40 mg/Kg DEN per week to rats resulted in the development of 120 nodules in rat liver. A notifiable reduction to 298 g of body weight was observed. A significant increase in the weight of the liver to 19.03 g was an important feature of these rats. Oral feeding of a single dose of Dawa-ul kurkum formulation per day (week 9-18) demonstrated dose-dependent restoration of these values. An effect matching close to the standard drug

was shown by a high dose of formulation and by the combination of medium dose and doxorubicin (Table 2).

Effect on biochemical parameters

The blood samples were analysed for biochemical parameters AST, ALT, ALP, LDH, AFP and CEA. The results are presented in Figure 7. The enzymes AST, ALT and ALP prominently correlate with inflammation in the liver. A More than 2.5-fold increase in their values was noted in DEN control rats. Treatment with standard drug doxorubicin resulted in a significant reversal of these values (Fig. 7). Even the oral feeding of low dose was effective in significant decrement of the enzyme values ($p<0.001$). Enzyme LDH is an indicator of cellular damage. Its high levels are associated with acute liver failure. Oral feeding of Dawa-ul-kurkum formulation was effective in restoring enzyme levels. An equivalent effect was observed with oral feeding of test formulation, the high dose being the maximum effector.

The tumor markers AFP and CEA are used in the diagnosis of HCC[14]. CEA in particular correlates with the spread of cancer in the body. Their values increase to 2.5-fold in HCC. Highly significant restoration of AFP and CEA values were observed after 5 weeks of oral formulation feeding. Most importantly, a significant effect was noted with low dose (250 mg/Kg dose) ($p>0.001$). Maximum effects were shown by high and medium plus doxorubicin doses.

The histopathology analysis of dissected liver tissue revealed a normal liver parenchyma with hepatocytes arranged in cords of 1 - 2 cell layers separated by sinusoids in group 1 normal rats (Fig. 8 a). After 40 mg/Kg DEN treatment neoplastic nodules were seen on necked eye examination. Microscopically, the neoplastic nodules showed abnormal hepatocytes merging with the surrounding normal liver cells (Fig. 8 b). Loss of sinusoidal pattern, arrangement of cells in compact sheets, presence of large polygonal cells with high N: C ratio, and condensed nuclear chromatin were noted. Many mitotic figures and multinucleated cells were seen. The intravenous treatment of doxorubicin resulted in reducing the size of tumor islands and ballooning degeneration. The cells and nuclei were smaller with normal chromatin patterns (Fig. 8 c). Similar alteration in histopathology was noted in high dose Dawa-ul kurkum and standard drug combined dose

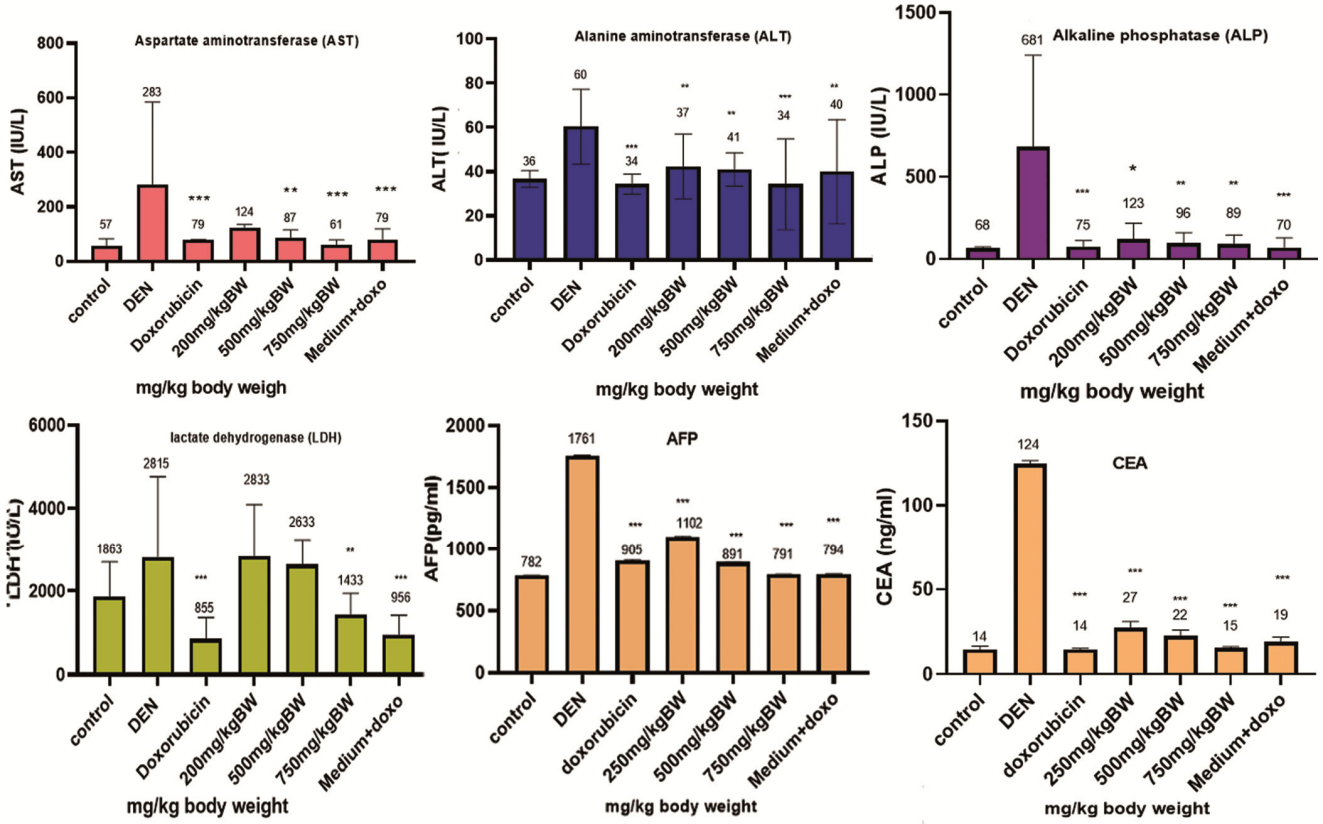

Fig. 7 — Levels of liver enzymes in blood samples of group 1 to group 7 study rats obtained prior to dissection at the end of study period

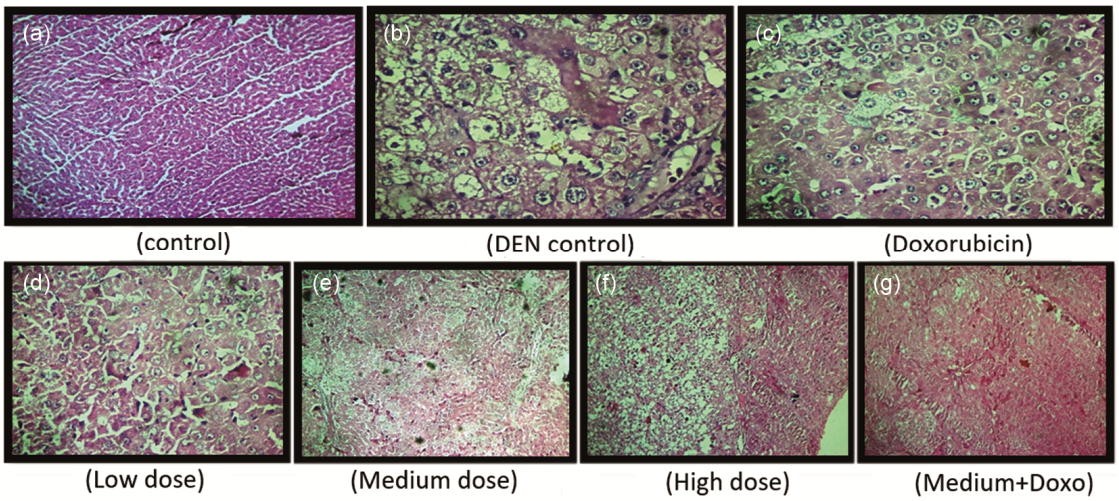

Fig. 8 — (a-g) shows the histopathology result of liver tissues isolated from group 1 to group 7 rats. The images show the effect of medium and high dose of formulation in reducing the number of tumor nodules and restoring the regular tissue morphology

treated rats (Fig 8 d-g). The Dawa-ul kurkum formulation thus displayed significant inhibition of tumor nodules, reversal of inflammation and biochemical parameters in DEN-induced rat HCC tumor models. The animal study endorsed a very promising anticancer effect of Dawa-ul-kurkum formulation in DEN-induced HCC experimental rats.

Discussion

Inflammation, fibrosis, hepatic obstruction, cirrhosis, and liver failure represent critical stages in determining the severity of hepatic damage across various chronic liver diseases. Treatment options are selected based on the degree of liver damage. Dawa-ul kurkum is one complex formulation having diuresis, deobstruction as well as astringent properties. It is used traditionally in the treatment of multiple liver ailments such as anorexia, hepatomegaly, ascites, obstruction, dropsy, and abdominal pain[15]. Case studies illustrating a substantial reduction in fibrosis among patients with decompensated liver cirrhosis have been documented[16]. Its hepatoprotective, anti-inflammatory, antitumor, and immunomodulatory properties are also documented[7-11]. The high efficacy and specificity of this formulation is probably due to the unique combination of herbs used. As per the classical texts, it is prepared by mixing fine powder of seven ingredient herbs in honey[6]. Each of the ingredient plants is known for its prominent hepatoprotective as well as anticancer properties[4]. An ancient formulation using this unique combination of herbs was investigated in present the study for its effect on hepatocellular carcinoma.

To assess the importance of honey in preparation of Dawa-ul kurkum formulation, aqueous and honey extracts of each plant drug were compared for cytotoxic effect on HCC (HepG2) cell line. All seven plant extracts demonstrated significant cytotoxic effects on HepG2 cell line. *Crocus sativus* and *Cinnamomum cassia* were most effective with IC_{50} values 36.15 and 38.18 µg/ml, respectively. The results correlated with earlier reports of antitumor studies. This study pointed out a drastic difference in IC_{50} values of aqueous and honey extracts. The honey extracts of all samples displayed much lower IC_{50} values than the aqueous extracts. The use of honey for the preparation of drugs is a traditional method applied in most alternative medicinal systems[17]. The results showed that it augments the drug significantly for antitumor effect. Most importantly, the study pointed out that Dawa-ul kurkum formulation with IC_{50} value of 8.3 µg/mL was much superior to any of the single drugs. The effect was close to that shown by standard drug doxorubicin.

Staining with Annexin V- FITC and PI revealed the potential of the drug to induce early apoptosis in HepG2 cells. Annexin V-FITC conjugate binds to negatively charged phosphatidylserine which are externalized in apoptotic cells and thus detects apoptosis at an early stage. These cells exclude PI as their membranes are still intact, whereas the membrane of dead or damaged cells is permeable to PI. Hence, staining of cells with FITC–annexin V and PI enables the detection of live, nonapoptotic cells (annexin V negative/PI negative), early apoptotic cells

(annexin V positive/PI negative), and late apoptotic or necrotic cells (both annexin V and PI positive) by cytometry[18]. 62.5 µg/mL concentration of each plant drug induced early apoptosis in 50-66% of HepG2 cells. The proportion of viable cells was only 1-15% compared to 96% in untreated control cells. The Dawa-ul kurkum formulation at the same concentration induced apoptosis in 94% of cells. The standard drug induced apoptosis in 97.8% of cells. A negligible number of viable cells were noted at this concentration.

Dysregulation of the cell cycle is a hallmark of most cancer types. Progression of the cell cycle is normally regulated by the phosphorylation of specific proteins by cyclin-dependent kinases (cdk) and their dephosphorylation by phosphatases followed by proteolytic degradation through ubiquitin–proteasome system. The process of transformation is commonly associated with over-expression of cyclins and loss of expression of cdk inhibitors. A major consequence is dysregulated cdk activity, provoking cells to continue in cell cycle[19]. The drugs that can arrest cell cycle have enormous therapeutic potential in cancer. The Dawa-ul kurkum formulation displayed a high potential to arrest cell cycle progression in HepG2 cell line. The results were in accordance with the anticancer properties of each study drug. All plant drugs arrested about 55-77% of the cell population in G0/G1 phase of cell cycle. The Dawa-ul kurkum formulation was the maximum effector of cell cycle arrest with 83% cell population in G0/G1 phase.

A rat model of HCC induced by diethylnitrosamine (DEN) was used to evaluate the *in vivo* efficacy of Dawa-ul kurkum formulation. A significant decrease in body weight and an increase in liver weight were prominent features of DEN-treated rats at the end of the 18-week study period. The animals orally fed with medium and high doses of test formulation restored the body weight as well as the liver weight to near normal. They reduced the number of tumor nodules from 120 to 55 and 22, respectively. Although their weights were slightly lower than those of doxorubicin-treated animals ($p>0.01$), they were very active throughout the study period compared to the standard chemotherapy drug-treated rats.

Oral feeding of Dawa-ul kurkum to DEN-treated rats resulted in significant restoration of liver enzymes. AST, ALT, and ALP are involved in protein metabolism and are released in the bloodstream in case of liver damage. ALP is also an indicator of blocked bile duct[20]. LDH is another liver enzyme whose levels are elevated in liver damage[21]. Alfa fetoprotein (AFP) is a gold standard for the diagnosis of HCC. Its elevated levels are associated with liver malignancies[14]. These tumor markers were reversed even in low-dose Dawa-ul kurkum fed rats. The effect was dose-dependent with high dose being most effective. A combination of doxorubicin and the medium dose of Dawa-ul kurkum did not pose any significant advantage over oral high-dose feeding except for the observation that the rats were much more active than the doxorubicin alone injected group. The restoration of histopathological parameters, tumor markers, serum liver function tests, body weight, liver weight, and reduction in tumor nodules defined a pronounced anti-tumor potential of Dawa-ul kurkum formulation.

Conclusion

A traditional honey-based Dawa-ul kurkum formulation was evaluated for anticancer effects against hepatocellular carcinoma. The honey in this formulation enhanced the cytotoxic effect making the formulation the maximum effector of cytotoxicity, induction of apoptosis, and cell cycle arrest. Oral feeding of the formulation to DEN-induced HCC rats resulted in regression of tumor growth comparable to the standard drug. Therefore, the present detailed study undoubtably demonstrated a high potential of Dawa-ul kurkum formulation in the prevention of HCC. It suggests consideration of this cost-effective and safe herbal composition for clinical evaluation as well as integration in the treatment of HCC.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_23(11)(2024)1044-1054_SupplData.pdf

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

ASM, MMD, and NSB conceived the study. SSK conducted investigations and data analysis. GM, MMD prepared the formulation and analysis. MNK performed histopathology. ASM drafted the manuscript.

Ethics Approval

Use of animals for this study was approved by Institutional animal ethics committee of Bharati Vidyapeeth Deemed to be University, Pune (Approval number: BVDUMC/563/2021/001/001) and the experiments were performed in accordance with CPCSEA guidelines

Data Availability

The authors confirm that the data supporting the findings of this study are presented within the article and/or its supplementary materials.

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